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SARS-CoV-2 Alpha, Beta and Delta variants display enhanced Spike-mediated Syncytia Formation

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Dear Olivier,

Thank you for submitting your manuscript to The EMBO Journal. Your study has now been seen by two referees and I am afraid that that the overall recommendation is not very positive.

While the referees appreciate that the analysis adds new insight, they also raise concerns with it that precludes publication here. They find that important controls are missing and that the analysis would have to be extended for further consideration here. Given these comments by good experts in the field and as it is unclear if they can be resolved, I am afraid that I see no other choice than to reject the MS.

I am sorry that I can't be more positive on this occasion.

With best wishes

Karin

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

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Referee #1:

Rajah et al. investigated the formation of syncytia mediated by the viral spike protein of different variants of SARS-CoV-2, namely B.1.1.7 (alpha, UK variant) and B.1.351 (beta, South African variant). The authors selected various cell lines, i.e., Vero, Caco-2, Calu-3, primary airway cells, and U2OS. They first assessed viral replication and release of viral progeny in most these cells, which were globally similar for all the viruses tested. Cell-cell fusion was then assayed using a GFP-split based system recently developed by the authors and published in EMBO J last year. They observed that the syncytia formation was faster, larger, and more numerous when cells expressed the variant spikes compared to the original SARS-CoV-2 spike or the previously identified D614G variant spike. Fusion in infected cells was similarly sensitive to IFN treatment, regardless of the expressed spike protein. Finally, the authors sought to correlate spike-specific mutations in these variants with cell-cell fusion, the capacity of SARS-CoV-2 to bind to the ACE2 receptor, and the ability of certain antibodies to bind to the spike. The results could be important and interesting, but the enthusiasm is damped by the many important controls that are missing, making the conclusions drawn by the authors and their discussion overstated in many places. I also have serious concerns about the appropriateness of several approaches used in the study. This study has too many flaws to be published under the current form.

Major comments

1. Line 70, strictly speaking, I disagree with the statement that syncytia formation is analogous to virus-cell fusion. This may be true for HIV that assembles at the plasma membrane but not for SARS-CoV-2 that buds from the ER-Golgi network. This has strong implications for spike folding and proteolytic processing on viral particles in producer and target cells and ultimately for infectivity. Overexpression of the spike to be secreted onto the cell surface to force the syncytia formation implies a different maturation process of the protein, and as a potential consequence, a different requirement for cellular proteases. The results obtained here are valid for syncytia formation but, overall, should not be extrapolated in the manuscript to the fusion of viral particles during normal infection, which remains the main route of host-to-host transmission.
2. TMPRSS2 has been shown to be important for spike activation, especially at the cell surface. This was notably one of the findings of a previous study published by the authors last year (Buchrieser et al., 2020). What is the expression level of TMPRSS2 on the surface of the different cell lines tested here? How do the variant mutations impact proteolytic processing of the spike by TMPRSS2, which is broadly expressed, especially in lung tissue? The role of TMPRSS2 needs to be documented as it has become clear in recent publications by independent groups that this protease is a key player in SARS-CoV-2 fusion and infection (Ou et al., PLoS Path, 2021; Dittmar et al., Cell Reports, 2021; Koch et al., EMBO J, 2021).
3. No comparison is possible between variants unless spike expression is carefully controlled on the cell surface in the different experiments and cell lines tested. The FACS dot plots shown in Figure EV4A are not convincing. They show the percentage of cells positive for the spike, not the extent to which the positive cells express the spike. Indeed, the MFIs (geometric mean would actually be more appropriate) indicate in Figures EV4B and EV4C that spike expression differs greatly between variants. In addition, the authors assessed spike expression only in 293T cells while experiments are mainly performed with other cell lines. All experiments should be normalized to the level of fully activated spike expressed on the cell surface. Please, see Major comment 1, the spike variant could in addition be processed and activated in a different proteolytic manner.

4. What the authors intend to demonstrate by testing IFN- 1 on infection and cell-cell fusion is not clear. It is expected that the spike is no longer expressed when viral replication is impaired by IFN and that no syncytia is formed as a result. It has been documented many times since last year. In addition, preprints suggest that IFN molecules others than IFN- 1 may be involved. General conclusions about IFN drawn from the assay of a single type of IFN molecule should be made with more caution.
5. Was the expression level of the different IFITMs controlled? What is the cellular localization of the different IFITMs evaluated in this study? What viral mechanisms do they disrupt? The fusion itself, viral replication, spike processing? Again, what the authors intend to demonstrate here is rather vague.
6. The general discussion of the effect of each point mutation is quite unclear, and I do not see what these results add to our general understanding of these mechanisms. Different combinations of mutations should be tested to try to learn more.
7. It is understandable that the rapid rate of emergence of new SARS-CoV-2 variants makes it difficult to include all of them in a study, but unfortunately the British and South African variants have already been replaced by the Indian (delta) variant in many countries, which is now the major cause for concern. The addition of the Indian variant to the study would significantly increase the interest of this work.

Specific comments

1. I would like to see the MOI indicated in all figure legends. Although I can easily imagine that the authors employed the same MOI, this information is important to compare the different variants.
2. What is the state-of-the-art about the formation of syncytial pneumocytes in patients infected with the different variants? It is not clear from the text which viruses the authors are referring to.
3. In Figure 1C, viral replication in Vero cells begins 24-48 hours post-infection, whereas fusion is readily visible after only 6-8 hours in Figure 3C. The authors should provide an explanation for this.
4. Figure EV1A, the y-axis of the three graphs should be similar to facilitate comparison of the values obtained for viral replication in the three cell lines.
5. Syncytia formation in Vero cells appears negligible compared to that in U2OS (Figures EV3A and EV3B). Could the authors comment on this point?
6. What was the control used in Figure EV5. I could not find the information in the text.
7. Disturbingly, the authors tested spike binding to ACE2 with the spike expressed on the cell surface and a soluble form of the receptor. The same is true for the antibody assays. Binding of viral particle to ACE2-expressing cells or assessment of the ability of antibodies to block binding or infection by the different variants would be more intuitive, except that the authors intend to correlate the ability of antibodies to block the different spikes with syncytia formation and protection of infected people from severe forms of COVID-19. This latter case should be discussed more clearly and explicitly. In the current form, the results with antibodies seem disconnected from the rest of the investigation.
8. Could the authors discuss their results on ACE2 in light of the most recent literature showing that SARS-CoV-2 can use alternative receptors to infect cells (Puray-Chavez et al., Cell Reports, 2021)?

Referee #2:

The manuscript submitted by Rajah, et al. is an informative study that explores the effects of SARS-CoV-2 spike protein variants on viral replication, binding to the ACE2 receptor and cell fusion. Additionally, the authors determine whether the mutations in these variants alter binding to human monoclonal antibodies. The experiments are well-controlled and the results are clearly presented. The data nicely demonstrate that certain mutations affect cell fusion and suggest that some of these mutations affect antibody binding. While interesting, additional experimentation would clarify the impact of these findings.

1. While the initial infection studies are performed in multiple cell types and in primary cells, subsequent studies are in U2OS and Vero cells. Additional studies in the more relevant cell lines, which express TMPRSS2, would support the results showing differences in cell fusion.

2. The authors do not determine whether any of these mutations affect processing of Spike by furin or TMPRSS2. These experiments would add additional biological relevance.

3. More information is required for the monoclonal antibody studies. What is the source of these antibodies and what is the evidence suggestion region of binding to Spike? Also, the results of these studies would be enhanced by the inclusion of virus neutralization assays for the antibodies recognizing the RBD.

Minor editorial changes should be made throughout the manuscript. For example:

Line 106: RT-qPCR for the SARS-CoV-2 N protein should be for the gene encoding the SARS-CoV2 N protein

Line 196: the word "boarder" should be replaced with "border"

Dear Editor,

We would like to thank you for your consideration of our manuscript entitled "Syncytia formation by SARS-CoV-2 infected cells" for publication in The EMBO Journal. We readily concede that the reviewers' criticisms improve the clarity and the scientific rigor of our manuscript and we thank them for their diligence. We have responded to reviewers by incorporating new experimental data, rewording for clarity and incorporating a few sentences to further discuss the limitations of our work. Below we address each of the reviewers concerns in point-by-point form:

Referee #1:

Rajah et al. investigated the formation of syncytia mediated by the viral spike protein of different variants of SARS-CoV-2, namely B.1.1.7 (alpha, UK variant) and B.1.351 (beta, South African variant). The authors selected various cell lines, i.e., Vero, Caco-2, Calu-3, primary airway cells, and U2OS. They first assessed viral replication and release of viral progeny in most these cells, which were globally similar for all the viruses tested. Cell-cell fusion was then assayed using a GFP-split based system recently developed by the authors and published in EMBO J last year. They observed that the syncytia formation was faster, larger, and more numerous when cells expressed the variant spikes compared to the original SARS-CoV-2 spike or the previously identified D614G variant spike. Fusion in infected cells was similarly sensitive to IFN treatment, regardless of the expressed spike protein. Finally, the authors sought to correlate spike-specific mutations in these variants with cell-cell fusion, the capacity of SARS-CoV-2 to bind to the ACE2 receptor, and the ability of certain antibodies to bind to the spike. The results could be important and interesting, but the enthusiasm is damped by the many important controls that are missing, making the conclusions drawn by the authors and their discussion overstated in many places. I also have serious concerns about the appropriateness of several approaches used in the study. This study has too many flaws to be published under the current form.

We thank the reviewer for her/his critical evaluation of our manuscript and for the suggestions. We readily concede that many of reviewers' criticisms improve the clarity and the scientific rigor of our manuscript and we appreciate their diligence. We agree that the concern regarding our controls could be further clarified with data in our possession and with a more detailed description in the manuscript. We have presented the control data in the point-by point response and have also included it in the figures. Furthermore, we have been more cautious about not overstating our result and we take the reviewer's advice and clearly state that this is a study on Spike protein mediated syncytia formation by altering the title to "SARS-CoV-2 variants Alpha, Beta and Delta display enhanced Spike mediated syncytia formation" and by rewriting the areas that they felt extrapolated too much from the data. Additionally, we obliged the reviewer by including some data on the Delta variant. The specific changes we made to the manuscript is documented below in point-by-point form.

