nature portfolio

Peer Review File

A foldon-free prefusion F trimer vaccine for Respiratory Syncytial Virus to reduce off-target immune responses

Corresponding Author: Dr Johannes P.M. Langedijk

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Bakkers et al.

In this study the authors have developed a novel prefusion-stabilized viral immunogen for RSV that does not require a heterologous trimerization domain, unlike the two existing FDA-approved vaccines. The authors use molecular dynamics to study the fusion protein and identify regions of charge repulsion that underly the well-documented instability. They identify instabilities at the stem, including a cluster of negatively charged residues at the 3-fold axis. Mutations in these residues to counteract the repulsion, in addition to other stabilizing mutations from a separate unpublished work and mutations in other MD-identified regions, improved trimer expression in the absence of foldon and prefusion conformational stability. Cryo-EM analysis identified mutated residues in contact with fusion machinery, potentially restricting fusion peptide conformational freedom. The stabilized trimer maintains immunogenicity and the prefusion conformation without a trimerization domain. Structural characterization with cryo-EM provides insights into the individual interactions contributing to the overall stabilized design. Overall, the work is innovative and would be of interest to the vaccine design community.
One major concern with the study that prevents recommending publication in Nature Microbiology in its current form. Through serum profiling, the authors demonstrate that inclusion of a heterologous multimerization domain creates a neo-epitope that induces off-target antibodies against the foldon domain. However, the presented data comparing the impact of a foldon domain on animal viral neutralization titers does not seem to be consistent with the suggestion in the discussion that such induction comes at the cost of viral neutralizing titers.

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Decision Letter:

5th June 2024

*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 Dr Langedijk,

Thank you for your patience while your manuscript "An efficacious foldon-free prefusion F trimer vaccine for Respiratory Syncytial Virus by stabilizing trimerization-induced regions of instability" was under peer-review at Nature Microbiology. It has now been seen by 3 referees, whose expertise and comments you will find at the end of this email. Although they find your work of some potential interest, they have raised a number of concerns that will need to be addressed before we can consider publication of the work in Nature Microbiology.

In particular, reviewer #1 raises the concern that neutralizing titres in vivo are not improved when the foldon domain is missing, thus calling into question whether usage of T4 containing heterotrimers comes with a cost.

Should further experimental data allow you to address these criticisms, we would be happy to look at a revised manuscript.

We are committed to providing a fair and constructive peer-review process. Please do not hesitate to contact us if there are specific requests from the reviewers that you believe are technically impossible or unlikely to yield a meaningful outcome.

We strongly support public availability of data. Please place the data used in your paper into a public data repository, if one exists, or alternatively, present the data as Source Data or Supplementary Information. If data can only be shared on request, please explain why in your Data Availability Statement, and also in the correspondence with your editor. For some data types, deposition in a public repository is mandatory - more information on our data deposition policies and available repositories can be found at https://www.nature.com/nature-research/editorial-policies/reporting-standards#availability-of-data.

Please include a data availability statement as a separate section after Methods but before references, under the heading "Data Availability". This section should inform readers about the availability of the data used to support the conclusions of your study. This information includes accession codes to public repositories (data banks for protein, DNA or RNA sequences, microarray, proteomics data etc…), references to source data published alongside the paper, unique identifiers such as URLs to data repository entries, or data set DOIs, and any other statement about data availability. At a minimum, you should include the following statement: "The data that support the findings of this study are available from the corresponding author upon request", mentioning any restrictions on availability. If DOIs are provided, we also strongly encourage including these in the Reference list (authors, title, publisher (repository name), identifier, year). For more guidance on how to write this section please see: http://www.nature.com/authors/policies/data/data-availability-statements-data-citations.pdf

If revising your manuscript:

* Include a "Response to referees" document detailing, point-by-point, how you addressed each referee comment. If no action was taken to address a point, you must provide a compelling argument. This response will be sent back to the referees along with the revised manuscript.

* If you have not done so already we suggest that you begin to revise your manuscript so that it conforms to our Article format instructions at http://www.nature.com/nmicrobiol/info/final-submission. Refer also to any guidelines provided in this letter.

* Include a revised version of any required reporting checklist. It will be available to referees (and, potentially, statisticians) to aid in their evaluation if the manuscript goes back for peer review. A revised checklist is essential for re-review of the paper.

When submitting the revised version of your manuscript, please pay close attention to our href="https://www.nature.com/nature-portfolio/editorial-policies/image-integrity">Digital Image Integrity Guidelines. and to the following points below:

-- that unprocessed scans are clearly labelled and match the gels and western blots presented in figures.
-- that control panels for gels and western blots are appropriately described as loading on sample processing controls
-- all images in the paper are checked for duplication of panels and for splicing of gel lanes.

Finally, please ensure that you retain unprocessed data and metadata files after publication, ideally archiving data in perpetuity, as these may be requested during the peer review and production process or after publication if any issues arise.

Please use the link below to submit a revised paper:

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href="http://www.springernature.com/orcid">www.springernature.com/orcid.

