

Immunological and transcriptomic profile of chimeric live-attenuated Zika vaccine linked to protection in non-human primates

Corresponding Author: Professor Kai Dallmeier

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)

This manuscript reports continued studies by this group on their candidate Zika vaccine based on a chimera with a yellow fever (YF) 17D vaccine backbone with YF sequences swapped for Zika sequences (YF-ZIK). The mouse data has been published and looks promising, and in this manuscript they continue their studies by investigating the vaccine in non-human primates (NHPs) with incorporation of system vaccinology.

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Lines 146-160 describe the passive protection data where sera for immunized NHPs is administered to AG129 mice and then challenged with wild-type ZIKV. The authors claim the results are impressive, but the titer of neutralizing antibodies administered was over 1 in 1000 yet only 7/9 mice were protected. I would argue this result is not as good as what other groups have reported previously with lower doses of neutralizing antibodies. This raises the question is the mCherry neutralization assay used by the authors too sensitive. Have the authors compared their neutralization assay vs a classical plaque reduction neutralization assay? Also, since the data reported is 7/9 mice, has the experiment been repeated?

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The systems vaccinology is interesting, but would not one expect there to be extensive similarity with 17D vaccine as this is the backbone of the Zika vaccine. Would not a focus on interpreting differences between the Zika vaccine and 17D be important in terms of interpretation. The animal numbers are small. Are there sufficient numbers for statistical analysis?

Lines 83-84 and 437-438: The authors need to expand their text on clinical trials to say that none have undergone phase III clinical trials as the low incidence of Zika disease means vaccine efficacy trials cannot be undertaken.

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Line 232: Is the limit of detection 10e4 copies of vRNA?

Lines 235-236: What does "very limited, if any, increase" mean? Either there is an increase or there is not.

Line 356: Why "remarkably"? Was the NHP study repeated to confirm the data?

Reviewer #2

(Remarks to the Author)

1. Please provide the reason for only including a subset of YF-ZIK vaccinated macaques (n=3) in the ICS assay. Why weren't all six vaccinated animals included?
2. Along these lines, I am not sure the T cell responses can be described as robust as indicated in the Discussion. None of the T cell responses are significantly different from the sham controls (although this may be a result of low animal numbers). Moreover, the CD8+ T cell responses are not consistent for the three animals tested, and one animal appears to be a non-responder.
3. Why is the data for Figure 3 not discussed until late in the manuscript? It may be beneficial to the reader to move the figure so that it follows Figure 6 and matches the text.
4. In Figure 6B, please explain the rationale for comparing D30 YF-ZIK to D2 YF17D. Do you consider the priming YF17D vaccination to be functionally equivalent to the boosting YF-ZIK vaccination? Is this due to species differences or artifacts of the respective vaccines?
5. In Figure 3E, IFN signaling increases at D2 following prime and boosting vaccinations, yet TLR signaling decreases after the priming vaccination. Is the IFN increase due to RIG-I or some other cytosolic RNA sensor?
6. In line 422, I believe a word is missing after serially. Perhaps collected?

Reviewer #3

(Remarks to the Author)

In this manuscript, the authors extend their investigation to test the chimeric vaccine YF-ZIKV in macaques. While the clinical evidence supports that the yellow fever YF17D is safe and efficacious, it remains unclear whether YF-ZIKV behaves in the same way.

Based on their data, I am not convinced that the YF-ZIKV stimulates a polyfunctional response, especially the cell-mediated responses. Also, based on current literature, most of the T cell epitopes are present in the NS genes, which means that it is very unlikely the YF-ZIKV will stimulate T cell responses as the NS genes belong to YF. Because of this, the data does not support their claims.

In addition, there are several other concerns that should be addressed:

1. The authors did not show the viremia of YF-ZIKV in the animals. This is important because YF17D is known to induce detectable viremia, and it is this viremia that drives vaccine immunogenicity. Moreover, knowing the peak viremia will help in interpreting the transcriptomics data better.
2. Figure 2D. The authors claim that they detected T cell responses. However, the data clearly shows that the T cell responses to ZIKV E peptides are not significantly increased. Thus, the data indicated that the mechanism of protection is primarily antibodies and not T cells. If this is indeed the case, then the authors should not claim that the vaccine can induce polyfunctional responses.
3. Figure 3B and 3D: It is unclear how the heatmaps are generated. Based on the volcano plot, it is clear that the host responses are resolved at day 7. Yet, the colours indicated that the pathways are in red. There are also no mention of the methods employed to generate the heatmaps.
4. Figure 3E: What is the pathway score based on? Is this based on a list of genes involved in the pathways or only DEGs?
5. Figure 3: There is a lot of mention about DEGs, but the authors did not indicate the fold-change and p-value thresholds to indicate DEGs.
6. Figure 3: Various critical genes involved in IFN/TLR/B cell related genes were mentioned but the authors did not plot the kinetics of these key genes to show their dynamics and extent of change.
7. Figure 6: Public data used are either RNAseq or scRNAseq data. However, the nCounter was used for the transcriptomic analysis. How did the authors process the data so that nCounter and RNAseq data can be compared? Were the correlations based on only the DEGs?
8. Since the primary mechanism for efficacy is likely neutralising antibodies, then the authors need to acknowledge the limitations, and highlight the potential risk of antibody-dependent enhancement.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The authors have provided a very comprehensive rebuttal to the reviewers' comments and this greatly improves the manuscript. There is one aspect that I do not agree with the authors, and I would suggest should be included in the limitations, namely the extensive comparisons with a "human dose of YF/17D vaccine". The dose of the candidate Zika vaccine was 10^5 pfu whereas 17D vaccine is usually administered at a lower dose to vaccinees for all but one producer. The authors are encouraged to read the 17D vaccine dose-sparing papers for information that include the quantity of 17D virus in a dose and parameters of induction of antibodies post-vaccination, which do show some differences depending on the dose of vaccine and particular producer. Indeed, the Brazilian 17DD substrain has been compared at many different doses in volunteers. Thus, I do not believe the authors can directly compare the human 17D dose to the dose of candidate Zika vaccine as different quantities of virus are being compared. The authors may also wish to look how much virus is in a dose of Imojev.

The English language needs to be carefully reviewed.

The term "flavivirus" should be updated to "orthoflavivirus"

Line 86: I would suggest that Zika virus is not found "globally", and this statement should be tempered.

Reviewer #3

(Remarks to the Author)

The revised version of the manuscript provides a more balanced view of the results and findings. I have no further comments as my previous comments were adequately addressed.

Editorial note: due to the absence of reviewer 2, this reviewer was additionally asked to comment on the authors response to the prior comments of reviewer 2: Yes, I think the reviewer's comments were adequately addressed.

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RESPONSE TO REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

This manuscript reports continued studies by this group on their candidate Zika vaccine based on a chimera with a yellow fever (YF) 17D vaccine backbone with YF sequences swapped for Zika sequences (YF-ZIK). The mouse data has been published and looks promising, and in this manuscript they continue their studies by investigating the vaccine in non-human primates (NHPs) with incorporation of system vaccinology.

1. The manuscript is interesting but views everything very positively with the vaccine candidate while this reviewer believes that, while the results are very interesting, the conclusions and interpretation need to be toned down.

R1/ We appreciate this overall supportive assessment of our study and agree that some conclusions drawn have been too strong. In the revised version, interpretations and wording have been toned down accordingly, as will become obvious from our point-by-point responses.

