nature portfolio

Peer Review File

Lineage-specific pathogenicity, immune evasion, and virological features of SARS-CoV-2 BA.2.86/JN.1 and EG.5.1/ HK.3

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

Liu and colleagues provide a comprehensive analysis of recently circulating Omicron subvariants. In brief, they report that variant JN.1, which currently dominates globally, shows augmented antibody evasion as compared to BA.2.86.1. and the recent Omicron subvariants are demonstrated to induce largely type specific antibody responses. Further, increased Calu-3 cell entry and TMPRSS2-dependence is reported for BA.2.86.1 and JN.1. Moreover, increased Calu-3 cell entry of BA.2.86.1 and JN.1 is shown to correspond to increased Calu-3 infection and BA.2.86.1 and JN.1 are demonstrated to infect nasal epithelial cells more efficiently than other circulating variants. Finally, insights into ACE2 binding are provided and it is demonstrated that despite increased infection of Calu-3 lung cells and nasal epithelial cells, BA.2.86.1 and JN.1 show more pronounced attenuation in a hamster model as compared to other Omicron subvariants. The study is of interest but some points remain to be addressed.

Major

Figure 3D: Using a single dose of inhibitor is not convincing. Please demonstrate concentration dependence.

Pseudotype data were normalized, which makes it very difficult to judge the quality of the assays. Unprocessed data must be shown in the supplement, ideally including a negative control (particles with no spike) and a positive control.

Minor

The robust cell entry phenotype of JN.1 markedly contrasts that reported by PMID: 38591890. Reasons for these discrepant findings should be discussed.

Increased infection of nasal epithelial cells by BA.2.86.1 as compared to other Omicron subvariants has not been found by a recent study published in Nature Communications (PMID: 38480689). Again, reasons for these differences need to be discussed.

Reviewer #2 (Remarks to the Author):

In this manuscript, authors aimed to clarify virological characteristics (factors) of the globally dominant SARS-CoV-2 JN.1 variant and the underlying mechanisms leading to such global dissemination. Authors addressed questions by investigating some virological properties of the JN.1 variant in comparison to parental (BA.2 and BA.2.86) as well as other variants (XBB.1, EG.5.1, and HK.3). The evaluated infectivity, entry, and immune resistance/evasion of JN.1 variant in susceptible cell lines and human nasal epithelial model showed distinct viral properties of such variant when compared to its parental variant BA.2.86. In addition, authors attempted to clarify the underlying

mechanisms controlling the distinct behavior of the JN.1 variant by showing that the acquired mutation L455S in the JN.1 spike protein can alter Spike-ACE2 interaction/binding. Overall, authors clearly addressed questions and the experimental design and flow are comprehensive. However, the following points are needed to be addressed. Moreover, the clarity, fluency, and consistency of the English used need to be addressed. I strongly suggest using a third-party editor to address this issue.

Major points

1. Line 2: rephrase to “SARS-CoV-2” BA.2.86/JN.1 and EG.5.1/HK.3
2. Authors need to clarify and precisely determine the use of these words (virus, variant, pseudovirus and spike protein) when affixed to individual SARS-CoV-2 variant (for example: lines: 81, 204, 218, 223, 242, 244, 246, 248 etc and suffixing “pseudovirus” to HK.3 in line 232).
3. Line131-135: authors should highlight the domains where the enumerated spike mutations belong (for example NTD, RBD). Are there any conserved sequences among mentioned variants? Is there any relevance of the conserved sequences in terms of vaccine tolerance and immune evasion?
4. Line 171: The word in “EG.5.1 constituted another” seems to be missing. Add cluster or group.
5. Line184: Authors need to clarify what exactly meant by antigenicity. Is it equal to immune evasion/resistance to antibody neutralization?
6. Line 206: Fusion assay. Did authors consider the spike protein expression levels of each variant? Authors used 293T cells for fusion assay. However, it is so hard to conclude spike fusogenicity using only one cell line. Authors should test respective fusion activity in Calu-3 cells, at least.
7. Line 213-215: SARS-CoV-2 can’t infect 293T and A549 cells. Authors need to mention precisely in main text, figures, and methods whether ACE2 (and TMPRSS2) is expressed in these cell lines.
8. Line 226: What is the reference for this conclusion “where coronavirus entry predominantly occurs at the plasma membrane”?
9. Line 228 What is the reference for this conclusion “where coronaviruses enter through the endosomal entry route”?
10. Line 230: What is the reference for this conclusion “in Caco2 that supports both entry pathways”?
11. Line 239-241: Have L455S and L455F mutations been reported to enhance variants transmissibility?
12. Line:250-252: JN.1 and HK.3 variants showed higher replication and pseudovirus entry. How are they correlated to viral transmissibility?
13. Line 264-268: One representative result is not enough to conclude S1/S2 cleavage of JN.1 spike protein is more efficient than that of BA.2.86 spike protein. Need to show biological replicates and statistical analysis.
14. Line 272: Are there reports supporting the conclusion of enhanced fitness and its impact?
15. Line 504: Fusion assay. Authors mentioned 293T cells as effector cells. However, VeroE6 cells were described as effector and target cells. What cell lines were used for this assay?
16. Line 548: what MOI is used for infecting VeroE6 cells?
17. Line 552: change “camostat” to Camostat.
18. Line 582: authors need to clarify the formula used to calculate the cleavage ratio.
19. In discussion: L455S mutation of JN.1 spike (S455) modulated the mode of JN.1 spike/ACE2 interaction to a smaller number of orientations. On the other hand, JN.1 spike showed increased spike cleavage efficiency over the BA.2.86 spike. Authors should recall/mention reports that show similar phenomena of other variant's spike protein, and the possible mechanisms inducing it.
20. JN.1 has L455S mutation in the spike as well as the other mutations in NSP6 and ORF7b. How did you know that the only L455S mutation in the spike is associated with the global spread of the JN.1 variant? In terms of this, authors should test the effect(s) of insertion of L455S mutation into

parental variants [and/or previous VOCs (e.g. B.1.1, Delta)] to highlight changes of virological characteristics of such variants. At least, authors need to discuss this point.

