nature portfolio

Peer Review File

The sequence and structural integrity of the SARS-CoV-2 Spike protein transmembrane domain is crucial for viral entry

Corresponding Author: Dr Luis Martinez-Gil

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

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The sequence and structural integrity of the SARS-CoV-2 Spike protein transmembrane domain is crucial for viral entry by Ortiz-Mateu J et al
This manuscript reports that the trans-membrane domain (TMD) is an important determinant of the SARS-CoV-2 spike protein functionality. The authors use Ala-mutagenesis to replace TMD residues that have been suggested by previous studies to be important for spike structure and function. They assayed the impact of these substitutions and insertions using a pseudovirus system.
The manuscript is quite interesting, in my opinion but can be substantially improved in its impact through a few focused experiments, using the authors' pseudovirus system.
1. Are the VSV-pseudovirus phenotypes specific to Ala, or do they hold if other residues are used? This is important to address experimentally with a few selected constructs (and not through rewriting the manuscript or using in silico predictions) to ensure that the study is not biased by Ala residues.
2. Lines 186-189: If the authors' hypothesis is correct, then swapping Leu1218-Ile1232 and Gly1219-Ala1226 should still hold the TMDs together. I would like to see the impact of these swapped residues in the VSV-pseudovirus system. If the authors' are incorrect, then the result they see could be an artifact of Ala-mutations. This goes back to my concern that we do not know if these otherwise exciting results are pointing toward a fundamental mechanism of TMD function, or if this is an Ala-effect. The data with Tyr substitution provides reason for optimism that these experiments should work out.
3. The manuscript needs to be evaluated for careless mistakes in sentence construction and punctuation- e.g., line 81: it should be 3.4 not 3,4.
4. I suggest not using "variant" to describe the constructs. These did not arise on their own through evolution, but were generated through site-directed mutagenesis.

Reviewer #2

(Remarks to the Author)
The manuscript entitled "The sequence and structural integrity of the SARS-CoV-2 Spike protein transmembrane domain is crucial for viral entry" by Ortiz-Mateu J et al. targets the TMD of the SARS-CoV-2 Spike protein with various mutations and demonstrates that the TMD is crucial for viral entry. The authors performed various experiments to verify their claim. However, I have several questions related to the experimental design and the results obtained from those experiments, which are listed below.
However, most of these results are not interconnected.
1. The authors have mentioned that several structures of the S protein have been solved. However, only one includes the TMD. A reference is required for the statement, "Several structures of the S protein have been solved."
2. "No infection was observed when the TMD of the SARS-CoV-2 S protein was eliminated (ΔTMD) or substituted by a scrambled version of the TMD (SCRBL), where the composition of the residues included in the TMD was preserved, but their positions were randomly varied, confirming the importance of the SARS-CoV-2 S protein TMD in the entry process and indicating a role beyond." – How did the authors confirm that the spike protein is attached to virus particles in the ΔTMD or scrambled version of the TMD (SCRBL)? Generally, TMDs play a vital role in the spike protein's attachment to the virus. It is obvious that if the TMD is eliminated, the spike protein should not attach to virus particles, and no infection would be