For convenience of assessment, we add a marked-up version of our updated manuscript with highlights in two font colors. Rearrangements comprising entire sections of old text (i.e., without major edits) are highlighted in **LIGHT BLUE**. Additions, corrections and major changes are highlighted in **RED**.

2. The introduction is weak on previous work on correlates of protection. Some is mentioned in the results and discussion, but a summary of the state-of-the-art of protection and neutralizing antibody titres in mouse and NHP models for Zika vaccine candidates would help readers understand where the data included in this paper sits with respect to other candidates. It is suggested this section includes text on different assays used by different groups and the limitations with making comparisons.

R2/ As suggested, the widely established concept of seroprotection has now been added to the *Introduction*, citing seminal ZIKV studies in mice and NHP (new lines 95-115), yet also emphasizing that cell mediated immunity (CMI) to ZIKV is not irrelevant yet simply highly understudied in NHP (lines 116-123). This (carefully critical) review of published evidence for the – as we still think – often overemphasized role of NABs for protective immunity is complemented by a more in-depth comparison of our data with those described in these seminal studies in the *Discussion* (lines 476-486).

Importantly, despite high NAB serum titres may be sufficient, growing evidence, including from controlled human challenge trials (Kalimuddin et al. Nat Microbiol 2025

PMID: 39794472), suggests that virus neutralization may be a redundant, partially dispensable mechanism for protection. Besides humoral responses, active immunization induces innate and CMI responses. Hence, NAb titres should be considered rather as diagnostic biomarker for immunization (i.e., formal *Correlate of Protection*, CoP) rather than the mechanism of protection [See also discussion in Barrett JID 2020 PMID: 31545366; added as new REF# 8].

For discussion of the AG129 model for passive serum transfer studies, please see our response R4/ to Comment 4.

3. The manuscript uses log₁₀ geometric neutralization titres. These are very difficult to interpret when a log scale is used. It is suggested arithmetic/real numbers are used with error bars and/or actual titres shown as, for example, Fig 2a has only lines. It is not possible to evaluate differences between animals.

R3/ In contrast to laboratory mice, NHPs are outbred and exhibit significantly higher variability. We therefore decided to treat each animal individually, showing their seroconversion kinetics and NAb trajectories as individual lines. Log₁₀ transformed neutralization titres (Supplementary Table 2) were used to visualize the relatively high or very high values observed in our study, especially in comparison to other reports (see also our R4b/ to Comment 4). Nevertheless, all original values are presented in the now provided Source Data file.

4. Lines 146-160 describe the passive protection data where sera for immunized NHPs is administered to AG129 mice and then challenged with wild-type ZIKV. The authors claim the results are impressive, but the titre of neutralizing antibodies administered was over 1 in 1000 yet only 7/9 mice were protected. I would argue this result is not as good as what other groups have reported previously with lower doses of neutralizing antibodies. This raises the question is the mCherry neutralization assay used by the authors too sensitive. Have the authors compared their neutralization assay vs a classical plaque reduction neutralization assay? Also, since the data reported is 7/9 mice, has the experiment been repeated?

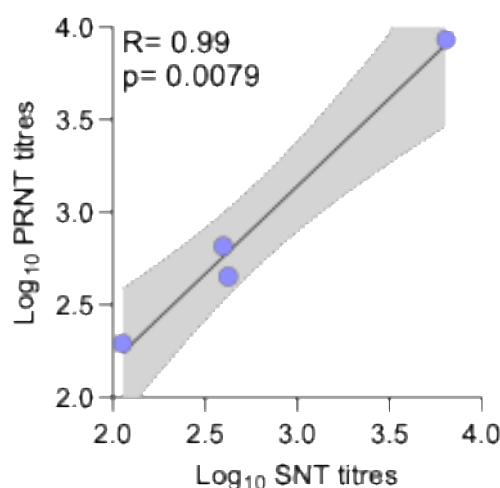
R4a/ Regarding choice of AG129 mouse model: Different mouse models have been used for ZIKV serum transfer experiments, including BALB/c and AG129 mice. Earlier seminal studies focused on the use of immunocompetent BALB/c mice: Abbink et al. (PMID: 27492477; REF#9) transferred purified IgG from immunized monkeys into BALB/c mice and challenged them with the very low dose of 10² PFU ZIKV. Under these conditions, 50% of mice were protected against RNAemia by a serum NAb titre of 1:100 or higher. Similar values have been reported by Larocca et al. (PMID: 27355570; REF#13) using purified IgG from immunized BALB/c mice.

However, immunocompetent BALB/c mice are poorly susceptible, requiring the use of a mouse-adapted ZIKV strain to induce disease (PMID: 28148798), while clinical isolates of ZIKV do not cause any disease in this semi-permissive wild-type mouse model. In contrast, IFN- α/β and γ receptor-deficient (AG129) mice are highly sensitive to lethal infection. See also Supplementary Fig. 3d, e, where we confirm this extraordinary sensitivity with virtually any dose of ZIKV (down to one infectious unit, 1 PFU) uniformly causing lethal infection.

To increase assay stringency for our serum adoptive transfer experiment, we hence chose AG129 mice, alike Maciejewski et al. 2020 (PMID: 32522807; REF# 12 cited as one of the seminal studies). Notably, our non-purified NHP serum pool conferred survival in 7/9 AG129 mice (78% protection) and was, with this regard, more potent than purified IgG enriched for highly functional NAb that provided only a 50% survival benefit in the study by Maciejewski et al. 2020. Small additions have been added throughout the main text (lines 95-115) to emphasize these peculiarities of the AG129 mouse model, plus an extra paragraph in the new *Limitations* section (lines 558-588).

R4b/ Regarding assay proficiency: For ZIKV NAb quantification, we used an in-house developed fluorescence-based seroneutralization assay (SNT_{Fluo}) that employs a mCherry-tagged fully infectious ZIKV reporter virus; details are described in the M&M (lines 642-651). Our SNT_{Fluo} approach for ZIKV is based on a methodology originally developed by us for the determination of YFV-specific NAb. This methodology was intensively cross-validated regarding reliability and accuracy, benchmarking against (i) classical PRNT assay results as well as (ii) data generated in two reference laboratories (Rasulova et al. Microbiol Spectrum 2022 PMID: 35670599; # 76).

For further validation of the respective ZIKV NAb assay, we now compared our SNT assay with standard PRNT by using the same serum samples from YF-ZIK immunized macaques in parallel in both assays (new Supplementary Fig. 3c).



Hereby, for both the SNT_{Fluo} and PRNT assays, each data point represents three technical replicates. Supplementary Fig. 3c shows a strong correlation between our SNT_{Fluo} and PRNT (R=0.99, p-value=0.0079). Importantly, our method of NAb quantification does not overestimate, but rather slightly underestimates, NAb titres compared to PRNT titres as further discussed in the new *Limitations* section (lines 558-588).

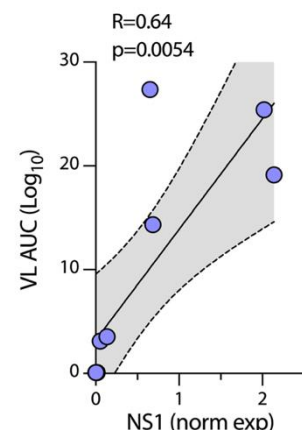
R4c/ Regarding reproducibility: Technically, the results are from two independent serum transfer experiments in AG129 mice. This has now been specified in the M&M section in lines 697-699.

