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Peer Review File

A Zika Virus Vaccine with E Protein Fusion Loop Mutations Protects via CD8+ T Cells

Corresponding Author: Professor Sujan Shresta

Version 0:

Decision Letter:

27th October 2025

Dear Professor Shresta,

Thank you for your patience while your manuscript "A Zika Virus Vaccine with E Protein Fusion Loop Mutations Fails to Elicit Neutralizing Antibodies Against Mature Virions but Protects via CD8+ T Cells" was under peer review at Nature Microbiology. I am sending this decision on behalf of[redacted] while is on annual leave. It has now been seen by our referees, whose expertise and comments you will find at the end of this email. In the light of their advice, we have decided that we cannot offer to publish your manuscript in Nature Microbiology.

From the reports, you will see that while they find your work of some potential interest, the referees raise concerns about the advance your findings represent over earlier work and the strength of the novel conclusions that can be drawn at this stage. In particular, while the reports vary somewhat in terms of enthusiasm for your work, the concerns regarding conceptual advance, the limited mechanistic insights provided and experimental design are sufficiently important as to preclude publication of your work in Nature Microbiology.

While we cannot continue with this manuscript at our journal, we have discussed the study with our colleagues at the EMBO Molecular Medicine. As you may know, EMBO Molecular Medicine is a non-profit, fully open-access journal published by EMBO Press and offers rapid and transparent peer review with a single major revision round policy. I am glad to say that the handling editor [redacted] would be happy to consider your manuscript for publication at EMBO Molecular Medicine. Please note, that the revised version that addresses reviewers' concerns in full will be sent to the original referees for re-evaluation. If you are interested in this option, please submit the manuscript to EMBO Molecular Medicine via the transfer link below. Please feel free to contact [redacted] if you have any questions. Please note that no reformatting of the manuscript is necessary for the transfer.

To transfer your manuscript please use our manuscript transfer portal. You will not have to re-supply manuscript metadata and files, unless you wish to make modifications. For more information, please see our manuscript transfer FAQ page.

I am sorry that we cannot be more positive on this occasion, but hope that you find the referees' comments helpful when preparing your paper for resubmission elsewhere.

Yours sincerely,

[redacted]

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Reviewer Expertise:

Referee #1: Flavivirus vaccines, DENV
Referee #2: T cell responses to viruses
Referee #3: Vaccinology

Reviewers Comments:

Reviewer #1 (Remarks to the Author):

This manuscript by Chuensirikulchai and colleagues reports a study that identified CD8+ T cells as a functional mediator of protection against ZIKV infection. Using different mouse models, the authors dissected the antibody response from DNA vaccine expressing wild-type prM-E of ZIKV (WT) or prM-E with 4 amino acid substitutions to modify the fusion loop of the E protein; this fusion-loop modified (FLM) construct was generated with the goal of reducing the likelihood of infection enhancing antibodies. Contrary to conventional assumptions, however, the authors showed that vaccination with FLM produced antibodies that, whilst neutralizing in vitro, did not offer any protection against ZIKV infection. Instead, protection in mice vaccinated with FLM vaccine was mediated by CD8+ T cells. In contrast, mice vaccinated with WT prM-E construct produced both antibodies and CD8+ T cells that could, on their own, protect against ZIKV challenge infection. The authors also showed that modification of the fusion loop of E protein interfered with prM cleavage and hence reduced the abundance of antibodies that bind epitopes concealed by uncleaved prM. They concluded that CD8+ T cells are essential for protection against ZIKV infection.

This is a well written manuscript reporting a very detailed dissection of humoral and cellular immunity against Zika. The findings are interesting and adds another important piece of evidence that challenges the conventional assumption that neutralizing antibodies are absolutely required for protection against disease caused by ZIKV and other related viral infections. Data from passive immunization and adoptive T cell transfer experiments are particularly compelling. Several areas could benefit from additional attention. These are:

1. The time interval between challenge infection and termination of experiment is very short (3 days). Moreover, ZIKV challenge infection was through inoculating the virus directly into retroorbital vein, which is unusual. Justification on such an experimental design, with reference to previous findings, would be helpful for readers to contextualise the informativeness of infection outcome through an unconventional route of inoculation and measured at day 3 post challenge infection.

2. Line 26. There has been a change to the nomenclature of this genus. Flavivirus is now Orthoflavivirus.

3. Lines 131-132. Please indicate the identity of the mAb that was used to detect domain III of ZIKV E protein.

4. The gating strategy for flow cytometry and ICS assay should be shown as a supplemental figure.

5. Lines 147-156 and Figures 2c and 2d. Please indicate if values plotted are after subtraction of values obtained from unstimulated negative controls. This would be helpful to differentiate Ag-specific T cell responses from overall T cell response.

6. Lines 169-172. Conventionally, 4-fold or greater rise in antibody titers following infection is measured at least 2 weeks apart. This 3-day interval is too early to support the interpretation that the vaccination induced nAb titers were already at maximal levels before infection.

7. Lines 178-183 and Figure 3c. Please show statistical test results between CD4+ T cells in FLM vs WT animals. Please also indicate, similar to comment #4, how the T cell data was processed for plotting on the graphs in 3c and 3d.

8. Lines 187-189. The authors have assumed the T cell response to the two vaccine constructs in C57Bl/6 mice, with and without IFNAR knockout, are similar in trend; pre-challenge T cell counts were not measured in IFNAR -/- mice. The effect of type-I IFN response on T cells has been shown previously (Crouse et al, Nat Rev Immunol 2015) and thus differences in CD8+ T cell response to FLM vs WT vaccination in IFNAR-/- mice, although unlikely, cannot be completely excluded. The assumption applied in this final sentence should be clearly stated to avoid misleading readers.

9. Lines 196-198 and figure 3e. The finding of complete abrogation of infectious ZIKV despite detectable ZIKV RNA in both organs and sera is not surprising although interesting, in light of the explanation offered in Figure 5. The authors suggest that antibodies from WT but not FLM infection are more potent in neutralizing mature ZIKV. The extended logic from this line of thinking is that there are more mature viral particles in vivo compared to ZIKV cultured in C6/36 cells. Would not the serum viremia (Figure 3e, upper row) and RNAemia (Figure 3e, lower row), argue against such an explanation?

10. Line 228. T cell counts shown in Figure 3d were measured after challenge, not after vaccination.

11. Line 238. Should “reduction” be “increase” instead?

12. Lines 240 and 243. It cannot be ascertained that the antibodies from WT vaccinated animals “cleared” infection instead of neutralizing infection. Please rephrase.

13. Lines 285-288 and Figure 4j. The interpretation of viral RNA elimination in HLAB*0702 ifnar-/- mice in FLM vaccinated animals with intact CD8+ T cell contrasts with findings shown in Figure 3e. The difference may be due to the detection limit shown in dotted lines in these respective figure panels. Is there an error here?

14. Figure 5. Could WT vaccination produce C8-like antibodies that have shown to correlate with protection, at least against dengue (Mpingabo et al, Sci Transl Med 2025)? Would it be possible to do a competition ELISA to determine if WT but not FLM vaccinated animals elicited C8-like antibodies that could have more precisely explain the findings?

Minor comments:
Line 27. Change neurological injury to neurological syndromes.
Line 145. Close bracket is missing at the end of this sentence.
Line 359. Perhaps “under-appreciated” is more accurate than “unappreciated”?
Lines 75-78: As far as I’m aware, there is no clinical or epidemiological evidence to show that prior DENV/WNV/JEV infection

worsens risk of severe ZIKV infection outcomes. This should be included as a caveat to the statements on experimental findings.

Reviewer #2 (Remarks to the Author):

In this study, the authors investigated the mechanisms of protection offered by DNA vaccines against Zika Virus which possess a mutated fusion loop region of the E protein EDII ectodomain (Z-prM/E-FLM). In previous studies, such vaccines have been shown to be promising in their capacity to protect against Zika virus infection, and are less likely to induce sub-neutralising antibodies, which can promote severe disease through antibody-dependent enhancement of infection. The findings show that while CD8+ T cells are not strictly required for protection conferred by Z-prM/E-WT vaccines, CD8+ T cells are indispensable for protection induced by Z-prM/E-FLM vaccines. Overall, the study is well executed and offers interesting new insights into the mechanisms of protection by FLM vaccines. The manuscript, however, lacks the depth of understanding of immune response kinetics following vaccination +/- infection to fully define vaccine-induced immunity. Comments are provided below.

Major comments:

•The authors utilise C57BL/6 Ifnar1-/- mice to perform challenge experiments with Zika virus following vaccination. While I appreciate that this is a common and necessary approach to investigate Flavivirus infections, the rationale for doing so needs to be made clearer in the results section for a broader audience. The authors should also reference previous studies in which this approach has been employed.

•In addition to the comment above, the use of Ifnar1-/- for the study of T cell responses has inherent limitations, given the importance of IFNa to T cell expansion and effector functions. Please address how these limitations potentially impact the findings of this study.

•In addition to investigating T cell responses in the spleen, the authors should also investigate CD4 and CD8 T cell responses in the draining lymph node following I.M. vaccination and whether responses at this site differ.

•Although the authors show that CD8+ T cells are necessary for the protective effect of Z-prM/E-FLM vaccines, it is not clear exactly how CD8+ T cells contribute to this. In the Discussion, the authors speculate that polyfunctional T cells are necessary for protection against vaccine-elicited immunity against other flaviviruses such as DENV. To further elaborate as to how CD8+ T cells elicited by FLM vaccination confer protection, the authors could assess whether the adoptive transfer of CD8+ T cells from IFNg deficient mice, for example, will still confer protection against ZIKAV. If this is not feasible, the use of an IFNg blocking antibody following adoptive transfer may also be informative.

•Protective immune responses towards vaccination and/or infection cannot be defined by the analysis of one time-point only:
- In Fig 2: why was only d14 post the 3rd vaccine dose investigated? While the authors found no differences in antibody responses between 2 vaccination protocols, the timing of these responses post vaccination as well as durability of these antibody responses may differ substantially across vaccine compositions. This needs to be investigated. Similarly, early recruitment and longevity of T cell responses should be investigated. Kinetics rather than one time-point analyses need to be shown. For example, what happens at 5 days post dose 3 and at 3 months after 3 vaccinations? Please also graph IFN-g single-producing CD8+ T cells to understand total (rather than polyfunctional) CD8+ T cell responses. Representative FACS plots across different cytokine-expressing T cells populations as well as across different vaccine regiments plus controls should be shown.
- Similar comments go for Figure 3 re immune response kinetics and single-IFNg producing CD8+ T cells.
-Fig 4: Why was only d3 after infection investigated?

•Gating strategy needs to be shown.
•Please show representative FACS plots, either as part of the main figure or as a supplementary figure. In particular, it would be good to see representative flow cytometry plots for the intracellular cytokine staining studies (including the relevant unstimulated controls or equivalent); also as per comments above.

Minor comments:
•The Introduction is too detailed; a more concise approach is recommended.
•Please be consistent with the virus nomenclature across the manuscript– either Flavivirus or Orthoflavivirus (I believe the latter is more up to date with current standards) when referring to the genus.