Minor points
1. Line 109: you should add cell type of Calu3 (e.g. bronchial adenocarcinoma cells).
2. Line 157: change “a XBB” to “an XBB”.
3. Line 188: Add figure citation after HK.3.
4. Line 198: add (Fig. 2i) after “and EG.5.1”.
5. Line 207: add (Fig. 3a) after “and HK.3”.
6. Line 214: add figure citation after 293T cells.
7. Line 230: add cells after Caco2.
8. Line 231: add pseudovirus entry after “against BA.2.86 and JN.1”.
9. Line 231-232: rephrase “effective against EG.5.1 and HK.3” to effective against entry of EG.5.1 and HK.3 pseudoviruses.
10. Line 245: change “protease sensitivity” to protease dependency or usage.
11. Line 246: change “(Fig. 3d and Fig.3d)” to (Fig.3b and Fig.3d).
12. Line 246: change “in Calu3 cells was” to “in Calu3 cells were”.
13. Line 247: change “that of EG.5.1” to those of EG.5.1 and HK.3.
14. Line 253: change “to understand if” into to understand whether.
15. Line 267: change “than that of EG.5.1” to than those of EG.5.1 and HK.3
16. Line 276: change “to evaluate if” into to evaluate whether. Change “bind” to binds.
17. Line 340: “rapid grow in numbers” is not grammatically correct.
18. Line 345: delete in hNECs after L455S.
19. Line 354: add lineage after “from BA.2”.
20. Line 480: “recent 6 months” is not a precise term. It is better to show in the designated date (e.g. August 2023).
21. Line 487: What do the asterisks mean?
22. Ref.27 (BioRxiv), Ref. 28, Ref. 31, Ref. 38 citations are incomplete.
23. Line 510: Can’t recognize open square.

Reviewer #3 (Remarks to the Author):

The authors report an extensive characterization in vitro and in vivo of the most recent SARS-CoV-2 variants, which includes immunological assays, fusion assays and structural biology. The efforts are impressive and timely, which makes the paper exciting; the cryo-EM analysis, however, is not the strongest part of the paper. The quality of the cryo-EM data is good, but the presentation of the structural data is not as impressive as the presentation of the functional/immunological data. The structural analysis could be pushed a little further, for example by describing a structural comparison with previous variants, not just between the two variants described in the paper. I still believe the manuscript is valuable and deserves publication, it just needs something more in terms of structural analysis with the current data that the authors have already collected.

Major issues:

The main maps, both for the trimer and the local refinement of RBD+ACE2 have been sharpened

using EMReady. It definitely improved the appearance of the maps but the program distorts the density of ACE2 in the trimer map. The authors should display and deposit the maps obtained using standard programs like cryoSPARC and deposit the EMReady maps as Additional maps in the PDB and EMDB.

The main figure (Fig. 5) has some issues:
-Showing the model as surface in the context of a new variant doesn't deliver any new message. What's new is the interface between ACE2 and the RBDs of the two variants. The authors could show the local refinement cryo-EM maps (which have a very good-looking density!) in the main figure, colored based on the protein chains, side by side with the structural models shown in panels e-g.
-The authors use two panels (e and f) to show the two conformers of His34. I suggest the authors show a single panel where the two orientations of His 34 are shown (like they have in the model) labelled as conformer A and B.
-The authors report a change in side-chain interaction, or rather a preferential orientation of a single residue, but a comparison with other variants is missing. I suggest adding panels that show the same RBD:ACE2 interface region in other recent variants, as well as describe differences and similarities in the result section and/or discussion section.
-Is there any difference in the trimer structures compared to previous variants? The resolution is very high for the trimer structure, so the authors should be able to point out differences between the new variants reported and the old variants.

Minor issues:

Protein expression and purification: mentions "full-length spike" but the protein is secreted. The authors should clarify whether their construct is the full-length transmembrane trimer or the extracellular portion of the trimer.

Cryo-EM methods: "frozen for 2-s", do the authors mean blotted for 2 s?

Line 280: replace "frozen" with "vitrified"
Line 380: remove "the"

Manuscript reference number: NCOMMS-24-22063

Title: Lineage-specific pathogenicity, immune evasion, and virological features of SARS-CoV-2 BA.2.86/JN.1 and EG.5.1/HK.3

REVIEWER COMMENTS

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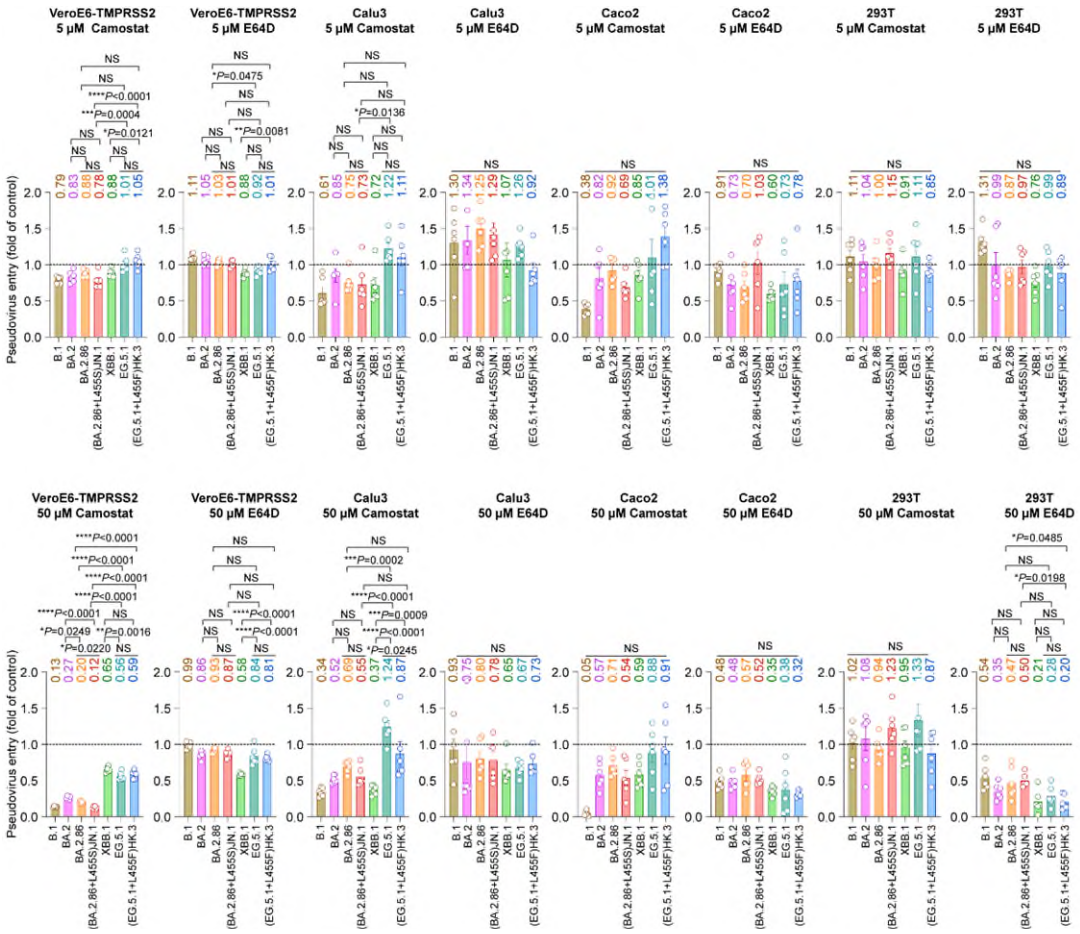
Response: We thank the Reviewer on the positive comments on our study.