observed. Similarly, the scrambled TMD (SCRBL) version would severely affect the transmembrane helicity. How would the particles be suitable for infection in this case?
3. The authors used AlphaFold for structure prediction, but nowhere in the methods section is it mentioned which version or tools were used (e.g., AlphaFold2, ColabFold) or how they were used.
4. Throughout the manuscript, the authors mention various mutations; did the authors use any computational tools to predict the effects of mutations in the TMD? I do not find any details about the mutations or primer design in the methods section.
5. It seems that the authors performed computational studies to incorporate Ala between Phe and Ile or to change hydrophobic amino acids to Ala. However, the authors claim that Figure 2D shows that Ins-Ala1221 significantly reduced the infectivity of pseudo-typed VSVDG-GFP particles. Were these changes expressed in cells? Otherwise, how could the authors claim that the infectivity of the pseudo-typed VSVDG-GFP particles was reduced? What experiments did the authors perform to support this claim? Most importantly, the authors performed numerous mutations, and it is demonstrated that, in all cases, the TMD remains intact. It is difficult to believe that the TMD is expressing under any conditions or that any changes occurred.
6. On what basis did the authors perform all the mutations in the TMD? What is the rationale behind this? Gly1223 to Ile, Gly1219 to Ile, Gly1223 to Ile, Ile1221 to Tyr, Ile1225 to Tyr, Met1229 to Tyr, Ala1222 to Ile, Gly1223 to Ile, Ala1222 to Tyr, Gly1223 to Tyr, Leu1224 to Tyr, Leu1224 to Tyr, and Val1228 to Tyr (G1223I, G1219I, G1223I, I1221Y, I1225Y, M1229Y, A1222I, G1223I, A1222Y, G1223Y, L1224Y, L1224Y, V1228Y, respectively). Did the authors randomly mutate so many amino acids in the TMD? Why were these specific mutations chosen? Is there any supporting literature? Are these mutations physiologically relevant?
7. What is the rationale behind replacing Ile1221, Ile1225, or Met1229 with tyrosine (I1221Y, I1225Y, and M1229Y)? Tyrosine has a much bulkier shape than Ile and Met, which could alter surrounding shapes and interactions. What is the structural reasoning behind the slight reduction in viral entry for the substitution of Ile1221 with Tyrosine (I1221Y)?
8. It is expected that substituting small amino acids, Gly1219 and Gly1223, with isoleucine (G1219I, G1223I) would affect the TMD, which may further decrease pseudo-typed VSVDG-GFP infectivity. Can the authors discuss the structural changes in the TMD due to this substitution and how it affects infectivity?
9. The authors also mentioned that double substitutions (Figure 2E) reduce the infectivity farther- reason behind this is small residues and the hydrophobic bulky residues aligned on opposing faces of the helix. However, from Figure 2E, it is not clear what they are trying to describe. Additionally, the authors mentioned that infectivity is reduced because the residues likely participate in protein-protein or TMD-lipid interactions. The authors should include proper figures to describe their hypothesis and claims. It is really difficult to correlate the figures with the text, and we have to assume what they are trying to represent.
10. The BiMuC assay showed that there was no alteration in oligomerization due to the single substitutions I1221Y, I1225Y, or M1229Y, and it also did not alter oligomerization. Then why did the authors find that the substitution of Ile1221Y slightly reduced viral entry? How is it structurally significant that the bulky tyrosine substitution does not affect or alter the structure of oligomerization? Could the authors explain the reason behind this?
11. The BiMuC assay suggested that the double substitution L1224Y V1228Y did not decrease the observed fluorescence. The authors claimed that the hydrophobic surface does not participate in the oligomerization of the SARS-CoV-2 S protein TMD. The authors already have structural information about the TMD. Can the authors explain why they believe the hydrophobic surface does not affect oligomerization?
12. In the methods section, the authors mentioned Ct of the VFP. What is Ct? I assume it refers to the C-terminus. Please clarify this.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The sequence and structural integrity of the SARS-CoV-2 Spike protein transmembrane domain is crucial for viral entry
By Ortiz-Mateu et al
The authors have satisfactorily addressed the questions I posed. However, I feel the Discussion section is not well written and does a disservice to the results. The authors have missed an opportunity to provide context for their results, to suggest what these mutations mean for spike trimerization, spike function, viral infectivity, and where the field should go next using their results as a launching pad. They correctly cite the Lemmon articles but do not provide any comparative discussion for the protein-protein interaction strategy adopted by the spike TMD relative to endogenous cellular proteins like glycophorins. Such comparisons will substantially elevate the manuscript in my opinion. I strongly urge the authors to expand the Discussion section.

Reviewer #2

(Remarks to the Author)
The authors have revised several figures and addressed most of the reviewers' suggestions. Most of the responses are convincing, and the manuscript has improved significantly.

