

Potential Role of Heterologous Flavivirus Immunity in Preventing Urban Transmission of Yellow Fever Virus

Corresponding Author: Professor Scott Weaver

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The influence of prior heterologous flavivirus immunity on infection with yellow fever virus has been studied a number of times, with the general conclusion that there is an element of cross-protection and potential influence on transmission, and this remains an attractive hypothesis to explain aspects of yellow fever epidemiology and pathogenesis. The authors have reinvestigated this hypothesis with a focus on two prevalent flaviviruses, DEN-2 and Zika, and have approached the experimental design in a substantially broader way than previous work, including both the effects of heterologous immunity on YF infection and clinical parameters in the host and its ability to serve as the source of mosquito infection and transmission. Unlike other studies, a long interval between prior immunization and challenge with time for affinity maturation is a major positive over previous reports. The results, methods, and interpretation are clear and comprehensive and the paper will be of wide interest. A few improvements are suggested below

Page 3, line 74. The authors acknowledge previous work showing that Zika immunity suppressed experimental YFV infection in nonhuman primates; however the reference cited is incorrect (ref 19 should be Henderson BE, Cheshire PP, Kirya GB, Lule M. Immunologic studies with yellow fever and selected African group B arboviruses in rhesus and vervet monkeys. *Am J Trop Med Hyg.* 1970 Jan;19(1):110-8.).

Page 3 para starting with line 76. Zika has been enzootic /endemic in Africa as well, where YF outbreaks, including *Ae aegypti* outbreaks, have been common. Alternating ZIK and YF epizootics have been documented and ZIK immunity and epidemiologic observations have not indicated ZIKV interfere with transmission of YF (McCrae AW, Kirya BG. Yellow fever and Zika virus epizootics and enzootics in Uganda. *Trans R Soc Trop Med Hyg.* 1982;76(4):552-62). Also, studies in Africa have documented severe YF disease and outbreaks in populations with a high background of flavivirus immunity, especially from prior ZIK infection.

The authors infer that the widespread transmission of DEN and ZIK virus may have interfered with urban YF transmission in Brazil. However, they also note that urban (*Ae. aegypti*) has not been reported in Brazil in many years (actually since 1942), long before recognition of DEN or ZIK and before wide use of YF vaccines.. Some discussion is warranted to reconcile these observations.

Page 3 Line 96-98, Fig 1b and Methods lines 309-310. The source, year of isolation and passage history of all virus strains used in the study should be provided. Details of the YF challenge strain are given in Methods.

Methods line 350-360 and Fig 1b. Clarify which DEN2 strain was used to measure antibody, homologous strain or homologous genotype? The titers following infection with the DEN-2 P8 virus were higher than for the NGC animals. Similarly the ZIK titers were quite variable. Results Fig 1. Did the authors determine the effect on viremia if the DEN-2 immune animals and ZIK-immune animals were considered together and then compared to the naïve controls? The paper would be improved by providing a regression analysis of pre-challenge DEN-2 and ZIK FRNT titer vs YF viremia peak titer. Anti-NS1 antibodies were measured against YFV. It is assumed that all pre-challenge sera were also negative for YF neutralizing antibodies, but apparently these were not measured.

The authors speculate that anti-NS1 elicited by DEN-2 and ZIK could have modulated YFV infection. Again the paper could be improved by regressing anti-NS1 ELISA titer against peak viremia. The NS1 data are presented graphically but the individual NHP data are not provided.

Reviewer #2

(Remarks to the Author)

This is high profile work performed by a very well-known and authorized group of scientists.

As authors acknowledged the limited circulation of YFV in LA and Asia regions despite the favorable epidemiological and ecological conditions is captivating from a scientific point of view. It is also interesting that previous reports on DENV and other arboviruses interfering with YFV replication in NHPs date back to 1970s.

The main hypothesis behind this work "...that prior flavivirus immunity, especially DENV, plays a major role in preventing urban YFV emergence by limiting the number of competent human amplifying hosts".

While the work instilled a high level of enthusiasm, there are some minor and major observations/suggestions we would like to bring to the authors attention.

Minor Comments:

Lines 78-79 vs. Line 86

"...ZIKV recently caused a major epidemic in the Americas from 2014 to 2016, infecting over 50% of most neotropical human populations²⁰. The peak of this outbreak preceded the 2016 YF epidemic, raising the question of whether preexisting ZIKV immunity may also have influenced YFV infection dynamics including viremia, which could influence interhuman mosquito transmission and YF disease progression.

Since ZIKV has been endemic to Asia for many decades²¹, its immunity could also have influenced the risk of YF there.

While this statement above explains itself, its meaning is not clear when compared to the statement in line 86. Are the authors acknowledging and underscoring a potential YF amplification by ZIKV?

Has prior ZIKV immunity allowed or created the immunological basis favoring a YF outbreak?

The statement "...preexisting ZIKV immunity may also have influenced YFV infection dynamics..." is somehow vague as it cannot be defined if the influence is towards amplification or reduction, as it is mentioned next in lines 86-87.

Line 86-87

Our findings, based on a cohort of NHPs with distinct flavivirus immunity profiles, revealed that prior immunity to DENV and ZIKV generally reduces YFV viremia, as well as mosquito infection and subsequent transmission potential.

Lines 95-97

The initial infections were described in a previous study to be published independently; 23 animals were exposed to Ae. albopictus mosquitoes sham-infected with PBS or infected with one of the four virus strains: DENV-2 P8-1407 or ZIKV DakAr 41525 (sylvatic strains), DENV-2 NGC or ZIKV PRVABC59 (human 97 endemic/epidemic strains).

The number of animals for each group could be included for better clarity.

Line 116

To investigate further if initial infections from mosquito feeding occurred with FRNT-seronegative animals, we performed ELISA to detect binding but non-neutralizing antibodies. Interestingly, both of the DENV-2 P8-1407-seronegative and the one ZIKV-seronegative animal had low levels of IgG antibodies by ELISA, suggesting that infection did occur despite the lack of homologous neutralizing antibody induction.

Please name here animal FR1565 as it is critically relevant for this work.

Line 129-130

Most mosquitoes (bodies) that fed on flavivirus-naïve animals had high rates 129 of YFV infection on peak viremia days 3 (Figure 2a) and 4 (Figure 2b) post-infection.

Could authors speculate on why mosquitoes that fed on flavivirus-naïve NV 259 were not infected with YFV despite having detectable viremia on days 3 and 4?

Line 140

Upon comparing the mosquito infectivity with NHP viremia for flavivirus-naïve and DENV-2-immune groups, we observed that the infectivity for mosquitoes that fed on a DENV-2-immune animal was 20-30% lower compared to flavivirus-naïve animals with similar YFV viremia levels (5.3 Log₁₀ FFU/ml) (Figure 3).