Major

Figure 3D: Using a single dose of inhibitor is not convincing. Please demonstrate concentration dependence.
Response: Thank you for the comment. In response to your suggestion, we conducted additional experiments to examine the concentration-dependent effects of the inhibitors. Specifically, we performed the protease inhibition experiment at two additional concentrations including 5μM and 50μM, which supplement our existing data performed at 10μM.

We found that in VeroE6-TMPRSS2 and Calu3 cells, the entry of BA.2.86 and JN.1 pseudoviruses were significantly more sensitive to Camostat treatment when compared to EG.5.1 and HK.3. In 293T cells, which predominantly supports endosomal entry, the entry of EG.5.1 and HK.3 pseudoviruses were more sensitive to E64D treatment than that of BA.2.86 and JN.1. Overall, these results are in keeping with our earlier findings, which collectively indicate that the BA.2 descendent variants BA.2.86 and JN.1 are more dependent on the TMPRSS2-mediated cell surface entry pathway than the XBB descendent variants EG.5.1 and HK.3. These results are illustrated in **Supplementary Figure 7** of the revised manuscript.



Supplementary Figure 7. Protease usage by pseudoviruses in cell lines.

Pseudotype data were normalized, which makes it very difficult to judge the quality of the assays. Unprocessed data must be shown in the supplement, ideally including a negative control (particles with no spike) and a positive control.

Response: Thank you for the suggestions. To address your concerns, we have now included the unprocessed raw data of pseudovirus entry in different cell lines in **Supplementary Table 2**. In addition, we have now provided raw data readings of negative control (particles with no spike) and positive control (VSV-G particles) in the same table (**Supplementary Table 2**). We hope that these added raw data will provide a better picture on our assay quality and reliability.

Minor

The robust cell entry phenotype of JN.1 markedly contrasts that reported by PMID: 38591890. Reasons for these discrepant findings should be discussed.

Response: Thank you for the suggestion. Among the different cell lines that we have evaluated, we observed cell-type dependent entry of JN.1 pseudoviruses. When compared to the pseudovirus entry results of that of PMID: 38591890 (Reference #18 in our revised manuscript), our pseudovirus entry data in Calu3 is different (we did not evaluate pseudovirus entry in 293T-ACE2 cells). Specifically, while JN.1 and BA.2.86 pseudoviruses entered Calu3 with similar efficiency on our hand, PMID: 38591890 reported a lower pseudovirus entry of JN.1 when compared to that of BA.2.86.

Pseudovirus entry data can vary based on a number of variables such as the backbone of pseudovirus, pseudovirus production cell-line, and modification of the spike expression construct. Therefore, it is not uncommon that pseudovirus entry results vary between different laboratories. In the context of JN.1 pseudoviruses, PMID: 38184005 reported a significantly higher pseudovirus entry of JN.1 when compared to BA.2.86. In addition, as we now demonstrate in **Supplementary Figure 8**, the introduction of L455S (which is present in JN.1) in the background of XBB.1 and HK.3 significantly increased pseudovirus entry. Similar observation has also been mentioned in a recent preprint article (doi.org/10.2139/ssrn.4874431). We have included these discussions in the discussion section.

Increased infection of nasal epithelial cells by BA.2.86.1 as compared to other Omicron subvariants has not been found by a recent study published in Nature Communications (PMID: 38480689). Again, reasons for these differences need to be discussed.

Response: Thank you for the suggestion. PMID: 38480689 (Reference #22 in our revised manuscript) evaluated viral RNA release from the apical side of hNECs between BA.2.86 and JN.1. On our hands, we investigated the replication, pseudovirus entry, protease usage, spike cleavage, and infection efficiency of BA.2.86, JN.1, EG.5.1, and HK.3 in hNECs. During manuscript revision, we performed additional experiments that introduced L455S (which is present in JN.1) in the background of XBB.1 and HK.3 spike. Our results further reveal that the spike L455S mutation significantly increases the entry of XBB.1 and HK.3 pseudoviruses in hNECs similar to what we observed for JN.1 (BA.2.86+L455S) pseudoviruses in hNECs (**Supplementary Figure 8**). The differential observation between PMID: 38480689 and our current study is not exactly clear but may be attributed to the protocol of hNEC preparation and/or the experimental procedures being performed. We have included these discussions in the discussion section.

Reviewer #2:

In this manuscript, authors aimed to clarify virological characteristics (factors) of the globally dominant SARS-CoV-2 JN.1 variant and the underlying mechanisms leading to such global dissemination. Authors addressed questions by investigating some virological properties of the JN.1 variant in comparison to parental (BA.2 and BA.2.86) as well as other variants (XBB.1, EG.5.1, and HK.3). The evaluated infectivity, entry, and immune resistance/evasion of JN.1 variant in susceptible cell lines and human nasal epithelial model showed distinct viral properties of such variant when compared to its parental variant BA.2.86. In addition, authors attempted to clarify the underlying mechanisms controlling the distinct behavior of the JN.1 variant by showing that the acquired mutation L455S in the JN.1 spike protein can alter Spike-ACE2 interaction/binding. Overall, authors clearly addressed questions and the experimental design and flow are comprehensive. However, the following points are needed to be addressed. Moreover, the clarity, fluency, and consistency of the English used need to be addressed. I strongly suggest using a third-party editor to address this issue.

Response: We thank the Reviewer on the overall positive comments on our study.

Major points

1. Line 2: rephrase to “SARS-CoV-2” BA.2.86/JN.1 and EG.5.1/HK.3

Response: Thank you for your comment. We have rephrased the sentence accordingly.

2. Authors need to clarify and precisely determine the use of these words (virus, variant, pseudovirus and spike protein) when affixed to individual SARS-CoV-2 variant (for example: lines: 81, 204, 218, 223, 242, 244, 246, 248 etc and suffixing “pseudovirus” to HK.3 in line 232).

Response: Thank you for your valuable feedback. We agree that it is important to clarify and precisely determine the use of these terms in our manuscript. We have double-checked and revised the above-mentioned lines accordingly. In addition, we have gone through the entire manuscript and have corrected similar issues.