One minor comment:

The manuscript is written or compiled in an unusual manner. In several instances, figure legends appear before the corresponding figures, and at times, legends for both main and supplementary figures are presented together with the result section. This formatting makes it somewhat difficult to follow the Results section coherently. The authors are requested to carefully organize the manuscript files to improve clarity and facilitate easier reading for reviewers and readers.

Version 2:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Manuscript#: COMMSBIO-25-1318B

Title: The sequence and structural integrity of the SARS-CoV-2 Spike protein transmembrane domain is crucial for viral entry

The authors have modified the manuscript to my satisfaction. I have no further questions.

We thank the reviewers for their insightful comments and suggestions. Below, we provide point-by-point responses to each comment, with our replies highlighted in **blue**. We believe the manuscript has been significantly improved as a result of these revisions. Major changes in the manuscript have been highlighted in the marked-up version.

Reviewer #1:

The sequence and structural integrity of the SARS-CoV-2 Spike protein transmembrane domain is crucial for viral entry by Ortiz-Mateu J et al.

This manuscript reports that the trans-membrane domain (TMD) is an important determinant of the SARS-CoV-2 spike protein functionality. The authors use Ala-mutagenesis to replace TMD residues that have been suggested by previous studies to be important for spike structure and function. They assayed the impact of these substitutions and insertions using a pseudovirus system.

The manuscript is quite interesting, in my opinion but can be substantially improved in its impact through a few focused experiments, using the authors' pseudovirus system.

1. Are the VSV-pseudovirus phenotypes specific to Ala, or do they hold if other residues are used? This is important to address experimentally with a few selected constructs (and not through rewriting the manuscript or using in silico predictions) to ensure that the study is not biased by Ala residues.

To ensure that the observed effects are not biased by the use of alanine residues, we substituted the segment Phe1220–Gly1223 with leucine residues (F_{1220}-G_{1223}L). We observed similar phenotypic outcomes with both alanine and leucine substitutions, indicating that the results are not specific to alanine. These data have been incorporated into Figure 2.

Additionally, we inserted a tryptophan at position 1221 (InsW1221) and a methionine at position 1230 (InsM1230). Once again, we observed comparable effects regardless of whether alanine or alternative residues were inserted (Fig. 2D), further supporting that the observed phenotype is not an artifact of alanine usage.

2. Lines 186-189: If the authors' hypothesis is correct, then swapping Leu1218-Ile1232 and Gly1219-Ala1226 should still hold the TMDs together. I would like to see the impact of these swapped residues in the VSV-pseudovirus system. If the authors' are incorrect, then the result they see could be an artifact of Ala-mutations. This goes back to my concern that we do not know if these otherwise exciting results are pointing toward a fundamental mechanism of TMD function, or if this is an Ala-effect. The data with Tyr substitution provides reason for optimism that these experiments should work out.

As suggested by the reviewer, we performed residue swaps between Leu1218 and Ile1232 ($L_{1218}I/I_{1232}L$), and between Gly1219 and Ala1226 ($G_{1219}A/A_{1226}G$) (Fig. 3C). Neither these swaps nor the corresponding single mutations negatively affected VSVDG-GFP entry. These findings suggest

that the observed results are not artifacts due to alanine substitution. We have included these new data, along with a schematic representation of the TMD, in the newly added Figure 3.

3. The manuscript needs to be evaluated for careless mistakes in sentence construction and punctuation- e.g., line 81: it should be 3.4 not 3,4.
We apologize for the mistakes. We have revised the manuscript accordingly.

4. I suggest not using “variant” to describe the constructs. These did not arise on their own through evolution, but were generated through site-directed mutagenesis.
We thank the reviewer for this suggestion. In response, we have replaced the term “variant” with “mutation” throughout the manuscript, except when referring to naturally occurring sequences, where "variant" remains appropriate.