Is this observation related to one animal? ID is not mentioned and in figure 3 refers to dengue immune group, not to one animal. As DENV infection and neutralization is very variable among animals the animal ID is relevant for correlation with the specific results from that animal.

Lines 141-143 Upon comparing the mosquito infectivity with NHP viremia for flavivirus-naïve and DENV-2- immune groups, we 141 observed that the infectivity for mosquitoes that fed on a DENV-2- immune animal was 20-30% lower compared 142

to flavivirus-naïve animals with similar YFV viremia levels (5.3 Log₁₀ FFU/ml) (Figure 3).

Mosquito infectivity % was not analyzed against NHP viremia for ZIKV-immune animals but no reason is mentioned.

Line 274

Twenty-three healthy adolescent cynomolgus macaques (11 males and 12 females, 2.5-5.5 years old, 3-5.5 kg)

Authors mention males and females were used in this study but their distribution among groups is unknown.

Line 269-270

No statistical methods were used to predetermine the sample size as the NHPs were originally utilized in a separate experiment in keeping with the ethical goal of reducing the total number of animals utilized in research.

Authors state that statistical power was not established because the animals used belonged to a separate experiment. However, the number of animals in most groups is low given the recommended NHP sample size, and the difference in sample size between the DENV2-P8 group and other groups is remarkable. Perhaps if sample size was more adequate, the significant difference between DENV2-P8 group and the naïve group would be lost, or other statistical differences would be gained. Author comments on this statistical bias are encouraged.

Under Ethical statement

As this is terminal experiment a statement on the humanized euthanasia is highly recommended.

Figures 1b-e, 2a-d and 5a-f could include a header for better clarity.

Major Comments:

Line 98

To ensure that our study was reflective of typical convalescent flavivirus immunity, and not an acute phase response, we allowed the immunity to develop for at least 6-9 months post-primary infection.

A topic limitedly discussed in flavivirus field, particularly for dengue is the optimal time between consecutive infections or vaccine dosing.

For this particular work, considering that the response among animals is very variable (including response to prior exposure to dengue or ZIKV) it is important to highlight the specific timelapse between initial flavi and the subsequent YF exposures. Also, in order to reinforce the value of this work, the reason why selecting a 6-9 month period between flavi infections should be better explained/justified

Line 112

However, the ZIKV-seronegative animal (NHP ID: FR1565, exposed to the bite of an infected mosquito but FRNT-negative) had higher and longer viremia compared to the flavivirus-naïve animals (Figure 1e). Surprisingly, unlike any other YFV-infected animal, FR1565 was laterally recumbent and had to be euthanized by day 9 post-challenge. No visible signs of illness were observed in any other of the infected animals.

Line 146

To assess this hypothesis, we tested the ability of NS1 antibodies generated in response to DENV and ZIKV infection to react with the YFV NS1. As shown in Extended Data Fig. 1, these crossreactions, although lower in magnitude than the homologous reactions in ELISA, were significantly above the levels of binding exhibited by naïve or seronegative sera.

As this is an important observation, can the authors elaborate on the differences in the reactivity to YFV NS1 protein between the DENV and ZIKV cohort? It looks significant. Also there are prior reports covering this cross-reactivity.

Line 195

High levels of IL-8 have been reported in convalescent ZIKV patients, suggesting that prior ZIKV immunity could be an important factor in elevating IL-8 levels post-YFV-infection.

Was IL-8 significantly elevated compared to the DENV group? It looks likely to be the case. If so, any comment on why ZIKV but not DENV predisposes to a "proinflammatory status" after YFV?

Line 225

Our finding of cross-binding antibodies by ELISAs from DENV and ZIKV-immune sera suggest a role for NS1 antibodies in restricting mosquito infection to limit human amplification potential. Additional experiments are needed to further test this

hypothesis.

Actually, this finding is highly relevant to support the hypothesis behind this work looking precisely for a mechanism restricting YF transmission. DENV and ZIKV anti-NS1 antibodies should be tested, at least in vitro, to reinforce this highly likely mechanism of YFV replication restriction. As per the results showed here, DENV anti-NS1 Abs may interfere more with YF replication than ZIKV-anti-NS1 Abs.

Line 236

The low levels of ZIKV non-neutralizing IgG we detected suggested the possibility of antibody-dependent enhancement, as has been reported in sequential flavivirus infections (mainly DENV). However, with only one animal with a severe outcome and an immunological profile of this kind in our study, we cannot conclude that low level ZIKV immunity exacerbated the YF outcome; future studies are required to investigate this hypothesis.

The potential of ZIKV-induced YF ADE is also highly significant for this study and of high relevance for the flavivirus field. Together with the epidemiological evidence that the major ZIKV 2014-2016 epidemic precedes the 2016 YF epidemic, the finding of ZIKV non-neutralizing IgG in the only animal that died to YF, deserves further attention in order to increase the scientific impact of this work.

While ADE in vitro is not necessarily an accurate measure of what can happen in vivo, by comparing the ADE properties of the binding non-neutralizing antibodies from animal FR1565 vs. other selected animals will shed light on the potential YF ADE by ZIKV and strengthen this manuscript.

Reviewer #3

(Remarks to the Author)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer #4

(Remarks to the Author)

In the present manuscript, Shinde et al. hypothesized that heterologous flavivirus immunity can prevent YFV transmission in urban areas where the main urban vector, *Aedes aegypti*, is present. To test their hypothesis, they first infected monkeys with DENV and ZIKV to build heterologous flavivirus immunity. Then, after a defined period, they infected the monkeys with YFV and exposed *Ae. aegypti* mosquitoes to the YFV-viremic monkeys. The results are very interesting and significant. The observation of a significant reduction in mosquito infection is remarkable.

The study represents a tremendous amount of work and is very well-written. The new findings in this study align with the mission and impact factor of Nature Communications.

In my opinion, this manuscript can be accepted after a minor review. Please see my comments below.

Title

The title of the manuscript broadly expresses the idea of the work, but I am wondering if it truly reflects the findings of the present study. The authors did not demonstrate the impact of their findings on preventing urban transmission. While I understand the main point, I would like to know the authors' opinion on this issue, and a reflection about the title.

Abstract

The abstract is very clear and concise. It provides enough background, states the main hypothesis, and presents the principal findings, supporting a new hypothesis.

Importance

The section is well-written and clearly states the relevance of the study.

Introduction

The Introduction provides sufficient and relevant background to support the study's context and hypothesis. It is a very objective and well-written section. I have only one comment below:

44-45 – The authors should also comment on the live-attenuated aspect of the vaccine and the controversy surrounding widespread vaccination, even though it has been proven to be safe. Additionally, the authors should address the lack of a massive vaccination program for YFV prevention in general populations to clarify the naïve immunity profile. This issue was nicely addressed for Brazil later in the introduction.