3. Line131-135: authors should highlight the domains where the enumerated spike mutations belong (for example NTD, RBD). Are there any conserved sequences among mentioned variants? Is there any relevance of the conserved sequences in terms of vaccine tolerance and immune evasion?

Response: Thank you for your comments and questions. Following your suggestion, we have identified the domains where the spike mutations belong in the revised text. Please refer to line 131-132.

A large number of previously identified mutations (such as D614G that enhances viral transmissibility and N501Y/Q498R that enhances immune evasion) are shared between the spike protein of the studied variants including SARS-CoV-2 BA.2.86/JN.1 and EG.5.1/HK.3. We feel that these mutations have been described quite frequently in previous literature and opt not to repeat them in the current manuscript.

BA.2.86 spike differs from that of BA.2 spike at over 30 amino acid positions. When compared to BA.2.86 spike, JN.1 only contains one additional mutation at L455S in spike RBD. EG.5.1 spike differs from that of XBB.1 spike at NTD Q52H and RBD F456L, while HK.3 spike RBD contains an additional L455F mutation when compared to that of EG.5.1 spike. We have now stated the difference in sequence between the studied variants clearly in the text. The shared and different amino acids in the spike protein of the studied variants are also illustrated in **Figure 1b**.

4. Line 171: The word in “EG.5.1 constituted another” seems to be missing. Add cluster or group.

Response: Thank you for your comment. We have corrected the sentence accordingly.

5. Line184: Authors need to clarify what exactly meant by antigenicity. Is it equal to immune evasion/resistance to antibody neutralization?

Response: Thank you for your comment. Generally, antigenicity is the ability of an antigen to provoke an immune response *in vivo*. In this study, the antigenicity refers to the capacity of

various SARS-CoV-2 variant generating anti-serum to neutralize different Omicron sub-lineages. We utilize ID_{50} values to quantify this neutralization ability, where higher values signify greater neutralization efficacy. In our analysis, we emphasize the antigenic differences among the sub-lineages using an antigenic map. These differences are calculated based on the antigenicity distances between different sub-lineages and serums, determined as follows:

$$D_{ij} = log_2(b_j)-log_2(H_{ij})$$

Where D_{ij} represents the antigenicity distance between sub-lineage i and serum j, b_j denotes maximum titer against one specific strain (normally, it is strain j itself) for serum generated by strain j as the base of antigenic distance. H_{ij} is the ID50 measurement of neutralizing titer of serum generated by j against strain i. Full documentation of the antigenic map can be found in Reference 1.

In practice, we use the Racmacs package to calculate all the antigenicity distances, summarize them into antigenic differences, and optimize all the differences into a single map. As we wrote in the "Antigenic cartography" section in Methods, "The constructed antigenic map was based on serum neutralization data utilizing the antigenic cartography methods, which are implemented in the Racmacs package (https://acorg.github.io/Racmacs/). The antigenic map was generated in R with 10000 optimization steps and other default parameters in a 2-dimensional space. The distances between positions of sub-lineages and serum on the antigenic map were optimized so that distances approach the fold decreases in neutralizing ID_{50} titer, relative to the maximum titer for each serum. Each unit of distance in arbitrary directions in the antigenic map represents a 2-fold change in the ID_{50} titer".

Thus, in the antigenic map, different dots indicate different antigens, and Euclidean distance between dots represent the antigenic distance between variants, representing the similarity of antigenicity. Antigenically similar variants will be close to each other on the map, while antigenically dissimilar variants will disperse on the map. In this way, the greater antigenic distance from a new variant to the previous one might suggest more significant immune evasion/resistance of this new variant.

Overall, though the antigenicity and immune escape/resistance to antibody neutralization are not synonymy, the antigenic distance can suggest potential immune evasion. In general, for antibody neutralization, the greater the antigenic distance from different sub-lineages, the weaker the antibody's neutralization ability.

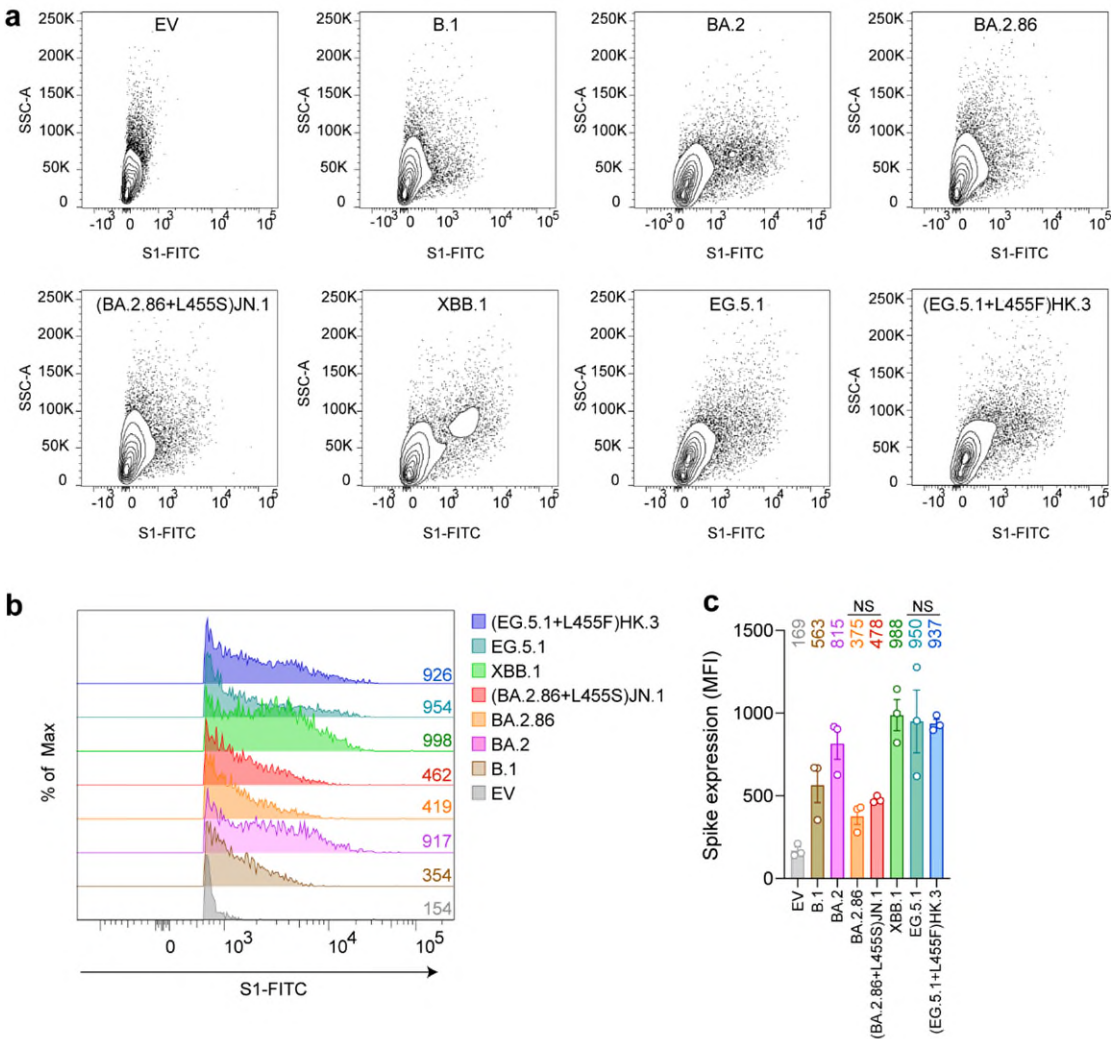
1 Smith, D. J. et al. Mapping the Antigenic and Genetic Evolution of Influenza Virus. Science 305, 371-376, doi:10.1126/science.1097211 (2004).