Major comments

1. Line 70, strictly speaking, I disagree with the statement that syncytia formation is analogous to virus-cell fusion. This may be true for HIV that assembles at the plasma membrane but not for SARS-CoV-2 that buds from the ER-Golgi network. This has strong implications for spike folding and proteolytic processing on viral particles in producer and target cells and ultimately for infectivity. Overexpression of

the spike to be secreted onto the cell surface to force the syncytia formation implies a different maturation process of the protein, and as a potential consequence, a different requirement for cellular proteases. The results obtained here are valid for syncytia formation but, overall, should not be extrapolated in the manuscript to the fusion of viral particles during normal infection, which remains the main route of host-to-host transmission.

Upon re-reading the manuscript in light of this comment, we believe that the reviewer is correct in their assessment that we did not contextualize our manuscript sufficiently and our phrasing in many places was improper. When we stated that "Syncytia formation was analogous to virus-cell fusion" in the introduction we intended to describe the mechanistic process in which the spike induces membrane fusion. The processes are broadly comparable mechanistically as both virus-cell fusion and cell-cell fusion involves the spike interacting with the ACE2 receptor, the fusion peptide being exposed and penetrating the membrane, followed by pore formation and the mixing of contents. While it is true that the spike protein is sequestered in ER-Golgi; recent work by Cattin-Ortola and colleagues suggest that the SARS-CoV-2 spike protein possesses a suboptimal COPI binding residues which allows for leakage from the ER -Gogli and accumulation at the plasma membrane and ultimately Cell-cell fusion (Cattin-Ortola et al, 2021 ; https://doi.org/10.1101/2020.10.12.335562). It is unclear at this point if different proteases are involved, but the aforementioned mechanism will sufficiently allow for cell-cell fusion. Indeed, we characterized increased syncytia formation by the variants during real virus infection in addition to the overexpression of just spike protein (Fig 2, EV2, EV3). As the reviewer agrees that our data is valid for syncytia formation but does not find the analogy between cell-cell fusion and virus-cell fusion appropriate we have rewritten section of the manuscript to address this:

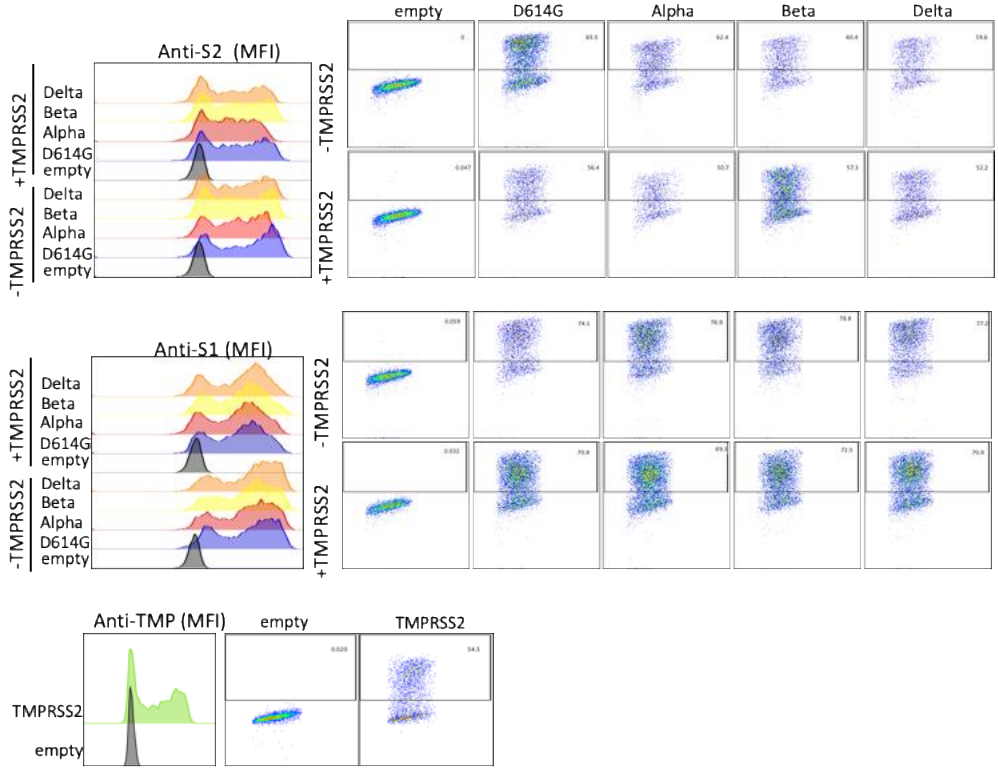
A) We changed the title of the manuscript to "SARS-CoV-2 variants Alpha, Beta and Delta display enhanced Spike mediated syncytia formation" from the previous "spike mediated fusion"
B) The introduction was rewritten to remove the offending statement and now better describes how the spike protein could be expressed at the plasma membrane during infection in order to induce syncytia formation and molecular mechanism in which the spike protein induces fusion. This section now reads as follows:

"The SARS-CoV-2 S protein is a viral fusogen. The interaction of trimeric S with the ACE2 receptor and its subsequent cleavage and priming by surface and endosomal proteases results in virus-cell fusion (Hoffmann *et al*, 2020). Merging of viral and cellular membranes allows for viral contents to be deposited into the cell to begin the viral life cycle. Within the cell, newly synthesized spike, envelope and membrane proteins are inserted into the endoplasmic reticulum (ER), and trafficked and processed through the ER-Golgi network (Cattin-Ortolá *et al*, 2021; Duan *et al*, 2020; Nal *et al*, 2005). Virion are formed by budding into ER-Golgi membranes and are then transported to the surface in order to be released from the cell (Klein *et al*, 2020). While the majority of the S protein is sequestered within the ER, motifs within its cytoplasmic tail allow for leakage from the Golgi apparatus and localization at the plasma membrane (Cattin-Ortolá *et al.*, 2021). The S protein at the surface of an infected cell interacts with receptors on adjacent cells, fusing the plasma membranes together and merging the cytoplasmic contents. We and others had previously shown that the S protein interacting with the ACE2 receptor induces cell-cell fusion (Braga *et al.*, 2021; Buchrieser *et al.*, 2020; Lin *et al*, 2021; Sanders *et al.*, 2021; Zhang *et al.*, 2021).The TMPRSS2 protease further augments cell-cell fusion (Barrett *et al*, 2021; Buchrieser *et al.*, 2020; Hornich *et al*, 2021)" (lines 78-93)

2. TMPRSS2 has been shown to be important for spike activation, especially at the cell surface. This was notably one of the findings of a previous study published by the authors last year (Buchrieser et al., 2020). What is the expression level of TMPRSS2 on the surface of the different cell lines tested here? How do

the variant mutations impact proteolytic processing of the spike by TMPRSS2, which is broadly expressed, especially in lung tissue? The role of TMPRSS2 needs to be documented as it has become clear in recent publications by independent groups that this protease is a key player in SARS-CoV-2 fusion and infection (Ou et al., PLoS Path, 2021; Dittmar et al., Cell Reports, 2021; Koch et al., EMBO J, 2021).

In terms of syncytia formation, our finding from last year (Buchriesser et al., 2020) suggest that the interaction between the Spike protein at the surface and the ACE2 receptor on the neighbouring cell is sufficient. The presence of the TMPRSS2 proteases further augments cell-cell fusion but is not a requirement. As such the cell lines we used here either contain no or very little endogenous TMPRS22 (Bertram et al., 2010; J virol)(Koch et al., EMBO J, 2021)(protein atlas: https://www.proteinatlas.org/ENSG00000184012-TMPRSS2/cell). This allowed as to focus on the fusogenicity of the variant Spike as they relate to the ACE2 receptor, and indeed we show that the variant spike induced cell-cell fusion nicely corelated with ACE2 affinity (Figure 3 and Fig 5A, EV7A). We agree with the reviewer that characterizing the impact of TMPRSS2 would benefit the manuscript. To this aim, we transfected the spike proteins without and with TMPRSS2 and performed western blots. We found no noticeable differences in the processing of the different spike's and we have included this data in the manuscript (Fig EV4E). We also co expressed the variant spike proteins with and without TMP and stained for either S1 or S2 and also found no real difference between the variants. This data is presented below for consideration:



We have also made the following additions to the text to both describe the results and acknowledge future interest in characterizing the effect of proteases on the variants:

"We then asked whether the TMPRSS2 protease, that cleaves S and facilitate viral fusion, may act differently on the variant S proteins. To this aim, we expressed the different variant S proteins without

or with TMPRSS2 in 293T cells. We examined the processing of the different S by western blot and the surface levels by flow cytometry. The cleavage profile induced by TMPRSS2 and the surface levels of the different variant S proteins were similar **(Fig EV4E)**." (Line 191-195)

"While we had previously shown that the interaction between the S protein on the plasma membrane with the ACE2 receptor on neighboring cells is sufficient to induce syncytia formation, there is compelling evidence of the importance of the TMPRSS2 protease in S activation (Buchrieser *et al.*, 2020; Dittmar *et al*, 2021; Koch *et al*, 2021; Ou *et al*, 2021). We did not detect any major differences in the processing of the variant spike proteins by TMPRSS2. It will be worth further characterizing how the fusogenicity of variant associated mutations are influenced by other cellular proteases." (line 382-388)

3. No comparison is possible between variants unless spike expression is carefully controlled on the cell surface in the different experiments and cell lines tested. The FACS dot plots shown in Figure EV4A are not convincing. They show the percentage of cells positive for the spike, not the extent to which the positive cells express the spike.