In our serum transfer experiment, we used pooled serum from YF-ZIK-immunized NHPs with a mean reciprocal neutralizing titre of 5492, (GMT of 3.7), which is comparable to or not higher than titres used in similar studies. Additionally, we used a uniformly lethal challenge dose of ZIKV MR766 (i.e., 10 PFU possibly representing a 10x LD₁₀₀) which causes 100% mortality and severe neurotropic disease (including hind limb paralysis, hunch posture, ruffled fur and weakness) in AG129 mice (see response R4a). Despite the stringency of this model, 7 out of 9 mice were fully protected and survived such a harsh challenge without any signs of disease. By using the stringent AG129 mouse model, our data suggest that both the quantity and quality of NAbs induced by YF-ZIK might alone be sufficient to protect against Zika virus infection.

5. The investigation of anti-NS1 antibodies is very interesting but appears to be only binding antibodies. Were any functions of these antibodies, such as complement, investigated?

R5/ NS1-specific antibody has been used as a sensitive biomarker for early diagnosis of flavivirus infections. In our correlation analysis, the level of ZIKV NS1-specific antibody in the serum also strongly correlates with the cumulative viral loads after ZIKV challenge (see inserted figure).

However, NS1-specific antibodies have shown elusive roles in flaviviruses infection, from eliciting a protective immune response in infected hosts to being pathogenic in different studies. The function of anti-NS1 antibodies indeed deserves more investigation in the future. However, we trust exploring this is beyond the scope of the current study and manuscript (see *Limitations*, line 559-588).



6. The systems vaccinology is interesting, but would not one expect there to be extensive similarity with 17D vaccine as this is the backbone of the Zika vaccine. Would not a focus on interpreting differences between the Zika vaccine and 17D be important in terms of interpretation. The animal numbers are small. Are there sufficient numbers for statistical analysis?

R6/ Based on our previous extensive studies on YF17D-vectored vaccine candidates, including those against ZIKV, SARS-CoV-2, Ebola and studies from other groups, there are indeed strong similarities between YF17D-based vaccines and the YF17D vaccine (see our review by Sanchez-Felipe et al. EJI 2024; REF#37). It is generally assumed that YF17D derivatives inherit favourable features from the YF17D legacy

vaccine vector. Our first objective was to test this hypothesis. We are delighted to read that for the reviewer we are “*carrying coals to Newcastle*”.

Though seemingly trivial, to the best of our knowledge, this *a priori* assumption has never been shown for any YF17D derivative in a step-up NHP model by systems approaches. While most published data have been generated in mice, our study might be the first complete transcriptional analysis using a NHP model to understand the (i) early (after prime, day 2 and 7), (ii) late (after boost, day 30 and 35) and (iii) post-infection (day 57 and 60) immunity of such a representative YF17D-vectored vaccine. These data could provide a more complete picture of how live-attenuated vaccines stimulate innate and adaptive immunity and shape the response to infection.

In our NHP study, we found that YF-ZIK induced, alike YF17D in humans, early and strong antibody responses, (evidence for) a balanced T cell response, and activation of key immune pathways, such as IFNs, to mount prime immunity. Such a live-attenuated vaccine that recapitulates key features of YF17D will more likely confer long-lasting immunity. In addition, our systems analysis indicates that some transcriptional markers may serve as common predictors for both YF-ZIK and YF17D vaccine immunogenicity and efficacy, e.g. B-cell receptor *TNFRSF17* as a predictor for strong antibody responses. These transcriptional patterns identified in NHP might translate into relevant biomarkers and deserve further validation in future clinical trials.

We also agree with the reviewer that differences at the molecular level between the two vaccines (secondary objective) might be equally relevant as the thus identified commonalities. We hence now also report some novel findings listed briefly below as (a)-(e). *Limitations* linked to these datasets are discussed in lines 562-569:

- a) In general, YF-ZIK induced an earlier peak of DEG (at day 2) than reported for YF17D immunization in humans, which peaks around day 7 (PMID:19047440; PMID: 36316475); in lines 184-186.
- b) Besides obvious commonalities, the comparison of YF-ZIK and YF17D also unveiled unique DEG sets (Supplementary Table 3). At day 2, YF-ZIK exclusively upregulated 26 genes that were not regulated by YF17D at any time point. These included several IFN-stimulated genes such as *SIGLEC1*, *IRF5*, *TMEM173* (the gene encoding IFN activator STING), *HLA-DRA*, and *HLA-DQA1*. Likewise, YF17D exclusively upregulated 45 genes, including several IFN-stimulated genes. Of interest, these genes were also upregulated by infection with unrelated viral pathogens such as SARS-CoV-2 in rhesus macaques and humans, as well as in ferrets, hamsters and mice. It is hence tempting to speculate whether the favorable safety profile of YF-ZIK (Supplementary Fig. 1), observed before as reduced neurovirulence in suckling mice and survival of AG129 mice as compared to parental YF17D (REF #6, #18, #36), might be linked to the absence of any these

transcripts. The 16 genes exclusively downregulated by YF17D were not significantly enriched in any particular pathway.

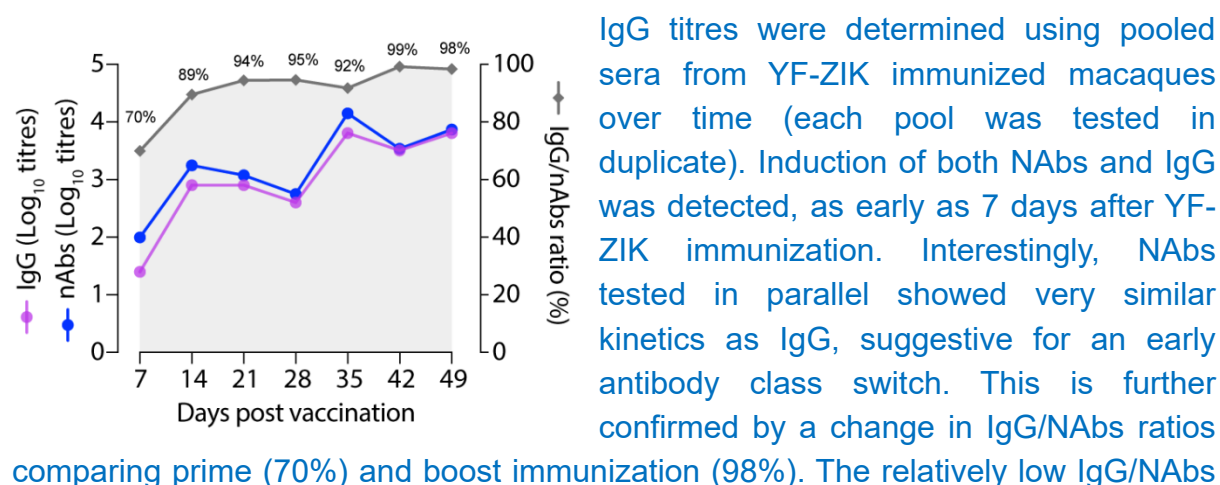
For four genes both vaccines showed an opposite behavior. *SOCS1* (Suppressor Of Cytokine Signaling 1, an inhibitor of type I and II IFN signaling) and *TICAM1* (TIR Domain Containing Adaptor Molecule 1, a TLR signaling adapter protein; highlighted in Fig. 4b), and anti-apoptotic *BLC2L1* (Bcl-2-Like Protein 1) were upregulated by YF17D and downregulated by YF-ZIK, while *PTPRC* (encoding pan-leukocyte marker CD45) was downregulated by YF17D and upregulated by YF-ZIK. An elevated *CD45* and diminished *BLC2L1* expression 2 days after YF-ZIK vaccination are in agreement with a simultaneous increase in monocytes/macrophages and a decrease in apoptosis (Fig. 2; Supplementary Fig. 2), a feature not shared with YF17D (PMID: 19029902, PMID: 36316475); lines 320-329.