We have now included more information on the antigenicity calculation in the method section for added clarity.

6. Line 206: Fusion assay. Did authors consider the spike protein expression levels of each variant? Authors used 293T cells for fusion assay. However, it is so hard to conclude spike fusogenicity using only one cell line. Authors should test respective fusion activity in Calu-3

cells, at least.

Response: Thank you for your comments. Following your comment, we assessed the spike protein expression level of each studied variant. We transfected 293T cells with spike plasmids from B.1, BA.2, BA.2.86, JN.1, XBB.1, EG.5.1, and HK.3, followed by flow cytometry analysis. Our results suggest that the increased fusion mediated by L455S and L455F was not due to increased surface expression of the expressed spike proteins. These results are now included in **Supplementary Figure 5** of the revised manuscript.

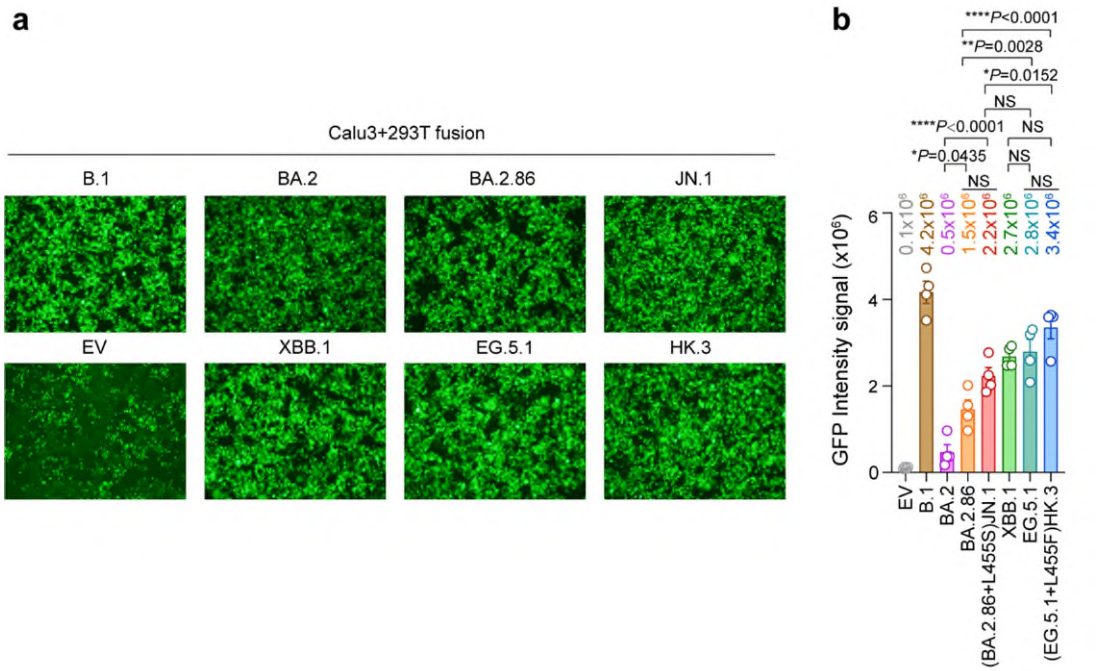


Supplementary Figure 5. Surface expression of B.1, BA.2, BA.2.86, JN.1, XBB.1, EG.5.1, and HK.3 spike protein on 293T cells.

Following your suggestion, we conducted additional experiments to test the respective fusion activity in Calu3 cells. For this purpose, we transfected spike-expressing plasmids and GFP-expressing plasmids in 293T cells, which were then co-cultured with Calu3 cells. The co-cultured cells were fixed after 24 hours of incubation and GFP signal intensity was quantified by ImageJ.

Overall, we found that the fusion pattern in Calu3 cells was largely in keeping with what we observed in 293T cells, with XBB descendent variants XBB.1, EG.5.1, and HK.3 demonstrated

higher fusogenicity than that of BA.2, BA.2.86, and JN.1. L455S and L455F appeared to increase fusogenicity of JN.1 and HK.3 when compared their parental variant BA.2.86 and EG.5.1, respectively. However, the magnitude of increase observed in Calu3 was smaller than that in 293T cells and did not reach statistical significance. We have added these results as **supplementary Figure 6** in the revised manuscript.



Supplementary Figure 6. B.1, BA.2, BA.2.86, JN.1, XBB.1, EG.5.1, and HK.3 spike protein mediated cell-cell fusion in Calu3 cells.

7. Line 213-215: SARS-CoV-2 can't infect 293T and A549 cells. Authors need to mention precisely in main text, figures, and methods whether ACE2 (and TMPRSS2) is expressed in these cell lines.

Response: Thank you for your comment. In our current study, we analysed pseudovirus entry in VeroE6-TMPRSS2, VeroE6, Calu3, Caco2, A549, Huh7, and 293T cells. For A549 and 293T cells, we evaluated pseudovirus entry without ACE2 or TMPRSS2 overexpression. We have double-checked the text in main text, figures, and methods to ensure the descriptions are correct.

While SARS-CoV-2 does not replicate in 293T and A549 cells, both cell types can support certain level of SARS-CoV-2 spike pseudovirus entry. Please refer to References #1-5 below for some examples.