Reviewer #2:
The manuscript entitled "The sequence and structural integrity of the SARS-CoV-2 Spike protein transmembrane domain is crucial for viral entry" by Ortiz-Mateu J et al. targets the TMD of the SARS-CoV-2 Spike protein with various mutations and demonstrates that the TMD is crucial for viral entry. The authors performed various experiments to verify their claim. However, I have several questions related to the experimental design and the results obtained from those experiments, which are listed below.
However, most of these results are not interconnected.
1. The authors have mentioned that several structures of the S protein have been solved. However, only one includes the TMD. A reference is required for the statement, "Several structures of the S protein have been solved."
We have included the necessary references.

2. "No infection was observed when the TMD of the SARS-CoV-2 S protein was eliminated (ΔTMD) or substituted by a scrambled version of the TMD (SCRBL), where the composition of the residues included in the TMD was preserved, but their positions were randomly varied, confirming the importance of the SARS-CoV-2 S protein TMD in the entry process and indicating a role beyond." – How did the authors confirm that the spike protein is attached to virus particles in the ΔTMD or scrambled version of the TMD (SCRBL)? Generally, TMDs play a vital role in the spike protein's attachment to the virus. It is obvious that if the TMD is eliminated, the spike protein should not attach to virus particles, and no infection would be observed. Similarly, the scrambled TMD (SCRBL) version would severely affect the transmembrane helicity. How would the particles be suitable for infection in this case?
To determine whether the absence of infectivity in certain VSVDG-GFP pseudoparticle constructs was due to a lack of surface expression of the SARS-CoV-2 S protein, we labeled the surface-expressed spike with an anti-RBD antibody and performed flow cytometry analysis.

These data are presented in Figure 4. All spike protein mutants with reduced pseudovirus infectivity were included in this assay, with the WT spike serving as a positive control.

As expected, the ΔTMD construct lacks membrane anchoring and thus should not reach the cell surface. We used this construct as a negative control. Our results indicate that all spike mutants, with the exception of Ins-A1228, were present at the plasma membrane at levels comparable to WT. These findings validate the infectivity results observed in the pseudovirus assay.

The SCRBL construct exhibited only a minor, statistically non-significant reduction in surface expression, likely because key biophysical properties such as hydrophobicity (analyzed in Figure 1) and, potentially, helical propensity are preserved.

3. The authors used AlphaFold for structure prediction, but nowhere in the methods section is it mentioned which version or tools were used (e.g., AlphaFold2, ColabFold) or how they were used.

We used AlphaFold3 for structural predictions. This is now explicitly stated in the figure legends of Figures 3A (previously 1C), Supplementary Figure 2, and Figure 6. It is also mentioned in the Results section on page 14.

The Method section has been expanded to include the required information.

4. Throughout the manuscript, the authors mention various mutations; did the authors use any computational tools to predict the effects of mutations in the TMD? I do not find any details about the mutations or primer design in the methods section.

We did not use any computational tool to predict the effect of the mutations. These were rationally design base on previous results.

5. It seems that the authors performed computational studies to incorporate Ala between Phe and Ile or to change hydrophobic amino acids to Ala. However, the authors claim that Figure 2D shows that Ins-Ala1221 significantly reduced the infectivity of pseudo-typed VSVDG-GFP particles. Were these changes expressed in cells? Otherwise, how could the authors claim that the infectivity of the pseudo-typed VSVDG-GFP particles was reduced? What experiments did the authors perform to support this claim? Most importantly, the authors performed numerous mutations, and it is demonstrated that, in all cases, the TMD remains intact. It is difficult to believe that the TMD is expressing under any conditions or that any changes occurred.

Yes, all mutations, including Ins-A_{1221}, were expressed in cells as part of our pseudoparticle infectivity assays shown in Figure 2. Expression of these mutants was confirmed in HEK293T cells by western blotting (Supp. Figure 1) and flow cytometry for surface expression (Figure 4). Several of the constructs were also tested in BiMuC and BiFC assays. Thus, our conclusions are supported by both functional and expression data.