- c) Several new transcriptional biomarkers defining infection outcome were identified; lines 550-556.
7. Lines 83-84 and 437-438: The authors need to expand their text on clinical trials to say that none have undergone phase III clinical trials as the low incidence of Zika disease means vaccine efficacy trials cannot be undertaken.

R7/ Added to *Introduction* as suggested (lines 84-91).

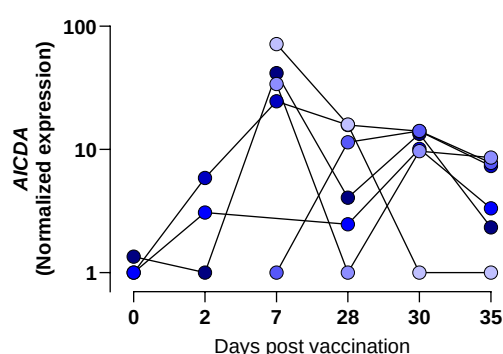
8. Line 187: the early production of neutralizing antibodies is interesting. Is this IgM as with 17D vaccine or another isotype?

R8/ Unfortunately, detection of ZIKV-specific IgM failed, partially due to a high assay background when using the available macaque-specific reagents (see *Limitations*, lines 562-566). In addition, rapid induction of high(er) affinity IgG may have overshadowed low-affinity IgM. Data for IgG detection has now been added as new Fig. 3b and lines 252-258.



ratio early during immunization can be interpreted as a higher prevalence of non-IgG NAb, i.e. likely virus-neutralizing IgM. Accordingly, later during the course of immunization NAb get dominated by IgG.

Furthermore, our transcriptomic analyses underscore early class switching as soon as day 7. *AICDA* (Activation Induced Cytidine Deaminase), a gene specifically expressed and active in germinal center-like B cells, is involved in somatic hypermutation, gene conversion, and Ig class-switch recombination. We found that *AICDA* RNA levels were almost undetectable before vaccination (as expected), peaked at d7 after vaccination and slowly declined thereafter, as shown in the new Fig. 3c.



9. Line 232: Is the limit of detection 10e4 copies of vRNA?

R9/ No, LLOD is $<10^2$ /mL (Fig. 5a). The value originally mentioned here ($< 10^4$ /mL) is clearly above LLOD. We thought that it would be useful to mention that there was detectable vRNA in at least one YF-ZIK-immunized macaque (R13010) at least at one time point. This “break-through” viremia was thus (i) delayed, very short-lived and lower than the average viremia at any other time points (1-5 dpi; range 3.96 to 5.72 $\log_{10}$ Supplementary Fig. 5a in the sham group. To avoid any confusion, the mention of the value has been removed. It can still be found in the Source Data table.

10. Lines 235-236: What does “very limited, if any, increase” mean? Either there is an increase or there is not.

R10/ We removed ‘if any’.

11. Line 356: Why “remarkably”? Was the NHP study repeated to confirm the data?

R11/ We removed ‘remarkably’. Due to ethical considerations and unaffordable costs, the NHP study cannot be repeated.

Seminal ZIKV vaccine studies have used groups of n=4-5 inbred mice (PMID: 27492477; PMID: 27355570) or NHPs (PMID: 32522807; PMID: 27492477) without

repeats; in contrast to n=6 NHPs in our study. We leveraged on the to-be-expected variability in NHPs regarding the different readouts for immunogenicity (innate, humoral, CMI and transcriptional) to derive correlations beyond binary Y/N responses (Fig. 7 and Supplementary Fig. 7). Nevertheless, despite any possible variation, uniform global protection has been observed in YF-ZIK immunized animals (Fig. 5).

Reviewer #2 (Remarks to the Author):

Note: For convenience of assessment, we add a marked-up version of our updated manuscript with highlights in two font colors. Rearrangements comprising entire sections of old text (i.e., without major edits) are highlighted in **LIGHT BLUE**. Additions, corrections and major changes are highlighted in **RED**.

1. Please provide the reason for only including a subset of YF-ZIK vaccinated macaques (n=3) in the ICS assay. Why weren't all six vaccinated animals included?

R1/ Three PBMC samples were of very low quality due to issues during the freezing procedure. These samples had low viable cell counts, did not meet quality criteria, and were therefore excluded from the flow cytometry analysis. This shortcoming is now explained in the M&M (lines 675-677) and new *Limitations* (lines 562-569) section.

2. Along these lines, I am not sure the T cell responses can be described as robust as indicated in the Discussion. None of the T cell responses are significantly different from the sham controls (although this may be a result of low animal numbers). Moreover, the CD8+ T cell responses are not consistent for the three animals tested, and one animal appears to be a non-responder.

R2/ We agree with the reviewer that the limited number of animals in our experimental groups (n=3; see R1) does not allow us to infer conclusions about the 'robustness' of the cellular immune response. Furthermore, to compensate for poor preservation of APCs in the cryopreserved PBMCs, stimulation of antigen-specific recall responses required massive co-stimulation (using a combination of IL-2, anti-CD28 and anti-CD49d for 72h; see M&M lines 654-659). This led to an overall high background in experimental controls, both (i) PBMC from sham-vaccinated individuals and (ii) PBMC from both groups without peptide stimulation; further reducing the dynamic range and increasing the experimental noise of the assay. As a consequence (small n; generally high background; reduced dynamic range), no formal statistical significance (using two-sided test) could be established as also indicated in Supplementary Fig. 7d. These shortcomings in CMI analysis are now further discussed in the new *Limitations* section (lines 562-569). The attribute 'robust' has been eliminated to tone our claims down accordingly.