(1) Hoffmann, M., Kleine-Weber, H., Schroeder, S., Krüger, N., Herrler, T., Erichsen, S., ... & Pöhlmann, S. (2020). SARS-CoV-2 cell entry depends on ACE2 and TMPRSS2 and is blocked by a clinically proven protease inhibitor. Cell, 181(2), 271-280.
Figure S1 of this study showed SARS-CoV-2-S pseudovirus entry in **293T and A549 cells**.

(2) Zhang L, Kempf A, Nehlmeier I, et al. SARS-CoV-2 BA.2.86 enters lung cells and evades neutralizing antibodies with high efficiency. Cell. 2024;187(3):596-608.e17.
Figure 1C of this study showed SARS-CoV-2-S pseudovirus entry in **293T cells**.

(3) Hoffmann M, Arora P, Groß R, et al. SARS-CoV-2 variants B.1.351 and P.1 escape from neutralizing antibodies. Cell. 2021;184(9):2384-2393.e12.
Figure 1B of this study showed SARS-CoV-2-S pseudovirus entry in **293T cells**.

(4) Zhang F, Li W, Feng J, et al. SARS-CoV-2 pseudovirus infectivity and expression of viral entry-related factors ACE2, TMPRSS2, Kim-1, and NRP-1 in human cells from the respiratory, urinary, digestive, reproductive, and immune systems. J Med Virol. 2021;93(12):6671-6685.
Figure 1 of this study showed SARS-CoV-2-S pseudovirus entry in **A549 cells**.

(5) Chen J, Fan J, Chen Z, et al. Nonmuscle myosin heavy chain IIA facilitates SARS-CoV-2 infection in human pulmonary cells. Proc Natl Acad Sci U S A. 2021;118(50):e2111011118.
Figure 3B and 3D of this study showed SARS-CoV-2-S pseudovirus entry in **A549 cells**.

8. Line 226: What is the reference for this conclusion “where coronavirus entry predominantly occurs at the plasma membrane”?
Response: Thank you for the comment. We have revised the sentence to “where SARS-CoV-2 entry predominantly occurs at the plasma membrane” to ensure the message is more precise.

The conclusion that SARS-CoV-2 entry predominantly occurs at the plasma membrane in VeroE6-TMPRSS2 and Calu3 is derived from earlier studies. Please Refer to references #1-3 below for some examples. We have also included these references in the text for better support of the message.

(1) Hoffmann, M., Kleine-Weber, H., Schroeder, S., Krüger, N., Herrler, T., Erichsen, S., Schiergens, T. S., Herrler, G., Wu, N. H., Nitsche, A., Müller, M. A., Drosten, C., & Pöhlmann, S. (2020). SARS-CoV-2 Cell Entry Depends on ACE2 and TMPRSS2 and Is Blocked by a Clinically Proven Protease Inhibitor. Cell, 181(2), 271–280.e8.

(2) Hoffmann, M., Kleine-Weber, H., & Pöhlmann, S. (2020). A Multibasic Cleavage Site in the Spike Protein of SARS-CoV-2 Is Essential for Infection of Human Lung Cells. Molecular cell, 78(4), 779–784.e5.

(3) Laporte M, Raeymaekers V, Van Berwaer R, Vandeput J, Marchand-Casas I, Thibaut HJ, Van Looveren D, Martens K, Hoffmann M, Maes P, Pöhlmann S, Naesens L, Stevaert A. The SARS-CoV-2 and other human coronavirus spike proteins are fine-tuned towards temperature and proteases of the human airways. PLoS Pathog. 2021 Apr 22;17(4):e1009500.

9. Line 228 What is the reference for this conclusion “where coronaviruses enter through the endosomal entry route”?
Response: Thank you for the comment. We have revised the sentence to “where SARS-CoV-2

primarily enter through the endosomal entry route" to ensure the message is more precise.

The conclusion that SARS-CoV-2 enters 293T cells primarily through the endosomal entry route is derived from earlier studies. Please refer to References #1-2 below for some examples. We have also included these references in the text for better support of the message.

(1) Hoffmann, M., Kleine-Weber, H., Schroeder, S., Krüger, N., Herrler, T., Erichsen, S., ... & Pöhlmann, S. (2020). SARS-CoV-2 cell entry depends on ACE2 and TMPRSS2 and is blocked by a clinically proven protease inhibitor. Cell, 181(2), 271-280.

(2) Mykytyn AZ, Breugem TI, Riesebosch S, Schipper D, van den Doel PB, Rottier RJ, Lamers MM, Haagmans BL. SARS-CoV-2 entry into human airway organoids is serine protease-mediated and facilitated by the multibasic cleavage site. Elife. 2021 Jan 4;10:e64508. doi: 10.7554/eLife.64508.

10. Line 230: What is the reference for this conclusion "in Caco2 that supports both entry pathways"?
Response: Thank you for the comment. Please refer to References #1-2 below. We have also included these references in the text for better support of the message.

(1) Hoffmann, M., Kleine-Weber, H., Schroeder, S., Krüger, N., Herrler, T., Erichsen, S., ... & Pöhlmann, S. (2020). SARS-CoV-2 cell entry depends on ACE2 and TMPRSS2 and is blocked by a clinically proven protease inhibitor. Cell, 181(2), 271-280.

(2) Attenuated replication and damaging effects of SARS-CoV-2 Omicron variants in an intestinal epithelial barrier model.
Volcic M, Nchioua R, Pastorio C, Zech F, Haußmann I, Sauter D, Read C, Walther P, Kirchhoff F. J Med Virol. 2024 Jul;96(7):e29783. doi: 10.1002/jmv.29783.

11. Line 239-241: Have L455S and L455F mutations been reported to enhance variants transmissibility?
Response: Thank you for the question. L455S and L455F have not been extensively studied for their capacity to enhance transmissibility. PMID: 38184005 and PMID: 38219759 have demonstrated the capacity of L455S and L455F, respectively, in increasing viral infectivity as well as immune evasion, which may contribute to enhanced transmissibility. We have included these references in our revised manuscript.

(1) PMID: 38184005. Kaku Y, Okumura K, Padilla-Blanco M, et al. Virological characteristics of the SARS-CoV-2 JN.1 variant. Lancet Infect Dis 2024; 24(2): e82.

(2) PMID: 38219759. Kosugi Y, Plianchaisuk A, Putri O, et al. Characteristics of the SARS-CoV-2 omicron HK.3 variant harbouring the FLip substitution. Lancet Microbe 2024; 5(4): e313.

12. Line:250-252: JN.1 and HK.3 variants showed higher replication and pseudovirus entry. How are they correlated to viral transmissibility?