Reviewer #3 (Remarks to the Author):

The manuscript evaluates two plasmid DNA vaccine candidates encoding Zika virus (ZIKV) prM and E proteins in Ifnar1$^{-/-}$ mice, comparing wild-type (E-WT) and fusion loop mutant (E-FLM) forms. The authors show that both vaccines protect against infection, but through different mechanisms: E-WT induces neutralizing antibodies against mature and immature virions, whereas E-FLM relies on CD8^{+} T-cell–mediated protection due to impaired prM cleavage. The study is methodically organized. Some results and interpretations are presented overly positive, not necessarily describing the data as presented in the figures, and at times erroneously concluding. In general, the conclusions in this manuscript should be tempered.
The novelty of the work is, however, limited. The authors have already shown the role of CD8^{+} T cells in response to ZIKV infection (PMID: 28081442). More recently, using an NS3-based vaccine, the authors have shown that CD8^{+} T cells are

necessary and sufficient for (i) preventing mortality in lethally infected adult mice and (ii) protecting fetuses from growth restriction in infected pregnant mice after ZIKV challenge (PMID: 33148638). In addition, Richner et al. (2017, Cell, DOI: 10.1016/j.cell.2017.02.017; already in collaboration with the lab from which current study originates) previously used an identical prM/E sequence and fusion loop mutation (yet as mRNA vaccine) to demonstrate protection from disease, lethal challenge, and induction of neutralizing antibodies, showing also that the effect was independent of MHC class I haplotype by using three different mouse lines). Similarly, Thompson et al. (2022, PLoS Negl Trop Dis, DOI: 10.1371/journal.pntd.0010588) reported that even a single amino acid substitution between neutral, nonpolar residues in the flavivirus fusion loop can significantly alter the generation of neutralizing antibodies. These prior findings capture the main conceptual and mechanistic points presented here, leaving the current work with limited mechanistic novelty.

Unlike their previous work and exhaustive evaluation of the animal model, in this manuscript the authors chose to use viremia/detection of viral RNA or virus dissemination as sole readout after challenge. Hereby analytical procedures seems often to be flawed, as discussed in details below, having direct consequences in what the authors decide as no viremia (as proxy for "protection"), hence contribution of specific T cell population or nAbs to protection against viremia. Additional readouts commonly used in this model, like clinical score, body weights loss, mortality, would have been more necessary to support the conclusions and proposed mechanisms.

Please find below specific comments and points of attention:

Limit of detection: The authors should provide information on how the limit of detection (LoD) was determined and, specially, why is it so variable across experiments?
As explained in the Methods section (line 545-547) "Viral RNA concentrations were determined from a standard curve composed of four 100-fold serial dilutions of in vitro-transcribed RNA from ZIKV strain FSS13025 and were normalized to the 18S rRNA levels in the same samples to account for variations in RNA input." This implies that ZIKV RNA copies can be determined with a defined LoD. Further normalization with 18S rRNA allows the comparison of samples between/across experiments, but should not affect the LoD. However, for instance in Figure 4h and Figure 4j there are big discrepancies regarding the LoD even when comparing same tissues (LoD in liver 10e-2 vs. 10e0; LoD in Serum 10e0 vs 10e4).
One such examples is a ~10.000 fold difference in the LoD of serum samples from Figure 4h and 4j, meaning that, technically, the authors cannot detect 9000 ZIKV RNA copies in the serum. Obviously, this doesn't mean that there is no viremia, it shows that the methodology is not reliable. Definitely, such discrepancies spanning several orders of magnitude do not allow to consistently score for vaccine mediated protection.
Similarly, the LoD for FFU/g of tissue should be consistent. As mentioned above, survival and clinical scores should have been measured in this study to more carefully conclude on the protective role of CD8+ T cells or antibodies.
This problem with the LoD seriously hinders the conclusions drawn in the whole manuscript.

Figure 2b vs 5b: The authors show that antibodies generated by Z-prM/E-FLM are much less efficient inhibiting mature virions produced in Vero cells (Figure 5b). However, in Figure 2b, the same serum from Z-prM/E-FLM perfectly inhibits both mature and immature virions produced in C6/36 cells. How do the authors reconcile this incoherence?

Figure 2c, d: Since the authors are reporting absolute numbers of specific cell populations, it should be clearly stated in the Methods, Figure legend, the total number of events acquired, or, as best practice, relative to a relevant population e.g. Live/CD3+ cells. In the same line, representative plots and gating strategies should be provided.
Also, CD4+CD44high T cells, as shown in the axis and Figure legend are antigen experienced, and include also the Tcm (CD62L+), not only effector cells. Please correct accordingly (lines 176, 744, 762), or alternatively, please provide the quantification in the effector (CD44highCD62L-) population, which should be in any case the main contributor to IFNg, TNF and IL2 production.
Out of curiosity, was there a specific rationale to check for a degranulation marker (CD107a) in CD4 T cells in the context of vaccination?

Figure 2: The authors emphasize that the effect is due to vaccination, but so far, their data support that both constructs elicit similar immune responses. What is different is likely the kinetics of viral replication in the presence of antibodies. The authors should discuss further on the impact of these antibodies in allowing viral replication and inducing differential stimulation of T cells, due to, for instance, antigen presentation.

Lines 171-172: In our opinion, Figure 3b shows that the infection did not boost antibody response up to 3 dpi. The authors do not provide an antibody kinetics to demonstrate that antibodies were at maximal level at day 48. Since this are longitudinal data, data points at day 48 and 52 should instead be analyzed using a Wilcoxon signed-rank test (likely not normally distributed data).

Lines 187-189: Based on the data provided this conclusion is overstated. The authors can conclude that animals vaccinated with the FLM had higher frequencies of T cell in response to infection. But this can still be induced by different replication kinetics of ZIKV after infection due to differences in anti E antibodies.

Figure 3c-e: Same remark regarding gating choice and data representation as for Figure 2c, d.

Line 199: What do the authors mean by 'total anti ZIKV E protein IgG'? What does 'total' mean in this context?

Line 238: Should be 'significant increase'.

Line 240: The authors do not provide evidence to support that the virus was cleared. It may have never infected the tissue. It was also not 'completely' (1 animal with detectable virus in serum).

Line 242: We would suggest replacing ‘full protection’ with a more descriptive, specific term e.g. protection against viremia. The authors did not check protection against lethal challenge, did not provide disease scores (e.g. body weight loss, clinical score), and one animal had detectable virus in the serum, hence not ‘full’ protection.

Figure 4f: In general, the authors should temper their conclusions and restrict them to the actual evidence. Figure 4f shows that CD8 T cells contributes to reducing viral titers, but it also clearly shows that it’s not sufficient to protect against viremia since ZIKV is still widespread found and in the case of the brain, equally high as the control group. Such widespread virus dissemination, although significantly lower, can still produce similar mortality after r.o. inoculation of ZIKV.

Line 254: It’s not evident that in brain tissue virla load is ‘markedly reduced’. Please adapt the sentence to reflect what the representative result actually shows.

Line 271 and Methods: Please provide the genetic information or reference of the HLA-B*0702 Ifnar1 KO. It is not clear, nor clearly stated, that these animals express HLA-B*0702 as the only class I MHC.

Line 266-269: In our opinion, the use of an HLA-transgenic mouse here would be only useful to show that HLA-B*0702-restricted epitopes from ZIKV structural proteins are also protective against viremia, hence an element of translational value. We do not see how a transgenic MHC class I will alter B cell epitope dominance or antibody responses in general.

Figure 5a: Please provide primary data e.g. the dilution curves.

Line 299-304: Please temper the interpretation here, which in this case is also not correct. The differences shown in Figure 5a are not striking, statistically speaking there are no difference at all between WT and FLM. Please also see that the AUC adds an additional arbitrary dimension that amplifies the numerical value, consider providing primary data showing actual differences in OD values at the different dilutions.

Line 314, 430: This conclusion is also incorrect, and doesn’t describe the data as presented in the figure. Z-prM/E-FLM vaccine is less efficient in generating nAbs, not ‘incapable’. Figure 5b shows that 4 out of 11 vaccinated animals (i.e., 36%) do have nAbs comparable to Z-prM/E-WT.

Figure 5c: A relevant control for total protein should be included.

Figure 2b, 3b, 5b: Please correct parenthesis size in y axis, right panel.

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Version 1:

Decision Letter:

11th May 2026

*Please ensure you delete the link to your author homepage in this e-mail if you wish to forward it to your co-authors.

Dear Professor Shresta,

Thank you for your patience while your manuscript "A Zika Virus Vaccine with E Protein Fusion Loop Mutations Fails to Elicit Neutralizing Antibodies Against Mature Virions but Protects via CD8+ T Cells" was under peer-review at Nature Microbiology. It has now been seen by the original 3 referees, whose expertise and comments you will find at the end of this email. You will see from their comments below that while they find your work of interest, some important points are raised. We are interested in the possibility of publishing your study in Nature Microbiology, but would like to consider your response to these concerns in the form of a revised manuscript before we make a final decision on publication.

The referees’ reports are clear and the remaining issues should be straightforward to address.

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Some reduction could be achieved by focusing any introductory material and moving it to the start of your opening ‘bold’ paragraph, whose function is to outline the background to your work, describe in a sentence your new observations, and explain

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Figure legends must provide a brief description of the figure and the symbols used, within 350 words, including definitions of any error bars employed in the figures.

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We hope to receive your revised paper within three weeks. If you cannot send it within this time, please let us know.

We look forward to hearing from you soon.

Yours sincerely,

[redacted]

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Reviewers Comments:

Reviewer #1 (Remarks to the Author):

This revised manuscript has addressed the issues present in the original version. The inclusion of new sets of data, particularly the lethal challenge ZIKV infection as well as challenge infection at 12 weeks post-vaccination, add insights. The edited text also provides a more balanced interpretation of the findings.

I have just two comments:

1. Lines 63-65. I am not sure this sentence is necessary to frame this study and can be removed to reduce an already lengthy introduction.

2. Lines 663-669. I do not fully agree that a retroorbital inoculation is a superior approach compared to subcutaneous inoculation for evaluating vaccines. Delivering ZIKV directly into the vein would indeed likely provide a more consistent systemic level infection. However, such a route of inoculation shortens the incubation period in which ZIKV, following entry into a host, need to replicate to titers sufficient for systemic dissemination. A challenge infection at day 49 after priming vaccination could, besides just IgG antibodies and T cells, also contain IgM antibodies that could be more potent in neutralizing infection given their pentavalent structure (Singh et al, Cell 2022). A longer interval could see waning of IgM antibody titers. Moreover, not only is there no protection from FLM vaccination induced antibodies, but there could also be, with reduced antibody titer, a hint of infection enhancement in the liver (Figure 6b); a T cell depletion control would have clarified the data in Figure 4e (and f?). Having said that, I am not suggesting that such a control is necessary for Figure 4 as it would involve even more mice in the experiment. What I think should be considered, however, is that the route of inoculation may compromise the extent of long-term protection from ZIKV-specific T cells. Replication sites in the skin and the draining lymph nodes after ZIKV inoculation from an infected mosquito are sites for ZIKV-specific T cells to control infection and reduce systemic ZIKV dissemination. Direct intravenous infection route would thus bypass these sites of infection where ZIKV-specific T cells likely exert their antiviral effects. Perhaps a more nuanced interpretation could be considered.

Reviewer #2 (Remarks to the Author):

The authors have reasonably addressed my queries in the revised manuscript.

I have one further comment about the new gating strategies included in Figs S1 and S2. These need to be clearly labeled on the figures with the information about the population gated and % frequency within the gate.