If you wish to submit a suitably revised manuscript we would hope to receive it within 6 months. If you cannot send it within this time, please let us know. We will be happy to consider your revision, even if a similar study has been accepted for publication at Nature Microbiology or published elsewhere (up to a maximum of 6 months).

In the meantime we hope that you find our referees' comments helpful.

Yours sincerely,

Julie

Julie Tai-Schmiedel, PhD
Associate Editor
Nature Microbiology

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Reviewer Expertise:

Referee #1: Vaccinology, virology
Referee #2: Structural biology, virology
Referee #3: Cryo-EM, vaccines, respiratory viruses

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Decision Letter:

Our ref: NMICROBIOL-24041064A

29th August 2024

Dear Hans,

Thank you for submitting your revised manuscript "An efficacious foldon-free prefusion F trimer vaccine for Respiratory Syncytial Virus by stabilizing trimerization-induced regions of instability" (NMICROBIOL-24041064A). 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.

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We are now performing detailed checks on your paper and will send you a checklist detailing our editorial and formatting requirements in about a week. 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,

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14th October 2024

Dear Hans,

I am delighted to accept your Article "A foldon-free prefusion F trimer vaccine for Respiratory Syncytial Virus to reduce off-target immune responses" 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.

As discussed please add: "Ethics approval was given through the Living Airway Biobank, administered through the UCL Great Ormond Street Institute of Child Health (REC reference: 19/NW/0171, IRAS project ID: 261511, Northwest Liverpool East Research Ethics Committee)." to the ethics section.

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Reviewer Comments:

Reviewer #1 (Remarks to the Author):

Bakkers et al.

In this study the authors have developed a novel prefusion-stabilized viral immunogen for RSV that does not require a heterologous trimerization domain, unlike the two existing FDA-approved vaccines. The authors use molecular dynamics to study the fusion protein and identify regions of charge repulsion that underly the well-documented instability. They identify instabilities at the stem, including a cluster of negatively charged residues at the 3-fold axis. Mutations in these residues to counteract the repulsion, in addition to other stabilizing mutations from a separate unpublished work and mutations in other MD-identified regions, improved trimer expression in the absence of foldon and prefusion conformational stability. Cryo-EM analysis identified mutated residues in contact with fusion machinery, potentially restricting fusion peptide conformational freedom. The stabilized trimer maintains immunogenicity and the prefusion conformation without a trimerization domain. Structural characterization with cryo-EM provides insights into the individual interactions contributing to the overall stabilized design. Overall, the work is innovative and would be of interest to the vaccine design community.

One major concern with the study that prevents recommending publication in Nature Microbiology in its current form. Through serum profiling, the authors demonstrate that inclusion of a heterologous multimerization domain creates a neo-epitope that induces off-target antibodies against the foldon domain. However, the presented data comparing the impact of a foldon domain on animal viral neutralization titers does not seem to be consistent with the suggestion in the discussion that such induction comes at the cost of viral neutralizing titers.

We would like to thank the reviewer for the constructive feedback on our manuscript and are pleased to hear that you found our work innovative and of interest to the vaccine community.

Reviewer #1 raises an important point regarding the apparent lack of improvement in the foldon-free preF design in animal models compared to foldon-containing designs. It is indeed challenging to conclusively assess the risk posed by the foldon component in a prophylactic vaccine, especially within the constraints of preclinical animal models.

We acknowledge that our current data do not directly demonstrate the superiority of the foldon-free RSV PreF in enhancing neutralizing titers in these models. This limitation is due to the nature of preclinical studies, which may not fully capture the nuances of immune responses requiring repeated vaccinations over an extended period to properly induce B cell memory. The advantage of the vaccine lies in the fact that it will not induce an off-target foldon response. Whether the boostable off-target responses impact vaccine efficacy after multiple vaccinations can likely only be evaluated in longitudinal clinical trials involving groups of vaccinees receiving either the PreF with or without a foldon. Such comprehensive trials are beyond the scope of the current study and have not been feasible thus far.

Our study's primary contribution is highlighting the potential drawbacks of the foldon domain and showing that a foldon-free RSV PreF design is possible, paving the way for future investigations. We believe our findings set the stage for subsequent clinical studies to explore this further and potentially validate the long-term benefits of a foldon-free design.

We would like to stress that our design of an RSV vaccine without a foldon domain is of significant interest and importance for the following reasons:

1. Our study demonstrates that rodents, NHP and humans develop an antibody response against the foldon domain, which is boosted upon repeated vaccine administrations.
2. We show for the first time that highly stable RSV prefusion proteins can be produced without heterologous trimerization domains.
3. These proteins induce comparable neutralization titers to those with a foldon domain.
4. Our design eliminates the need for the foldon domain, reducing potential risks associated with auxiliary components in prophylactic vaccines. All non-target specific sequences of a vaccine need to be accounted for because they pose a potential risk, particularly in prophylactic vaccines repeatedly administered to tens of millions of healthy individuals. Since vaccine hesitancy is a growing concern, minimizing even small risks by avoiding foreign or redundant sequences is crucial.
5. Although the chance of side effects is small, off-target responses could impact the potency of repeated RSV PreF-foldon vaccinations or future non-RSV vaccines containing a foldon domain, especially if the sequence of the vaccine would need to be adapted to antigenically drifting RSV strains while the foldon remains identical.
6. Clinical development of foldon-trimerized therapeutic proteins may be obstructed by inclusion of foldon-containing prophylactic vaccines into vaccination campaigns.
7. The PreF-Δfoldon shows superior manufacturability with higher Tm_{50} and higher expression levels compared to earlier generation PreF designs.