Response: Thank you for the question. At Line 250-252 (Line 265-267 of the revised manuscript) we described the higher replication and pseudovirus entry of JN.1 over BA.2.86, EG.5.1, and HK.3 in the human nasal epithelial cells. We believe the increased fitness of JN.1 in the human nasal epithelial cells will positively impact the transmissibility of JN.1.

13. Line 264-268: One representative result is not enough to conclude S1/S2 cleavage of JN.1 spike protein is more efficient than that of BA.2.86 spike protein. Need to show biological replicates and statistical analysis.

Response: Thank you for the suggestion. Following your comment, we have performed additional repeats of the experiment. We similarly quantified spike cleavage from the new experiments and the summarized results are now included as **Figures 4f** of the revised manuscript.

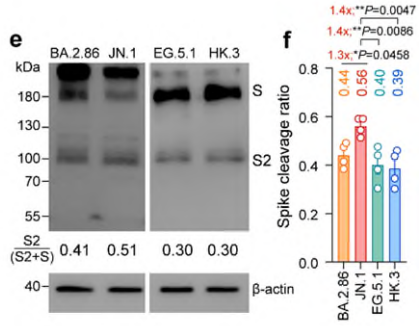


Figure 4f. Spike cleavage of BA.2.86, JN.1, EG.5.1 and HK.3 in hNECs.

14. Line 272: Are there reports supporting the conclusion of enhanced fitness and its impact?

Response: Thank you for your question. We have revised the sentence to: "the further elevated infectivity of JN.1 in hNECs conferred by the L455S mutation may contribute to its dominance over other variants in humans".

There has been no report that directly illustrates the impact of L455S on enhanced fitness other than PMID: 38184005 that demonstrated the capacity of L455S to increase viral infectivity and immune evasion. However, the most recent variants under monitoring (VUM) including JN.1.7, KP.2. KP.3, JN.1.18, and LB.1 all carry L455S, suggesting the impact of L455S on enhanced fitness, which further supported the importance of our findings on L455S.

15. Line 504: Fusion assay. Authors mentioned 293T cells as effector cells. However, VeroE6 cells were described as effector and target cells. What cell lines were used for this assay?

Response: Thank you for catching this. It should be 293T and we have revised the text accordingly.

16. Line 548: what MOI is used for infecting VeroE6 cells?

Response: Thank you for the question. 60 PFU per well was used for VeroE6 infection and we have clarified that in the text accordingly.

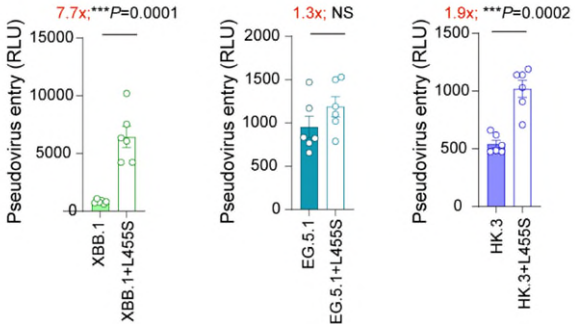
17. Line 552: change “camostat” to Camostat.
Response: Thank you for catching this. It has been corrected.

18. Line 582: authors need to clarify the formula used to calculate the cleavage ratio.
Response: Thank you for the suggestion.
The formula used to calculate the cleavage ratio is: $S_2/ (S_{full\ length}+S_2)$
We have included the formula in **Figure 4e** of the revised manuscript.

19. In discussion: L455S mutation of JN.1 spike (S455) modulated the mode of JN.1 spike/ACE2 interaction to a smaller number of orientations. On the other hand, JN.1 spike showed increased spike cleavage efficiency over the BA.2.86 spike. Authors should recall/mention reports that show similar phenomena of other variant's spike protein, and the possible mechanisms inducing it.
Response: Thank you for the suggestion. Mutations on spike may alter spike cleavage efficiency. For example, the P681R mutation is well known to increase spike cleavage (PMID: 35550680 and PMID: 37043872). In our case, how L455S increases spike cleavage is not known. By further analyzing the structure of JN.1 spike, we found that RBD^{S455} in JN.1 spike can form 5 pairs of hydrogen bonds with neighboring residues F456, Q493, P491 and Y453, but RBD^{L455} in other mutants can only form 2 pairs of hydrogen bonds with P491 and Y453. However, exactly how the increased number of hydrogen bonds and a more stable internal structure may impact spike cleavage will require further investigation. We have now included the new structural analysis as **Supplementary Figure 11** of the revised manuscript.

20. JN.1 has L455S mutation in the spike as well as the other mutations in NSP6 and ORF7b. How did you know that the only L455S mutation in the spike is associated with the global spread of the JN.1 variant? In terms of this, authors should test the effect(s) of insertion of L455S mutation into parental variants [and/or previous VOCs (e.g. B.1.1, Delta)] to highlight changes of virological characteristics of such variants. At least, authors need to discuss this point.
Response: Thank you for your comments. Following your suggestions, we introduced the L455S mutation into the background of XBB.1, EG.5.1, and HK.3 spike constructs, and generated XBB.1+L455S, EG.5.1+L455S, and HK.3+L455S pseudoviruses. We found that the L455S mutation significantly increased the pseudovirus entry of XBB.1 and HK.3 pseudoviruses in hNECs. There was a similar trend of increase for EG.5.1 pseudoviruses but the difference did not reach statistical significance. These added results have further confirmed the importance of L455S in facilitating virus entry in the hNECs. The results are now included as **Supplementary Figure 8** of the revised manuscript.

In addition to our data, a recently available preprint similarly suggests that L455S can increase pseudovirus entry fitness (doi.org/10.2139/ssrn.4874431; Figure 6). As also mentioned in Response #14, the most recent variants under monitoring (VUM) including JN.1.7, KP.2. KP.3, JN.1.18, and LB.1 all carry L455S. Together, these findings highlight the importance of L455S on the enhanced viral fitness and global spread of JN.1.



Supplementary Figure 8. Pseudovirus entry of XBB.1, EG.5.1, and HK.3 with the L455S mutation in hNECs.

Minor points

1. Line 109: you should add cell type of Calu3 (e.g. bronchial adenocarcinoma cells).

Response: Thank you for your suggestion. We have added the cell type information accordingly.

2. Line 157: change “a XBB” to “an XBB”.

Response: Thank you for your suggestion. We have revised the text accordingly.

3. Line 188: Add figure citation after HK.3.

Response: Thank you for your suggestion. We have added (Fig. 2f) after HK.3.

4. Line 198: add (Fig. 2i) after “and EG.5.1”.

Response: Thank you for your suggestion. We have added (Fig. 2i) after EG.5.1.