Methods

271-272 – The authors should explain why they designed the study with these parameters: “The experiments were not randomized. The investigators were not blinded to allocation during experiments and outcome assessment.” What are the limitations in the interpretation of the data in this case, if there are any.

275 -Is there a reference for the previous study? “...were transferred from a previous study...”. Is it the reference 22 that was cited later?

275-276 – In “where they were exposed to mosquitoes infected with PBS (3/23), DENV-2 NGC (3/23), DENV-2 P8-1407 (10/23), ZIKV PRVABC59 (4/23), and ZIKV DakAr 41525 (3/23).”:

1-Please, fix “Infected with PBS”

2-How did the authors decide for DENV-2 instead of other DENV serotypes?

3-What was the strain of the mosquitoes? I assume they were *Ae. aegypti* but it is not stated in the sentence above. (This information was in the results section; it was odd to know that it was *Ae. albopictus*).

4-How did the authors test for the infection status of the mosquitoes, and monkeys after exposure to DENV and ZIKV?

5-Why is the number of the infected monkeys with DENV-2 P8-1407 so discrepant from the other ones?

6-What is the susceptibility to YFV of the mosquitoes used in the infection assay? Even though the experiments give us a clue, it would be nice to have it established before the experiments.

277- How did the authors decide for a dose of 1×10^4 FFU and 0.5 mL of the inoculum?

279- In “Temperatures were recorded at 15-minute intervals on loggers (Star Oddi, Iceland) placed in the abdominal cavity.”, for how long?

281-commercial chow (manufacturer?)

289- “A cardboard carton with a mesh top containing 100 female *Ae. aegypti* (mosquitoes from Salvador, Brazil, maintained for 18 generations at the UTMB Insectary) were allowed to feed on NHP ears for 5-15 minutes”:

1-Is there a reference for this strain (Salvador, Brazil)?

2-What is the susceptibility level of this strain for the viruses?

3-Why did the authors decide for 5-15 minutes of feeding? Was it enough time for the females to be fully engorged. How was this aspect verified?

4-What is the age range of the female mosquitoes that were used in the infection assays?

Could the authors describe the containment protocol for the monkeys? This is not detailed in the methods.

317-318- “Fully engorged mosquitoes were maintained at 28°C with a 16-hour light/8-hour dark cycle with ad libitum access to 10% sucrose.” Please, describe how the engorged ones were separated from the non-engorged ones.

Results

92-93- “Dengue virus type-2 (DENV-2) and ZIKV-immune non-human primates (NHPs) have modulated YFV viremia.” should not be “Dengue virus type-2- (DENV-2) and ZIKV-immune non-human primates (NHPs) have modulated YFV viremia?”

95-96- Why did the authors use *Ae. albopictus* instead of *Ae. aegypti* mosquitoes for DENV- and ZIKV-infections? This was not addressed in the methods and the species was not cited in that section as well. Is there an explanation for that?

109-110- “DENV-2- and ZIKV-FRNT-positive animals” please, be more specific on what positive is about.

112- “However, the ZIKV-seronegative animal (NHP ID: FR1565, exposed to the bite of an infected mosquito but 113 FRNT-negative) had higher and longer viremia compared to the flavivirus-naïve animals (Figure 1e).” Why did the authors state this result without statistical significance on Figure 1e? Did I miss anything in here? I am sorry if I did.

Figure 1

Figures 1d and 1e:

1-Could the authors offset the y axis from x axis? The overlapped data on the y axis makes it hard to read the figures and significance (asterisks).

2-The x legend should be labeled as “Days post YFV exposure” instead of just “Days Post-infection”.

3-Figure 1d should have a headline for DENV-2-exposed, and Figure 1e for ZIKV-exposed”.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The manuscript is improved and the authors have addressed reviewer comments.

The difficulty with the paper overall is that it is based on very limited data given the constraints of the sample size in the experimental groups, there is a substantial and unexplained difference in the effect size on viremia suppression between two strains of the same dengue serotype 2 (NGC and P81407), and a combined analysis of DEN2+ZIK immune animals did not show a statistical result. Since this paper will be cited many times in the future as evidence for heterologous protection by DEN and ZIK immunity, this reviewer would like to see a cautionary conclusion in the Abstract regarding the limitations of the study.

Figure 1. It is not clear what is displayed in panel (d). Are these geometric means $\pm$ 95% CIs?

Figure 3: Are all animals included in the analysis displayed? Extended Table 2 shows some animals with higher viremia levels.

Reviewer #2

(Remarks to the Author)

Thank you for the comprehensive answers provided. Those cover all of my observations.

Reviewer #3

(Remarks to the Author)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer #4

(Remarks to the Author)

The authors have addressed all the points raised in my review and have clarified the experimental descriptions and other textual issues. I believe this is an improved version that can be accepted as is.

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Thank you for giving us the opportunity to submit a revised version of our manuscript titled “Yellow Fever Emergence: Role of Heterologous Flavivirus Immunity in Preventing Urban Transmission” to *Nature Communications*. We appreciate the time and effort that you and the reviewers have dedicated to providing your valuable feedback on our manuscript. We are grateful to the reviewers for their insightful comments on our paper. We have been able to incorporate changes to address most of the suggestions provided by the reviewers. We have highlighted the changes within the manuscript.

Here is a point-by-point response to the reviewers’ comments and concerns.

Reviewer 1

The influence of prior heterologous flavivirus immunity on infection with yellow fever virus has been studied a number of times, with the general conclusion that there is an element of cross-protection and potential influence on transmission, and this remains an attractive hypothesis to explain aspects of yellow fever epidemiology and pathogenesis. The authors have reinvestigated this hypothesis with a focus on two prevalent flaviviruses, DEN-2 and Zika, and have approached the experimental design in a substantially broader way than previous work, including both the effects of heterologous immunity on YF infection and clinical parameters in the host and its ability to serve as the source of mosquito infection and transmission. Unlike other studies, a long interval between prior immunization and challenge with time for affinity maturation is a major positive over previous reports. The results, methods, and interpretation are clear and comprehensive and the paper will be of wide interest. A few improvements are suggested below

Comment 1:

Page 3, line 74. The authors acknowledge previous work showing that Zika immunity suppressed experimental YFV infection in nonhuman primates; however the reference cited is incorrect (ref 19 should be Henderson BE, Cheshire PP, Kirya GB, Lule M. Immunologic studies with yellow fever and selected African group B arboviruses in rhesus and vervet monkeys. *Am J Trop Med Hyg.* 1970 Jan;19(1):110-8.).

Response:

We thank the reviewer for pointing out the error. We have corrected the reference #19.