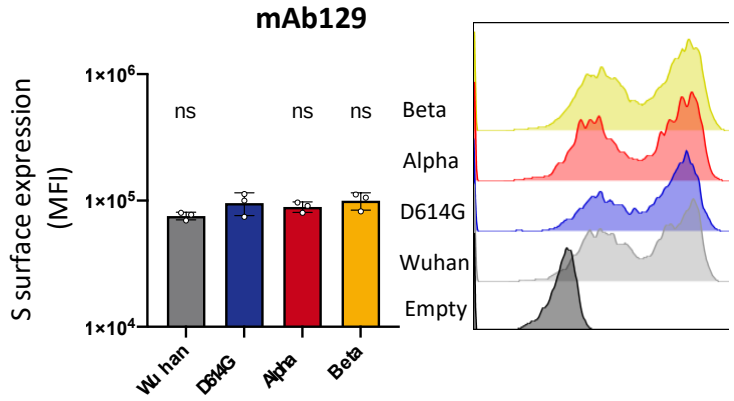
We agree that on its own the FACS plots (EV4A) showing that variant spike proteins are expressed equally between cells is not sufficient to support the argument for greater fusogenicity and that MFI is necessary. However, equal expression within the system (percent of cells) is necessary for quantifying syncytia formation so we chose to include this control data, as well as MFIs. We now used two different pan-coronavirus mAbs to carefully measure the surface levels of the different spike variants. The new results (MFI) appear in the novel Figure EV4C.

Indeed, the MFIs (geometric mean would actually be more appropriate) indicate in Figures EV4B and EV4C that spike expression differs greatly between variants.

In addition to the percent of cell (EV4A) we have also shown the MFI (EV4B) which as the reviewer correctly stresses are crucial to show to the extent to which spike positive cells express the variant spike. We, however, must respectfully disagree with the statement that "spike expression differs greatly between variants". Upon reviewing the disputed figure (below EV4B) it is clear to us that our presentation is not conducive to conveying our message without further clarification.

We intended to show with this figure that the surface expression (MFI) of the variant spikes is similar despite the B.1.1.7 (Alpha) and B.1.351 (Beta) forming more syncytia (Fig.3). Indeed, The D614G reference spike to which everything else is compared to is similar in MFI to the B.1.1.7 and B.1.351. The main discrepancy is the MFI of the Wuhan which is significantly higher than the D614G and the other variants. This is a result of the pan-coronavirus antibody (mAb10) used for this comparison which was isolated form patient infected with the Wuhan strain during the early stages of the epidemic and seems to have a higher affinity for the Wuhan spike. However, despite the higher antibody affinity this the Wuhan strain is the least fusogenic (Fig3). Our manuscript mainly focuses on comparing the D614G, B.1.1.7 and B.1351 which are all recognized equally by this antibody. To remedy this situation, we have presented in the supplement (EV4C, and below for consideration) results from another pan-coronavirus antibody (mAb129), which we had on hand, which clearly shows that the surface expression of the S protein is the

same for variants. We have also tested a panel of other antibodies which support our conclusions (data not shown). We have also added the description of mAB10 to the material and methods for clarification.



We have all added the following section to the materials and method describing the antibodies:

"The antibodies were derived from convalescent individuals by the Mouquet lab at the Institut Pasteur. mAb10 was generated during the early stages of the epidemic from a patient infected with the Wuhan strain and thus has a higher affinity for the Wuhan spike (Planas *et al.*, 2021a)." (line 498-501)

In addition, the authors assessed spike expression only in 293T cells while experiments are mainly performed with other cell lines. All experiments should be normalized to the level of fully activated spike expressed on the cell surface. Please, see Major comment 1, the spike variant could in addition be processed and activated in a different proteolytic manner.

We here suggest the 293T cells are the appropriate control for this system as they lack ACE2 and do not form syncytia. The cell used for syncytia formation experiments are Vero cells, which endogenously express ACE2, as well as U2OS-ACE2 cells; both of which start to fuse extensively upon Spike transfection and are unsuitable for quantitative flowcytometry to examine surface levels. Our goal here is to characterize the spike protein of the variants itself and to demonstrate that they are equally express both inter- and intra-cellularly. We checked for proteolytic processing of with TMP as the reviewer requested (comment 2) and found no significant difference. Here, we are more focused on the spike interaction with the ACE2 receptor as Ace2 is the only necessary component to allow the Spikes to be active in our cell-cell fusion system (Buchrieser et al., 2020). The text has been modified to explain why 293 T cells have been used to study surface expression of the different spikes:

"We then verified that the variation in S mediated fusion was not due to differential cell surface levels. We transfected 293T cells, which lack ACE2 and thus do not fuse upon S expression, with the different variant plasmids in order to assess S protein surface levels by flow cytometry. The variants S proteins were equally expressed after transfection **(Fig. EV4A-C).** (line 180-184)

4. What the authors intend to demonstrate by testing IFN-β1 on infection and cell-cell fusion is not clear. It is expected that the spike is no longer expressed when viral replication is impaired by IFN and that no syncytia is formed as a result. It has been documented many times since last year. In addition, preprints

suggest that IFN molecules others than IFN-β1 may be involved. General conclusions about IFN drawn from the assay of a single type of IFN molecule should be made with more caution.

We intended to show that IFN treatment can effectively reduce syncytia formation as efficiently as replication and it is equally effective between variants. We agree with the reviewer that this information is tangential to the story; however, we believe it important to know how discuss how components of the innate immunity and spike mediated syncytia formation is related. As such we combined all the IFN-B1 data with the IFITM data into one supplemental figure (Fig EV5). The reviewer is also correct in their assessment that we extrapolated too much from our results. We have now combined all of the IFN and IFITM information into a single section of the results and we have taken care to phrase our conclusions more cautiously throughout the manuscript.

The paragraph describing eth effect of IFN and IFITMs on page 11 and 12 now reads:

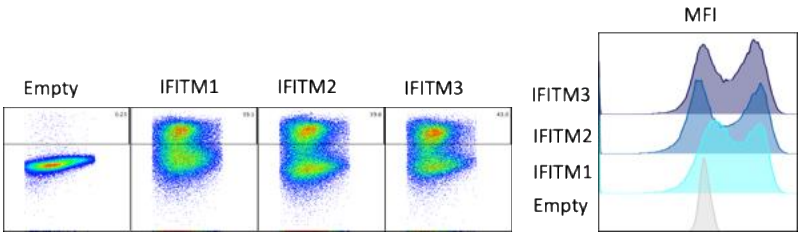
"Restriction of S mediated syncytia formation by IFN-B1and IFITMs

As the variants did not show any major difference in replication under basal conditions, we next investigated if they were differently sensitive to the interferon response. To this aim, we pre-treated Vero cells or U2OS-ACE2 (S-Fuse) cells with increasing doses of IFN-B1 and infected them with the different variants. IFN-□□ was equally effective at reducing viral replication of D614G, Alpha, and Beta variants in Vero cells **(Fig. EV5A)**. Preincubation of S-Fuse cell with IFN-B1 also abrogated infection and syncytia formation to the same extent with the different variants (**Fig. EV5B**). Therefore, IFN-B1similarly inhibited viral replication and reduced syncytia formation by D614G, Alpha and Beta variants.

IFITMs are interferon stimulated transmembrane proteins that restrict early stages of the viral life cycle by inhibiting virus-cell fusion; likely by modifying the rigidity or curvature of membranes (Compton *et al*, 2014; Shi *et al*, 2017; Zani & Yount, 2018). IFITM1 localizes at the plasma membrane while IFITM2 and 3 transit through surface and localize in endo-lysosomal compartments (Buchrieser *et al.*, 2020). We previously reported that IFITMs restrict Wuhan S mediated cell-cell fusion and that their activity was counteracted by the TMPRSS2 protease (Buchrieser *et al.*, 2020). As infection with Alpha and Beta induce more syncytia, we further investigated if this resulted in an increased resistance to IFITM restriction. We thus characterized the impact of IFITMs on syncytia formed upon expression of D614G, Alpha and Beta S proteins in 293T cells. The variants were effectively restricted by IFITMs **(Fig. EV5C-G)**. Of note, the three IFITMs were expressed at similar levels (not shown). The presence of TMPRSS2 increased fusion of all S proteins and reverted the restriction by IFITMs **(Fig. EV5C-G)**. Taken together, our data show that Alpha and Beta variants induce more syncytia, but their S proteins remain similarly sensitive to IFITMs." (Line 198 – 220)

5. Was the expression level of the different IFITMs controlled? What is the cellular localization of the different IFITMs evaluated in this study? What viral mechanisms do they disrupt? The fusion itself, viral replication, spike processing? Again, what the authors intend to demonstrate here is rather vague.

IFITM1, 2 and 3 plasmids are all equally expressed and are ubiquitously used (Shi et al, 2021: EMBO J). These IFITMs plasmids are also functional and have been shown to prevent the fusion and spread of HIV (Compton et al., 2014; Cell Host & Microbe), are capable of inhibiting the formation of placental syncytiotrophoblast (cell-cell fusion) (Buchrieser et al., 2019; Science) and also restrict S mediated fusion (Buchrieser et al., 2020; EMBO J). A more thorough description of the plasmids could we found in our previous publication which we have referenced in the manuscript. Below is the control data showing equal expression of IFITMs with the system and this is now mentioned in line 216-217.



IFITM1 localizes at the cell membrane while IFITM2 and 3 cycle through the cell membrane to the ER and Gogli. Their ability to restrict syncytia formation occurs at the cell membrane, likely by increasing the rigidity of the cell membrane prevent fusion. Thus, we suggest it is of value to understand this process in the context of the variants. The reviewer was correct in criticizing us for not providing adequate background information and context and we have now added and section to the introduction describing the function of IFITMs in syncytia formation. This we believe will make our point more understandable to potential readers.