Reviewer #3 (Remarks to the Author):

The study by Chuensirikulchai and colleagues as been revised to large extent in scope and effort to explain, incorporating most of our earlier concerns, in particularly our criticism on LOD determinations and lack of challenge data in a lethal model.
Some minor oversights may still to be corrected as listed in below:
(1) Line 144, 482 and 931: CD107a is not a marker of cytotoxicity yet of degranulation. The study shows involvement of IFNg (that is also secreted by degranulation) but no expression of granzymes or perforins. As stated now, the authors imply CTL activity by CD8+ and CD4+ T cells. Likewise, in line 972, CD107a should not be called an “effector molecule”.
(2) Line 297: Claim of “full protection” is wrong. Protection from lethal infection isdemonstrated yet still with clear evidence for an elevated disease score (Fig-2H) and several break-through events (detection of viral RNA of FFU) in multiple organs throughout the study. Likewise, even the more potent WT vaccines lost markedly in potency within only 3 month after the last boost (+Fig-4E).
(3) Line 539: Item, there is no evidence for “sterilizing” immunity consistently demonstrated. Even in the low-dose infections there are repeatedly events of virus detection. Likewise, sometimes sterilizing immunity is defined by “no elevation of Ab titers following challenge/re-exposure”. This criterium cannot be used here as animals were not followed up beyond 3dpi.
(4) The discussion is massively expanded arguing for novelty, i.e. mainly a more in-depth mechanism-of-action study with higher resolution than earlier studies using the same test items (pDNA or saRNA vaccines) or comparable FL construct. We feel that this could be contracted again.
(5) Line 706: We doubt that a Ct value of >>40 or even 50 (i.e, a 10e15-times amplification!) can be considered reliable at all.
(6) In Fig-6B and D, there is still this odd 3log10 difference in LOD values for normalized vRNA counts between tissues (e.g. RNA isolated from liver vs. brain).
(7) The western blot shown in Fig-7B is of very poor quality, whereby band resolution and quantification remain difficult. This is getting critical as large parts of the expanded discussion argue along differential prME processing that should be supported by these data. I suggest at least discussing this technical shortcoming as a limiting factor possibility prohibiting a more vigorous interpretation of the data. Therefore, also the different E-domain-specific ELISAs (line383 and Fig-7) lack validation, specificity of analyses is rather announced.

****

Version 2:

Decision Letter:

Our ref: NMICROBIOL-25093320B

30th June 2026

Dear Dr. Shresta,

Thank you for submitting your revised manuscript "Antibody quality versus CD8+ T cell protection in a fusion loop mutated Zika virus vaccine" (NMICROBIOL-25093320B). It has now been seen by the original referees and their comments are below. The reviewers find that the paper has improved in revision, and therefore we'll be happy in principle to publish it in Nature Microbiology, pending minor revisions to satisfy the referees' final requests and to comply with our editorial and formatting guidelines.

If the current version of your manuscript is in a PDF format, please email us a copy of the file in an editable format (Microsoft Word or LaTex)-- we can not proceed with PDFs at this stage.

We are now performing detailed checks on your paper and will send you a checklist detailing our editorial and formatting requirements in a couple of weeks. Please do not upload the final materials and make any revisions until you receive this additional information from us.

Thank you again for your interest in Nature Microbiology Please do not hesitate to contact me if you have any questions.

Sincerely,

[redacted]

Reviewer #3 (Remarks to the Author):

The authors have carefully edited the manuscript, mainly by a more nuanced interpretation of results, by a more detailed M&M narrative explaining key quantitative analytic parameters and, importantly, a rewritten more focussed discussion. Our main concerns have been addressed appropriately. We are grateful for the consideration of our criticism.

Two minor points that can be addressed by modifying the text are:

(a) In the rebuttal to our point (1) it is announced that the discussion contains a note that "direct cytolytic activity has not been formally assessed". This statement is not to be found. Instead, in Line 419-420, a less explicite "acting at least in part through IFNg production" wording has been used. For us this suffices for the conclusions. I suggest to still add former statement to the result section.
(b) In lines 409-414 the importance of FL epitopes for protection from experimental ZIKV and JEV infection is highlighted and compared, endorsing a "careful design" of VLP vaccines, including considerations of reducing ADE risk. This statement is a logical and important consequence of current study yet remains vague. In particular, in the following EDE-specific humoral responses to DENV1-4 are compared to those described in current manuscript for Z-prM/E-WT (lines 414-418). For us it remains unclear, which design principle the authors finally endorse for a potent ZIKV vaccine antigen; VLP w/ or w/o FL, VLP with "FL-mimic" that avoids ADE yet enables prM/E cleavage and processing, EDE-based antigens, etc.?

INTERNAL USE ONLY AIP modules required: ["Life Science"]

Version 3:

Decision Letter:

24th July 2026

Dear Professor Shresta,

I am pleased to accept your Article "A Zika Virus Vaccine with E Protein Fusion Loop Mutations Protects via CD8+ T Cells" for publication in Nature Microbiology. Thank you for having chosen to submit your work to us and many congratulations.

Over the next few weeks, your paper will be copyedited to ensure that it conforms to Nature Microbiology style. We look particularly carefully at the titles of all papers to ensure that they are relatively brief and understandable.

Once your paper is typeset, you will receive an email with a link to choose the appropriate publishing options for your paper and our Author Services team will be in touch regarding any additional information that may be required. Once your paper has been scheduled for online publication, the Nature press office will be in touch to confirm the details.

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[redacted]

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Response to Reviewers
Reviewer #1 (Remarks to the Author):

This manuscript by Chuensirikulchai and colleagues reports a study that identified CD8+ T cells as a functional mediator of protection against ZIKV infection. Using different mouse models, the authors dissected the antibody response from DNA vaccine expressing wild-type prM-E of ZIKV (WT) or prM-E with 4 amino acid substitutions to modify the fusion loop of the E protein; this fusion-loop modified (FLM) construct was generated with the goal of reducing the likelihood of infection enhancing antibodies. Contrary to conventional assumptions, however, the authors showed that vaccination with FLM produced antibodies that, whilst neutralizing in vitro, did not offer any protection against ZIKV infection. Instead, protection in mice vaccinated with FLM vaccine was mediated by CD8+ T cells. In contrast, mice vaccinated with WT prM-E construct produced both antibodies and CD8+ T cells that could, on their own, protect against ZIKV challenge infection. The authors also showed that modification of the fusion loop of E protein interfered with prM cleavage and hence reduced the abundance of antibodies that bind epitopes concealed by uncleaved prM. They concluded that CD8+ T cells are essential for protection against ZIKV infection.

This is a well written manuscript reporting a very detailed dissection of humoral and cellular immunity against Zika. The findings are interesting and adds another important piece of evidence that challenges the conventional assumption that neutralizing antibodies are absolutely required for protection against disease caused by ZIKV and other related viral infections. Data from passive immunization and adoptive T cell transfer experiments are particularly compelling. Several areas could benefit from additional attention. These are

We thank the reviewer for their thoughtful and constructive feedback, which has helped us to clarify and strengthen our manuscript. The following bullet points provide a brief summary of our responses, which are discussed in more detail below:

- We have provided additional justification for our experimental design, including the rationale for assessing viral burden at day 3 post-challenge and for using the retro-orbital inoculation route, and referenced established precedents in the field and our prior work.
- We have clarified how T cell and Ab responses were analyzed and presented, including the treatment of background controls, statistical comparisons, and gating strategies.
- Where appropriate, we have toned down interpretations, particularly regarding the kinetics of nAb titers, and clarified that the different responses to vaccine constructs should not be assumed to be the same across mouse strains with distinct interferon signaling profiles.
- We have also refined our discussion of the relationship between viral RNA and infectious particles, emphasizing the complementary roles of antibodies and T cells in early viral control.
- Finally, we have corrected wording related to Ab “clearance” versus “neutralization” and expanded the discussion to include the potential contribution of C8-like (EDE-like) antibodies as a mechanistic hypothesis for the distinct outcomes observed with the WT and FLM vaccines.
- We hope that the reviewer will agree that, collectively, these revisions improve the clarity, precision, and scientific context of our manuscript while maintaining its original scope and conclusions.

1. The time interval between challenge infection and termination of experiment is very short (3 days). Moreover, ZIKV challenge infection was through inoculating the virus directly into retroorbital vein, which is unusual. Justification on such an experimental design, with reference to

previous findings, would be helpful for readers to contextualise the informativeness of infection outcome through an unconventional route of inoculation and measured at day 3 post challenge infection.

We welcome the opportunity to clarify the rationale behind our experimental design. Day 3 post-challenge is a well-established time point for assessing the influence of vaccination on viral burden, particularly in studies focusing on T cell-mediated immune responses. The goal of vaccination is to enable rapid mobilization of memory T and B cells upon viral challenge; therefore, measuring viral load at this early stage allows vaccine-induced memory responses to be evaluated before the onset of the primary adaptive immune response, which typically develops later and could otherwise obscure the contribution of vaccine-elicited immunity. Assessing viral burden at an early time point therefore provides a more accurate representation of the protective efficacy conferred by vaccine-elicited pre-existing memory T cells, rather than by newly generated (primary) effector cells. Our observation that infectious viral particle burden was substantially reduced at day 3 post-challenge further supports the validity and appropriateness of this time point. We have added an explanation for the selection of this time to the Results section (Lines 175–177, page 6 and 7).

Regarding the route of inoculation, retro-orbital (RO) injection ensures precise viral delivery and ensures experimental consistency across all animals, especially when compared with more variable routes of viral administration (e.g., *via* subcutaneous or intra-footpad). Importantly, our previous work has demonstrated that comparable T cell response profiles are induced following ZIKV challenge *via* the RO and subcutaneous (intra-footpad) challenge (PMID: 30677097). We have added a justification for the use of the RO route in the Results and Methods sections (Lines 172–174, page 6; Lines 663–665, page 22).

In summary, the use of the RO administration route and evaluation of viral burden at day 3 post-challenge not only facilitate consistency between experiments and allow specific assessment of the memory response to vaccine-induced memory immune protection, but they are also consistent with accepted practices in the field.

2. Line 26. There has been a change to the nomenclature of this genus. Flavivirus is now Orthoflavivirus.

We thank the reviewer for pointing this out. The nomenclature has been corrected throughout the manuscript.

3. Lines 131-132. Please indicate the identity of the mAb that was used to detect domain III of ZIKV E protein.

We employed an anti-ZIKV EDIII mAb (clone 09), which cross-reacts with the EDIII sequence encoded by the vaccines as well as ZIKV strain SD001 used here (MyBiosource, #MBS2564004). We have added this to the first mention of the mAbs used in the Results section and to the Methods section (Lines 118, page 5 and 779–780, page 25).

4. The gating strategy for flow cytometry and ICS assay should be shown as a supplemental figure.

Thank you for this suggestion. We have added the gating strategy used for flow cytometry of cell subsets and cytokine-producing cells as Supplementary Figures S1 and S2, respectively.

Descriptions of the strategies have also been added to the figure legends (Lines 1089–1111, page 36)

5. Lines 147-156 and Figures 2c and 2d. Please indicate if values plotted are after subtraction of values obtained from unstimulated negative controls. This would be helpful to differentiate Ag-specific T cell responses from overall T cell response.

The values presented for the abundance of T cells are not the net counts after subtraction of unstimulated negative controls (medium/DMSO-treated cells) because the values were negligible relative to the numbers in the peptide-stimulated cultures. We have clarified this point in the Methods section to ensure transparency (Lines 831–839, page 27). The non-specific *vs* Ag-specific T cell responses were differentiated by comparing the responses in cells from Z-prM/E-WT- and Z-prM/E-FLM-vaccinated mice with those from the pVAX1 control-vaccinated mice. The raw values from unstimulated controls can be supplied to further support this decision, if requested.