We have now added additional background in the updated manuscript on the potential risk of off-target foldon responses in line 355-358. (Please also see our answer to your question #4).

Major points:

1. In the section Counteracting native trimer repulsion through stabilizing substitutions, several mutations are listed. Some are described to have been previously used in the preF+foldon control, while others are mentioned without introduction. Was a screen performed to identify these substitutions, or are all the mutations inspired from other preF-stabilizied designs from literature? If the former, how were the mutations rationally selected?

In the section "Counteracting Native Trimer Repulsion through Stabilizing Substitutions," we mostly employed novel substitutions that we designed based on the regions of instability identified by the MD simulations, as well as some stabilizing substitutions from literature, and two substitutions (D486N and D489Y) informed by both sources.

The MD-based design efforts focused on three regions of increased RMSF upon trimerization:

1. Region around the ring of negative charge repulsion

2. Regions around the fusion peptide, including the 353-357 loop

3. The 326-331 loop, which also showed increased mobility according to MD. However, none of the substitutions in this loop resulted in increased stability or expression.

As the reviewer noted, some stabilizing substitutions were derived from previous studies. In the revised manuscript a clearer distinction between the different origins are made and a clear description of the proposed stabilization mechanism was added to the main text in lines 178-190.

a. Related point: this section was generally harder to follow than the rest of the manuscript and would benefit from substantial clarification regarding the design process

We agree with the comment of the reviewer and have now thoroughly revised this section. We have included more details on known stabilizing substitutions described for RSV-A (Krarup et.

al. 2015), RSV B (WO2022175477), and known stabilizing substitutions derived from literature that match positions indicated by the MD approach are discussed (D486N and D489Y).

Clarifications are included in the revised manuscript for better understanding and distinction of the sources and mechanisms of the stabilizing substitutions.

Please see line 181-185 of the updated manuscript "…. S215P is stabilizing the hinge loop in the membrane-distal apex between α4 and α5 [2]. L203I is located in the apex and stabilizes the triple helix formed by α1, α4 and α5. Stabilizing substitution P101Q and I152M are located at the protomer interface at the pore where the F2 C-terminus and the fusion peptide enter and exit the trimer cavity, respectively. D486N and D489Y are reducing the repulsion in the ring of negative charge and stabilize the interaction with the fusion peptide respectively. …"

2. The framing of the paper describes the MD simulations as uncovering the regions of positive and negative charge repulsion, and neutralization of these as being the primary driver of prefusion trimer stability. It would be helpful to see MD simulations of the preF-Δfoldon construct to compare the regions of instability with the charged residue substitutions and the full set of substitutions, to support that the change in the stabilization of those residues could be driving the stability of the prefusion trimer. A similar simulation on RSV-B, additionally, would be useful to confirm the relative impact of these charged regions across strains.

The reviewer makes an excellent point regarding the inclusion of MD simulations for the preF-Δfoldon construct and RSV-B as a means to support our findings. We appreciate the insights such simulations could provide. To address your point, within the scope of our current study, we have performed additional MD simulations for the D486N variant, one of the most impactful substitutions (Fig. 3A right panel, Supplementary Figure 3B). This new data conclusively demonstrates the significant stabilizing effect of the D486N mutations on the fluctuations at the base of the RSV F protein. However, extending these simulations to cover all substitutions and RSV strains is beyond our current resources, as running long MD simulations for large systems such as RSVF protein are prohibitively costly and time-consuming. Our primary aim was to use MD to complement the static X-ray structures of the F protein, and allow us to specifically target regions that exhibit instability in molecular dynamics. To address the reviewer's question, we have added line 167-170 in the main text:

"*To validate the MD-based approach, another set of MD was performed on a trimer PreF with the stabilizing D486N substitution that neutralizes the electrostatic repulsion in the repulsive ring. The mutation significantly decreases the RMSF in the trimer, alleviating the wildtype instability (Fig 3A right panel, Fig S3B)*). "

With regards to RSV-B, we would like to highlight that the successful transfer of stabilizing substitutions from RSV-A to RSV-B supports the proposed mechanism of destabilization upon trimerization across different strains. This indicates a broader applicability of our findings without the necessity of performing exhaustive simulations for each variant and strain. We have now added this statement regarding RSV-B to the main text (see lines 214-215).

We appreciate your understanding and hope our additional data and clarifications address your concerns.

3. Of the residues described in Fig. 3, it appears that only one of the three positively charged residues was mutated in Fig. 4 (Q354).