We have added a background section to the introduction which now reads:

"S-mediated cell-cell fusion is sensitive to innate immunity components. The interferon response to SARS-CoV-2 is one of the key factors down-modulating viral entry and replication, and deficiencies in the interferon response are associated with severe or critical COVID-19 (Arunachalam *et al*, 2020; Bastard *et al*, 2021; Bastard *et al*, 2020; Hadjadj *et al*, 2020; van der Made *et al*, 2020). SARS-CoV-2 induced syncytia formation by the Wuhan strain is restricted by innate immunity, in part through the action of interferon induced transmembrane proteins (IFITMs) (Buchrieser *et al.*, 2020). IFITM1, 2 and 3 are restriction factors which display antiviral activity against a variety of enveloped viruses including SARS-CoV-2; likely by increasing membrane rigidity and hindering virus-cell fusion (Shi *et al*, 2021). Their effectiveness at restricting cell-cell fusion of novel variants has yet to be assessed." (109-118)

We have also described the localization of the IFITMs and stated that they are equally expressed in our system in the new results section:

"IFITMs are interferon stimulated transmembrane proteins that restrict early stages of the viral life cycle by inhibiting virus-cell fusion; likely by modifying the rigidity or curvature of membranes (Compton *et al*, 2014; Shi *et al*, 2017; Zani & Yount, 2018). IFITM1 localizes at the plasma membrane while IFITM2 and 3 transit through surface and localize in endo-lysosomal compartments (Buchrieser *et al.*, 2020)." (Line 207-211)

6. The general discussion of the effect of each point mutation is quite unclear, and I do not see what these results add to our general understanding of these mechanisms. Different combinations of mutations should be tested to try to learn more.

The mutations provide a higher resolution of understanding why the variants form more syncytia and by examining their individual contribution. We were able show that several antibody escape mutations are detrimental to syncytia formation (suggesting a trade-off between fusogenicity and antibody escape) and

we identified several mutations as increase fusogenicity in both the B.1.1.7 (Alpha) and B.1.351 (Beta) variant, helping to understand why we see more syncytia formation with these variants. This information will further our understanding how the fusion machinery of the spike protein is affected by the mutations accumulated by the variants. To better contextualize this, we have added a section to the introduction describing the different domains of the spike protein how they function. It reads as follows:

"The S protein is comprised of S1 and S2 subunits. The S1 subunit includes the N-terminal domain (NTD) and the receptor binding domain (RBD). The function of the NTD has yet to be fully elucidated but it may be associated with glycan binding, receptor recognition and pre-fusion-to-post fusion conformational changes. The NTD is also targeted by neutralizing antibodies (Chi *et al*, 2020; Krempl *et al*, 1997; Zhou *et al*, 2019). The RBD interacts with the ACE2 receptor and is the main target for neutralizing antibodies (Huang *et al*, 2020b). The S2 domain consists of the fusion peptide (FP), heptapeptide repeat sequences 1 and 2, (HR1 and HR2), the transmembrane anchor, and the C-terminal domain. The FP inserts into the target membrane by disrupting the lipid bilayer and anchors the target membrane to the fusion machinery (Huang *et al.*, 2020b). This exposes regions of HR1 that interact with HR2, forming a flexible loop that brings the membranes together to facilitate fusion (Huang *et al.*, 2020b). The versatility of the S protein suggests that any mutations that have arisen are of particular concern as they can affect fusogenicity, antibody recognition, affinity to ACE2, proteolytic processing and incorporation into virions. There is a general paucity of information regarding how the mutations associated with variant S proteins contribute to cell-cell fusion." (Line 94-108)

While we find that the variant fusion directly correlates with ACE2 affinity, the individual mutations play differential roles suggesting an overalls structural change in the spike elicited by the mutations. We believe that the next step would be to understand these structural changes that the mutations (or as the reviewer suggests, combinations of them) induce in the spike protein. However, this is beyond our scope and expertise and hope that the characterizations we provide in terms of fusogenicity and ACE2 binding will assist those who venture to do so. We have indicated in the revised discussion the interest of further combing the different mutations. The text now reads as follows:

"Despite the insights we provide into the S-mediated fusogenicity of the variants, we did not address the conformational changes that the mutations individually or in combination may elicit." (line 401-403)

7. It is understandable that the rapid rate of emergence of new SARS-CoV-2 variants makes it difficult to include all of them in a study, but unfortunately the British and South African variants have already been replaced by the Indian (delta) variant in many countries, which is now the major cause for concern. The addition of the Indian variant to the study would significantly increase the interest of this work.

We are grateful that the reviewer acknowledges that the time it takes to prepare and experimentally characterize the variants is not easily synchronized with the emergence of new variants. We, however, understand that Indian variant will benefit this work and we have done our best to oblige the reviewer. We have generated and characterized the B.1.617.2 (Indian; Delta) spike protein for its ability to induce cell-cell fusion, its relative fusion kinetics to the other variants and ACE2 binding. We find that the Delta variant fuses more and faster than D614G and similarly to Alpha (B.1.1.7). The delta variant also binds to ACE2 more than D614G but less that B.1.1.7. We have added a new main figure with this data to the manuscript (Figure 6). The results section in now reads:

"Spike mediated syncytia formation by the Delta variant

With the emergence and rapid spread of the Delta variant, we sought to characterize its potential to form syncytia. We recently shown that the Delta variant induce large syncytia in S-Fuse cells (Planas *et al.*, 2021b). We thus compared the fusogenicity of the Delta S protein to that of D614G and Alpha. We transiently expressed the three S proteins in Vero-GFP split cells. The Delta S protein triggered more cell-cell fusion than the D614G variant but was similar to the Alpha S protein **(Fig 6A).** The fusion kinetic of the Delta S was also similar to Alpha but more rapid than D614G **(Fig 6B).** We confirmed that that the variant S proteins were equally expressed on the surface by transfecting them into non-fusogenic 293T cells and performing flow cytometry upon staining with the pan-SARS-CoV-2 mAb129 (**Fig EV4D)**. We next examined the ACE2 binding potential of Delta S protein using our aforementioned soluble biotinylated ACE2. The Delta S protein has a higher binding capacity to ACE2 than the D614G S protein, but the binding was lower than the Alpha S protein **(Fig 6C)**." (line 297-309)

Specific comments

1. I would like to see the MOI indicated in all figure legends. Although I can easily imagine that the authors employed the same MOI, this information is important to compare the different variants.
The reviewer is correct in assuming we used the same MOI for the comparisons, and we have added the MOI in the figure legends for clarity.

2. What is the state-of-the-art about the formation of syncytial pneumocytes in patients infected with the different variants? It is not clear from the text which viruses the authors are referring to.
There is a paucity of information on how the variants contribute differential to syncytia formation. We have clarified our aims better in the rewritten introduction.

"The versatility of the S protein suggests that any mutations that have arisen are of particular concern as they can affect fusogenicity, antibody recognition, affinity to ACE2, proteolytic processing and incorporation into virions. There is a general paucity of information regarding how the mutations associated with variant S proteins contribute to cell-cell fusion." (lines 104-108)

3. In Figure 1C, viral replication in Vero cells begins 24-48 hours post-infection, whereas fusion is readily visible after only 6-8 hours in Figure 3C. The authors should provide an explanation for this.

The reviewer here has compared figure 1 (viral replication after infection) to figure 3 (individual spike protein transfection). In figure 2C we show that syncytia form after viral infection at 24h-48h and that the variant form more syncytia. We initially infected the cells and saw more syncytia, so we proceeded to look at the main fusogen, the spike protein by transfection. In the infection context, there are other viral proteins (eg the M protein) and host factors that sequester the Spike in the ER-Golgi. The solitary transfection of the spike protein, in the absence of other protein, speeds up syncytia formation. This data is coherent within our study and with the literature. We have clarified this point in the text:

"Within the cell, newly synthesized spike, envelope and membrane proteins are inserted into the endoplasmic reticulum (ER), and trafficked and processed through the ER-Golgi network (Cattin-Ortolá

et al, 2021; Duan *et al*, 2020; Nal *et al*, 2005). Virion are formed by budding into ER-Golgi membranes and are then transported to the surface in order to be released from the cell (Klein *et al*, 2020). While the majority of the S protein is sequestered within the ER, motifs within its cytoplasmic tail allow for leakage from the Golgi apparatus and localization at the plasma membrane (Cattin-Ortolá *et al*., 2021)." (Lines 81-97)

4. Figure EV1A, the y-axis of the three graphs should be similar to facilitate comparison of the values obtained for viral replication in the three cell lines.

We have changed the axis of all the graphs so that they are comparable.

5. Syncytia formation in Vero cells appears negligible compared to that in U2OS (Figures EV3A and EV3B). Could the authors comment on this point?

U2OS cells are much larger and flatter than the Vero cells and form syncytia that are conducive to imaging. U2OS cell also transduced to over-express ACE2 whereas Vero cells have an endogenous ACE2 level. We believe that the higher levels of ACE2 in U2OS relative to Vero cells explain those differences.

6. What was the control used in Figure EV5. I could not find the information in the text.

The control is a transfection of the same plasmid that encodes only for PuroR. It is mentioned in line 408 but we agree it not clear and we have added this clarification to the material and methods:

"10 ng of phCMV-SARS-CoV2-S and/or 25 ng of pCDNA3.1-hACE2, 25 ng of pCSDest-TMPRSS2, and 40 ng of pQCXIP-IFITM were used and adjusted to 100 ng DNA with pQCXIP-Empty (control plasmid)" (lines 472-474)

7. Disturbingly, the authors tested spike binding to ACE2 with the spike expressed on the cell surface and a soluble form of the receptor. The same is true for the antibody assays. Binding of viral particle to ACE2-expressing cells or assessment of the ability of antibodies to block binding or infection by the different variants would be more intuitive, except that the authors intend to correlate the ability of antibodies to block the different spikes with syncytia formation and protection of infected people from severe forms of COVID-19. This latter case should be discussed more clearly and explicitly. In the current form, the results with antibodies seem disconnected from the rest of the investigation.