6. On what basis did the authors perform all the mutations in the TMD? What is the rationale behind this? Gly1223 to Ile, Gly1219 to Ile, Gly1223 to Ile, Ile1221 to Tyr, Ile1225 to Tyr, Met1229 to Tyr, Ala1222 to Ile, Gly1223 to Ile, Ala1222 to Tyr, Gly1223 to Tyr, Leu1224 to Tyr, Leu1224 to Tyr, and Val1228 to Tyr (G1223I, G1219I, G1223I, I1221Y, I1225Y, M1229Y, A1222I, G1223I, A1222Y, G1223Y, L1224Y, L1224Y, V1228Y, respectively). Did the authors randomly mutate so many amino acids in the TMD? Why were these specific mutations chosen? Is there any supporting literature? Are these mutations physiologically relevant?

The mutations were not chosen randomly. As described in the Results section, our strategy focused on dissecting the role of GxxxG and hydrophobic zipper motifs in TMD oligomerization. For instance, the G1223I substitution was selected based on its ability to disrupt dimerization in Glycophorin A, as reported previously (refs. 1,2). Substitutions such as I1221Y, I1225Y, and M1229Y were selected due to their documented influence on TMD homo-oligomerization (ref. 3). Additional substitutions target key residues along either the hydrophobic face (e.g., Leu1218, Val1228) or the small residue-rich face (e.g., Gly1219, Ala1222), informed by helical wheel projections (Figure 3A) and relevant literature.

7. What is the rationale behind replacing Ile1221, Ile1225, or Met1229 with tyrosine (I1221Y, I1225Y, and M1229Y)? Tyrosine has a much bulkier shape than Ile and Met, which could alter surrounding shapes and interactions. What is the structural reasoning behind the slight reduction in viral entry for the substitution of Ile1221 with Tyrosine (I1221Y)?

These substitutions were designed to test whether disruption of the hydrophobic interface impacts oligomerization or infectivity. Although tyrosine is indeed bulkier, prior studies have shown that such substitutions can alter TMD oligomerization (ref. 3). Our data show that these substitutions slightly reduce infectivity (Figure 2), but do not impair oligomerization as measured by the BiFC assay. This suggests that the hydrophobic surface may not play a dominant role in TMD-TMD interactions.

8. It is expected that substituting small amino acids, Gly1219 and Gly1223, with isoleucine (G1219I, G1223I) would affect the TMD, which may further decrease pseudo-typed VSVDG-GFP infectivity. Can the authors discuss the structural changes in the TMD due to this substitution and how it affects infectivity?

These substitutions target the small-residue face of the helix, which may be critical for tight TMD-TMD packing. Replacing glycine with bulkier isoleucine is expected to disrupt such interactions. Our infectivity assays show a significant reduction upon these substitutions, supporting the hypothesis that these residues contribute structurally to TMD-mediated functions.

9. The authors also mentioned that double substitutions (Figure 2E) reduce the infectivity farther- reason behind this is small residues and the hydrophobic bulky residues aligned on opposing faces of the helix. However, from Figure 2E, it is not clear what they are trying to

describe. Additionally, the authors mentioned that infectivity is reduced because the residues likely participate in protein-protein or TMD-lipid interactions. The authors should include proper figures to describe their hypothesis and claims. It is really difficult to correlate the figures with the text, and we have to assume what they are trying to represent.

We have reorganized the data previously presented in Figure 2E into a new figure (Figure 3), which now includes additional experiments and a schematic representation of the TMD. This new figure clarifies the rationale behind the mutations and better aligns with the accompanying text.

10. The BiMuC assay showed that there was no alteration in oligomerization due to the single substitutions I1221Y, I1225Y, or M1229Y, and it also did not alter oligomerization. Then why did the authors find that the substitution of Ile1221Y slightly reduced viral entry? How is it structurally significant that the bulky tyrosine substitution does not affect or alter the structure of oligomerization? Could the authors explain the reason behind this?