Comment 2:

Page 3 para starting with line 76. Zika has been enzootic /endemic in Africa as well, where YF outbreaks, including *Ae aegypti* outbreaks, have been common. Alternating ZIK and YF epizootics have been documented and ZIK immunity and epidemiologic observations have not indicated ZIKV interfere with transmission of YF (McCrae AW, Kirya BG. Yellow fever and Zika virus epizootics and enzootics in Uganda. *Trans R Soc Trop Med Hyg.* 1982;76(4):552-62). Also, studies in Africa have documented severe YF disease and outbreaks in populations with a high background of flavivirus immunity, especially from prior ZIK infection. The authors infer that the widespread transmission of DEN and ZIK virus may have interfered with urban YF transmission in Brazil. However, they also note that urban (*Ae. aegypti*) has not been reported in Brazil in many years (actually since 1942), long before recognition of DEN or ZIK and before wide use of YF vaccines. Some discussion is warranted to reconcile these observations.

Response: We thank the reviewer for the comment and agree that the history of epizootics in Uganda is important to include. We added on line 57 the reference on Zika and YF in Uganda, and information on *Ae. aegypti* eradication on lines 60-61. We also added text to the discussion on lines 242-246 regarding possible explanations for the lack of cross protection evident from the Ugandan studies.

Comment 3:

Page 3 Line 96-98, Fig 1b and Methods lines 309-310. The source, year of isolation and passage history of all virus strains used in the study should be provided. Details of the YF challenge strain are given in Methods.

Response: We thank the reviewer for catching this oversight and have included the details of all the viral strains including source, year of isolation, and passage history in the Methods section. (See lines 358-67)

Comment 4:

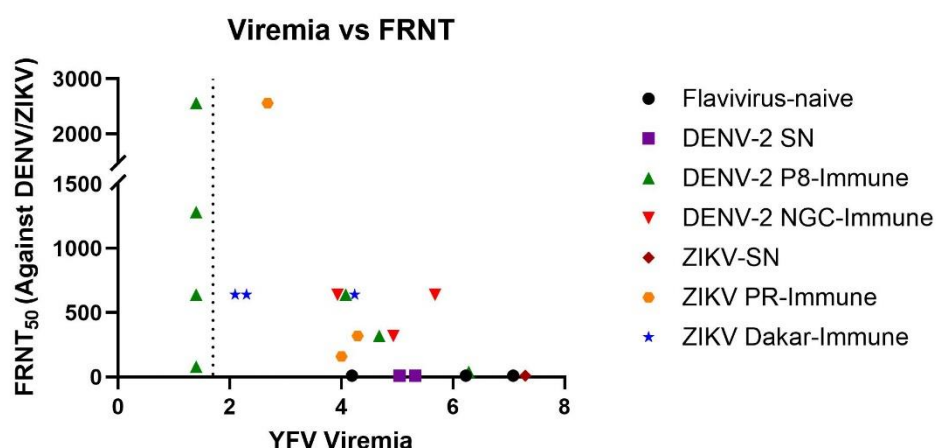
Methods line 350-360 and Fig 1b. Clarify which DEN2 strain was used to measure antibody, homologous strain or homologous genotype? The titers following infection with the DEN-2 P8 virus were higher than for the NGC animals. Similarly the ZIK titers were quite variable. Results Fig 1. Did the authors determine the effect on viremia if the DEN-2 immune animals and ZIK-immune animals were considered together and then compared to the naïve controls? The paper would be improved by providing a regression analysis of pre-challenge DEN-2 and ZIK FRNT titer vs YF viremia peak titer.

Response: We agree and have included a sentence in the methods section to clarify that DENV-2 homologous strains were used to measure antibodies. See lines 413-414. The primary exposure of animals to DENV or ZIKV were done as a part of another study (Ref#24). The animals were exposed to DENV/ZIKV via mosquito bites at high or low dose. An explanation for variable levels of DENV/ZIKV antibodies could be the level of infection during the primary exposure.

We did in fact compare the flavivirus-immune animals together (DENV and ZIKV groups) with flavivirus-naïve animals and did not find a significant difference in viremia levels. Since a significant difference is not observed, we deemed it would not add value to the manuscript. We have now included the figure as Supplementary Figure 1 and addressed in lines 116-118.

Upon statistically comparing the FRNT titers against peak YFV viremia, we observe variable results. The r values were as follows: -0.481, -0.083, and -0.973 for the DENV P8, DENV-2 NGC, and ZIKV PR-immune groups respectively. This suggests a moderate negative correlation, no correlation and a strong negative correlation between neutralizing antibodies and viremia from the groups mentioned above. See lines 278-281.

For the reviewers benefit, please find the viremia vs FRNT graph is below. We have also included as Supplementary Figure 2.



Comment 5:

Anti-NS1 antibodies were measured against YFV. It is assumed that all pre-challenge sera were also negative for YF neutralizing antibodies, but apparently these were not measured.

Response: We thank the reviewer for pointing this out. The pre-challenge sera were measured against YF neutralizing antibodies and were found to be negative. The data are included in Extended Data Table 1.

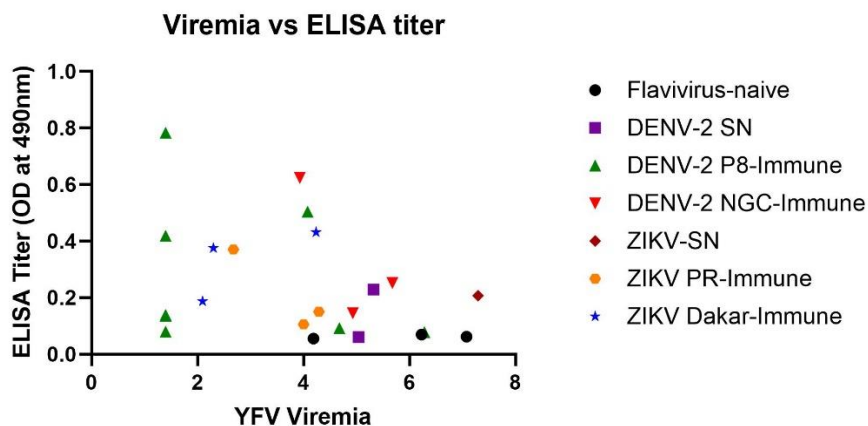
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The authors speculate that anti-NS1 elicited by DEN-2 and ZIK could have modulated YFV infection. Again the paper could be improved by regressing anti-NS1 ELISA titer against peak viremia. The NS1 data are presented graphically but the individual NHP data are not provided.

Response: Upon analyzing peak viremia titer against anti-NS1 ELISA titer, we did not find a significant correlation as shown below. We speculate this based on a previously reported study with mosquitoes. They showed that upon injecting anti-NS1 antibodies in mice, followed by a challenge with DENV did not reduce viremia, but it significantly reduced the mosquito infectivity, suggesting the possibility that anti-NS1 antibodies may not necessarily affect viremia levels, but may affect the ability of a mosquito to get infected by interfering with midgut infection. Therefore, we believe to demonstrate the role of NS1 antibodies, similar studies with mosquitoes should be conducted. See lines 251-253. The referred paper is attached below:

Liu J, Liu Y, Nie K, Du S, Qiu J, Pang X, Wang P, Cheng G. Flavivirus NS1 protein in infected host sera enhances viral acquisition by mosquitoes. Nat Microbiol. 2016 Jun 20;1(9):16087. doi: 10.1038/nmicrobiol.2016.87. PMID: 27562253; PMCID: PMC5003325.