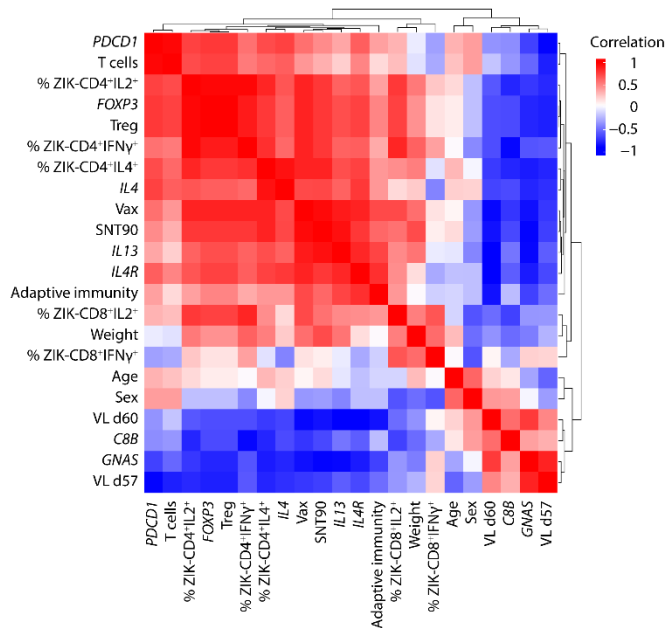
Nonetheless, the reviewer may appreciate that ZIKV E-specific CD8+ T cells, as well as CD4+ T cells with both Th1 (IFN- γ) and Th2 (IL4) polarisations, can be clearly detected. Lack of established benchmarks makes it difficult to assess the biological relevance of our findings. Studies reporting ZIKV E-specific T cell responses in NHPs are scarce. Abbink et al. (Science 2016 PMID: 27492477; STM 2017 PMID: 29237759) report T cell responses by IFN- γ ELISPOT in rhesus monkeys after a multi-dose

immunization comparing a range of different ZIKV prM/E-containing vaccine candidates. Generally, immunization led to the production of no more than 100 ZIKV E-specific SFU per 10^6 PBMCs (0.01%) as assessed by IFN- γ ELISpot. These seminal studies are now explicitly mentioned in the extended *Introduction* (lines 116-123). Polyfunctionality (CD4+/CD8+; IL-2/IFN- γ /IL-4) has not been assessed in macaques before.

By contrast, we provide evidence for such a diversified CMI response, at least for a selection of YF-ZIK vaccinated macaques (see R1). In our case, ZIKV E-specific T cells, as assessed by ICS reach a 0.5-2 % range (max. 4%) in all tested subgroups (Supplementary Fig. 7d), which may suggest a stronger CMI response, though methodologies are difficult to compare quantitatively.

In support of our ICS data, in our transcriptional analysis, we have observed a robust activation of T cell-related immune genes and pathways, including:

- 1) T cell activation (*MR1*, *CD4*, *KLRB1*, *PSMB8*, *CD74*, *TAP2*) (Fig. 2a, c).
- 2) Leveraging publicly available single-cell RNAseq data (Tabula Sapiens), we found that YF-ZIK and YF17D induced comparable gene expression across all major blood leukocyte populations (dendritic cells, macrophages, neutrophils; naive and memory B cells, plasmablasts, plasma cells, and NK cells), including CD4⁺ T, and CD8⁺ T cells (Fig. 4a; Supplementary Fig. 4b).
- 3) As shown in the new Fig. 7f, we found that ZIKV E-specific cellular responses (quantified by flow cytometry after stimulation with ZIKV E protein peptide pool) are significantly correlated to corresponding transcriptomic signatures: the level of ZIKV-stimulated CD4+IL4⁺ T-cells is significantly correlated with *IL4* RNA level measured by nCounter ($p=0.0067$, $R=0.89$, Spearman), while ZIKV-stimulated CD4⁺ IL2⁺ T-cells were significantly correlated with *FOXP3* RNA ($p=0.0028$, $R=0.96$), a proxy for Treg cell levels. Of note, RNA levels of *IL13* and *IL4R* were positively correlated with neutralizing antibody levels ($p=0.0048$ and $p=0.057$, respectively) and negatively correlated with viral load ($p=0.013$ and $p=0.0095$, respectively), underscoring a protective effect of YF-ZIKV-induced Th2 signaling. From a mathematical point of view, we have more power for these non-parametric (Spearman) correlations using a linear (rank-based) model with $n=7$, in contrast to the lack of power in a non-parametric Mann-Whitney analysis as shown in Supplementary Fig. 7d (which can only reach significance with $n=12$, i.e. with 6 animals in each experimental group).



In summary these data confirm that YF-ZIK immunization does induce measurable T cell responses in NHPs and that T cell responses are significantly correlated to both antibody production (SNT90) and protection from ZIKV challenge (virus load at peak of viremia, VL d60).

3. Why is the data for Figure 3 not discussed until late in the manuscript? It may be beneficial to the reader to move the figure so that it follows Figure 6 and matches the text.

R3/ As suggested we reorganized the order in which data are presented. In the updated version of the manuscript, data sets are lined up according to a less bumpy storyline that now comprises three major parts: A-mounting of polyfunctional immunity (i-iii), B-infection and protection (iv-vi), and C-prediction of biomarkers/correlation analysis; each aspect organized in series of main Figures and accompanying Supplementary Figures as follows:

- (i) new Fig. 2 (old Fig. 3) shows the transcriptional profile after YF-ZIK prime and boost; discussed in lines 496-506.
- (ii) new Fig. 3 (with parts from old Fig. 2 and new data) focuses on the kinetics, quantification and quality of the humoral immune response following vaccination.
- (iii) new Fig. 4 (formerly part old Fig. 6) compares the pattern and compartmentalization of the transcriptional profiles seen for YF-ZIK in NHP and parental YF17D in humans
- (iv) new Fig. 5 (old Fig. 4) presents the virological and serological analysis of ZIKV challenge in vaccinated NHP in comparison to sham controls

- (v) new Fig. 6 (with parts from old Fig. 5) presents the transcriptional response to ZIKV challenge infection in vaccinated NHP in comparison to sham controls
- (vi) new Fig. 7 and new Supplementary Fig. 7 (with parts from old Fig. 5 and new analyses) links DEG networks and the expression of particular marker genes to explain and predict vaccine immunogenicity and efficacy.

We agree that such an order might be a more logical way to present our study.

4. In Figure 6B, please explain the rationale for comparing D30 YF-ZIK to D2 YF17D. Do you consider the priming YF17D vaccination to be functionally equivalent to the boosting YF-ZIK vaccination? Is this due to species differences or artifacts of the respective vaccines?

R4/ We would like to point out that this is probably a misunderstanding. Since in original Fig. 6b (now Fig. 4b) all comparisons were made regarding YF-ZIK D2: values for YF-ZIK D2 on the X-axis, compared to those for YF17D at D3 and D7 (for YF17D no public D2 data are available) on the Y-axis. In the same graph we also plotted YF-ZIK D2 vs YF-ZIK D30, which confirms that the overall transcriptional response to YF-ZIK is similar between the prime and boost (lines 303-305). Thus, we do not make any claims that the prime only YF17D vaccination might be functionally equivalent to the prime/boost YF-ZIK vaccination, especially because our 2-dose study design does not allow us to separate prime/boost response and protective efficacy upon ZIKV challenge of our vaccinated animals. To clarify this, a respective disclaimer was added (lines 472-473).

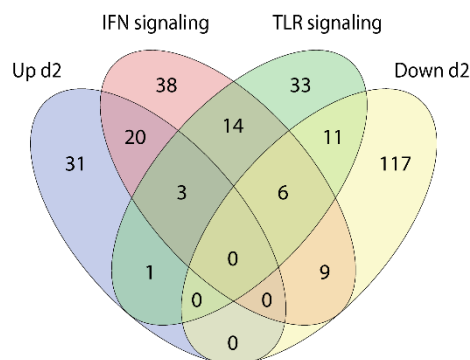
However, we have exhaustively tested all relevant YF-ZIK time points to all publicly available time points for YF17D transcriptional responses (Supplementary Table 3), which shows similar fold-changes and significant overlaps at all time points except D14 for YF17D. As elaborated above in reply to Q6 from Reviewer 1, we have compared both similarities (lines 283-307) and “exclusive” effects (lines 308-329) of each vaccine. Regarding possible differences arising from a comparison of data generated in humans versus NHP, we observed that YF17D exclusively up-regulated 45 genes, including several IFN-stimulated genes. Of interest, these genes were significantly upregulated by infection with other unrelated viruses such as SARS-CoV-2 besides in macaques as well as in mice, hamsters, ferrets and humans (new Supplementary Table 3), thus arguing rather for vaccine differences rather than species differences.