The ACE2 binding experiments provide an understanding on how the variant and their mutation spike proteins bind to ACE2, and a higher receptor affinity could potentially be a mechanism for the increased levels of syncytia formation seen with the variant spikes. For syncytia formation we find that Wuhan > D614G > B.1.351 > B.1.1.7 which matches the ACE2 binding profile, providing a potential mechanism as to why the new variants for more syncytia. We had previously shown that some of these neutralizing antibodies are not able restrict infection by the variants (Planas et al, 2021; Nature Medicine). Neutralizing mAb48 does not restrict B.1.1.7 (alpha) or B.1.351 (Beta) infection induced fusion but does restrict D614G. Our intention was to see which specific mutations in the spike protein itself hinder neutralizing antibody recognition and thus cell-cell fusion. We find that many of the antibody escape mutations are also detrimental to the fusogenicity of the spike suggesting that there many trade-offs between antibody escape and greater fusogenicity. We have rewritten the section of the results to provide better context and to not over extrapolate. The modified paragraph reads as follows:

"**Antibody binding to Spike proteins bearing individual variant-associated mutations.**

We had previously found that certain neutralizing antibodies differentially affect SARS-CoV-2 D614G, Alpha and Beta variants (Planas *et al.*, 2021a). For instance, neutralizing monoclonal antibody 48 (mAb48) restricts D614G virus but not Alpha or Beta variants (Planas *et al.*, 2021a). We sought to determine which mutations in variant S proteins contributed to the lack of recognition by the neutralizing antibodies. To this aim, we assessed by flow cytometry the binding of a panel of four human monoclonal antibodies (mAbs) to the different S mutants. As a control we used mAB10, a pan-coronavirus antibody that targets an unknown but conserved epitope within the S2 region (Planchais, manuscript in preparation). mAb10 equally recognized all variants and associated individual mutations **(Fig. 5C)**. mAb48 and mAb98 target the RBD and mAb71 the NTD (Planas *et al.*, 2021b)(Planchais, manuscript in preparation). mAb48 did not recognize the Beta variant, and more specifically did not bind to the K417N mutant **(Fig. 5C)**. The mAb71 recognized neither Alpha nor Beta variants and did not bind to their respective NTD ΔY144 and Δ242-244 mutations. The K417N and Δ242-244 mutations were also responsible for decreasing S-mediated fusion, suggesting a tradeoff between antibody escape and fusion **(Fig. 5C)**. mAb98 did not recognize the Beta variant. However, none of the associated mutations were specifically responsible for the lack of binding **(Fig. 5C)**, suggesting a combined effect on the structure of the S protein that may affect antibody escape.

Therefore, several of the mutations found in the variants spike proteins are advantageous in terms of antibody escape despite slightly reducing the ability the proteins to fuse." (line 277 -296)

8. Could the authors discuss their results on ACE2 in light of the most recent literature showing that SARS-CoV-2 can use alternative receptors to infect cells (Puray-Chavez et al., Cell Reports, 2021)?

We thank the review for drawing our attention to this article and we have included a discussion point on how the mutations may confer advantage in an ACE2 independent manner, the potential interest of novel receptors to the syncytia field.

"Variant mutations may also confer advantages in an ACE2 independent manner. Indeed, recent work has suggested that the E484 mutation may facilitate viral entry into H522 lung cells, requiring surface heparan sulfates rather than ACE2 (Puray-Chavez *et al*, 2021). It would be of future interest to examine the syncytia formation potential of the variant mutations in other cell types." (Line 358-362)

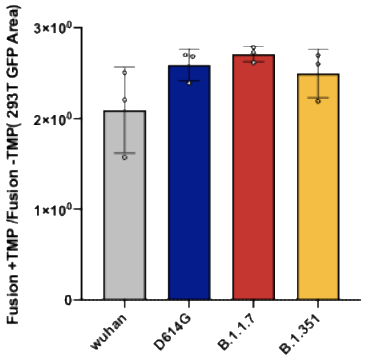
Referee #2:

The manuscript submitted by Rajah, et al. is an informative study that explores the effects of SARS-CoV-2 spike protein variants on viral replication, binding to the ACE2 receptor and cell fusion. Additionally, the authors determine whether the mutations in these variants alter binding to human monoclonal antibodies. The experiments are well-controlled and the results are clearly presented. The data nicely demonstrate that certain mutations affect cell fusion and suggest that some of these mutations affect antibody binding. While interesting, additional experimentation would clarify the impact of these findings

We thank the reviewer for the kind reception of our work and their suggestions for improving our manuscript. We have added data on TMPRSS2 processing of the variants and provided more detailed explanations and background information for the antibodies.

1. While the initial infection studies are performed in multiple cell types and in primary cells, subsequent studies are in U2OS and Vero cells. Additional studies in the more relevant cell lines, which express TMPRSS2, would support the results showing differences in cell fusion.

While we initially analyzed replication in variety of cell lines, we found the that the Vero cells were the among the mots fusogenic. As such we continued our experiments by generating the GFP split system in Vero cells which express endogenous ACE22 and the U2OS human cell line which we transduced with ACE2. This allowed us to quantify syncytia formation. While other cell like the Human airway epithelial cells form syncytia upon infection (Zhu et al, 2010; Nature Communications), it is difficult to quantify differences. We have added this as a limitation to our discussion. To see if TMPRSS2 differentially affect variant fusion, we transfected TMPRSS2 along with each of the variant spikes into 293T cells and compared it to a condition without TMPRSS2. The addition of TMPRSS2 increased fusion drastically for the D614G, B.1.1.7 and B.351 variants with no noticeable difference (below). We have included the results for the reviewer's consideration. We haven't included the figure in the revised version, since the manuscript is already busy.



2. The authors do not determine whether any of these mutations affect processing of Spike by furin or TMPRSS2. These experiments would add additional biological relevance.

We thank the reviewer for their suggestion, we have examined the processing of the variant spike by TMPRSS2 by western blot (Fig EV4E) and by flow cytometry (please see our answer to point 2 of reviewer 1). We find that the variants are similarly processed by TMPRSS2. In this article we are primarily focused on the interaction between the S protein and ACE2, as we find that the presence of ACE2 is sufficient for syncytia formation. We agree it would be of interest to study the processing of each of the individual mutations, but the amount of mutations puts this slightly beyond our scope and we have added this to the discussion as a limitation. All of our experiments were performed in Furin positive cells. https://www.proteinatlas.org/ENSG00000140564-FURIN/celltype.

3. More information is required for the monoclonal antibody studies. What is the source of these antibodies and what is the evidence suggestion region of binding to Spike? Also, the results of these studies would be enhanced by the inclusion of virus neutralization assays for the antibodies recognizing the RBD.

The antibodies originate from Hugo Mouquet's lab at Institut Pasteur and were derived from convalescent individuals; we have added this information to the material and methods. We have characterized them in terms virus neutralization assays in recent publications (Planas et al, 2021; Nature Med) (Planas et al, 2021; Nature). We have added a section to the results summarizing the relevant findings. A more precise characterization of the binding regions of the antibody will be published the Mouquet lab at a later date (Planchais manuscript in preparation). There is internal consistency to our claims regarding the binding regions of the antibodies. mAb48 does not recognize the K417N mutation which is in the RBD and mAb71 does not recognize DeltaY144 and Delta242-244 which are both in the NTD. We have added the description of the antibodies to the materials and methods (Line 498-501). We have also added the following context regarding the neutralization assays to our results section on the antibodies:

"We had previously found that certain neutralizing antibodies differentially affect SARS-CoV-2 D614G, Alpha and Beta variants (Planas *et al.*, 2021a). For instance, neutralizing monoclonal antibody 48 (mAb48) restricts D614G virus but not Alpha or Beta variants (Planas *et al.*, 2021a). We sought to determine which mutations in variant S proteins contributed to the lack of recognition by the neutralizing antibodies." (lines 278-282)

Minor editorial changes should be made throughout the manuscript. For example:

We have made all of the suggested minor editorial changes. We thank the reviewer for the diligence.

Line 106: RT-qPCR for the SARS-CoV-2 N protein should be for the gene encoding the SARS-CoV2 N protein

Line 196: the word "boarder" should be replaced with "border"

Dear Olivier,

Thank you for submitting your revised manuscript to The EMBO Journal. This submission is an invited re-submission of manuscript # 108944 that was rejected post review back in July.

Your study has now been seen by the original two referees and I am afraid that they both are not convinced that the added data has clarified their previous concerns. Referee #2 questions the biological significance of the reported findings as in the presence of TMPRSS2, there is no difference in the fusogenicity of the variants. The experiments would have to repeated in a cell line that express endogenously ACE2 and TMPRSS2 to see if there is a difference in the fusogenicity of the variants. Referee #1 raises the concern if there are equal levels of the spike variants expressed on the cell surface and if not that this could have an impact of the outcome of the results.

I have discussed the issues with the referees and they are both in agreement that the above raised issues would have to be addressed with additional experiments to get more clarity what is going on.

Given that this is already a revised version and that the manuscript has not improved to the level that we expert for an EMBO Journal paper, I am afraid that I can't be more positive on this occasion.

I am sorry that I can't be more positive on this occasion.

With kind wishes

Karin

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

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Referee #1:

The authors provided a detailed rebuttal, including new results in a very short period, and convincingly addressed all my concerns, apart from major comments 2 and 3 for which I still have concerns.
1. The authors present new data regarding the possible processing of spike proteins by TMPRSS2 using a co-transfection assay. While I agree that there is no significant difference between the different spike proteins, there is a clear difference for each spike variant when TMPRSS2 is expressed (Figure EV4E). This finding should be discussed further.
2. It is still not convincing how the authors control the expression of the spike variants, i.e., in a cell line, 293T, that is different from those used for cell-cell fusion, U2OS and Vero. This does not ensure that each spike variant is expressed on the cell surface in a similar manner (U2OS, Vero). The authors justify the inability to control spike expression in U2OS and Vero cells because these cells fuse extensively. Based on the IF images shown in Figures 2B and EV3B, and with all due respect to the authors, I disagree that Vero cells fuse extensively. I am convinced that it is possible to measure the expression of spike proteins, at least on the surface of Vero cells. This is an important limitation, which should be, at least, considered in the discussion if the authors are not willing to show these results.
3. The authors have provided results for consideration by reviewers, but these results are important and should be shown in the manuscript.