The viremia vs ELISA titer is below and description mentioned in lines 281-287, and has been included as Supplementary Figure 3.



Reviewer 2:

This is high profile work performed by a very well-known and authorized group of scientists.

As authors acknowledged the limited circulation of YFV in LA and Asia regions despite the favorable epidemiological and ecological conditions is captivating from a scientific point of view. It is also interesting that previous reports on DENV and other arboviruses interfering with YFV replication in NHPs date back to 1970s.

The main hypothesis behind this work "...that prior flavivirus immunity, especially DENV, plays a major role in preventing urban YFV emergence by limiting the number of competent human amplifying hosts".

While the work instilled a high level of enthusiasm, there are some minor and major observations/suggestions we would like to bring to the authors attention.

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Since ZIKV has been endemic to Asia for many decades²¹, its immunity could also have influenced the risk of YF there.

While this statement above explains itself, its meaning is not clear when compared to the statement in line 86. Are the authors acknowledging and underscoring a potential YF amplification by ZIKV?

Has prior ZIKV immunity allowed or created the immunological basis favoring a YF outbreak?

The statement “...preexisting ZIKV immunity may also have influenced YFV infection dynamics...” is somehow vague as it cannot be defined if the influence is towards amplification or reduction, as it is mentioned next in lines 86-87.

Line 86-87

Our findings, based on a cohort of NHPs with distinct flavivirus immunity profiles, revealed that prior immunity to DENV and ZIKV generally reduces YFV viremia, as well as mosquito infection and subsequent transmission potential.

Response: We thank the reviewer for pointing out this ambiguity and we acknowledge that the statement can be confusing in context of amplification or reduction of YFV. As such, we modified the statement as follows to coincide with lines 86-87. *“The peak of this ZIKV outbreak preceded the 2016 YF epidemic, raising the question of whether preexisting ZIKV immunity may also have influenced YFV infection dynamics by reducing viremia, which could reduce interhuman mosquito transmission as well as YF disease progression.”* (See lines 83-86)

Comment 2:

Lines 95-97

The initial infections were described in a previous study to be published independently; 23 animals were exposed to *Ae. albopictus* mosquitoes sham-infected with PBS or infected with one of the four virus strains: DENV-2 P8-1407 or ZIKV DakAr 41525 (sylvatic strains), DENV-2 NGC or ZIKV PRVABC59 (human 97 endemic/epidemic strains).

The number of animals for each group could be included for better clarity.

Response: Thank you for pointing out this oversight, we have included the n-values for each group in the text. (See lines 101-104)

Comment 3:

Line 116

To investigate further if initial infections from mosquito feeding occurred with FRNT-seronegative animals, we performed ELISA to detect binding but non-neutralizing antibodies. Interestingly, both of the DENV-2 P8-1407-seronegative and the one ZIKV-seronegative animal had low levels of IgG antibodies by ELISA, suggesting that infection did occur despite the lack of homologous neutralizing antibody induction.

Please name here animal FR1565 as it is critically relevant for this work.

Response: We have included the name FR1565 in this section. (See line 130)

Comment 4:

Line 129-130

Most mosquitoes (bodies) that fed on flavivirus-naïve animals had high rates 129 of YFV infection on peak viremia days 3 (Figure 2a) and 4 (Figure 2b) post-infection.

Could authors speculate on why mosquitoes that fed on flavivirus-naïve NV 259 were not infected with YFV despite having detectable viremia on days 3 and 4?

Response: Flavivirus-naïve animal NV259, although it generated detectable viremia, exhibited relatively low ($4.19 \log_{10}$ FFU/mL) levels compared to the other two flavivirus-naïve animals (Extended Data Table 2). Based on the OID_{50} curve (Figure 3), it appears that a viral titer of $5.7 \log_{10}$ FFU/mL in flavivirus-naïve animals is required to infect 50% of mosquitoes and the infection rate drops to negligible levels with viremia below $4 \log_{10}$ FFU/mL. See the following revision on lines 132-136. Since YFV infection in humans and primates present variability in pathogenesis ranging from mild to severe, we expected that our low passage, contemporary YFV strain, not highly adapted to cell culture or animal hosts, would mimicked natural variation in outcomes. This was indeed seen in our data, as one of the naïve animals had a relatively mild infection and low viremia compared to the other two animals.

Comment 5:

Line 140

Upon comparing the mosquito infectivity with NHP viremia for flavivirus-naïve and DENV-2-immune groups, we observed that the infectivity for mosquitoes that fed on a DENV-2-immune animal was 20-30% lower compared to flavivirus-naïve animals with similar YFV viremia levels ($5.3 \log_{10}$ FFU/ml) (Figure 3).

Is this observation related to one animal? ID is not mentioned and in figure 3 refers to dengue immune group, not to one animal. As DENV infection and neutralization is very variable among animals the animal ID is relevant for correlation with the specific results from that animal.

Response: We have modified the sentence to include the NHP ID as well as the specific infection rate and corresponding viremia levels. (See lines 158- 161)

Comment 6:

Lines 141-143 Upon comparing the mosquito infectivity with NHP viremia for flavivirus-naïve and DENV-2- immune groups, we 141 observed that the infectivity for mosquitoes that fed on a DENV-2- immune animal was 20-30% lower compared 142 to flavivirus-naïve animals with similar YFV viremia levels ($5.3 \log_{10}$ FFU/ml) (Figure 3).

Mosquito infectivity % was not analyzed against NHP viremia for ZIKV-immune animals but no reason is mentioned.

Response: We decided to primarily show the analysis for DENV-2 groups since DENV-2 NGC animals that had higher YFV viremia had lower mosquito infection, which was an unexpected outcome and led us to test NS1 binding. To specifically highlight this occurrence with DENV NGC group, we presented the data in an OID_{50} curve. However, with regards to the ZIKV-immune group, all animals had suppressed YFV viremia and an intuitively corresponding lower mosquito infection rate. We did not observe an unusual result with the ZIKV group, therefore did not present the data in the OID_{50} graph. We have summarized this information on lines 167-168.

Comment 7:

Line 274

Twenty-three healthy adolescent cynomolgus macaques (11 males and 12 females, 2.5-5.5 years old, 3-5.5 kg)

Authors mention males and females were used in this study but their distribution among groups is unknown.