This is indeed correct. We have not been able to find stabilizing substitutions that directly stabilize the positive charge cluster around F137. One of the positive charges is the F1 N-

terminus, and the second positive charge is R106 which forms part of the furin cleavage site, neither of these residues can thus be mutated. R339 is the only positive charge that could be targeted for substitutions, however, none of our attempts was successful and all lead to loss of F expression.

Upon the reviewer's request we could include the negative data regarding R399 in a supplemental figure or refer in the text as ‘(negative) data not shown)? Importantly, although we could not identify a stabilizing substitution at position 339, this does not mean that R339 is not part of this metastable cluster. It is likely that the R399 substitutions we investigated are either impacting folding or post translational modification of the F protein. In the end, we decided to explore and employ alternative strategies to stabilize the N-terminus of the fusion peptide:

- Interaction of D489Y with F137 and F140 in the fusion peptide.
- Interaction of Q354L with G139 and L142 stabilizing a loop in the fusion peptide.

D489, in the negatively charged region, is not described throughout as a single mutant (unlike D486, in Fig. 2) but is included in the final construct.

To improve the design section, we included more details on the stabilizing effect of this substitution (see lines 185-186 of the revised manuscript). In short, although D489 is close to D486 and E487, it is not part of the negatively charged region we described in the manuscript, but together with F488 it is positioned between the aromatic cluster (F137 and F140) and the negatively charged ring (D486, E487), see Figure 3B.

For the positively charged mutants, why was Q354L chosen? Is Q354L sufficient to mitigate the positive charge repulsion, or does that charge region not materially contribute to the instability?

In order to preserve the precise interaction of F137 at the fusion peptide N-terminus, it is important to stabilize the turn in the fusion peptide (139-142). In the wildtype PreF the N-terminus is stabilized by the transient interaction of Q354 with F137 and G139 which is very unstable according to the MD simulations. In the updated manuscript, the transient interaction of Q354 is also illustrated in the novel supplementary figure S6. Therefore, the Q354L substitution is a strategy to stabilize the fusion peptide N-terminus by interacting with G139 and L142 without touching the positive charges. Although the positively charged R106 is not visible in the current stabilized PreF, the interaction of F137 and R339 seems a bit more favorable but we do not want to overinterpret the cryoEM structure with the current resolution and therefore decided not to describe this in the manuscript. In summary, stabilization of the N-terminal fusion peptide turn by Q354L seems a strong stabilizing substitution as shown in figure 4B and explained by figure 5C. Therefore, we think the positively charged region contributes to the instability, although direct substitution of the charges was not possible.

It would be good to include some clarifying notes on how the non-charged region stabilizing mutants were selected; the manuscript refers to a separate manuscript in prep, but as this is not viewable it leaves the story at that point incomplete/unclear as to what mutations are truly driving the effect.

A few of the non-charged substitutions were derived from literature or a manuscript in preparation. As the manuscript in preparation is not viewable we added a reference to the published patent application and, in order to improve the design section, we have now included a more in-depth descriptions of the stabilization strategy. In short, the PreF stabilizing substitution S215P was described for RSV-A (Krarup et al. 2015) and is stabilizing the hinge loop in the membrane-distal apex between α4 and α5. We have added the following description

in the Results section line 182-185: "L203I is located in the apex and stabilizes the triple helix formed by α1, α4 and α5. Stabilizing substitution P101Q and I152M are located at the protomer interface at the pore where the F2 C-terminus and the fusion peptide enter and exit the trimer cavity, respectively."

4. The discussion posits that the foldon binding antibody responses may explain the poor VNT increase after boost. This a central and motivating concept for the study. How should readers evaluate this claim in the context of the presented in vivo data that shows viral neutralization titers are not improved without the foldon domain?

Please also see our answer at the start of this rebuttal to your general concern. In short, the central motivating concept of the study was twofold: i) understanding RSV PreF destabilization upon trimerization, and ii) development of a vaccine candidate that would not induce off-target anti-foldon immune responses. We do not believe that a foldon-free RSV preF would directly lead to improved neutralization responses compared with a foldon-containing RSV PreF vaccine. However, there is a general perception to avoid any non-target sequences (e.g. foldon) in vaccines to minimize risk of cross-reactivity and auto-immunity. Additionally, a boosted off-target response against foldon could impact the immune response against the target, especially upon repeated administrations of the vaccine or when the vaccine sequence is updated to negate antigenic drift in the F protein. These antibodies might increase the chance of an imbalanced skewing of the immune response to foldon in some cases and an immunodominant foldon response against foldon-containing future vaccines. Likely, these hypotheses can only be proven in human trials in which two groups of adults are vaccinated with either a foldon-based trimer or a foldon-free trimer. While such trials are now possible with the herein described preF proteins, they are well beyond the scope of the current study. Of note, an additional reason to avoid foldon-based trimers is because clinical development of foldon-trimerized therapeutic proteins (e.g. cytokine therapeutics or receptor (ant)agonists) may be obstructed.

To accommodate the reviewer's feedback, we now added a more thorough rationale for the study, as well as a more elaborate explanation on the need for foldon-free vaccine components.