The infectivity reduction observed for I1221Y in pseudovirus assays (Figure 2) is not mirrored by an effect on oligomerization. This suggests that while oligomerization is maintained, other properties—such as TMD-mediated membrane fusion or interaction with lipids—may be affected. This interpretation is included in the Results section.

11. The BiMuC assay suggested that the double substitution L1224Y V1228Y did not decrease the observed fluorescence. The authors claimed that the hydrophobic surface does not participate in the oligomerization of the SARS-CoV-2 S protein TMD. The authors already have structural information about the TMD. Can the authors explain why they believe the hydrophobic surface does not affect oligomerization?

Our BiFC assay data show that substitution of key hydrophobic residues (e.g., L1224Y, V1228Y) with bulky residues does not impair oligomerization. This suggests that the hydrophobic surface is not essential for TMD oligomerization in the SARS-CoV-2 spike protein. This conclusion is discussed in both the Results and Discussion sections.

12. In the methods section, the authors mentioned Ct of the VFP. What is Ct? I assume it refers to the C-terminus. Please clarify this.

We thank the reviewer for pointing this out. We have now clarified that "Ct" and "Nt" refer to the C-terminal and N-terminal regions, respectively, of the Venus fluorescent protein. These abbreviations are now defined in the Methods section.

References

1. Lemmon, M. A., Flanagan, J. M., Treutlein, H. R., Zhang, J. & Engelman, D. M. Sequence specificity in the dimerization of transmembrane alpha-helices. *Biochemistry* **31**, 12719–12725 (1992).

2. Orzáez, M., Lukovic, D., Abad, C., Pérez-Payá, E. & Mingarro, I. Influence of hydrophobic matching on association of model transmembrane fragments containing a minimised glycophorin A dimerisation motif. *FEBS Lett.* **579**, 1633–1638 (2005).
3. Fu, Q. & Chou, J. J. A Trimeric Hydrophobic Zipper Mediates the Intramembrane Assembly of SARS-CoV-2 Spike. *J. Am. Chem. Soc.* **143**, 8543–8546 (2021).

We thank the reviewers for their comments and suggestions. Below, we provide point-by-point responses to each comment, with our replies highlighted in **blue**.

Reviewer #1
The sequence and structural integrity of the SARS-CoV-2 Spike protein transmembrane domain is crucial for viral entry
By Ortiz-Mateu et al
The authors have satisfactorily addressed the questions I posed. However, I feel the Discussion section is not well written and does a disservice to the results. The authors have missed an opportunity to provide context for their results, to suggest what these mutations mean for spike trimerization, spike function, viral infectivity, and where the field should go next using their results as a launching pad. They correctly cite the Lemmon articles but do not provide any comparative discussion for the protein-protein interaction strategy adopted by the spike TMD relative to endogenous cellular proteins like glycophorins. Such comparisons will substantially elevate the manuscript in my opinion. I strongly urge the authors to expand the Discussion section.

We have re-organize and expand our discussion section to better summarize the conclusions of our work. We have also clarify some concepts in the Results section. However, we will like to remain cautious about our conclusions as more research must be done to precisely identify the role of the different amino acids in the S protein biogenesis and function.

Reviewer #2 (Remarks to the Author):
The authors have revised several figures and addressed most of the reviewers' suggestions. Most of the responses are convincing, and the manuscript has improved significantly.

One minor comment:
The manuscript is written or compiled in an unusual manner. In several instances, figure legends appear before the corresponding figures, and at times, legends for both main and supplementary figures are presented together with the result section. This formatting makes it somewhat difficult to follow the Results section coherently. The authors are requested to carefully organize the manuscript files to improve clarity and facilitate easier reading for reviewers and readers.

We have included the figures (except supplemental figures) in the main article file as suggested by the journal peer review guidelines. Figures now appear together with the figure legend. Additionally figures and supplemental figures have been included as separated files for publications purposes. We hope the new organization improves clarity and facilitates the reading for the reviewers.