5. In Figure 3E, IFN signaling increases at D2 following prime and boosting vaccinations, yet TLR signaling decreases after the priming vaccination. Is the IFN increase due to RIG-I or some other cytosolic RNA sensor?

R5/ We have now added Supplementary Table 1, which lists all genes and their respective signaling pathways. As indicated in Supplementary Table 1, 90 genes in our panel are associated to IFN signaling, and 69 genes with TLR signaling, with 23 genes overlapping between both pathways.

As shown in the Venn diagram (see below), 20 out of 55 genes (36%) upregulated at day 2 belong to the IFN signaling pathway (Supplementary Table S1), in contrast to only one gene (*ITGB2*) in the TLR signaling pathway. In addition, 3 upregulated genes (*IRF7*, *NFKBIA*, *APP*) were shared between both pathways. These 20 IFN-related genes include RIG-I (encoded by *DDX58*), as well as other key antiviral genes (*EIF2AK2*, *STAT2*, *OAS3*, *OAS2*, *OAS*, *OASL*, *STAT1*, *MX1*, *MX2*, *IFIT3*, *IFIT2*, *JAK*, *JAK2*, *IFIH1*, *IFIT1*, *IRF5*) and HLA class II encoding genes (*HLA-DRA*, *HLA-DQA1*, *HLA-DRB1*).

Among the 143 genes downregulated at day 2, 9 genes (6%) correspond exclusively to IFN signaling (*IFNGR2*, *IFNAR1*, *B2M*, *TYK2*, *IFNA2*, *IFN- γ* , *IFNAR2*, *SOCS3*, *PTPN2*) and 11 genes (8%) to TLR signaling (*TLR4*, *TLR6*, *TLR9*, *TIRAP*, *MAPK*, *MAPK14*, *FOS*, *MAP2K4*, *MAPK8*, *JUN*, *BIRC2*). Six downregulated genes (4%) were shared between both pathways (*TRAF6*, *CHUK*, *FADD*, *MAPK3*, *CASP8*, *SOCS1*). Thus, our results are in agreement with *RIG-I/DDX58* and *MDA5/IFIH1* acting as major cytosolic RNA sensors in flavivirus infections. A brief mention of this observation is added in lines 192-201.



Venn diagram showing the overlap between up- and down-regulated genes by YF-ZIKV at day 2 with the IFN and TLR signaling pathways as defined in nCounter analysis.

6. In line 422, I believe a word is missing after serially. Perhaps collected?

R6/ Yes, one word was missing, it should be serial samples.

Reviewer #3 (Remarks to the Author):

Note: For convenience of assessment, we add a marked-up version of our updated manuscript with highlights in two font colors. Rearrangements comprising entire sections of old text (i.e., without major edits) are highlighted in **LIGHT BLUE**. Additions, corrections and major changes are highlighted in **RED**.

In this manuscript, the authors extend their investigation to test the chimeric vaccine YF-ZIKV in macaques. While the clinical evidence supports that the yellow fever YF17D is safe and efficacious, it remains unclear whether YF-ZIKV behaves in the same way. Based on their data, I am not convinced that the YF-ZIKV stimulates a polyfunctional response, especially the cell-mediated responses. Also, based on current literature, most of the T cell epitopes are present in the NS genes, which means that it is very unlikely the YF-ZIKV will stimulate T cell responses as the NS genes belong to YF. Because of this, the data does not support their claim.

Indeed, most T cell epitopes are present in NS genes as part of the YF17D vector; consistent with their cytoplasmic exposure, access to the proteasome and processing pathway towards MHC-I presentation (prM/E and NS1 reside in the ER lumen). However, growing evidence suggests that NS T cell responses also contribute to protection. Recently, Kalimuddin et al. (PMID: 39794472) conducted a clinical trial using YF17D and chimeric YF17D-Japanese encephalitis (IMOJEV) vaccine to demonstrate that vaccine-induced T cell responses might be sufficient to contain a systemic viral challenge, independent of neutralizing antibodies.

Regarding the YF-ZIK induced polyfunctional cellular responses, please see our responses to Reviewer 2, Q2/R2. Particularly, the transcriptional signatures observed both (i) after prime and boost vaccination as well as (ii) after challenge infection clearly indicate a broad activation of T cell-related immune genes and pathways in a ZIKV specific manner. This expression analysis is experimentally supported by ICS analyses of peptide stimulated PBMC; revealing the induction of ZIKV prM/E specific CD4⁺ and CD8⁺ T cells expressing both Th1 (IFN- γ) and Th2 (IL-4) cytokines as unequivocal evidence for polyfunctionality.

In addition, there are several other concerns that should be addressed:

1. The authors did not show the viremia of YF-ZIKV in the animals. This is important because YF17D is known to induce detectable viremia, and it is this viremia that drives vaccine immunogenicity. Moreover, knowing the peak viremia will help in interpreting the transcriptomics data better.

R1/ Seroconversion rates for original YF17D have been reported to be almost 100%, irrespective of detectable virus RNA. By contrast, viremia has not consistently been

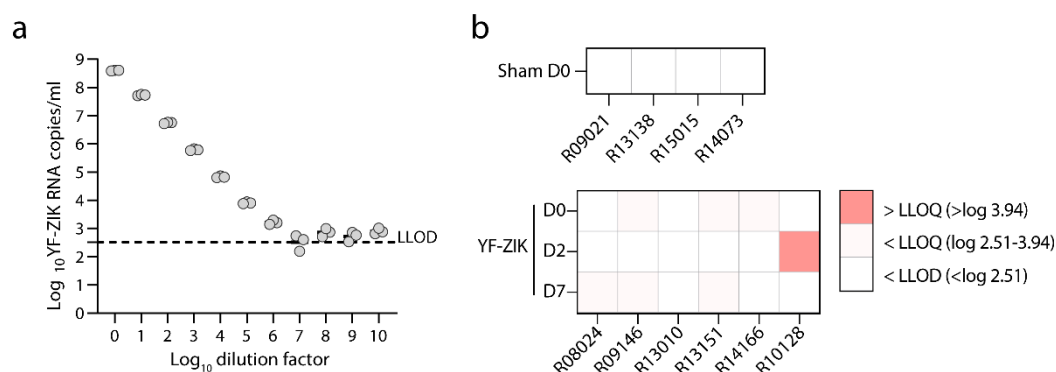
detected between studies, with individual detection rates may have been maximally 30-70%; often for a singular timepoint and at barely detectable viral RNA levels. Hence, subclinical replication (rather than detectable viremia) is sufficient for immunogenicity, and lack of detectable viremia does not defy vaccine efficacy. By contrast, elevated levels and persistence of viremia (resulting from prolonged viral replication and systemic spread) is frequently seen as a safety concern (PMID: 25446819).