Line 355-358 "Apart from the possibility of side-effects, the off-target response could impact vaccine potency after repeated administrations or the potency of future vaccines that contain a foldon domain and even possible future therapeutics that contain a foldon domain".

Moreover, in line 367 we wrote that the foldon antibody responses might play a role, and in line 368-369 we wrote that further investigation is required to elucidate the role of the trimerization domain in this observation.

If the reviewer thinks that this is still not careful enough, we are open for suggestions or if needed, delete lines 362-369.

5. There seems to be substantial difference in the melting temperatures between the designs with and without foldon (Fig. S3B), presumably because the +foldon design has fewer stabilizing mutations. Would the addition of the foldon domain to the same design with more stabilizing mutations in the del-foldon design lead to even higher titers?

We agree with the reviewer that the increased melting temperature of the foldon-free designs is due to the additional stabilizing substitutions. While an interesting proposition, we do not expect that the foldon-free proteins would improve further by once again adding a foldon domain since stability is already very high (with an astounding Tm for RSV-A PreF of 75°C), and antigenicity is as desired. While an interesting idea, we did not test it since our primary

aim was to generate a protein that no longer depends on foldon for stability. If even further stability improvement would be the aim, we would propose to introduce stabilizing disulfides instead of a foldon domain.

6. Is the antigenicity of the designed construct with several additional mutations different from the +foldon construct? Are all relevant epitopes retained?

Indeed, all neutralizing epitopes are retained in the foldon-free PreF designs, please see antigenicity data, as determined by BLI, in figures 4H and S7C (previous S3C).

7. Relevant to Fig. S3C, are mAbs CR9501+RSV90 known to only bind RSV-A? If not, does this imply that the prefusion conformation is significantly different from the native prefusion conformation in the RSV-B-Δfoldon construct? This question would be answered with an RSV-B-(+)foldon control for the antigenic analysis.

CR9501 also binds to RSV-B F but with somewhat lower affinity. RSV90 does not bind currently circulating strains of RSV-B F, likely due to the 172/173 substitutions that are in the center of the RSV90 epitope.

8. Does the newly exposed base of the fusion protein form a new epitope, as has been observed with designed immunogens for other viral targets?

The reviewer makes an excellent point regarding the antigenicity of the base/HR2 region of our foldon-free PreF design. It will indeed be different from previously published designs on various points: 1) our design is 11 amino acids longer in the C-terminal HR2 stem compared with designs used in licensed vaccines and thus contains more antigenic information, leading to an additional RSV F epitope. 2) the absence of linker and foldon in our design will prevent induction or boosting of off-target antibodies against this region and therefore not interfere with future novel vaccines or therapeutic proteins. 3) Like all soluble proteins derived from membrane proteins, the exposed terminus at the base does form a neo-epitope that could induce non-relevant responses. However, this region is very small and likely not immunodominant. In contrast to e.g. highly glycosylated recombinant HIV-1 gp140 trimers, which only expose the non-glycosylated base.

We believe that future studies aimed at isolating antibodies that target the near-native HR2 region, and determining their neutralizing potential (e.g. by preventing HR2 helix dissociation), would be of considerable interest.

Minor points

1. The hand-drawn red lines on the elution peaks in Figure S3A are confusing.

We apologize for the confusion. The red line is not hand-drawn but is the actual data coming from the MALS detector. We appreciate the need for clarification and have added an explanatory sentence to the legend.

2. Why was the binding rate reported in the antigenic analysis in Fig. 4h, while R-Eq is reported previously in Fig. 2? It may be useful to include the full binding/dissociation curves.

While we generally plot binding rates (as we did in Fig 4H), the low expression levels of wildtype / minimally stabilized RSV F proteins makes the initial binding values rather variable. We therefore opted to show the end point shift instead. The full binding/dissociation curves will be added to the source data file that will accompany the manuscript.

3. While D486 is explored as a single mutant in Fig. 2, for clarity it would be beneficial to include it in Fig. 4b.

We apologize for the confusion, but the screen in Fig 2B was actually performed in a semi-stable background that included a foldon and the D486N substitution. Thus, all constructs shown contain D486N. For clarity we now highlighted this in lines 192-194 and in the legend.

4. Is the near/below 1:1 ratio of preF:postF antibodies expected, given the observed prefusion stability? If so, is this another potential cause for the poor VNT boosting?

A preF:postF ratio of near 1:1 actually does not mean that equal amounts of preF and postF antibodies are present in a sample. The ELISA assays do not assess absolute amounts of antibodies. Instead, the amounts are measured as relative potency, calculated relative to amounts present in a standard serum sample taken along in each assay, as explained in the Methods section. Therefore, the preF:postF ratio can only be used in a relative manner, as we did to demonstrate that there is no difference in the ratio between the response to the preF proteins with and without foldon. Speculations on the effect of a below 1:1 ratio on the VNT boosting should therefore be avoided.

To make it more clear that we did not measure absolute antibody levels, we added that preF and postF binding antibodies were measured as relative potency to the legend of Figure S4 where the ratio is shown.