Response: We have now included the sex for each animal in “Extended Data Table 1”. (See line 563)

Comment 8:

Line 269-270

No statistical methods were used to predetermine the sample size as the NHPs were originally utilized in a separate experiment in keeping with the ethical goal of reducing the total number of animals utilized in research.

Authors state that statistical power was not established because the animals used belonged to a separate experiment. However, the number of animals in most groups is low given the recommended NHP sample size, and the difference in sample size between the DENV2-P8 group and other groups is remarkable. Perhaps if sample size was more adequate, the significant difference between DENV2-P8 group and the naïve group would be lost, or other statistical differences would be gained. Author comments on this statistical bias are encouraged.

Response: We agree that a larger cohort in all the immune groups might yielded a significant difference. Since the NHPs were primarily exposed as a part of a separate study, we did not contribute to determining the allocation and number of animals in each group.

Comment 9:

Under Ethical statement

As this is terminal experiment a statement on the humanized euthanasia is highly recommended.

Response: We have included a statement that mentions “animals were humanely euthanized”. (See line 335)

Comment 10:

Figures 1b-e, 2a-d and 5a-f could include a header for better clarity.

Response: We thank the reviewer for catching this oversight and have included headers on the figures for increased clarity.

Major Comments:

Comment 11:

Line 98

To ensure that our study was reflective of typical convalescent flavivirus immunity, and not an acute phase response, we allowed the immunity to develop for at least 6-9 months post-primary infection.

A topic limitedly discussed in flavivirus field, particularly for dengue is the optimal time between consecutive infections or vaccine dosing.

For this particular work, considering that the response among animals is very variable (including response to prior exposure to dengue or ZIKV) it is important to highlight the specific timelapse between initial flavi and the subsequent YF exposures.

Also, in order to reinforce the value of this work, the reason why selecting a 6-9 month period between flavivirus infections should be better explained/justified

Response: The primary exposures to DENV and ZIKV was conducted under a separate study (Ref#24). After the animals were transferred to our protocol, we decided on utilizing the 6-9 month time frame to ensure convalescence and to satisfy IACUC limitations on logger surgery. Moreover, we wanted to maintain realistic time period accounting for cost of animal maintenance in containment facilities and available animal resource services. We have added a sentence on lines 318-321 to acknowledge the complications in selection of the most relevant time after primary infection.

Comment 12:

Line 112

However, the ZIKV-seronegative animal (NHP ID: FR1565, exposed to the bite of an infected mosquito but FRNT-negative) had higher and longer viremia compared to the flavivirus-naïve animals (Figure 1e). Surprisingly, unlike any other YFV-infected animal, FR1565 was laterally recumbent and had to be euthanized by day 9 post-challenge. No visible signs of illness were observed in any other of the infected animals.

Line 146

To assess this hypothesis, we tested the ability of NS1 antibodies generated in response to DENV and ZIKV infection to react with the YFV NS1. As shown in Extended Data Fig. 1, these cross reactions, although lower in magnitude than the homologous reactions in ELISA, were significantly above the levels of binding exhibited by naïve or seronegative sera.

As this is an important observation, can the authors elaborate on the differences in the reactivity to YFV NS1 protein between the DENV and ZIKV cohort? It looks significant. Also there are prior reports covering this cross-reactivity.

Response: Although visually it appears like there is a significant difference between the groups, upon statistically analyzing each DENV group against ZIKV groups using two-way ANOVA Tukey's multiple comparisons test, we did not find a significant difference between the groups ($p > 0.05$). It is however important to note that these animals were primarily exposed to varying levels of DENV or ZIKV via mosquito bites during the previous study. This could affect the level of antibodies against homologous as well as heterologous viruses. Therefore, in our opinion it would be inaccurate to compare between groups. Overall, our results show that previous DENV or ZIKV exposure influences YFV infection (viremia) and resulting mosquito infectivity, irrespective of the antibody levels. To specifically test the direct correlation between level of immune response and its effect on course of YFV infection, animal experiments with controlled primary exposure must be executed. We have revised the text on lines 285-287 to clarify our interpretation.

Comment 13:

Line 195

High levels of IL-8 have been reported in convalescent ZIKV patients, suggesting that prior ZIKV immunity could be an important factor in elevating IL-8 levels post-YFV-infection.

Was IL-8 significantly elevated compared to the DENV group? It looks likely to be the case. If so, any comment on why ZIKV but not DENV predisposes to a "proinflammatory status" after YFV?

Response: Upon statistically comparing by one-way ANOVA, we found a significant elevation in the ZIKV Dakar group compared to the DENV-2 P8 group on day 5. On day 14, we found a significant elevation on both ZIKV PR- and ZIKV Dakar-immune groups compared to DENV-2 P8-immune group ($p = 0.007$ and $p = 0.02$ respectively). Although not conclusive, one possible explanation could be the long-term persistence of ZIKV potentially resulting in

persistent immune activation and predisposition to pro-inflammatory state. Since viral persistence is not observed in DENV infection, that could mark the difference between the two groups. We have revised the text on lines 218-220 to clarify our interpretation.

Comment 14:

Line 225

Our finding of cross-binding antibodies by ELISAs from DENV and ZIKV-immune sera suggest a role for NS1 antibodies in restricting mosquito infection to limit human amplification potential. Additional experiments are needed to further test this hypothesis.

Actually, this finding is highly relevant to support the hypothesis behind this work looking precisely for a mechanism restricting YF transmission. DENV and ZIKV anti-NS1 antibodies should be tested, at least in vitro, to reinforce this highly likely mechanism of YFV replication restriction. As per the results showed here, DENV anti-NS1 Abs may interfere more with YF replication than ZIKV-anti-NS1 Abs.

Response: We agree on the relevance of the finding, especially from a mechanistic viewpoint. However, as previously mentioned in Comment 12, we believe a direct comparison between groups would be inaccurate to infer a mechanism since these animals were previously exposed to varying levels of DENV or ZIKV, that may contribute to the level of immune response. Therefore, we believe future studies with controlled primary infections (via an injection rather than mosquito exposure) would directly address mechanistic questions. We have revised the text on lines 256-257 to clarify our interpretation.

Comment 15:

Line 236

The low levels of ZIKV non-neutralizing IgG we detected suggested the possibility of antibody-dependent enhancement, as has been reported in sequential flavivirus infections (mainly DENV). However, with only one animal with a severe outcome and an immunological profile of this kind in our study, we cannot conclude that low level ZIKV immunity exacerbated the YF outcome; future studies are required to investigate this hypothesis.

The potential of ZIKV-induced YF ADE is also highly significant for this study and of high relevance for the flavivirus field. Together with the epidemiological evidence that the major ZIKV 2014-2016 epidemic precedes the 2016 YF epidemic, the finding of ZIKV non-neutralizing IgG in the only animal that died to YF, deserves further attention in order to increase the scientific impact of this work.