6. Lines 169-172. Conventionally, 4-fold or greater rise in antibody titers following infection is measured at least 2 weeks apart. This 3-day interval is too early to support the interpretation that the vaccination induced nAb titers were already at maximal levels before infection.

While we agree with the reviewer that the 3-day interval post-challenge is too early to capture the rise of a de novo Ab response, memory Ab responses typically occur more rapidly. As for the T cell response, we selected this early time point because we anticipated that the Z-prM/E vaccine-induced memory response would occur more rapidly, and we wished to ensure that this was the only response captured. In light of the reviewer's concern, we have revised the text to highlight the expected lower Ab response in control *vs* vaccinated mice (Lines 179–183, page 7).

7. Lines 178-183 and Figure 3c. Please show statistical test results between CD4+ T cells in FLM vs WT animals. Please also indicate, similar to comment #4, how the T cell data was processed for plotting on the graphs in 3c and 3d.

We apologize for this oversight and have now added the statistical results (P value or not significant [ns]) to all panels in which differences between the WT *vs* FLM as well as CON *vs* WT or FLM conditions were missing. Although these comparisons were done, we chose to omit the "not significant" symbols to maintain figure clarity and readability. This has now been corrected. The gating strategy used for T cell analysis has been provided as Supplementary Figure S1 and explained in the legend, which we hope will clarify the data processing procedure for Figures 2c/d and 3c/d.

8. Lines 187-189. The authors have assumed the T cell response to the two vaccine constructs in C57Bl/6 mice, with and without IFNAR knockout, are similar in trend; pre-challenge T cell counts were not measured in IFNAR -/- mice. The effect of type-I IFN response on T cells has been shown previously (Crouse et al, Nat Rev Immunol 2015) and thus differences in CD8+ T cell response to FLM vs WT vaccination in IFNAR-/- mice, although unlikely, cannot be completely excluded. The assumption applied in this final sentence should be clearly stated to avoid misleading readers.

We agree with the reviewer that T cell responses to vaccination cannot be assumed to be equal in C57BL/6 WT and C57BL/6 *Ifnar1*$^{-/-}$ mice, given the documented role of type IFNs on T cells, and we did not intend to imply that. Rather, our statement referred specifically to the differences in quality and quantity of the T cell response post-challenge between the mice vaccinated with Z-

prM/E-WT and those vaccinated with Z-prM/E-FLM. To address the reviewer's comment, we have now included an evaluation of T cell immunogenicity (i.e., T cell responses before challenge) in the C57BL/6 *Ifnar1*$^{-/-}$ mice. We observed that the vaccines elicited comparable CD4^{+} and CD8^{+} T cell responses in the C57BL/6 *Ifnar1*$^{-/-}$ mice (revised Fig 2e and f) as previously shown in C57BL/6-WT mice (original and revised Figure 2c and d) (Lines 152–165, page 6).

9. Lines 196-198 and figure 3e. The finding of complete abrogation of infectious ZIKV despite detectable ZIKV RNA in both organs and sera is not surprising although interesting, in light of the explanation offered in Figure 5. The authors suggest that antibodies from WT but not FLM infection are more potent in neutralizing mature ZIKV. The extended logic from this line of thinking is that there are more mature viral particles in vivo compared to ZIKV cultured in C6/36 cells. Would not the serum viremia (Figure 3e, upper row) and RNAemia (Figure 3e, lower row), argue against such an explanation?

The reviewer correctly notes that if WT-elicited nAbs were the dominant mechanism of early viral control *in vivo*, one would expect lower serum viremia in WT- versus FLM-vaccinated mice, yet Figure 3e shows comparable levels at day 3 post-challenge. We believe this apparent discrepancy is explained by the fact that both vaccines elicit protective immune responses, albeit through different mechanisms. In WT-vaccinated mice, both nAbs and CD8^{+} T cells contribute to viral control (as demonstrated by our serum transfer and T cell adoptive transfer experiments). In FLM-vaccinated mice, CD8^{+} T cells compensate for the impaired nAb response, achieving equivalent early viral control. The comparable day 3 viremia thus reflects the net outcome of mechanistically distinct but functionally equivalent immune responses at this early time point. Importantly, the distinction between these mechanisms becomes apparent at later time points: Z-prM/E-FLM-vaccinated mice fail to maintain long-term protection (Figure 4), consistent with the loss of T cell-mediated control in the absence of nAbs.

10. Line 228. T cell counts shown in Figure 3d were measured after challenge, not after vaccination.

This error has been corrected (now Line 185).

11. Line 238. Should "reduction" be "increase" instead?

This error has been corrected (now Line 291).

12. Lines 240 and 243. It cannot be ascertained that the antibodies from WT vaccinated animals "cleared" infection instead of neutralizing infection. Please rephrase.

We agree with the reviewer and have modified the wording accordingly (Lines 295 and 298).

13. Lines 285-288 and Figure 4j. The interpretation of viral RNA elimination in HLAB*0702 ifnar-/- mice in FLM vaccinated animals with intact CD8+ T cell contrasts with findings shown in Figure 3e. The difference may be due to the detection limit shown in dotted lines in these respective figure panels. Is there an error here?

In response to the reviewer's query, we re-evaluated the limit of detection (LoD) in original Figure 4j (now Figure 6d) and discovered that a few values had been miscalculated. We have corrected the figures accordingly in the revised version. Importantly, although the corrected LoD changes the visual threshold, it does not alter the overall interpretation of the data. If we could not calculate a Ct value or, in a few rare cases, the Ct value was high and failed to generate reliable

amplification curves, the serum samples are represented as being at the LoD. Thus, they were considered to have undetectable viral RNA levels regardless of the corrected LoD. The main conclusion, that viral RNA was not detectable in these samples, remains unchanged. We have also clarified this in the Methods (Lines 705–716, page 23).

14. Figure 5. Could WT vaccination produce C8-like antibodies that have shown to correlate with protection, at least against dengue (Mpingabo et al, Sci Transl Med 2025)? Would it be possible to do a competition ELISA to determine if WT but not FLM vaccinated animals elicited C8-like antibodies that could have more precisely explain the findings?

The recent work by Mpingabo et al. (*Sci. Transl. Med.*, 2025) demonstrated that C8-like Abs recognizing a quaternary envelope dimer epitope (EDE) are associated with broad neutralization of mature virions from all four DENV serotypes and correlate with protection against DENV infection, which was reported as reduced risks of symptomatic dengue and severe dengue. It is therefore plausible that the Z-prM/E-WT vaccine could elicit analogous Abs, potentially explaining the superior viral control in mice receiving serum from Z-prM/E-WT *vs* FLM-vaccinated mice. We have acknowledged this possibility in the revised Discussion and propose to perform competition ELISAs to evaluate whether WT, but not FLM, vaccination induces EDE-like Abs in future studies (Lines 590–599, page 19).

Minor comments:

Line 27. Change neurological injury to neurological syndromes. Done.

Line 145. Close bracket is missing at the end of this sentence. Done.

Line 359. Perhaps “under-appreciated” is more accurate than “unappreciated”? Done.

Lines 75-78: As far as I’m aware, there is no clinical or epidemiological evidence to show that prior DENV/WNV/JEV infection worsens risk of severe ZIKV infection outcomes. This should be included as a caveat to the statements on experimental findings.

We agree that clinical and epidemiological evidence linking prior DENV, WNV, or JEV infection to increased ZIKV severity is currently lacking. However, given the links between exacerbation of disease and prior immunity for other flaviviruses, we feel that it is plausible for this to occur with ZIKV infection and/or vaccination. We suspect the current data gap is due to the limited scale of recent ZIKV outbreaks, which has restricted the opportunity for comprehensive clinical evaluation and sample collection to assess links between prior orthoflavivirus infections and pre-existing immunity. Following the reviewer's suggestion to keep the introduction focused, we have removed these lines to avoid a lengthy discussion of this caveat and to shorten the overall length of the section.

Reviewer #2 (Remarks to the Author):

In this study, the authors investigated the mechanisms of protection offered by DNA vaccines against Zika Virus which possess a mutated fusion loop region of the E protein EDII ectodomain (Z-prM/E-FLM). In previous studies, such vaccines have been shown to be promising in their capacity to protect against Zika virus infection, and are less likely to induce sub-neutralising antibodies, which can promote severe disease through antibody-dependent enhancement of infection. The findings show that while CD8+ T cells are not strictly required for protection conferred by Z-prM/E-WT vaccines, CD8+ T cells are indispensable for protection induced by Z-prM/E-FLM vaccines. Overall, the study is well executed and offers interesting new insights into the mechanisms of protection by FLM vaccines. The manuscript, however, lacks the depth of understanding of immune response kinetics following vaccination +/- infection to fully define vaccine-induced immunity. Comments are provided below.

We thank the reviewer for their thoughtful and constructive feedback, which has allowed us to clarify and strengthen several aspects of our study. The following bullet points provide a brief summary of our responses, which are discussed in more detail below:

- We have expanded the rationale for using C57BL/6 *Ifnar1*$^{-/-}$ mice, explained the necessity for this model to allow productive ZIKV infection, and referenced prior work that established its validity.
- We also acknowledge the limitations of this model for studying T cell responses and have clarified how this does not affect our comparative conclusions between vaccinated groups; in particular, we have added T cell immunogenicity data obtained in C57BL/6 *Ifnar1*$^{-/-}$ mice as well as C57BL/6-WT mice.
- We have addressed the reviewer's concern regarding the depth of understanding by included new data on IFNγ single-producing CD8^{+} T cells, as well Ab and T cells responses at later time points, thereby providing a more comprehensive view of the immune response.
- To complement these experiments, we evaluated potential effector mechanisms of CD8^{+} T cell-mediated protection by performing *in vivo* IFNγ blockade experiments.
- We have justified the choice of day 3 post-challenge as an established time point to evaluate vaccine-induced memory responses, while recognizing that future studies examining later time points and local lymph node responses will further enrich our understanding of localized immune kinetics and protection mechanisms.
- We hope that the reviewer will agree that, taken together, these revisions improve the clarity, rigor, and interpretability of the manuscript while remaining consistent with the study's scope and objectives.

Major comments:

•The authors utilise C57BL/6 Ifnar1-/- mice to perform challenge experiments with Zika virus following vaccination. While I appreciate that this is a common and necessary approach to investigate Flavivirus infections, the rationale for doing so needs to be made clearer in the results section for a broader audience. The authors should also reference previous studies in which this approach has been employed.

We agree with the reviewer and have now addressed this by clarifying the rationale for using C57BL/6 *Ifnar1*$^{-/-}$ mice in the Results section ((Lines 152–165, page 6; Figure 2e–f). This

explanation facilitates the transition from the existing C57BL/6 WT mice immunogenicity data to the new C57BL/6 *Ifnar1*$^{-/-}$ mice immunogenicity data (Figure 2e–f) prior to the use of C57BL/6 *Ifnar1*$^{-/-}$ mice for the viral challenge experiments.

•In addition to the comment above, the use of Ifnar1-/- for the study of T cell responses has inherent limitations, given the importance of IFNa to T cell expansion and effector functions. Please address how these limitations potentially impact the findings of this study.