5. It could be useful to include a figure similar to Fig. 5C of the fusion peptide in the wild-type/WT-foldon construct to show the difference in fusion peptide contacts.

For clarity we wanted to reserve Figure 5 for structural cryoEM data of the stabilized PreF but we do understand a comparison would be helpful. The same region is also shown in Figure 3D but with a different view showing different highlights. In order not to add too much redundancies in the manuscript, we have added a new Supplementary figure (Fig S6) to show the differences of this region for the wildtype and stabilized PreF.

Reviewer #2 (Remarks to the Author):

In the manuscript titled “An efficacious foldon-free prefusion F trimer vaccine for Respiratory Syncytial Virus by stabilizing trimerization-induced regions of instability” Bakkers, M. J., et al reported that the current Respiratory syncytial virus (RSV) vaccines that incorporate a foldon trimerization domain induce unwanted anti-foldon antibodies, which can cause problems when it comes to repeated vaccination or the use of multiple foldon-containing vaccines. To address this issue, the team designed a foldon-free RSV prefusion-stabilized F (PreF) trimer that is further stabilized by addressing regions of instability identified using MD approaches. This foldon free construct induced neutralizing antibodies and demonstrated protection in animal models. A cryo-EM structure was determined to visualize the engineered mutations.

This is a well-written and easy to read paper that addresses an important and highly significant issue of off-target responses to the foldon trimerization motif present in the RSV and other vaccines. The cryo-EM structure is of high quality with the positions of the stabilizing mutations clearly visualized.

We would like to thank the reviewer for the positive feedback on our manuscript. We are pleased to hear that you found our paper well-written, easy to read, and addressing an

important issue. Your comments on the quality of the cryo-EM structure and the significance of our approach are greatly appreciated.

A few relatively minor comments and suggestions for improving the clarity of the figures and the paper:

• Figure 2C, 4H, S3C: the individual data points shown in the figures are hard to find immediately. It may be better to use filled circles to indicate the points instead of the open circles.

Agreed, we have now filled the circles with a semi-transparent gray color (as not to be too distracting from the error bars).

• Line 28/670/943: Cryo-EM (capitalized C) in text, whereas cryo-EM (lower case C) in lines 243/247/662/937; can be fixed for consistency.

Thank you for the observation, we have now changed the C to lower case throughout the manuscript.

• Line 270: place a comma after “For preclinical evaluation”.

Added.

Reviewer #3 (Remarks to the Author):

The authors present a robust and innovative study on the structure-guided engineering of the RSV prefusion F protein into a stable prefusion state. This work is important because RSV prefusion F is the antigen needed to elicit protective immune responses. Prefusion F is the major antigen in novel and next-generation RSV vaccines. The authors used several diverse and robust methods in structural biology, cryo-EM, molecular dynamics, protein expression/purification/characterization, immunoassays/binding assay, and animal studies and or sera from mice, NHP, and humans in their work. The authors found that mutations can aid in the production and stability of RSV prefusion F without the T4-based foldon trimerization domain which produced off target (i.e. non preF) immune responses. The study is innovative and is important to a broad scientific audience.

We would like to thank the reviewer for the detailed and encouraging feedback on our manuscript. We are delighted that you found our study robust, innovative, and important for the field. Your recognition of our use of diverse methods and the significance of our findings regarding the RSV prefusion F protein is highly appreciated.

See below for minor comments.

Figure 1. add immunization schedule to the figure.

The immunization time points are added with arrows to the Figure where needed, and explained in the legend.

Maybe label somehow what antigen was on the ELISA plate? Foldon?

Indeed, a biotinylated foldon peptide was used. The requested information is added in more detail in the legend of Figure 1.

AREXVY data points (Fig. 1C) are the same bluish color as panels A and B. Should AREXVY data point be a different color?

Adjusted, AREXVY data points are now in a different color

Each panel should have its own legend below it.

Agreed, added below the panels where missing

Also, the animal/sera source (mice, NHP, human etc) of each panel should be labelled with the origin of the sera, NHP, mice, etc.

Agreed, added at the top of the panels

In summary, label Figure 1 a bit more.

The layout of Figure 1 has been adjusted based on the useful comments to improve readability

Figure 1A need molecular weight standards. Panel 1B 1C, SEC standards, a new supplemental figure can do.

We believe the reviewer is referring to figure 2. We have now added a stabilized, foldon-containing preF protein that was previously published as size control to figure 2A. For SEC we actually do not use standards since they often do not accurately reflect the behavior of the specific protein being studied due to differences in shape and potential column interactions. Therefore, we always purify our lead candidates and rigorously test them using SEC-MALS to provide us with the exact mass (confirming trimer formation), antigenicity using BLI (confirming prefusion conformation), and cryo-EM to visualize the structure.

For panel 3B the overlays are hard to read.

Make some supplemental figures with each panel for each day.

This might be many panels (27), but in supplemental readers would appreciate this data.

We appreciate the suggestion and have now included the individual panels in supplementary figure S1.

Figure 2C.

Is R-Equilibrium the right way to report biolayer interferometry (BLI) data?