Nevertheless, the magnitude and duration of viremia following YF17D immunization have been discussed as determinants of immunogenicity (PMID: 25713354). Where detectable, viremia from YF17D vaccination has been shown to be transient and low in both humans (detectable between day 3 and 9 post-immunization, $<10^4$ genome RNA copies/mL peripheral blood/plasma) (PMID: 25713354, PMID: 39794472) and macaques (detectable between day 3 and 7 post-immunization, $<10^4$ genome RNA copies/mL peripheral blood/plasma) (PMID: 38567951, PMID: 20607226). Thereby viremia (i.e. viral replication) is believed to drive type I interferon (IFN) responses as well as other innate immune responses that shape the development of adaptive immunity.

Seeing the high degree of attenuation for the YF17D-derived YF-ZIK candidate, both *in vitro* and *in vivo* (Kum et al. NPJ Vaccines. 2018 PMID: 30564463 REF# 22; Göbel et al. Vaccines 2024 PMID: 39066393 REF#36, we did not expect strong viremia from YF-ZIK immunization, alike reported for other YF17D-derivatives IMOJEV and DENGVAIXA also induce (if at all) lower viremia in NHPs and humans (PMID: 25446819).

To detect any viremia from YF-ZIK immunization, we followed a combined nested RT-PCR/qPCR approach adapted from a sensitive in-house methodology (validated by Li et al. Mol Ther Methods Clin Dev. 2022 PMID: 35313504): We first ran RT-PCR with primers targeting the YFV NS3 gene, followed by qPCR with primers and probes targeting the same region. RNA extract from a serial dilution of YF-ZIK virus stock was used as a positive control and to determine the sensitivity (or LLOD) of the assay. The results show that our assay is sensitive enough to detect vRNA until a 10^7 times dilution of the virus stock with a LLOD of 2.52 log₁₀ RNA copies/mL (about 1 copy per qPCR reaction; new Supplementary Fig. 3a, b).

When analyzing the NHP sera we only detect very transient and minimal viremia in macaques after prime, either at day 2 or 7 (new Supplementary Fig. 3b).



Only one macaque (R10128) showed transient vRNA above LLOQ at day 2. This animal was also among those 3/6 individuals (R13010, R13151 and R10128) that were “early responders” and seroconverted to high NAb titres (log₁₀ GMT of 2.5) already 7 days post immunization. On the other hand, we did observe a pronounced activation of IFN signaling after both prime and boost (Fig. 2b, d), which is driven by (transient) virus replication. Likewise, the absence of viremia further supports the excellent safety profile of our ZIKV vaccine candidate.

The detection (new Supplementary Fig. 3a, b) and likely consequences of viremia are now mentioned in lines 225-233 and in the new *Limitations* section.

2. Figure 2D. The authors claim that they detected T cell responses. However, the data clearly shows that the T cell responses to ZIKV E peptides are not significantly increased. Thus, the data indicated that the mechanism of protection is primarily antibodies and not T cells. If this is indeed the case, then the authors should not claim that the vaccine can induce polyfunctional responses.

R2/ Regarding questions on YF-ZIK induced polyfunctional cellular responses, please see our responses to Reviewer 2, Q2/R2.

We agree that due to high background in non-stimulated PBMCs and the limited number of specimens of sufficiently high quality amenable for ICS analysis, formal significance is not reached. These data have now been moved to new Supplementary Fig. 7d and more critically discussed in the new *Limitations* section (lines 558-588).

Importantly, though more indirect, our *in silico* whole blood transcriptomics analysis of vaccinated animals after ZIKV challenge shows a clear pattern that can be unequivocal linked to a virus-specific T-cell recall response. The thus observed transcriptional T-cell signature suggests CMI to be polyfunctional (CD4/CD8) and balanced (Th1/Th2/Treg). Such CMI recall is fully in line (and in our view difficult to explain without) priming/induction of decent T-cell memory during original vaccination and prior to first virus encounter (new Fig. 7f).

3. Figure 3B and 3D: It is unclear how the heatmaps are generated. Based on the volcano plot, it is clear that the host responses are resolved at day 7. Yet, the colours indicated that the pathways are in red. There are also no mention of the methods employed to generate the heatmaps.

R3/ We agree with the reviewer that, at first glance, the visual impressions of the Volcano plots and pathway heatmaps seem distinct. However, it should be noted that each pathway consist of multiple genes (as detailed below in reply to Q4, specified in Supplementary Table 1), with some genes being upregulated and others downregulated within the same pathway; as a result, a net zero change at the pathway level is possible. In addition, heatmaps only provide an overall visual overview of all data and are not a statistical tool: as indicated in Fig. 2, the colours are individually scaled per row (row minimum= white, row maximum= red), with each row representing an individual animal. Thus, if IFN signaling is strongly upregulated across a large number of genes (e.g., at d2 after prime and boost), all other pathways will appear visually downregulated due to the effect of the scaling. Thus, colours in the heatmap give a visual impression for each animal at each time point across all pathways, independent of statistical significance across experimental groups.

4. Figure 3E: What is the pathway score based on? Is this based on a list of genes involved in the pathways or only DEGs?

R4/ Pathways are predefined by the nCounter design; the specific lists of genes in each pathway are detailed in Supplementary Table 1; referred to in M&M section (line 740).

5. Figure 3: There is a lot of mention about DEGs, but the authors did not indicate the fold-change and p-value thresholds to indicate DEGs.

R5/ Due to the low number of animals in each experimental group, uncorrected p-values ($p < 0.05$) were used for systems vaccinology analyses, to enhance discovery and to avoid false negatives (as discussed below). However, FDR-corrected p-values are shown by dotted lines in Volcano plots where appropriate (Figs. 2c and 6b). No fold-changes were pre-specified since we observed in this study as well as several previous papers from our group (see below) that *bona fide* IFN-stimulated genes often display a fold-change lower than 2 (which is “traditionally” used in transcriptomics, although this number is not evidence-based), even with significant p- and q-values.

7. Figure 3: Various critical genes involved in IFN/TLR/B cell related genes were mentioned but the authors did not plot the kinetics of these key genes to show their dynamics and extent of change.

R6/ The kinetics of major predictive genes for both antibody production and viral challenge outcome are shown in the figure below. Myeloid-expressed gene *TNFSF13B/BAFF* and its B-cell-specific receptor *TNFRSF17* (plotted in Fig. 7c) follow similar kinetics as *IFI44* (the strongest IFN-stimulated gene/ISG in the YF17D dataset) and *CD74* (expressed in B-cells and other APC), respectively; increasing at d2 and further up at d30; or increasing at d2 and remaining on a higher plateau from d2 to d35. In contrast, *CD207* and *C8B* increased at d2, peaked at d7 and then steadily declined until d35. CD8+ T-cell and NK cell-expressed *CCL5* showed an exceptional kinetic profile: decreasing at d2 but remaining highly expressed from d7 to d35.