We agree that type I IFNs play well-documented roles in CD8$^+$ T cell priming, expansion, and effector differentiation across multiple viral systems, and we acknowledge that the absence of IFNAR signaling in our challenge model may alter the magnitude or quality of T cell responses compared with an immunocompetent host. Importantly, however, our study design mitigates this concern in two ways. First, our conclusions are based on *comparative* differences between Z-prM/E-WT- and Z-prM/E-FLM-vaccinated mice within the same *Ifnar1*$^{-/-}$ background, meaning that any effect of IFNAR deficiency on T cells applies equally to both groups and does not confound the comparison. Second, we now show that the two vaccines elicit comparable T cell responses in both C57BL/6-WT and C57BL/6 *Ifnar1*$^{-/-}$ mice (Figure 2c–f), demonstrating that the relative immunogenicity of the vaccines is preserved regardless of IFNAR status. We have added an acknowledgment of this limitation in the Discussion (Lines 541–555, page 17).

•In addition to investigating T cell responses in the spleen, the authors should also investigate CD4 and CD8 T cell responses in the draining lymph node following I.M. vaccination and whether responses at this site differ.

We agree that characterizing CD4$^+$ and CD8$^+$ T cell responses in the draining lymph nodes would be informative, particularly for understanding the early local dynamics of T cell priming following IM vaccination. However, the central question of our study is the mechanism of *systemic* protection, assessed by measuring viral burden in distal tissues (liver, brain, testes, and serum) following systemic challenge. For this purpose, the spleen was selected as the representative secondary lymphoid organ because it is the primary site of systemic immune surveillance and is where effector T cells that traffic to and protect distal tissues are maintained. Importantly, the functional relevance of splenic T cells in our model is directly validated by our adoptive transfer experiments (Figure 5e–f), in which purified splenic CD8$^+$ T cells from vaccinated mice conferred significant protection against ZIKV in recipient mice. These data confirm that the spleen contains the CD8$^+$ T cell population responsible for the protective efficacy observed in our study. We acknowledge that draining lymph node analysis could reveal differences in early T cell priming kinetics, tissue-resident memory precursor generation, or germinal center dynamics between the WT and FLM vaccines that are not captured by splenic analysis alone. We have added this as a limitation in the Discussion and propose it as a priority for future investigation (Lines 541–555, page 17).

•Although the authors show that CD8+ T cells are necessary for the protective effect of Z-prM/E-FLM vaccines, it is not clear exactly how CD8+ T cells contribute to this. In the Discussion, the authors speculate that polyfunctional T cells are necessary for protection against vaccine-elicited immunity against other flaviviruses such as DENV. To further elaborate as to how CD8+ T cells elicited by FLM vaccination confer protection, the authors could assess whether the adoptive transfer of CD8+ T cells from IFNg deficient mice, for example, will still confer protection against

ZIKAV. If this is not feasible, the use of an IFNg blocking antibody following adoptive transfer may also be informative.

We thank the reviewer for this suggestion to further investigate the contribution of $CD8^+$ T cells to protective efficacy. We have now performed the suggested *in vivo* IFN-γ blockade experiments during adoptive transfer of $CD8^+$ T cells from vaccinated mice to evaluate the role of IFN-γ in protective efficacy (Figure 5g-h). We transferred purified $CD8^+$ T cells from Z-prM/E-FLM-vaccinated mice into naïve C57BL/6 *Ifnar1*$^{-/-}$ mice and challenged the mice with ZIKV the next day. The mice were treated twice with a depleting anti-mouse IFN-γ mAb or IgG1 isotype control mAb, once 1 day before ZIKV challenge and once 1 day after. Tissues were harvested for analysis on day 3 post-challenge, as in all other experiments. Our data show that IFN-γ depletion significantly increased the level of ZIKV infectious particles in the brain, spleen and serum compared to the levels in isotype mAb-treated mice. We did not observe any effect of IFN-γ depletion in the pVAX1 control mice. These data confirm that the mechanism of $CD8^+$ T cell-mediated protection involves IFN-γ production, at least in part. In the revised manuscript, we have added the IFN-γ blocking experiment as Figure 5g-h and modified the text accordingly in the Results section (Lines 317–328, page 11), Methods section (Lines 764–766, page 25), and Figure legends (Lines 1004–1011, page 32 and 33).

•Protective immune responses towards vaccination and/or infection cannot be defined by the analysis of one time-point only:

- In Fig 2: why was only d14 post the 3rd vaccine dose investigated? While the authors found no differences in antibody responses between 2 vaccination protocols, the timing of these responses post vaccination as well as durability of these antibody responses may differ substantially across vaccine compositions. This needs to be investigated. Similarly, early recruitment and longevity of T cell responses should be investigated. Kinetics rather than one time-point analyses need to be shown. For example, what happens at 5 days post dose 3 and at 3 months after 3 vaccinations? Please also graph IFN-g single-producing CD8+ T cells to understand total (rather than polyfunctional) CD8+ T cell responses. Representative FACS plots across different cytokine-expressing T cells populations as well as across different vaccine regiments plus controls should be shown.
- Similar comments go for Figure 3 re immune response kinetics and single-IFNg producing CD8+ T cells.

We thank the reviewer for the insightful suggestion to evaluate later time points, which are indeed critical for understanding T cell recruitment and memory formation. We also agree that the durability of vaccine-induced protection is a vital metric. We had originally focused on the 2-week time point because of the expected peak of the vaccine-induced adaptive immune response. In the revised manuscript, we have added endpoint Ab titers (Figure 4b) and T cell analyses (Figure 4c–d) measured 12 weeks after the final vaccine boost. Notably, the results reveal a striking difference between the two vaccines in eliciting short-term protection (14 days) compared with long-term protection (12 weeks) (Figure 4e–f). Specifically, we demonstrate that, despite there being no significant differences at 12 weeks in the magnitude of $CD4^+$ and $CD8^+$ T cell responses in mice vaccinated with Z-prM/E-WT *vs* Z-prM/E-FLM, the FLM vaccine failed to protect against infection, as measured by tissue burden of ZIKV infectious particles or ZIKV RNA. Additionally, in response to the reviewer's suggestion, we have included data for IFNγ single-producing CD4+ and $CD8^+$ T cells in addition to the polyfunctional T cell populations previously presented. Although it appears that changes in polyfunctional $CD8^+$ T cells are more closely associated with vaccine-induced protective immunity, we agree that presenting both datasets offers a more

comprehensive view of the T cell response. We have also added representative FACS plots illustrating the analysis of cytokine-producing T cell subsets in peptide-stimulated splenocytes from CON, WT, and FLM-vaccinated mice as well as negative and positive control conditions (medium/DMSO and PMA/ionomycin, respectively) (Supplementary Figure S2).

-Fig 4: Why was only d3 after infection investigated?

Day 3 post-challenge is a well-established time point for assessing the influence of vaccination on viral burden, particularly in studies focusing on T cell-mediated immune responses. Measuring viral load at this early stage allows the focus to be on vaccine-induced memory responses, before the onset of the primary adaptive immune response (typically developing at ~day 4–5 and peaking at ~day 7 after ZIKV infection in mice), which could obscure the contribution of vaccine-elicited immunity. Assessing viral burden at an early time point therefore provides a more accurate representation of the protective efficacy conferred by pre-existing memory T cells, rather than by newly generated effector cells. We note that we do observe a substantial reduction in infectious viral particles at day 3 in vaccinated mice, further supporting the relevance of this time point.

•Gating strategy needs to be shown.

We have included the gating strategy as Supplementary Figure S1 and the process is explained in detail in the figure legend.

•Please show representative FACS plots, either as part of the main figure or as a supplementary figure. In particular, it would be good to see representative flow cytometry plots for the intracellular cytokine staining studies (including the relevant unstimulated controls or equivalent); also as per comments above.

We have included these data as Supplementary Figure S2, which contains representative FACS plots illustrating analysis of the cytokine-producing $CD4^+$ and $CD8^+$ T cell subsets in peptide-stimulated splenocytes from CON, WT, and FLM-vaccinated mice, as well as negative and positive control conditions (cells treated with medium/DMSO and PMA/ionomycin, respectively).

Minor comments: The Introduction is too detailed; a more concise approach is recommended.
•Please be consistent with the virus nomenclature across the manuscript– either Flavivirus or Orthoflavivirus (I believe the latter is more up to date with current standards) when referring to the genus.

We have reduced the size of the introduction by condensing the second paragraph, and corrected the nomenclature to use orthoflavivirus throughout the manuscript.

Reviewer #3 (Remarks to the Author):
The manuscript evaluates two plasmid DNA vaccine candidates encoding Zika virus (ZIKV) prM and E proteins in Ifnar1$^{-/-}$ mice, comparing wild-type (E-WT) and fusion loop mutant (E-FLM) forms. The authors show that both vaccines protect against infection, but through different mechanisms: E-WT induces neutralizing antibodies against mature and immature virions, whereas E-FLM relies on CD8$^+$ T-cell–mediated protection due to impaired prM cleavage. The study is methodically organized. Some results and interpretations are presented overly positive, not necessarily describing the data as presented in the figures, and at times erroneously concluding. In general, the conclusions in this manuscript should be tempered.

The novelty of the work is, however, limited. The authors have already shown the role of CD8$^+$ T cells in response to ZIKV infection (PMID: 28081442). More recently, using an NS3-based vaccine, the authors have shown that CD8$^+$ T cells are necessary and sufficient for (i) preventing mortality in lethally infected adult mice and (ii) protecting fetuses from growth restriction in infected pregnant mice after ZIKV challenge (PMID: 33148638). In addition, Richner et al. (2017, Cell, DOI: 10.1016/j.cell.2017.02.017; already in collaboration with the lab from which current study originates) previously used an identical prM/E sequence and fusion loop mutation (yet as mRNA vaccine) to demonstrate protection from disease, lethal challenge, and induction of neutralizing antibodies, showing also that the effect was independent of MHC class I haplotype by using three different mouse lines). Similarly, Thompson et al. (2022, PLoS Negl Trop Dis, DOI: 10.1371/journal.pntd.0010588) reported that even a single amino acid substitution between neutral, nonpolar residues in the flavivirus fusion loop can significantly alter the generation of neutralizing antibodies. These prior findings capture the main conceptual and mechanistic points presented here, leaving the current work with limited mechanistic novelty.

We thank the reviewer for their thorough and constructive feedback, which helped us to clarify methodological details, refine our interpretations, and highlight the novelty of our findings. The following bullet points provide a brief summary of our responses, which are discussed in more detail below:

- We present new data on short- versus long-term protection to extend our findings and show that Z-prM/E-FLM can elicit CD8$^+$ T cell-mediated short-term protection but fails to maintain this long-term. This observation highlights the interplay between Ab and T cell responses.
- We have evaluated potential effector mechanisms of CD8$^+$ T cell-mediated protection by performing in vivo IFN-γ blockade experiments and complemented this with new data on IFN-γ single-producing CD8$^+$ T cell responses. In combination with data on Ab kinetics at later time points, these new observations provide a more comprehensive view of the immune response.
- We have re-evaluated and standardized the limits of detection (LoD) for both qRT-PCR and focus-forming assays, added detailed explanations for their determination, and corrected any inconsistencies across figures. We note that this does not impact our conclusions.
- We have clarified the observed differences in neutralization capacity between C6/36 and Vero-derived virions.
- We have added details regarding the HLA-B*0702 *Ifnar1*$^{-/-}$ mouse strain and clarified their use for assessing human-relevant T cell responses.
- We now include survival data from vaccinated mice.
- We have adjusted figure formatting errors and tempered our interpretations where statistical differences were not significant.

- We hope that the reviewer will agree that, collectively, these revisions strengthen the transparency, accuracy, and interpretability of our results while preserving the overall novelty and conclusions of the study.