While ADE in vitro is not necessarily an accurate measure of what can happen in vivo, by comparing the ADE properties of the binding non-neutralizing antibodies from animal FR1565 vs. other selected animals will shed light on the potential YF ADE by ZIKV and strengthen this manuscript.

Response: We agree that this observation together with epidemiological evidence is highly relevant. We measured neutralizing and non-neutralizing antibodies in the sera from all animals. However, as mentioned above (Comment 14,15), we do not believe we can directly compare the antibody levels between groups since they had varying levels of previous exposure. Therefore, this preliminary observation is interesting and generates an avenue for future elaborate studies that specifically address the ADE hypothesis.

Also, we conducted controlled experiments in murine model as well to address the role of prior DENV-2 and ZIKV immunity on YFV, and observed a possible YF-disease enhancing phenotype in ZIKV-immune mice. Reference below:

Shinde, D.P.; Walker, J.; Reyna, R.A.; Scharton, D.; Mitchell, B.; Dulaney, E.; Bonam, S.R.; Hu, H.; Plante, J.A.; Plante, K.S.; et al. Mechanisms of Flavivirus Cross-Protection against Yellow Fever in a Mouse Model. *Viruses* **2024**, *16*, 836. <https://doi.org/10.3390/v16060836>

We have revised the text on lines 285-287 to clarify our interpretation.

Reviewer 4:

In the present manuscript, Shinde et al. hypothesized that heterologous flavivirus immunity can prevent YFV transmission in urban areas where the main urban vector, Aedes aegypti, is present. To test their hypothesis, they first infected monkeys with DENV and ZIKV to build heterologous flavivirus immunity. Then, after a defined period, they infected the monkeys with YFV and exposed Ae. aegypti mosquitoes to the YFV-viremic monkeys. The results are very interesting and significant. The observation of a significant reduction in mosquito infection is remarkable. The study represents a tremendous amount of work and is very well-written. The new findings in this study align with the mission and impact factor of Nature Communications. In my opinion, this manuscript can be accepted after a minor review. Please see my comments below.

Comment 1:

Title

The title of the manuscript broadly expresses the idea of the work, but I am wondering if it truly reflects the findings of the present study. The authors did not demonstrate the impact of their findings on preventing urban transmission. While I understand the main point, I would like to know the authors' opinion on this issue, and a reflection about the title.

Response: We agree that we did not directly assess the impact on urban YF, and have revised the title to: Yellow Fever Emergence: Potential Role of Heterologous Flavivirus Immunity in Preventing Urban Transmission

Comment 2:

Abstract

The abstract is very clear and concise. It provides enough background, states the main hypothesis, and presents the principal findings, supporting a new hypothesis.

Response: We thank the reviewer for their positive feedback on the abstract.

Comment 3:

Importance

The section is well-written and clearly states the relevance of the study.

Response: We thank the reviewer for the feedback.

Comment 4:

Introduction

The Introduction provides sufficient and relevant background to support the study's context and hypothesis. It is a very objective and well-written section. I have only one comment below:

44-45 – The authors should also comment on the live-attenuated aspect of the vaccine and the controversy

surrounding widespread vaccination, even though it has been proven to be safe. Additionally, the authors should address the lack of a massive vaccination program for YFV prevention in general populations to clarify the naïve immunity profile. This issue was nicely addressed for Brazil later in the introduction.

Response: We thank the reviewer for pointing out this weakness and have included a brief statement in the introduction to address the live-attenuated aspect of the vaccine and lack of vaccination. (See lines 44-48)

Methods

Comment 5:

271-272 – The authors should explain why they designed the study with these parameters: “The experiments were not randomized. The investigators were not blinded to allocation during experiments and outcome assessment.” What are the limitations in the interpretation of the data in this case, if there are any.

Response: We agree with the reviewer that under normal circumstances, the experiments should have been randomized. However, since these NHPs were repurposed from another study (published separately), it was not possible to maintain blinding and randomization. Additionally, the staff collecting the samples were also running the assays, further making it difficult to truly stay blinded. We agree that there may be certain limitations, however, the primary data collected in the experiment were quantitative (viral titers, mosquito infections, serum chemistries, CBCs). Therefore, we believe there was limited opportunity for bias. We now state this limitation on lines 307-308.

Comment 6:

275 -Is there a reference for the previous study? “...were transferred from a previous study...”. Is it the reference 22 that was cited later?

Response: We thank the reviewer for pointing it out. We have now included the reference in this section. And yes, it is the same reference that is cited later (Ref 22).

Comment 7:

275-276 – In “where they were exposed to mosquitoes infected with PBS (3/23), DENV-2 NGC (3/23), DENV-2 P8-1407 (10/23), ZIKV PRVABC59 (4/23), and ZIKV DakAr 41525 (3/23).”:

Responses:

1-Please, fix “Infected with PBS”

1- Thank you, we have amended the statement to “inoculated with PBS” (See lines #311-312)

2-How did the authors decide for DENV-2 instead of other DENV serotypes?

2-Since these animals were primarily exposed to DENV-2 and ZIKV in a previous unrelated study under a different protocol, the DENV serotype and strain were determined in that study previous to the design of this experimental plan. We now explain this on lines 314-315.

3-What was the strain of the mosquitoes? I assume they were *Ae. aegypti* but it is not stated in the sentence above. (This information was in the results section; it was odd to know that it was *Ae. albopictus*).

3-Thank you for noticing this shortfall, we have now included the strain *Ae. albopictus* that were used under the previous study (Reference #24). (See line# 311)

4-How did the authors test for the infection status of the mosquitoes, and monkeys after exposure to DENV and ZIKV?

4-The detailed experiments to test the infection status of the mosquitoes and monkeys after exposure is described in Ref#24, the previous study where the animals were initially exposed. In our study, we measured the resulting neutralizing and binding antibodies against homologous viruses to ensure seroconversion. We now more clearly state this limitation on lines 315-316.

5-Why is the number of the infected monkeys with DENV-2 P8-1407 so discrepant from the other ones?

5-The previous study (Ref#24) utilized a larger cohort for the DENV-2 P8 group that was further divided into two groups of “low-dose” and “high-dose”, where animals were exposed to variable number of mosquitoes. Since these two groups were only utilized for DENV-2 P8 infections, they required a larger cohort for it, and not for all other groups.

6-What is the susceptibility to YFV of the mosquitoes used in the infection assay? Even though the experiments give us a clue, it would be nice to have it established before the experiments.

6- Unfortunately due to limitations on the timeline for this study and our desire to use Brazilian mosquitoes, we were not able to do this type of pilot study.

Comment 8:

277- How did the authors decide for a dose of 1×10^4 FFU and 0.5 mL of the inoculum?

Response: Thank you for this question, we decided the dose to maximize infection and viremia to ensure mosquito infection and we referred to previous studies in NHPs (rhesus macaques) mentioned below. We have also included a statement in the manuscript. See line 317-318.