Referee #2:

The revised manuscript submitted by Rajah, et al. is a much-improved version of the previous manuscript with many additional controls and clarifications in the text, as requested by reviewers. It is noted that the authors were very responsive to all reviewer comments. Both reviewers brought up the effect of TMPRSS2 on fusion of the different variants. The authors have added data suggesting that the variants are comparably processed by TMPRSS2, suggesting there is no effect on proteolytic processing. However, the data provided by the authors (as requested by reviewers) suggests that in the presence of TMPRSS2, there is no difference in the fusogenicity of the variants. This calls into question the biological relevance of the findings presented in the manuscript. During infection in vivo, all cell types infected will express ACE2 and TMPRSS2. This data has not been incorporated into the manuscript and is only provided to reviewers. This may be due to overexpression of both TMPRSS2 and ACE2 but this would be answered by assessing fusogenicity in another (more relevant) cell line that endogenously expresses both proteins.

Point-by-point response

The editor and reviewers have been very generous with their time and offered pertinent advice throughout the review process. We are grateful that the reviewers acknowledged that many of their concerns were addressed in the previous round and through the addition of data on Delta variant, antibodies and a rewrite of the manuscript. We, however, had erroneously assumed that the experiments with TMPRSS2 was more for the reviewers consideration rather than for the manuscript. This point was made clear in the second round of comments and we have since performed and finalized new experiments to address these questions. We apologize for the previous oversight. We have also added new experiments to validate our controls. We readily concede that many of reviewers' criticisms improve the clarity and the scientific rigor of our manuscript and we appreciate their diligence. We hope that we have not exhausted their patience and that they will continue to engage with us.

The editor highlighted two remaining major points: (1) the need for replicating the results in a cell line that endogenously expresses TMPRSS2 and (2) The levels of Spike at the surface of the cells in which we perform the fusion assays. We have performed experiments that address both points and the results are very positive.

1. We generated our GFP split system in the Caco2 cell line which endogenously expresses TMPRSS2 and ACE2. We found that the variants S proteins, including Delta, induce more syncytia. This was the major comment from Reviewer 2 which we have now fully addressed. The enhanced fusogenicity is thus observed in different cell types, expressing either endogenously or ectopically ACE2 and TMPRSS2.
2. We show that the surface levels of the different Spikes are similar in 293T cells that we have now used as "donor" cells in coculture experiments with acceptor Vero cells. In this system, there is again an enhanced fusion associated with the variant Spikes. These novel experiments confirm and strengthen our initial conclusion that the enhanced fusion observed with variants is not due to increased surface levels of the spike.

Referee #1:

The authors provided a detailed rebuttal, including new results in a very short period, and convincingly addressed all my concerns, apart from major comments 2 and 3 for which I still have concerns.

We thank the reviewer for their advice and diligence. We acknowledge that by addressing their first round of criticisms, we were able to drastically improve the scientific clarity and rigor of our manuscript. Here we endeavour to further clarify the point that we overlooked by providing experimental evidence and further clarifications.

1. The authors present new data regarding the possible processing of spike proteins by TMPRSS2 using a co-transfection assay. While I agree that there is no significant difference between the different spike proteins, there is a clear difference for each spike variant when TMPRSS2 is expressed (Figure EV4E). This finding should be discussed further.

We believe that these results suggest that difference we see in fusogenicity between the variant spikes is not to differential TMPRSS2 processing but due to the mutations within the variant spikes that alter receptor binding and the fusion machinery. We added new experiments using Caco2 cells which have endogenous TMPRSS2 and ACE2 (Fig 6D) and support our previous findings. The variants may also be differentially processed by other proteases like Furin and a couple of new preprints explore this subject. The reviewer is correct in saying that this is an important discussion point and we included a paragraph in the results and discussion.

Page 17:

"S protein mediated syncytia formation and TMPRSS2.
We then asked whether the TMPRSS2 protease, that cleaves S and facilitate viral fusion, may act differently on the variant S proteins. To this aim, we generated a Caco2 GFP-split cell line and then expressed the different S proteins to examine fusogenicity. Human Caco2 cells express endogenous levels of TMPRSS2 and ACE2. In line with our results in Vero cells which lack endogenous TMPRSS2 (Fig 3B), we found that the Alpha, Beta, and Delta variants fused more than the D614G, and the Wuhan S protein fused the least (Fig 6D). Thus, the differences in variant fusogenicity can also be visualized in the presence of TMPRSS2. In order to see if TMPRSS2 differentially processes the variants S proteins, we expressed each of them without or with TMPRSS2 in 293T cells without ACE2. We examined the processing of the different S by western blot and the expression levels of S1 and S2 by flow cytometry. While all S proteins are processed the cleavage profile induced by TMPRSS2 and the expression levels between the different variant S proteins were similar (Fig EV8A)."

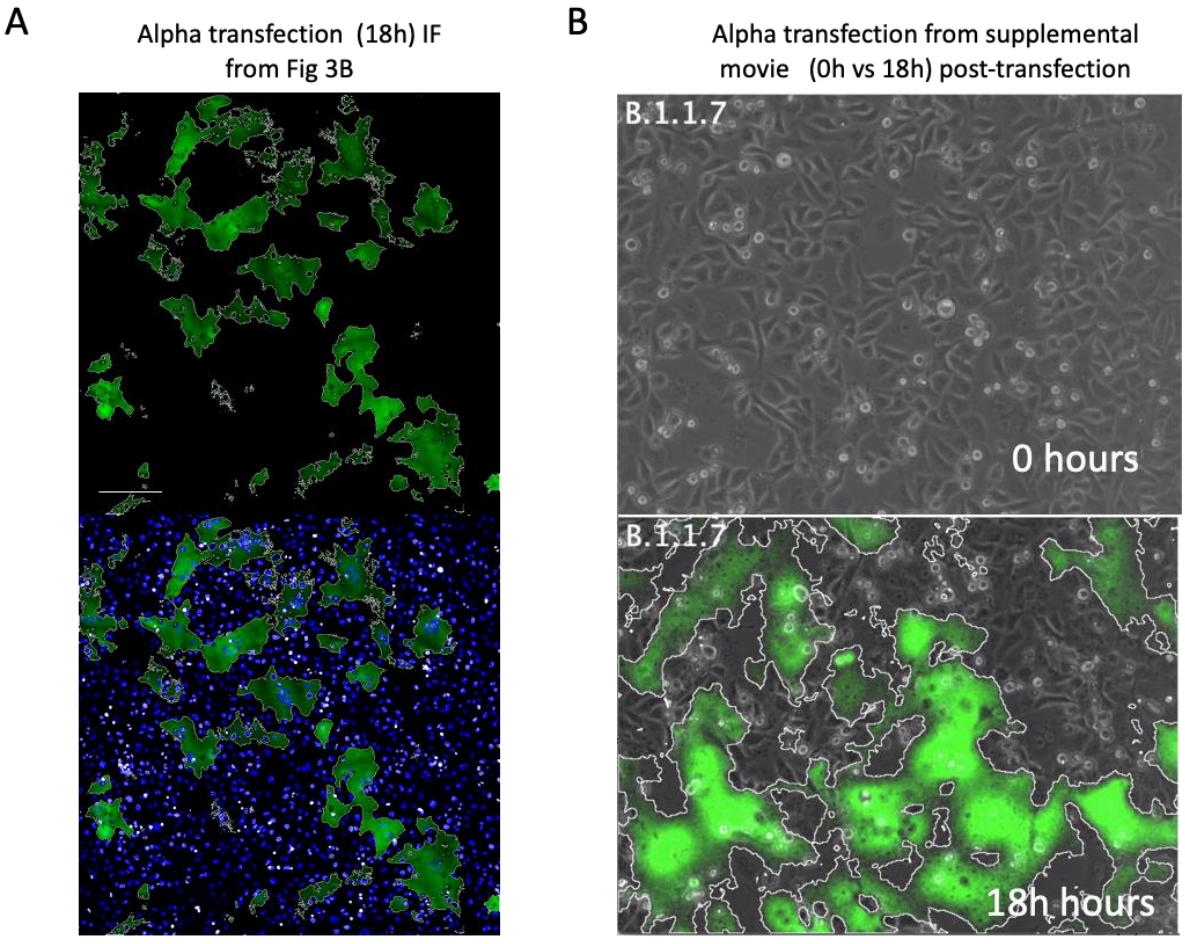
Page 21:

"While we had previously shown that the interaction between the S protein on the plasma membrane with the ACE2 receptor on neighboring cells is sufficient to induce syncytia formation, there is compelling evidence of the importance of the TMPRSS2 protease in S activation (Buchrieser et al., 2020; Dittmar et al, 2021; Koch et al, 2021; Ou et al, 2021). We found that the S protein of the novel variants induced more syncytia formation than the D614G and Wuhan S proteins in human Caco2 cells which express endogenous ACE2 and TMPRSS2. However, we did not detect any major differences in the processing of the variant S proteins by TMPRSS2. It will be worth further characterizing how the fusogenicity of variant associated mutations are influenced by other cellular proteases like furin."

2. It is still not convincing how the authors control the expression of the spike variants, i.e., in a cell line, 293T, that is different from those used for cell-cell fusion, U2OS and Vero. This does not ensure that each spike variant is expressed on the cell surface in a similar manner (U2OS, Vero). The authors justify the inability to control spike expression in U2OS and Vero cells because these cells fuse extensively. Based on the IF images shown in Figures 2B and EV3B, and with all due respect to the authors, I disagree that Vero cells fuse extensively. I am convinced that it is possible to measure the expression of spike proteins, at least on the surface of Vero cells. This is an important limitation, which should be, at least, considered in the discussion if the authors are not willing to show these results.

We understand this to be a major point and it is important to us to convince the reviewer of the validity of our controls. Below is a detailed response with new experiments and more thorough description of our experiments.