We acknowledge that prior work, including our own, has established the critical role of $CD8^+$ T cells in protection against ZIKV infection and following vaccination (PMID: 28081442; 33148638). However, there are important specific differences between that and the present study:

First, our previous vaccine study primarily focused on non-structural antigen NS3, which is a target of T cells, but not B cells, and demonstrated $CD8^+$ T cell-mediated protection in the context of the NS3 vaccine. In contrast, the present study reveals that the structural antigen prM/E, commonly associated with Ab-mediated immunity, also elicits protective $CD8^+$ T cell responses. This finding provides new mechanistic insight into how ZIKV prM/E-FLM vaccines confer protection when neutralizing Ab responses are suboptimal. To our knowledge, this is the first demonstration that a ZIKV prM/E-FLM vaccine drives $CD8^+$ T cell-dependent protection *in vivo*.

Second, we agree that Richner et al. (PMID: 28222903) used a similar prM/E construct with identical FL mutations in an mRNA platform and demonstrated that prM/E-FLM vaccine-induced protection in mice. However, that study only measured the vaccine-elicited Ab response in terms binding and nAb titers and did not evaluate T cell responses or the mechanisms of vaccine-induced protection. Our study therefore goes beyond the work of Richner et al. by directly dissecting the mechanistic role of vaccine-elicited T cells and specificity of anti-E protein Abs; demonstrating cellular rather than expected humoral mechanism of protection; and by examining mechanistic events contributing to protection.

Third, our findings provide new insight into the development of Z-prM/E mRNA-based vaccines in the event that they need to be rapidly deployed during large ZIKV outbreaks. Specifically, our study goes beyond the work of Thompson et al. (PMID: 35793354) by revealing that FL mutations not only alter nAb profiles but, importantly, they also elicit $CD8^+$ T cells that confer $CD8^+$ T cell-dependent short-term protection (Figure 3) but fail to maintain long-term protection (Figure 4). This is a crucial aspect of Z-prM/E-based vaccine development. We also show that the short-term protection is dependent on IFN-γ production. Therefore, while we acknowledge the vital contribution of prior studies to related aspects of our work, we maintain that our study significantly advances ZIKV vaccine development by providing novel mechanistic evidence linking antigen structural modifications to $CD8^+$ T cell-mediated protection and its impact on the durability of protection.

Unlike their previous work and exhaustive evaluation of the animal model, in this manuscript the authors chose to use viremia/detection of viral RNA or virus dissemination as sole readout after challenge. Hereby analytical procedures seems often to be flawed, as discussed in details below, having direct consequences in what the authors decide as no viremia (as proxy for “protection”), hence contribution of specific T cell population or nAbs to protection against viremia. Additional readouts commonly used in this model, like clinical score, body weights loss, mortality, would have been more necessary to support the conclusions and proposed mechanisms. Please find below specific comments and points of attention:

We acknowledge that additional readouts of protection can provide complementary information on vaccine efficacy. In the revised manuscript, we have added a new study evaluating survival, clinical scores, and body weights in Z-prM/E-vaccinated mice following challenge with a lethal dose of ZIKV (Figure 3f–i). Importantly, this study confirmed our overall conclusions and showed that, while mice immunized with the control pVAX1 vaccine succumbed rapidly (within 6 days) of

challenge with a lethal dose of ZIKV, all of the mice immunized with ZIKV prM/E-FLM or Z-prM/E-WT vaccines not only survived the challenge, but also exhibited reduced disease scores and had no significant body weight loss, consistent with robust vaccine-mediated protection. We have included these data in Figure 3 and modified the text in the Results section (Lines 211–220, page 8), Methods section (Lines 665–669, page 22), and Figure legends (Lines 953–956, page 31) accordingly.

Limit of detection: The authors should provide information on how the limit of detection (LoD) was determined and, specially, why is it so variable across experiments?

As explained in the Methods section (line 545-547) “Viral RNA concentrations were determined from a standard curve composed of four 100-fold serial dilutions of in vitro-transcribed RNA from ZIKV strain FSS13025 and were normalized to the 18S rRNA levels in the same samples to account for variations in RNA input.” This implies that ZIKV RNA copies can be determined with a defined LoD. Further normalization with 18S rRNA allows the comparison of samples between/across experiments, but should not affect the LoD. However, for instance in Figure 4h and Figure 4j there are big discrepancies regarding the LoD even when comparing same tissues (LoD in liver 10e-2 vs. 10e0; LoD in Serum 10e0 vs 10e4). One such examples is a ~10.000 fold difference in the LoD of serum samples from Figure 4h and 4j, meaning that, technically, the authors cannot detect 9000 ZIKV RNA copies in the serum. Obviously, this doesn’t mean that there is no viremia, it shows that the methodology is not reliable. Definitely, such discrepancies spanning several orders of magnitude do not allow to consistently score for vaccine mediated protection. Similarly, the LoD for FFU/g of tissue should be consistent. As mentioned above, survival and clinical scores should have been measured in this study to more carefully conclude on the protective role of CD8+ T cells or antibodies. This problem with the LoD seriously hinders the conclusions drawn in the whole manuscript.

We thank the reviewer for raising this important point, which prompted us to carefully re-examine our LoD determinations across all figures. We identified and corrected numerical errors in the LoD calculations in the original Figures 4h and 4j (now Figures 6b and 6d), which were the primary source of the large discrepancies noted by the reviewer. Specifically, the LoD values for serum qRT-PCR in the original Figure 4j had been miscalculated. To clarify the underlying methodology: for the FFA, the LoD is defined as the virus dilution yielding a single focus in duplicate wells at the lowest sample dilution, normalized per gram of average organ weight for that experiment. Because organ weights differ between tissue types, the LoD differs across organs but should be (and now is) around the same values for the same organ across experiments. For qRT-PCR, viral RNA was quantified from a standard curve of *in vitro*-transcribed ZIKV RNA and normalized to 18S rRNA to account for variation in RNA input. The LoD was determined from the Ct corresponding to the lowest reliably detectable RNA concentration (Ct ≈40.1 or 50.1), adjusted by the average 18S rRNA Ct for each organ type. Samples for which no Ct was obtained or that yielded extremely high Ct values without valid amplification curves are plotted at the LoD and represent undetectable viral RNA.

We acknowledge the reviewer's broader point that viral burden measurements alone have limitations as a readout for protection. In response, we have now included survival and clinical score data for both Z-prM/E-WT- and Z-prM/E-FLM-vaccinated mice (new Figure 3f–i), which independently confirm the protective efficacy observed in the viral burden assays. We have also added a detailed description of the LoD determination for each assay and tissue type to the Methods section (Lines 705–716, page 23) and corrected all figures for consistency. We believe these additions (corrected LoDs, transparent methodology, and orthogonal clinical endpoints) collectively address the reviewer's concern.

Figure 2b vs 5b: The authors show that antibodies generated by Z-prM/E-FLM are much less efficient inhibiting mature virions produced in Vero cells (Figure 5b). However, in Figure 2b, the same serum from Z-prM/E-FLM perfectly inhibits both mature and immature virions produced in C6/36 cells. How do the authors reconcile this incoherence?

We thank the reviewer for pointing this out. The apparent discrepancy arises primarily from the cell lines used for the virus production, as noted by the reviewer, which has been shown to directly influence the maturation state of the virions and therefore their susceptibility to Ab neutralization. Maciejewski et al. (PMID: 32522807) showed that the ability to neutralize mature ZIKV virions is the Ab property that correlates with efficacy *in vivo*. Virions produced by Vero-hfurin cells are predominantly fully mature, whereas those produced by C6/36 mosquito cells contain a higher proportion of immature or partially mature particles. Our data are consistent with the findings of Maciejewski et al. and suggest that FLM vaccination elicits Abs that preferentially recognize immature or partially mature virions. This is the likely explanation for their strong neutralizing activity against C6/36-derived virus and reduced potency against mature virions produced by Vero-hfurin cells. In contrast, WT vaccination appears to induce antibodies capable of neutralizing mature and immature virions, consistent with a broader neutralizing capacity.

Figure 2c, d: Since the authors are reporting absolute numbers of specific cell populations, it should be clearly stated in the Methods, Figure legend, the total number of events acquired, or, as best practice, relative to a relevant population e.g. Live/CD3+ cells. In the same line, representative plots and gating strategies should be provided.

We thank the reviewer for this suggestion, and we have now added more detail on the absolute number of events collected to the Methods section (Lines 832–839, page 27). Briefly, 2 million splenocytes per sample were stained, and data were acquired for 30,000 live T cells (gated on viable CD3+ cells) per sample. The gating strategy and representative plots have been added as Supplementary Figures S1 and S2, and the details have been added to the legends.

Also, CD4+CD44high T cells, as shown in the axis and Figure legend are antigen experienced, and include also the Tcm (CD62L+), not only effector cells. Please correct accordingly (lines 176, 744, 762), or alternatively, please provide the quantification in the effector (CD44highCD62L-) population, which should be in any case the main contributor to IFNg, TNF and IL2 production. Out of curiosity, was there a specific rationale to check for a degranulation marker (CD107a) in CD4 T cells in the context of vaccination?

We agree that the $CD4^+$ $CD44^h$ population does not exclusively identify effector cells ($CD62L^-$) and includes central memory ($CD62L^+$) cells. We have corrected the terminology throughout the Results and Methods sections. Regarding the inclusion of CD107a as a marker, our rationale was to explore the cytolytic potential of the T cells induced by the Z-prM/E vaccines, because CD107a expression has been reported to correlate with protective immunity in the context of DENV and ZIKV infections (PMID: 28003809, PMID: 23580623, PMID: 26195744, PMID: 39989460). Although this parameter was not central to our conclusions, its inclusion captures the broader functional heterogeneity of vaccine-induced T cells and this may be of interest to other researchers in the field. We clarify this rationale in the revised Discussion section (Lines 481–485, page 15).

Figure 2: The authors emphasize that the effect is due to vaccination, but so far, their data support that both constructs elicit similar immune responses. What is different is likely the kinetics of viral replication in the presence of antibodies. The authors should discuss further on the impact of

these antibodies in allowing viral replication and inducing differential stimulation of T cells, due to, for instance, antigen presentation.

We thank the reviewer for this insightful comment and have expanded our Discussion to address how the quality and timing of the Ab response may differentially shape viral dynamics and, consequently, T cell stimulation (Lines 523–540, page 17). We speculate that antibodies elicited by the Z-prM/E-FLM vaccine, which are less efficient neutralizers of mature virions than those elicited by Z-prM/E-WT, may permit transient low-level viral replication that enhances antigen presentation and T cell activation without leading to uncontrolled infection. In contrast, the more potent antibodies induced by the Z-prM/E-WT vaccine may be able to restrict viral replication earlier after challenge, resulting in reduced antigen persistence and T cell stimulation. This hypothesis aligns with our data and supports a scenario in which Ab quality influences antigen exposure and the breadth of the T cell response, rather than being a simple difference in immunogenicity between the two vaccines.

Lines 171-172: In our opinion, Figure 3b shows that the infection did not boost antibody response up to 3 dpi. The authors do not provide an antibody kinetics to demonstrate that antibodies were at maximal level at day 48. Since this are longitudinal data, data points at day 48 and 52 should instead be analyzed using a Wilcoxon signed-rank test (likely not normally distributed data).