Engelmann F, Josset L, Girke T, Park B, Barron A, Dewane J, Hammarlund E, Lewis A, Axthelm MK, Slifka MK, Messaoudi I. Pathophysiologic and transcriptomic analyses of viscerotropic yellow fever in a rhesus macaque model. PLoS Negl Trop Dis. 2014 Nov 20;8(11):e3295. doi: 10.1371/journal.pntd.0003295. PMID: 25412185; PMCID: PMC4238990.

Comment 9:

279- In “Temperatures were recorded at 15-minute intervals on loggers (Star Oddi, Iceland) placed in the abdominal cavity.”, for how long?

Response: We thank the reviewer for pointing this out and we have included the statement “throughout the course of the study” (See lines# 322)

Comment 10:

281-commercial chow (manufacturer?)

Response: We have included the manufacturer details in the text. See line# 324

Comment 11:

289- “A cardboard carton with a mesh top containing 100 female *Ae. aegypti* (mosquitoes from Salvador, Brazil, maintained for 18 generations at the UTMB Insectary) were allowed to feed on NHP ears for 5-15 minutes”:

1-Is there a reference for this strain (Salvador, Brazil)?

Response 1- We do not have a reference for the strain since it was acquired from our collaborators in Brazil prior to any other experiments being published, and maintained as a unique colony here at the University of Texas Medical Branch insectary.

2-What is the susceptibility level of this strain for the viruses?

Response 2- Since we used a recent contemporary strain of YFV, this is the first time that mosquitoes were exposed to this strain under experimental conditions, therefore we did not know the susceptibility of mosquitoes to YFV BR. However, through our studies with flavivirus-naïve animal, we were able to measure mosquito infection of various groups with varying levels of viremia. We showed that at viremia levels of 5.7 log₁₀ FFU/mL are required to infect 50% of the mosquitoes.

3-Why did the authors decide for 5-15 minutes of feeding? Was it enough time for the females to be fully engorged. How was this aspect verified?

Response 3-First, we decided to optimize the time upon discussing with other scientists that have used the same species of animal (*cynomolgus* macaques) and the *A. aegypti* mosquitoes in previous studies. We also visually confirmed high rates of feeding during every individual feeding. As the size of the animals, and various other features cause small variations in rates of engorgement.

4-What is the age range of the female mosquitoes that were used in the infection assays?

Response 4: Thank you for this question, the female mosquitoes used were 2-4 days post-eclosion. We have clarified this in the text (Line 333)

Comment 12:

Could the authors describe the containment protocol for the monkeys? This is not detailed in the methods.

Response: We have included a sentence that mentions that animals were individually housed at UTMB ABSL3 primate facility. See lines #323

Comment 13:

317-318- “Fully engorged mosquitoes were maintained at 28°C with a 16-hour light/8-hour dark cycle with ad libitum access to 10% sucrose.” Please, describe how the engorged ones were separated from the non-engorged ones.

Response:We thank the reviewer for pointing out this unclear statement, a cardboard carton containing the mosquitoes was placed in the refrigerator until the mosquitoes were fully anesthetized. The carton was transferred to a glove box and placed on ice. The mosquitoes were then placed in a petri dish and visually engorged (bloated red abdomen) mosquitoes were transferred to a fresh petri dish on ice and counted. The

non-engorged mosquitoes were eliminated from the study by dropping them in 70% ethanol. We now state this information on lines 372-377.

Results

Comment 14:

92-93- “Dengue virus type-2 (DENV-2) and ZIKV-immune non-human primates (NHPs) have modulated YFV viremia.” should not be “Dengue virus type-2- (DENV-2) and ZIKV-immune non-human primates (NHPs) have modulated YFV viremia?”

Response: Thank you for highlighting this error, we have modified the text. See lines# 98

Comment 15:

95-96- Why did the authors use *Ae. albopictus* instead of *Ae. aegypti* mosquitoes for DENV- and ZIKV-infections? This was not addressed in the methods and the species was not cited in that section as well. Is there an explanation for that?

Response: Thank you for noting this. The use of *Ae. albopictus* was only for a separate study for which we had no role in the design (Ref#24).

Comment 16:

109-110-“ DENV-2- and ZIKV-FRNT-positive animals” please, be more specific on what positive is about.

Response: Thank you for pointing this out. We have modified it to be clearer in the manuscript. (See lines 114-115). We replaced FRNT-positive with animals had detectable neutralizing antibodies when measured by FRNTs.

Comment 17:

112- “However, the ZIKV-seronegative animal (NHP ID: FR1565, exposed to the bite of an infected mosquito but 113 FRNT-negative) had higher and longer viremia compared to the flavivirus-naïve animals (Figure 1e).” Why did the authors state this result without statistical significance on Figure 1e? Did I miss anything in here? I am sorry if I did.

Response: We could not statistically analyze the result since there was only one animal in the ZIKV-seronegative group. Nevertheless, the result was important to note due to the peculiarity of the case wherein the ZIKV-FRNT-negative animal that had low levels of binding antibody in fact experienced a more severe form of the YF disease which suggestive of an enhancement due to low level of previous immunity.

Comment 18:

Figure 1

Figures 1d and 1e:

1-Could the authors offset the y axis from x axis? The overlapped data on the y axis makes it hard to read the figures and significance (asterisks).

- 2-The x legend should be labeled as “Days post YFV exposure” instead of just “Days Post-infection”.
- 3-Figure 1d should have a headline for DENV-2-exposed, and Figure 1e for ZIKV-exposed”.

Response: We have modified Figure 1 to incorporate all the suggestions offered by the reviewer.

We are grateful for the insightful comments from the reviewers and thank them for their time and expertise. We have included our responses to the reviewer.

Reviewer #1 (Remarks to the Author):

The manuscript is improved and the authors have addressed reviewer comments.

The difficulty with the paper overall is that it is based on very limited data given the constraints of the sample size in the experimental groups, there is a substantial and unexplained difference in the effect size on viremia suppression between two strains of the same dengue serotype 2 (NGC and P81407), and a combined analysis of DEN2+ZIK immune animals did not show a statistical result. Since this paper will be cited many times in the future as evidence for heterologous protection by DEN and ZIK immunity, this reviewer would like to see a cautionary conclusion in the Abstract regarding the limitations of the study.

Response: We thank the reviewer for their comments and understand that the information must be carefully presented in the manuscript. We have now included a cautionary statement in the abstract that mentions the low sample size and no statistical difference.

Figure 1. It is not clear what is displayed in panel (d). Are these geometric means $\pm$ 95% CIs?

Response: We have now included in the figure legend that the data are presented as means $\pm$ SD.

Figure 3: Are all animals included in the analysis displayed? Extended Table 2 shows some animals with higher viremia levels.

Response: We have included all DENV-2 immune animals in the figure