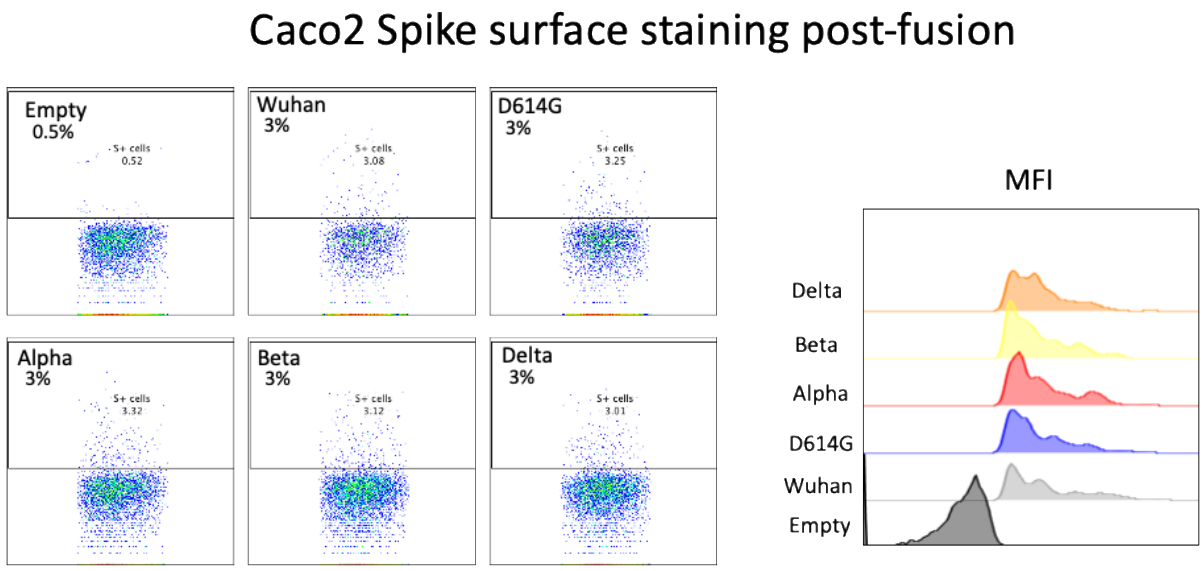
In this manuscript we use two complementary approaches for assessing fusion of the variants, infection with SARS-CoV-2 and transfection of S. Figure 2B, which is mentioned by the reviewer are IF images of infected Vero cells at 48h, in which the fusion is indeed less extensive than during transfection of Vero cells, the kinetic of replication for infection has been controlled in Figure 1 (and figure EV1A) at different timepoints using different cell lines. The 293T surface stainings, which the reviewer find unsatisfactory, shown in EV4C are the controls for S Vero transfection of figure 3B. The extent of fusion observed during transfection of Vero cells is much greater and it is in these transfection experiments where we claim that the cells fuse extensively. Below we provide a larger IF image of the syncytia formation by the alpha S protein derived from figure 3B and a screenshot from the supplemental movie at 0 h, where individual cells are visible, and at 18 h where syncytia are in green, both of which demonstrate the degree to which the Vero cells fuse when transfected (response figure 1).



Response Figure 1:Degree of syncytia formation in Vero cells (Derived from figure 3B and Supplemental movie 1)

It is because of these high levels of fusion and possibility of losing the S+ syncytia that we turned to the 293T cells which lack ACE2 and do not fuse (we confirmed this in our GFP split system before (Buchrieser et al, 2020 EMBO J) as our transfection control.

In an attempt to fully answer the reviewers concern, we tried flow cytometry and surface staining for S on both Vero and Caco2 cells (both of which express ACE2). The results of Caco2 fusion can be found in the new Figure 6D, and the fusion is less extensive than in Vero. We left the GFP laser open and found that we lost all the syncytia (that were previously visible in the IF) and syncytia represent a large population of S+ cells. Only a few non-fused cells remained for all the conditions (they were equal in percentage and MFI); however, we believe this strategy to be unreliable because an uncontrolled proportion of transfected cells is lost due to syncytia (recent Caco2 results can be found in response figure 2). The fragile syncytia are likely damaged and lost through the lifting, staining and washing process. This problem is more pronounced in the larger syncytia of the Vero cells. It is because of this limitation that we turned to the 293T transfection strategy as a control.



Response Figure 2: Surface staining on Caco2 cells post-fusion

Above we explained our reasoning for using the 293T as a control with experimental evidence, but the reviewer is in essence correct in that preforming the fusion experiments in Vero and then using 293T for surface staining represents a weakness in our approach. As such we devised a strategy to unify the 293T control to the fusion experiments. To this aim, we transfected the 293T GFP1-10 cells with the variant spikes in suspension and then set some aside for surface staining and the remaining cells we added in equal number to Vero GFP11 cells in an acceptor/donor experiment. The 293T GFP1-10 cells will not fuse with one another as they lack ACE2 (which allowed us to confirm equal surface expression without losing fused cells) but they will fuse with the Vero GFP11 cells which express endogenous ACE2 and give off a GFP signal (allowing for fusion quantification). The fusion results matched our previous experiments with the novel variants fusing more than the D614G and Wuhan spikes. The cells that were set aside showed that the variants spikes are equally expressed on the surface of the 293T donor cells. The results and methodology of the acceptor donor experiment is shown below and in the manuscript (Fig EV4E).

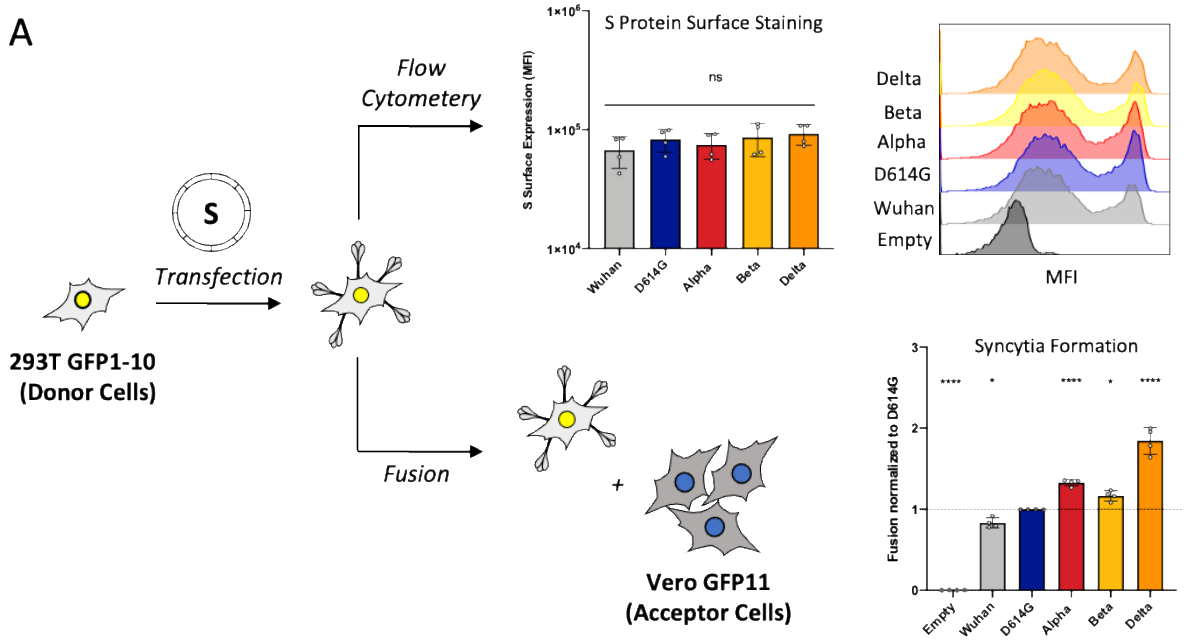


Figure EV 4E: Acceptor/donor experiments where 293T cells are transfected and surface stained to show equal surface Spike expression and also added to Vero cells to demonstrate variants induce more Syncytia

We hope with these explanations and new experiments the reviewer will be convinced of the validity of our controls and observations. We have now included these results in the novel Figure EV4E. They are described pages 10-11:

"We then verified that the variation in S mediated fusion was not due to differential cell surface levels. Due to extensive fusion in transfected Vero cells we were unable to compare S protein surface expression (Figure 3B and Movie E1), as the S protein-positive syncytia were damaged and lost during the flow cytometry procedure. As such, we transfected 293T cells, which lack ACE2 and thus do not fuse with one another upon S expression, with the different variant plasmids in order to assess S protein surface levels by flow cytometry. The variants S proteins were equally expressed after transfection ***(Fig. EV4A-C).*** *In order to unify the surface expression control with our fusion results, we performed an acceptor/donor experiment. 293T GFP1-10 (donor) cells were transfected with each of the variant S plasmids. The transfected donor cells were then co-cultured with Vero GFP11 (acceptor) cells* ***(Fig. EV4E)****. Some of the transfected 293T donor cells were set aside and stained for S protein to show equal surface expression between the variants* ***(Fig. EV4E)****. In the acceptor/donor co-culture we found that the 293T transfected with the novel variant S proteins formed more syncytia with the Vero acceptor cells than D614G and that the Wuhan S formed the least* ***(Fig. EV4E)****. The results of the acceptor/donor co-culture experiment matched our fusion results in Vero cells."*

We also took the reviewer's advice and discussed the limitation of not being able to look at surface expression in the Vero cells. It reads as follows page 18-19:

"One limitation of our study resides in the fact that were unable to look at surface expression of the variant S proteins in Vero and Caco2 without losing the large S protein positive syncytia. We thus used the non-fusogenic 293T cells to control for surface expression. We

further show that S-expressing 293T cells fuse with Vero cells in donor/acceptor experiments. The experiments confirmed the enhanced fusogenicity of the variants in cells with similar levels of S protein at their surface."

3. The authors have provided results for consideration by reviewers, but these results are important and should be shown in the manuscript.