We thank the reviewer for this important observation. We agree on both points. First, we acknowledge that our original interpretation that vaccine-induced nAb titers were already at maximal levels before challenge was not supported by the data, as we did not perform Ab kinetic measurements between vaccination and challenge. The data in Figure 3b show only that nAb titers did not increase between day 48 (pre-challenge) and day 51 (3 days post-challenge), which indicates the absence of a rapid anamnestic Ab response within this short window but cannot speak to whether titers had peaked prior to challenge. We have revised the text to reflect this more limited interpretation (Lines 179–183, page 7).

Second, we agree that because the day 48 and day 51 measurements represent paired observations from the same animals, a Wilcoxon signed-rank test is the appropriate statistical comparison. We have reanalyzed the data accordingly and updated Figure 3b and the associated text. The conclusions remain unchanged: there was no significant boost in nAb titers within 3 days of challenge for either vaccine group.

Lines 187-189: Based on the data provided this conclusion is overstated. The authors can conclude that animals vaccinated with the FLM had higher frequencies of T cell in response to infection. But this can still be induced by different replication kinetics of ZIKV after infection due to differences in anti E antibodies.

We apologize for the confusion; we did not intend to draw a conclusion regarding the FLM-induced Abs but were stating the results observed. We have rephrased this interpretation in the Results section (Lines 197–199, page 8)

Figure 3c-e: Same remark regarding gating choice and data representation as for Figure 2c, d.

We have provided the gating strategy used for Figure 2 in Supplementary Figure S1 and S2.

Line 199: What do the authors mean by ‘total anti ZIKV E protein IgG’? What does ‘total’ mean in this context?

We used this phrase to clarify that this was enumeration of “all anti-ZIKV E protein Abs” (by E monomer ELISA) rather than just nAbs, but we agree that the wording could be confusing. We have now deleted “total”.

Line 238: Should be ‘significant increase’.

We have corrected this error.

Line 240: The authors do not provide evidence to support that the virus was cleared. It may have never infected the tissue. It was also not ‘completely’ (1 animal with detectable virus in serum).

We used the term “cleared” in comparison to the pVAX1-vaccinated mice; however, the reviewer is correct and we have rephrased our results to avoid confusion.

Line 242: We would suggest replacing ‘full protection’ with a more descriptive, specific term e.g. protection against viremia. The authors did not check protection against lethal challenge, did not provide disease scores (e.g. body weight loss, clinical score), and one animal had detectable virus in the serum, hence not ‘full’ protection.

In the revised manuscript, we have added a new study assessing clinical scores, body weights, and survival for 15 days following challenge with a lethal dose of ZIKV (Figure 3f–i). Importantly, we observed that 100% of mice in both ZIKV prM/E-vaccinated groups survived and also exhibited reduced clinical scores and no significant body weight loss compared with the pVAX1-vaccinated infected mice. These data are consistent with robust vaccine-mediated protection over this time period. In addition to the figure, we have modified the text in the Results section (Lines 211–220, page 8), Methods section (Lines 665–669, page 22), and Figure legends (Lines 953–956, page 31).

Figure 4f: In general, the authors should temper their conclusions and restrict them to the actual evidence. Figure 4f shows that CD8 T cells contributes to reducing viral titers, but it also clearly shows that it’s not sufficient to protect against viremia since ZIKV is still widespread found and in the case of the brain, equally high as the control group. Such widespread virus dissemination, although significantly lower, can still produce similar mortality after r.o. inoculation of ZIKV.

We agree with the reviewer and have tempered our descriptions and conclusions throughout the revised manuscript.

Line 254: It’s not evident that in brain tissue virla load is ‘markedly reduced’. Please adapt the sentence to reflect what the representative result actually shows.

We have changed the wording to more accurately reflect the changes.

Line 271 and Methods: Please provide the genetic information or reference of the HLA-B*0702 Ifnar1 KO. It is not clear, nor clearly stated, that these animals express HLA-B*0702 as the only class I MHC.

HLA-B*0702 *Ifnar1*$^{-/-}$ mice express both HLA-B*0702 and mouse MHC I, but not mouse β2M; they express human β2M. We added this information in the manuscript (Lines 650–651, page 21).

Line 266-269: In our opinion, the use of an HLA-transgenic mouse here would be only useful to show that HLA-B*0702-restricted epitopes from ZIKV structural proteins are also protective against viremia, hence an element of translational value. We do not see how a transgenic MHC class I will alter B cell epitope dominance or antibody responses in general.

We agree with the reviewer and did not intend to draw conclusions regarding B cell or Ab responses from the HLA-transgenic mice experiments. Our objective was to evaluate whether the protective efficacy of the vaccines could also be observed in the context of ZIKV epitopes relevant to human T cell specificities, thereby strengthening the translational relevance of our findings. In addition, the data further demonstrate that the protection conferred by the Z-prM/E-FLM vaccine is observed in both C57BL/6 and HLA-B*0702 mouse strains.

Figure 5a: Please provide primary data e.g. the dilution curves.

As per the policy of the journal, we will share the raw data, including the primary dilution curves, to allow full transparency, for our ELISA results.

Line 299-304: Please temper the interpretation here, which in this case is also not correct. The differences shown in Figure 5a are not striking, statistically speaking there are no difference at all between WT and FLM. Please also see that the AUC adds an additional arbitrary dimension that amplifies the numerical value, consider providing primary data showing actual differences in OD values at the different dilutions.

We agree that the differences between WT and FLM groups in Figure 5a (now Figure 7a) are not statistically significant, and we have toned down our interpretation accordingly in both the Results and Discussion sections. We have retained mention of the observed trend while clarifying that it does not reach statistical significance. We present AUC because it allows capture of the broad binding ability across a range of serum dilutions, rather than a snapshot at one specific dilution. We will provide the raw OD values at each dilution to accompany the AUC analysis data, as per the journal policy.

Line 314, 430: This conclusion is also incorrect, and doesn't describe the data as presented in the figure. Z-prM/E-FLM vaccine is less efficient in generating nAbs, not 'incapable'. Figure 5b shows that 4 out of 11 vaccinated animals (i.e., 36%) do have nAbs comparable to Z-prM/E-WT.

We have revised the conclusion to more accurately reflect the data by stating that the group mean level of nAbs elicited by the vaccines is lower for Z-prM/E-FLM than for Z-prM/E-WT vaccines, and we have also added that a subset of Z-prM/E-FLM-vaccinated animals (4/11, 36%) developed nAb titers comparable to those seen in response to the WT-vaccinated animals. (Lines 402–408, page13).

Figure 5c: A relevant control for total protein should be included.

We agree that including a loading control is important for accurate normalization. In this experiment, however, we analyzed the supernatants of transfected cells producing WT or FLM virus-like particles (VLPs), rather than total cell lysates. As such, conventional intracellular loading controls (actin, GAPDH or tubulin) are not applicable. Instead, we probed with an EDIII-specific Ab as the internal protein reference, because this (unmutated) domain is present in the VLP and ZIKV preparations, allowing direct comparison across samples. We did not observe any detectable differences in the EDIII signal intensity between WT and FLM VLPs, nor between ZIKV

produced in C6/36 and Vero-hfurin cells. We have added this point to the Results section (Lines 417–421, page 14) and the Methods section (Lines 799–804, page 26).

Figure 2b, 3b, 5b: Please correct parenthesis size in y axis, right panel.

We have corrected this oversight.

Response to Reviewers Comments:

Reviewer #1 (Remarks to the Author):

This revised manuscript has addressed the issues present in the original version. The inclusion of new sets of data, particularly the lethal challenge ZIKV infection as well as challenge infection at 12 weeks post-vaccination, add insights. The edited text also provides a more balanced interpretation of the findings.

I have just two comments:

We thank Reviewer #1 for the positive assessment of our revisions and for confirming that the additional lethal challenge and 12-week post-vaccination experiments add valuable insights. We are particularly grateful for the recognition that the revised text offers a more balanced interpretation of our findings. The two specific comments are addressed individually below.

1. Lines 63-65. I am not sure this sentence is necessary to frame this study and can be removed to reduce an already lengthy introduction.

We agree with the Reviewer that this sentence is not essential for framing the present study, and we have removed it. The Introduction has been further tightened by trimming additional non-essential descriptive content (e.g., the sentence describing Guillain-Barré syndrome in adults), in line with the Reviewer's suggestion and the *Nature Microbiology* length guidance. These edits improve the flow of the Introduction without compromising the framing of the study (Introduction, lines 44–82).

2. Lines 663-669. I do not fully agree that a retroorbital inoculation is a superior approach compared to subcutaneous inoculation for evaluating vaccines. Delivering ZIKV directly into the vein would indeed likely provide a more consistent systemic level infection. However, such a route of inoculation shortens the incubation period in which ZIKV, following entry into a host, need to replicate to titers sufficient for systemic dissemination. A challenge infection at day 49 after priming vaccination could, besides just IgG antibodies and T cells, also contain IgM antibodies that could be more potent in neutralizing infection given their pentavalent structure (Singh et al, Cell 2022). A longer interval could see waning of IgM antibody titers. Moreover, not only is there no protection from FLM vaccination induced antibodies, but there could also be, with reduced antibody titer, a hint of infection enhancement in the liver (Figure 6b); a T cell depletion control would have clarified the data in Figure 4e (and f?). Having said that, I am not suggesting that such a control is necessary for Figure 4 as it would involve even more mice in the experiment. What I think should be considered, however, is that the route of inoculation may compromise the extent of long-term protection from ZIKV-specific T cells. Replication sites in the skin and the draining lymph nodes after ZIKV inoculation from an infected mosquito are sites for ZIKV-specific T cells to control infection and reduce systemic ZIKV dissemination. Direct intravenous infection route would thus bypass these sites of infection where ZIKV-specific T cells likely exert their antiviral effects. Perhaps a more nuanced interpretation could be considered.

We are grateful to the Reviewer for this thoughtful and nuanced critique. We fully agree that the retro-orbital (RO) inoculation route is not biologically superior to subcutaneous challenge and that it bypasses anatomical sites at which tissue-resident ZIKV-specific T cells likely exert important antiviral effects following natural mosquito-borne transmission. We have softened the original framing of RO inoculation throughout the manuscript so that this route is described as a methodological choice favoring reproducibility and systemic uniformity for comparative vaccine studies, rather than as a superior approach to subcutaneous challenge.

Specifically, we have revised the Methods (lines 469-479) to (i) acknowledge that intravenous/RO challenge shortens the dissemination window and bypasses the skin and draining lymph nodes,

where ZIKV-specific resident-memory T cells are likely to contribute to early viral control; (ii) note that our model may therefore underestimate the magnitude of T cell-mediated protection that would be observed after a natural mosquito-bite infection; and (iii) explicitly acknowledge that IgM antibodies present at the short-term challenge time point (day 49) may contribute to early neutralization in Z-prM/E-FLM–vaccinated mice given their pentavalent, avidity-enhanced neutralization capacity (Singh et al., Cell 2022, PMID 36402135), and that subsequent waning of IgM titers may be one of several factors contributing to the loss of long-term protection observed in this group at 12 weeks.

Reviewer #2 (Remarks to the Author):

The authors have reasonably addressed my queries in the revised manuscript.

We thank Reviewer #2 for the positive assessment and for confirming that our previous revisions have satisfactorily addressed their earlier queries.

I have one further comment about the new gating strategies included in Figs S1 and S2. These need to be clearly labeled on the figures with the information about the population gated and % frequency within the gate.