For BLI it is more standard to show the series of binding sensograms in supplemental figures.

Then report the different KDs for the glycoprotein-antibody pairs in a table/figure of some type.

When stabilizing fusion proteins, we aim to not introduce mutations into the binding sites of antibodies, so we do not expect different KD values for the different proteins. The stabilizing mutations may have an effect on the exposures of epitopes, though, so the V0 is a good measure of the quantity of those epitopes, instead of what the binding affinity (KD value) is. Furthermore, KD values cannot be calculated well, because the trimers are in solution. Therefore, if multiple concentration were tested, the curves cannot be fitted using binding equations describing a 1:1 or a 2:1 interaction. The sensorgrams showing the full binding/dissociation curves will be added to the source data file that will accompany the manuscript.

So, panel 2C. could be (1) Schematics of each different RSV F proteins and where the location of each epitope for testing antibody binds. (2) Table of Kd for binding pairs.

We appreciate the suggestion for a figure highlighting the binding epitopes of the different antibodies and have now made a new supplemental figure (Fig S2) that describes these in detail.

Figure 2: Are the blue color data in Panel B and C related to the blue colors in panel C?

Try to make the same and different data/RSV F proteins a consistent color theme.

We have changed the color scheme of figure 2 to make the panels more consistent and intuitive.

Figure 3 Figure caption

Would it be important to just show a monomer panel.

We agree with the reviewer that including a monomer panel would improve clarity; we have now updated the figure to show the RMSF in both the monomer and trimer context.

Is there some energy score or metric that comes from Molecular Dynamics? How are you scoring “region of instability”? By its red color (i.e. higher RMSF?).

For assessing stability, we primarily examine the Root Mean Square Fluctuation (RMSF), which is analogous to the B-factor in crystallography. The RMSF is calculated per residue, allowing us to compare how this property varies across different regions of the RSV F structure. This analysis is illustrated in Figure 3A. Additionally, we provide further insights into these fluctuations in Supplementary Figure 3A and B. In these figures, we compare the fluctuations of all residues for the wild-type monomer versus the trimer (Fig. 3A) and for the wild-type versus the, now also included, D486N variant (Fig. 3B). As depicted, trimerization induces destabilization in specific areas of the protein, while the D486N substitution introduces stabilization. To identify unstable regions, we employed an arbitrary threshold: regions with fluctuations one standard deviation above the average fluctuation of the entire protein were considered unstable. This method helps to systematically pinpoint areas of instability. We hope this clarifies our approach and the metrics we used to evaluate stability in our MD simulations.

Please discuss possible caveats, PDB 4MMS is at acidic pH, crystal contacts.

The reviewer makes an excellent point regarding the potential caveats of using PDB 4MMS, which was determined at acidic pH. We believe that our use of the 4MMS structure is valid since Molecular Dynamics (MD) simulations explore the local energy minima of the structure. Therefore, unless the protein is in a different conformational state, separated from the pre-fusion structure by a large free-energy barrier, every F WT crystal structure should exhibit similar RMSF values if sampled long enough. Our MD simulations were conducted at neutral pH. To avoid artifacts associated with protonation, we gradually released the constraints on the protein backbone over the course of 60 ns after system preparation. We consider this duration sufficient for the structure to adjust to neutral pH protonation states. Regarding the potential stabilizing effects of crystal lattice contacts, we also accounted for these during our simulations. By allowing the protein structure to evolve freely, without these artificial stabilizations, we believe we generate a more realistic representation of fluctuations for these regions, than obtained via analysis of the B-factors.

We hope this addresses your concerns and supports the validity of our approach. In the revised manuscript we have now also included the per residue analysis of the root mean square

fluctuations for the wild-type and D486N mutant for both monomer and trimer – see Supplementary figure 3.

Figure 4A. caption. Is the data display an expression patter or SEC profile? Looks like a SEC profile. For expression pattern on expects an SDS-PAGE gel.

It is indeed data from analytical SEC performed on clarified supernatant. Since there is no purification step involved, it is a more informative measure of expression than SDS-PAGE since it also directly details oligomerization status (monomers vs trimers).

Figure 4H, if available it might be helpful to show the structure (from PDB or EM database)

Of the complexes of RSV F with the Fab of the antibody probe (e.g. CR99501) to see the epitope. Also, BLI data, usually sensograms and KD table are given.

We appreciate the suggestion and have now added a novel supplemental figure 2 that depicts the antibody epitopes. Regarding KD and sensorgrams, please see our answer above.

Figure 5. Panel A shows the TM and CT. Did the designed protein have TM and CT? It might be helpful for readers to show some the actual regions in the RSV-A preFdeltaFoldon construct. Another schematic of the actual construct perhaps?

As not to overcrowd the figure, we have instead added an additional explanation to the legend " The soluble F ectodomain used in experiments consists of residues 1-524 and lacks the TM/CT regions."

Figure 6, caption confusion. “Foldon binding antibodies” Is “Foldon” the T4 foldon protein alone, or RSV F with foldon? What was the antigen on the ELISA plate to detect anti-foldon antibodies.