We have added the new results of the variant spike processing and surface levels in the revised manuscript as a novel Fig EV8. They are now described in the text pages 17-18:

"S protein mediated syncytia formation and TMPRSS2

We then asked whether the TMPRSS2 protease, that cleaves S and facilitate viral fusion, may act differently on the variant S proteins. To this aim, we generated a Caco2 GFP-split cell line and then expressed the different S proteins to examine fusogenicity. Human Caco2 cells express endogenous levels of TMPRSS2 and ACE2. In line with our results in Vero cells which lack endogenous TMPRSS2 (Fig 3B), we found that the Alpha, Beta, and Delta variants fused more than the D614G, and the Wuhan S protein fused the least (Fig 6D). Thus, the differences in variant fusogenicity can also be visualized in the presence of TMPRSS2. In order to see if TMPRSS2 differentially processes the variants S proteins, we expressed each of them without or with TMPRSS2 in 293T cells without ACE2. We examined the processing of the different S by western blot and the expression levels of S1 and S2 by flow cytometry. While all S proteins are processed the cleavage profile induced by TMPRSS2 and the expression levels between the different variant S proteins were similar ***(Fig EV8A)****. "*

Referee #2:

The revised manuscript submitted by Rajah, et al. is a much-improved version of the previous manuscript with many additional controls and clarifications in the text, as requested by reviewers. It is noted that the authors were very responsive to all reviewer comments. Both reviewers brought up the effect of TMPRSS2 on fusion of the different variants. The authors have added data suggesting that the variants are comparably processed by TMPRSS2, suggesting there is no effect on proteolytic processing. However, the data provided by the authors (as requested by reviewers) suggests that in the presence of TMPRSS2, there is no difference in the fusogenicity of the variants. This calls into question the biological relevance of the findings presented in the manuscript. During infection in vivo, all cell types infected will express ACE2 and TMPRSS2. This data has not been incorporated into the manuscript and is only provided to reviewers. This may be due to overexpression of both TMPRSS2 and ACE2 but this would be answered by assessing fusogenicity in another (more relevant) cell line that endogenously expresses both proteins.

We thank the reviewer for their assessment of our work and acknowledge that the advice has benefited the manuscript. The role of TMPRSS2 is a key component that we should have

characterized more thoroughly in the previous version and we have now taken the reviewer suggestions and preformed the requested experiments. The reviewer is correct in assessing that the flaw in our previous experiment was the over expression of TMP and ACE2 and that a cell line expressing endogenous levels of both proteins was necessary. To this aim we took Cao2 cells which express endogenous levels of TMPRSS2 and ACE (https://www.proteinatlas.org/ENSG00000184012-TMPRSS2/cell, also confirmed in-house) and are commonly used in SARS-CoV-2 studies for this purpose. We generated Caco2 cells expressing the GFP split system. We then proceed to transfect the spike protein of each variant and compared the levels of syncytia formation. We found that in the endogenous TMPRSS2+ACE2 expressing cells our observations held true. The novel variants fused more than the D614G S and the Wuhan fused the least (see below). We have included this figure in our manuscript.
A new panel has been added in the Figure 6D:

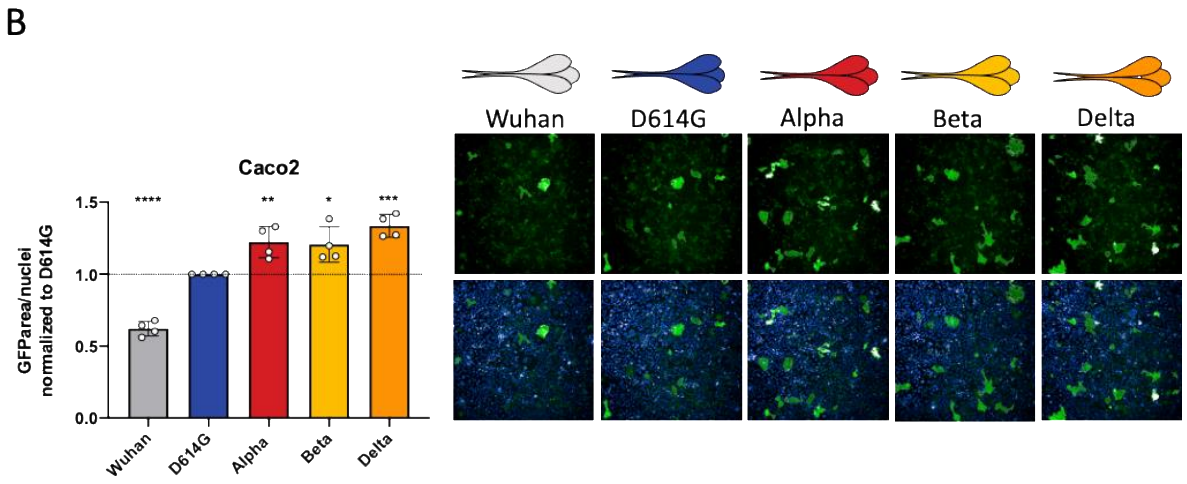


Figure 6D: Variants induce more fusion in endogenous TMP and ACE2 expressing Caco2 cells upon S transfection

The text now reads page 17-18:

"S protein mediated syncytia formation and TMPRSS2

We then asked whether the TMPRSS2 protease, that cleaves S and facilitate viral fusion, may act differently on the variant S proteins. To this aim, we generated a Caco2 GFP-split cell line and then expressed the different S proteins to examine fusogenicity. Human Caco2 cells express endogenous levels of TMPRSS2 and ACE2. In line with our results in Vero cells which lack endogenous TMPRSS2 (Fig 3B), we found that the Alpha, Beta, and Delta variants fused more than the D614G, and the Wuhan S protein fused the least (Fig 6D). Thus, the differences in variant fusogenicity can also be visualized in the presence of TMPRSS2. In order to see if TMPRSS2 differentially processes the variants S proteins, we expressed each of them without or with TMPRSS2 in 293T cells without ACE2. We examined the processing of the different S by western blot and the expression levels of S1 and S2 by flow cytometry. While all S proteins are processed the cleavage profile induced by TMPRSS2 and the expression levels between the different variant S proteins were similar ***(Fig EV8A)****."*

Dear Olivier,

Thanks for submitting you revised version, which has now been seen by the two referees. As you can see from the comments below, the referees appreciate the introduced changes and support publication here.

I am therefore very happy to let you know that we will accept your manuscript for publication here. Before sending you, the formal acceptance letter there are just a few editorial things to resolve:

- The grant ANR-10-INSB-04-01 and Région Ile-de-France program DIM1-Health are missing in the online submission system.

- I think author contributions are missing for Delphine Planas and Marija Zivaljic. Please check

- The legend from the movie file needs to be removed from MS and zipped with movie file. The movie should be called out in the text as movie EV1 (currently Movie E1)

-We can only have 5 EV figures - the other should be either added as main figures or to the appendix.

- You are missing a Data Availability Section. This is the place to enter accession numbers etc. If no data is generated that needs to be deposited in a database then please state: This study includes no data deposited in external repositories. Place it after the Materials and methods and before Acknowledgements

- Our publisher has also done their pre-publication check on your manuscript. When you log into the manuscript submission system you will see the file "Data Edited Manuscript file". Please look at the word file and the comments regarding the figure legends and respond to the issues. PS They did the check on the previous version.

That should be all you can use the link below to upload the revised version.

Let me know if you have any further questions

With best wishes

Karin

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

Guide For Authors: https://www.embopress.org/page/journal/14602075/authorguide

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Referee #1:

The authors have finally addressed the important issues raised by the reviewers. With the new results shown, readers will have all the data and discussion necessary to better appreciate the authors' conclusions.

Referee #2:

I have looked over the new data. I would have preferred a more relevant cell line, such as Calu3 but they are quite difficult to work with and I think Caco2 is an acceptable choice. Given this new data, I am comfortable recommending that the paper be accepted

- The grant ANR-10-INSB-04-01 and Région Ile-de-France program DIM1-Health are missing in the online submission system.

Region Ile-de-France program DIM1-Health; has been added

Région Ile-de-France program DIM1-Health are funding for the Institut Pasteur imaging platform used in this study and are not affiliated with any individual authour.

- I think author contributions are missing for Delphine Planas and Marija Zivaljic. Please check

Contribution for DP and MZ have been added.

- The legend from the movie file needs to be removed from MS and zipped with movie file. The movie should be called out in the text as movie EV1 (currently Movie E1)

Movie file has been renamed and zipped with the text.

-We can only have 5 EV figures - the other should be either added as main figures or to the appendix.

Figures EV1, 3 and 8 have been moved to the appendix. Figure numbering has been updated accordingly.

- You are missing a Data Availability Section. This is the place to enter accession numbers etc. If no data is generated that needs to be deposited in a database then please state: This study includes no data deposited in external repositories. Place it after the Materials and methods and before Acknowledgements

"This study includes no data deposited in external repositories." Statement has been added.

- Our publisher has also done their pre-publication check on your manuscript. When you log into the manuscript submission system you will see the file "Data Edited Manuscript file". Please look at the word file and the comments regarding the figure legends and respond to the issues. PS They did the check on the previous version.

We have responded to the issues and corrected them in the resubmitted manuscript.

3rd Revision - Editorial Decision **28th Sep 2021**

Dear Olivier,

Thank you for submitting your revised manuscript to The EMBO Journal. I have now had a chance to look at the revised version and all looks good. I am therefore very pleased to accept the manuscript for publication here.

Congratulations on a nice study!

With best wishes

Karin

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

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Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
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  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	At least 3 indpendent replicates were preformed for each experiment to ensure statistical power
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	N/A
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	N/A
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	images were processed using automated analysis on all conditions simultaniously to avoid subject bias
For animal studies, include a statement about randomization even if no randomization was used.	N/A no animal studies
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	No but image analysis was preformed automatically to avoid investigator bias. Several experimenters preformed and assessed replicates.
4.b. For animal studies, include a statement about blinding even if no blinding was done	N/A
5. For every figure, are statistical tests justified as appropriate?	Yes
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Is the variance similar between the groups that are being statistically compared?	Yes

C- Reagents

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7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	All cell lines are tested for mycoplasma twice a month. The origin of the cells and viruses is indicated in the manuscript.

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9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	N/A
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11. Identify the committee(s) approving the study protocol.	N/A
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	N/A
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F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	N/A
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