We agree that clearer labeling will improve interpretability of the gating strategy and ICS data. Extended data Figures S1 and S2 have been updated so that each gate is explicitly annotated with (i) the parent population, (ii) the marker combination used for the gate, and (iii) the percentage frequency within the parent gate. The figure legends (Extended data Figure S1 and Figure S2) have been updated accordingly to define each labeled population in the text. These changes have been propagated consistently across all dot-plot panels.

Reviewer #3 (Remarks to the Author):

The study by Chuensirikulchai and colleagues as been revised to large extent in scope and effort to explain, incorporating most of our earlier concerns, in particularly our criticism on LOD determinations and lack of challenge data in a lethal model.

We sincerely thank Reviewer #3 for the rigorous and constructive critique throughout the review process, and for acknowledging the substantial revisions, including the new lethal-challenge data and the refined LOD analysis. We have addressed each of the seven remaining points below.

Some minor oversights may still to be corrected as listed in below:
(1) Line 144, 482 and 931: CD107a is not a marker of cytotoxicity yet of degranulation. The study shows involvement of IFNg (that is also secreted by degranulation) but no expression of granzymes or perforins. As stated now, the authors imply CTL activity by CD8+ and CD4+ T cells. Likewise, in line 972, CD107a should not be called an “effector molecule”.

We agree, and we thank the Reviewer for this important correction. CD107a (LAMP-1) is a marker of lysosomal mobilization and surface exposure following degranulation, and in the absence of granzyme/perforin staining it cannot be used as a direct readout of cytolytic activity. We have systematically revised the manuscript so that CD107a is described exclusively as a degranulation marker and is no longer grouped with cytokines as an "effector molecule." Specifically: (i) in the Results sections and figure legends describing T cell phenotyping (lines 113; 759-760; 778; 801), CD107a-positive T cells are now described as "degranulating" rather than "cytotoxic", the previous statement that we analyzed CD107a as a "cytotoxic marker" has been changed to "degranulation marker reflecting mobilization of lysosomal contents," and the term "effector molecule" applied to CD107a has been removed; and (iii) the figure legends for Figures 2, 3, and 4 have been updated so that CD107a is consistently labeled as a "degranulation marker" rather than grouped with cytokines as an "effector molecule." We have also added a sentence to the Discussion explicitly noting that direct cytolytic activity (e.g., granzyme B/perforin expression or in vivo killing assays) was not assessed in this study, and that the functional consequence of CD107a mobilization in our model therefore remains to be formally established.

(2) Line 297: Claim of “full protection” is wrong. Protection from lethal infection is demonstrated yet still with clear evidence for an elevated disease score (Fig-2H) and several break-through events (detection of viral RNA of FFU) in multiple organs throughout the study. Likewise, even the more potent WT vaccines lost markedly in potency within only 3 month after the last boost (+Fig-4E).

We agree that "full protection" overstates the data, and we apologize for this imprecise wording. The text at line 297 has been revised to remove the claim of "full" or "complete" protection. The relevant passage now reads that “the $CD8^+$ T cell response induced by Z-prM/E-FLM vaccination was sufficient to provide robust protection against ZIKV.” (lines 242) noting that even the more potent Z-prM/E-WT vaccine showed a clear decline in potency by 12 weeks post-boost (Fig. 4e), and that durability of protection is a remaining limitation of both constructs.

(3) Line 539: Item, there is no evidence for “sterilizing” immunity consistently demonstrated. Even in the low-dose infections there are repeatedly events of virus detection. Likewise, sometimes sterilizing immunity is defined by “no elevation of Ab titers following challenge/re-exposure”. This criterium cannot be used here as animals were not followed up beyond 3dpi.

We agree, and we have removed all references to "sterilizing" immunity from the manuscript. We also note explicitly in the Methods (lines 477-479) that animals were terminated at 3 days post-challenge

in the short-term protection studies, precluding any assessment of sterilizing immunity by serological anamnesis.

(4) The discussion is massively expanded arguing for novelty, i.e. mainly a more in-depth mechanism-of-action study with higher resolution than earlier studies using the same test items (pDNA or saRNA vaccines) or comparable FL construct.

The Discussion has been substantially condensed and refocused. Specifically: (i) repetitive statements about the conceptual novelty of the prM/E-FLM construct have been removed or merged so that novelty is now claimed only once, in the context of the mechanism-of-action work; (ii) overlapping paragraphs comparing the present study with prior pDNA and saRNA FL vaccine studies have been consolidated into a single, shorter comparison paragraph; and (iii) the Discussion now opens with the principal mechanistic finding: the necessity and sufficiency of $CD8^+$ T cells, acting in part through IFN-γ, for Z-prM/E-FLM-mediated protection in the absence of mature-virion nAbs, before turning to interpretation, limitations, and implications. Overall, the Discussion has been reduced by approximately 25% (now lines 378-431), and the manuscript word count has been brought down to 4,700 words. We hope that these changes deliver the more focused, mechanism-centered narrative the Reviewer requested.

(5) Line 706: We doubt that a Ct value of >>40 or even 50 (i.e, a 10e15-times amplification!) can be considered reliable at all.

We agree that raw Ct values above ~40 cannot be considered reliable detection events. To avoid any confusion on this point, we have rewritten the description of the limit-of-detection (LoD) calculation in the Methods (lines 518-523). The revised text now makes clear that: (i) 40.1 (and, for instruments configured with a 50-cycle program, 50.1) is not a measurement but a conservative numerical placeholder programmed on the qPCR instrument to represent samples with undetectable amplification; (ii) the LoD per organ was computed by taking this conservative non-amplification value and normalizing it to the average 18S rRNA Ct of that organ, yielding an upper-bound LoD on the normalized vRNA scale; and (iii) samples with no detectable amplification or with raw Ct values >40 that failed to generate a sigmoidal amplification curve were censored at this LoD rather than treated as quantitative. The revised description therefore relies on the standard 40-cycle threshold and explicitly does not treat raw Ct >40 values as reliable measurements.

(6) In Fig-6B and D, there is still this odd 3log10 difference in LOD values for normalized vRNA counts between tissues (e.g. RNA isolated from liver vs. brain).

We thank the Reviewer for raising this important point. The apparent inter-tissue difference in normalized LoD is a direct mathematical consequence of the 18S normalization step rather than a measurement artifact. The 18S rRNA Ct values differ substantially between tissues in our experiments. For example, average 18S Ct values are ~14 in liver, 17–18 in testes, and 17–20 in brain, reflecting differences in cellular density and total RNA yield. Because the normalized vRNA value is calculated as a difference of Ct values (ΔCt between the ZIKV signal and the 18S internal control), tissues with abundant cellular RNA (and therefore lower 18S Ct) produce a more sensitive (lower) normalized LoD, while tissues with less cellular RNA produce a less sensitive (higher) normalized LoD, even when the underlying raw qPCR sensitivity is identical. Critically, the normalized LoD is highly consistent within each organ across animals and experiments (see dotted lines in Fig. 3e, Fig. 4e, Extended data Fig. 4b/d/f, and Extended data Figure S5), so within-tissue comparisons between vaccinated and control groups remain valid. We have added a brief explanatory sentence to the Methods (lines 518) to make this normalization-driven inter-tissue difference explicit to the reader.

(7) The western blot shown in Fig-7B is of very poor quality, whereby band resolution and

quantification remain difficult. This is getting critical as large parts of the expanded discussion argue along differential prME processing that should be supported by these data. I suggest at least discussing this technical shortcoming as a limiting factor possibility prohibiting a more vigorous interpretation of the data. Therefore, also the different E-domain-specific ELISAs (line383 and Fig-7) lack validation, specificity of analyses is rather announced.

Western blot: We thank the Reviewer for raising this legitimate technical concern. We acknowledge that the high background of the original blot reflects a technical limitation of our concentration step: secreted VLPs from transfected 293T supernatants were concentrated by ultrafiltration (Amicon Ultra), which co-concentrates serum and secreted background proteins. To improve resolution, we first concentrated the VLP supernatants using a larger pore size membrane (100 kDa instead of 3 kDa) to minimize the presence of irrelevant background proteins. Next, we titrated the loading amounts of all four samples to determine the ideal concentration for clear detection. Finally, we optimized the voltage parameters and run times to ensure optimal protein separation. These changes substantially reduce background and yield cleaner separation of prM and M bands. The updated blot replaces the original panel in Fig. 7c (renumbered 6c). Source images and uncropped scans will be provided in the Source Data file.

E-domain ELISAs: The recombinant E monomer, cysteine-cross-linked stable E dimer, EDI, and EDIII antigens used in this study were produced and characterized as previously described (Premkumar et al., refs. 73 and 74) and have been validated for orthoflavivirus serology in multiple published studies. To independently validate antigen integrity and inter-plate reproducibility in the present study, we have now included an 8-point serially diluted anti-ZIKV EDIII murine monoclonal antibody (clone ZV-67) as a calibrator on every ELISA plate. ZV-67 binds ZIKV E monomer, E dimer, and EDIII but not EDI, and therefore serves simultaneously as a positive control for the E-monomer, E-dimer, and EDIII assays and as a negative-control benchmark for the EDI assay. The ZV-67 standard curve confirmed the expected binding profile across all assay plates (Extended data Figure S5) and was used to normalize plate-to-plate variation in the calculation of area-under-the-curve binding titers. The ZV-67 calibrator protocol is now described in the Methods (lines 672–675) and referenced in the relevant figure (Extended data Figure S5).

Point-by-point response to reviewer, Nature Microbiology: NMICROBIOL-25093320B

Reviewer #3 (Remarks to the Author):

The authors have carefully edited the manuscript, mainly by a more nuanced interpretation of results, by a more detailed M&M narrative explaining key quantitative analytic parameters and, importantly, a rewritten more focussed discussion. Our main concerns have been addressed appropriately. We are grateful for the consideration of our criticism.

Two minor points that can be addressed by modifying the text are:

(a) In the rebuttal to our point (1) it is announced that the discussion contains a note that "direct cytolytic activity has not been formally assessed". This statement is not to be found. Instead, in Line 419-420, a less explicite "acting at least in part through IFNg production" wording has been used. For us this suffices for the conclusions. I suggest to still add former statement to the result section.
We thank the Reviewer for their careful scrutiny of our manuscript and apologize for the oversight in our previous revision. We have now explicitly incorporated the statement “direct cytolytic activity was not assessed” into the Results section of the revised manuscript (Line 114).

(b) In lines 409-414 the importance of FL epitopes for protection from experimental ZIKV and JEV infection is highlighted and compared, endorsing a "careful design" of VLP vaccines, including considerations of reducing ADE risk. This statement is a logical and important consequence of current study yet remains vague. In particular, in the following EDE-specific humoral responses to DENV1-4 are compared to those described in current manuscript for Z-prM/E-WT (lines 414-418). For us it remains unclear, which design principle the authors finally endorse for a potent ZIKV vaccine antigen; VLP w/ or w/o FL, VLP with "FL-mimic" that avoids ADE yet enables prM/E cleavage and processing, EDE-based antigens, etc.?
We have clarified our endorsed vaccine design principle in the Discussion section (Lines 411-414/425-428). We endorse a hybrid design principle that pairs fusion loop mutations (to abrogate ADE) with the stable presentation of quaternary envelope dimer epitopes (EDE). This strategy ensures both the absence of ADE-promoting antibodies and the induction of potent broadly neutralizing antibodies. Concurrently, future development strategies should investigate methods to enhance the durability of protective $CD8^+$ T cell responses, potentially through the selection of novel adjuvants or by combining structural humoral antigens with T cell epitopes or target proteins.