To detect anti-foldon antibodies we used biotinylated T4 foldon peptides, there was no RSV F present. We have made this now clearer in the methods section (lines 491-494).

Figure 7. panel C and D have: cynomolgus macaques: If panel A and B use mice, then label Balb/c mice. Make animal labels consistent so reader can quickly see what animal the data came from.

We have added the requested labels to panels A and B.

Figure S1. Should you say an “Expression pattern”. Those are SEC profiles that you are using to access expression of trimers and monomers? Make consistent in supplemental and main figure captions.

Agreed, we have now changed the caption to read: " Analytical SEC analysis of RSV F proteins without a foldon domain (Δfoldon), as determined by analytical SEC in the supernatant of Expi293F cells."

The adjustment was made throughout main and supplemental figures.

Figure S2. Should the word “ternary complex” be used? Usually, a complex of three biochemically different sequence molecules (e.g. A, B, C) are call ternary complexes.

Use “trimer”

We agree with the reviewer and have replaced 'ternary' by 'trimer' in current Figure S5 and throughout the manuscript.

Figure S3. What is the red line in panels A, B, C. Mention in caption.

The red line in current Figure S7 is the actual data coming from the MALS detector. We appreciate the need for clarification and have added an explanatory sentence to the corresponding legend.

Figure S4 caption. Extra space in caption.

All double spaces have been removed throughout the manuscript and we have added more space to the top captions of Fig S8 (previous Fig S4).

Cryo-EM work appears to be of good quality.

Line 121 “Native-Page analysis” but Figure 2A caption says western blot.

We have changed the sentence at line 121 to read “…shown by native PAGE followed by Western blot analysis of supernatants of transiently transfected Expi293F cells (Fig 2A)”, as to clarify the experimental procedure. To keep the text consistent, the Figure 2A caption was changed to “A) Multimerization profile of RSV-A F protein in the supernatant of Expi293F cells, as determined by native PAGE followed by Western blot and probed with anti-RSV F antibody CR9506.”

Line 813 says “Native PAGE Western blot” Is Figure 2A an image of a Gel or Western blot membrane. Make clear.

The image is indeed of a Western blot membrane. Following native PAGE, the proteins were transferred to a PVDF membrane and probed using anti-RSV F antibody CR9506. In analogy to line 121, we have now also modified the sentence to be clearer.

Note : You are saying the foldon is bad, but don’t lose focus on the value added that your mutations add to RSV PreF.

We agree and stressed this point more in the current version of the manuscript in line 148-149.

Line 147 “Altogether, these results demonstrate that the addition of a foldon trimerization domain destabilizes the prefusion conformation of the soluble RSV F protein.”

I would rephrase this sentence to “Altogether, these results demonstrate that the addition of our stabilizing substitutions increases the stability of the prefusion conformation of the soluble RSV F protein.”

We appreciate the suggestion but would also like to underline that the foldon-induced destabilization is one of the key messages of this paragraph. To accommodate the reviewer, we have now made a more detailed concluding sentence that captures both conclusions:

"Altogether, these results demonstrate that the addition of a foldon trimerization domain destabilizes the prefusion conformation of the soluble RSV F protein and that this effect can be reduced by the introduction of stabilizing substitutions."

Line 151 “151 Having established that foldon-driven trimerization destabilizes RSV PreF”

I would delete this text. Start with “The trimer interface…”

The requested modification to the text was made.

Figure: 3. Is it possible to show another panel with ribbon diagram of just a monomer.

Seeing a trimer and the monomer RMSF is a bit confusing (mentioned above).

Agreed, we have now updated the figure to show the RMSF in both the monomer and trimer context.

Structure-guided design has been done with influenza HA, HIV env, SARS S.

Can the authors add some reference on others who used MD for help design trimers? It will be good for readers to have some refences on the use of MD to help stabilize proteins, if available.

In recent years, molecular dynamics has become a popular method to help understand dynamics of proteins beyond the static picture of X-ray structures. In our study we aimed at employing the method to elucidate unstable regions of the protein, which could then subsequently be targeted with substitutions. There are multiple examples in literature, where MD is used to understand the dynamic nature of proteins, one of the more recent examples being the full-virion simulations of the influenza virus (Lorenzo *et al.* ACS Central Science (2022)). MD simulations were also used for the stabilization of herpesvirus gB (Vollmer *et al.* Sci. Adv. 2022), we have added this reference to line 157. Due to being costly and time consuming, unbiased MD simulations have not been extensively used for mutation screening. However, full atom MD simulations can be employed with enhanced sampling techniques, that allow for the calculation of mutation's ΔΔG. One of such methods, called free-energy perturbation (FEP) has been shown to work well for this purpose (Sergeeva *et al.* JMB (2023), Zhu *et al.* Scientific Reports (2022), Antonietta *et al.* J. Chem. Inf. Model. (2022)). Our approach uses MD primarily to focus mutation efforts to the destabilizing regions in the protein, but does not use MD to generate specific mutation ideas, like in the case of FEP.