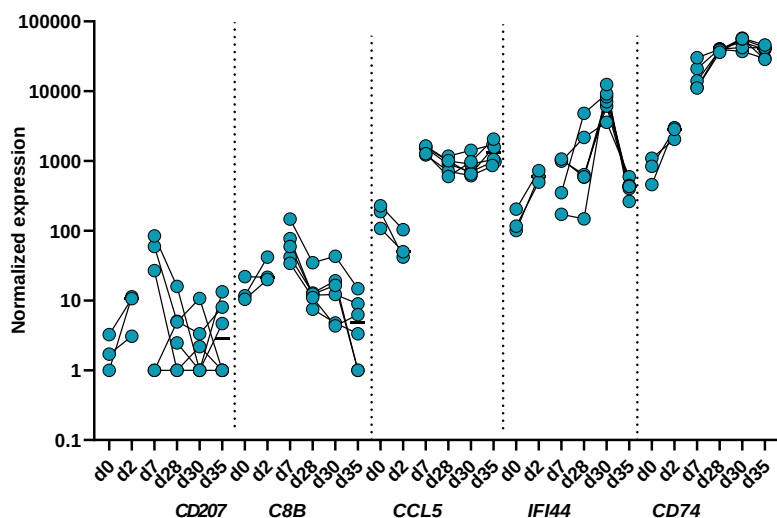


Figure legend: Kinetics of major genes predictive of viral challenge outcome. Gene expression quantified by digital transcriptomics over the course of vaccination (d0-d35), with prime at d0 and boost at d28.

8. Figure 6: Public data used are either RNAseq or scRNAseq data. However, the nCounter was used for the transcriptomic analysis. How did the authors process the data so that nCounter and RNAseq data can be compared?

R7a/ Due to its digital nature (hybridization and direct counting without enzymatic amplification), nCounter data (normalized for internal positive and negative controls, as well as housekeeping genes) represent an unbiased and robust quantification of the transcriptome. Upon its inception (Geiss et al. Nature Biotechnology, 2008 PMID: 18278033), comparison of the NanoString nCounter gene expression system with microarrays and TaqMan PCR demonstrated that the nCounter system is more sensitive than microarrays and similar in sensitivity to real-time PCR. During the last decade, several papers from our group (Feys et al. Lancet Microbe. 2024 PMID: 38280387; de Moraes et al. Front Microbiol. 2023 PMID: 37601355; Cuyper et al. Nature Aging. 2023 PMID: 37217661; Assone et al. J Neuroinflammation. 2022 PMID: 36482436; Feys et al. Lancet Respir Med. 2022 PMID: 36029799; Dirkx et al. Commun Biol. 2022 PMID: 35752645; Lloréns-Rico Nature Commun. 2021 PMID: 34716338;

Bouzas et al. J Clin Virol. 2018 PMID: 30031351) and others have cross-validated nCounter digital transcriptomics to both older technologies (microarrays and qPCR) and newer technologies such as RNAseq, single-cell RNAseq and spatial transcriptomics. Moreover, two recent manuscripts have successfully used nCounter analysis in macaque models of Ebola virus, in comparison to spatial transcriptomics (Wanninger et al. 2024 PMID: 39635525) and RNAseq, the latter being slightly less robust than nCounter in this model (Speranza et al. 2017 PMID: 29116224).

Were the correlations based on only the DEGs?

As shown in Fig. 7, a strong positive correlation ($R > 0.70$, $p < 10^{-15}$) was found across all genes that classified as DEG in YF17D (d3 and d7) and their corresponding RNA levels quantified by nCounter after YF-ZIKV “prime” vaccination (d2), i.e. without selection of being DEG upon YF-ZIKV vaccination, for the following reasons:

- a. A low number of animals in our study limited the statistical power and hence a stringent selection of FDR-corrected p-values would result in a large number of false negatives.
 - b. As outlined above, we and others have extensively cross-compared nCounter data with other technologies using transcriptome-wide fold-changes as quantitative readout, which resulted in highly significant cross-platform and cross-species correlations (e.g. Dirkx et al. Commun Biol 2022 PMID: 35752645).
8. Since the primary mechanism for efficacy is likely neutralising antibodies, then the authors need to acknowledge the limitations, and highlight the potential risk of antibody-dependent enhancement.

The risk of ADE and some considerations on how that risk could be mitigated during implementation have been added to the discussion (lines 581-588).

RESPONSE TO REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

The authors have provided a very comprehensive rebuttal to the reviewers' comments and this greatly improves the manuscript. There is one aspect that I do not agree with the authors, and I would suggest should be included in the limitations, namely the extensive comparisons with a "human dose of YF/17D vaccine". The dose of the candidate Zika vaccine was 10^5 pfu whereas 17D vaccine is usually administered at a lower dose to vaccinees for all but one producer. The authors are encouraged to read the 17D vaccine dose-sparing papers for information that include the quantity of 17D virus in a dose and parameters of induction of antibodies post-vaccination, which do show some differences depending on the dose of vaccine and particular producer. Indeed, the Brazilian 17DD substrain has been compared at many different doses in volunteers. Thus, I do not believe the authors can directly compare the human 17D dose to the dose of candidate Zika vaccine as different quantities of virus are being compared. The authors may also wish to look how much virus is in a dose of Imojev.

R/ We fully agree that comparing YF17D in humans and our YF-ZIK vaccine candidate in NHP under different immunization dosages comes with limitations. We gratefully acknowledge the suggestion of the Reviewer hinting towards other (pre)clinical studies which are taken up and now discussed in lines 580-592 of an extended 'Limitations' section.

Also, there might be a misunderstanding, as we did not intend to compare a "human dose of YF17D vaccine" with our "macaque dose of YF-ZIK immunization". In our manuscript, our comparison is made based on:

- 1) Similar immunization schedule (prime and boost), as explained in lines 223-225: "The immunogenicity was evaluated following a two-dose regimen (Fig. 1b) analogous to the schedule recommended for the chimeric YF17D-based JEV vaccine (Imojev®)."
- 2) Our comparison mostly focuses on vaccine-induced transcriptional responses after prime and boost immunization (Fig. 2 and 4), instead of antibody and cellular responses, as latter are more likely to be impacted by viral dosages and likewise replication characteristics (see e.g. Monath et al. PNAS 2006).
- 3) The public transcriptional data of human YF17D immunization were likely pooled from different YF17D strains and dosages (see e.g. Gaucher et al. JEM 2008 using both YF-VAX and Stamaril), under the condition that YF17D immunization was confirmed successful in recipients. We explained in lines 284-286: "To validate and translate our NHP data to the human model, we compared the gene expression profile triggered by YF-ZIK vaccination of macaques with what can be expected for YF17D vaccination following meta-analysis of available human data."

We trust that by clearly stating these caveats, our comparative meta-analyses will be interpreted with appropriate caution.

The English language needs to be carefully reviewed.

R/ Language reviewed and refined.

The term “flavivirus” should be updated to “orthoflavivirus”

R/ Updated throughout the manuscript to ‘*Orthoflavivirus*’ (with capital and in italics) when referring to the original Latin genus name, and ‘orthoflaviviruses’ when referring to several members from that genus.

Line 86: I would suggest that Zika virus is not found “globally”, and this statement should be tempered.

R/ ‘Globally’ has been changed for ‘across multiple regions’ (line 78-79).

Reviewer #3 (Remarks to the Author):

The revised version of the manuscript provides a more balanced view of the results and findings. I have no further comments as my previous comments were adequately addressed.

R/ Thank you. Much appreciated.

Editorial note: due to the absence of reviewer 2, this reviewer was additionally asked to comment on the authors response to the prior comments of reviewer 2: Yes, I think the reviewer's comments were adequately addressed.

R/ Many thanks for stepping in and the final assessment.