5. Line 207: add (Fig. 3a) after “and HK.3”.

Response: Thank you for your suggestion. We have added (Fig. 3a) after HK.3.

6. Line 214: add figure citation after 293T cells.

Response: Thank you for your suggestion. We have added (Fig. 3b) after 293T cells.

7. Line 230: add cells after Caco2.

Response: Thank you for your suggestion. We have added “cells” after Caco2.

8. Line 231: add pseudovirus entry after “against BA.2.86 and JN.1”.

Response: Thank you for your suggestion. We have added “pseudovirus entry” after JN.1.

9. Line 231-232: rephrase “effective against EG.5.1 and HK.3” to effective against entry of EG.5.1 and HK.3 pseudoviruses.

Response: Thank you for your suggestion. We have rephrased it accordingly.

10. Line 245: change “protease sensitivity” to protease dependency or usage.

Response: Thank you for your suggestion. We have changed it to “protease dependency”.

11. Line 246: change “(Fig. 3d and Fig.3d)” to (Fig.3b and Fig.3d).
Response: Thank you for catching this. We have revised this accordingly.

12. Line 246: change “in Calu3 cells was” to “in Calu3 cells were”.
Response: Thank you for catching this. We have revised this accordingly.

13. Line 247: change “that of EG.5.1” to those of EG.5.1 and HK.3.
Response: Thank you for catching this. We have revised this accordingly.

14. Line 253: change “to understand if” into to understand whether.
Response: Thank you for your comment. We have revised this accordingly.

15. Line 267: change “than that of EG.5.1” to than those of EG.5.1 and HK.3
Response: Thank you for catching this. We have revised this accordingly.

16. Line 276: change “to evaluate if” into to evaluate whether. Change “bind” to binds.
Response: Thank you for catching these issues. We have revised them accordingly.

17. Line 340: “rapid grow in numbers” is not grammatically correct.
Response: Thank you for catching this. We have revised the text to: “Recent studies including the current one, have demonstrated enhanced immune evasion of JN.1, which is believed to contribute to its rapid growth in numbers”.

18. Line 345: delete in hNECs after L455S.
Response: Thank you for catching this. We have removed the redundant words.

19. Line 354: add lineage after “from BA.2”.
Response: Thank you for catching this. We have revised it accordingly.

20. Line 480: “recent 6 months” is not a precise term. It is better to show in the designated date (e.g. August 2023).
Response: Thank you for your comment. We have put down the exact data, which is from September 1, 2023 to March 1, 2024.

21. Line 487: What do the asterisks mean?
Response: Thank you for your question. In this study, all variants are classified and named using the Pango lineage nomenclature system. Lineages are designated hierarchically. For example, the lineage BA (an alias of B.1.1.529) indicates that it is a sub-lineage of B.1.1, which is itself a sub-lineage of B.1. In this nomenclature system, adding an asterisk at the end of a Pango lineage indicates the inclusion of all its sub-lineages. For instance, BA.2 refers to the specific Pango lineage BA.2, while BA.2* indicates all BA.2-like variants including BA.2 and all its sub-lineages, such as BA.2.1, BA.2.12.1, BA.2.86, etc. To analyse variant-specific mutations in this study, we exclude BA.2.86* from BA.2* to prevent contamination of BA.2* data with BA.2.86* sequences. Similarly, XBB.1* does not include EG.5.1*, EG.5.1* does not

include HK.3*, and BA.2.86* does not include JN.1* for sequence and mutation analysis.

22. Ref.27 (BioRxiv), Ref. 28, Ref. 31, Ref. 38 citations are incomplete.
Response: Thank you for the comment. We have carefully reviewed and corrected the citations as follows:

Reference 27 (BioRxiv), now 28: [Wang X, Jiang S, Ma W, Zhang Y, Wang P. Robust neutralization of SARS-CoV-2 variants including JN.1 and BA.2.87.1 by trivalent XBB vaccine-induced antibodies. Signal Transduct Target Ther 2024; 9(1): 123.]

Reference 28, now 34: [Kosugi Y, Plianchaisuk A, Putri O, et al. Characteristics of the SARS-CoV-2 omicron HK.3 variant harbouring the FLip substitution. Lancet Microbe 2024; 5(4): e313.]

Reference 31, now 38: [Du P, Wu C, Hu S, Fan R, Gao GF, Wang Q. The omicron BA.2.86 subvariant as a new serotype of SARS-CoV-2. Lancet Microbe 2024; 5(6):e516.]

Reference 38, now 46: [Ai J, Wang X, He X, et al. Antibody evasion of SARS-CoV-2 Omicron BA.1, BA.1.1, BA.2, and BA.3 sub-lineages. Cell Host Microbe 2022; 30(8): 1077-83.e4.]

These corrections have been implemented in the References section of the revised manuscript.

23. Line 510: Can't recognize open square.
Response: Thank you for your comment. We have adjusted the color of the open squares. They should now be more easily recognized.

Reviewer #3:

The authors report an extensive characterization in vitro and in vivo of the most recent SARS-CoV-2 variants, which includes immunological assays, fusion assays and structural biology. The efforts are impressive and timely, which makes the paper exciting; the cryo-EM analysis, however, is not the strongest part of the paper. The quality of the cryo-EM data is good, but the presentation of the structural data is not as impressive as the presentation of the functional/immunological data. The structural analysis could be pushed a little further, for example by describing a structural comparison with previous variants, not just between the two variants described in the paper. I still believe the manuscript is valuable and deserves publication, it just needs something more in terms of structural analysis with the current data that the authors have already collected.
Response: We thank the Reviewer for the positive comments on our manuscript.

Major issues:

The main maps, both for the trimer and the local refinement of RBD+ACE2 have been

sharpened using EMReady. It definitely improved the appearance of the maps but the program distorts the density of ACE2 in the trimer map. The authors should display and deposit the maps obtained using standard programs like cryoSPARC and deposit the EMReady maps as Additional maps in the PDB and EMDB.

Response: Thank you for your comments. We have re-uploaded the map obtained at CryoSPARC as requested and stored the EMReady map as an additional map in PDB and EMDB.

The main figure (Fig. 5) has some issues:

-Showing the model as surface in the context of a new variant doesn't deliver any new message. What's new is the interface between ACE2 and the RBDs of the two variants. The authors could show the local refinement cryo-EM maps (which have a very good-looking density!) in the main figure, colored based on the protein chains, side by side with the structural models shown in panels e-g.

Response: Thank you for your suggestions. In the main figure, we have added the local refined cryo-EM maps around the interface and colored it according to protein chains (**Figure 5e-5f**).

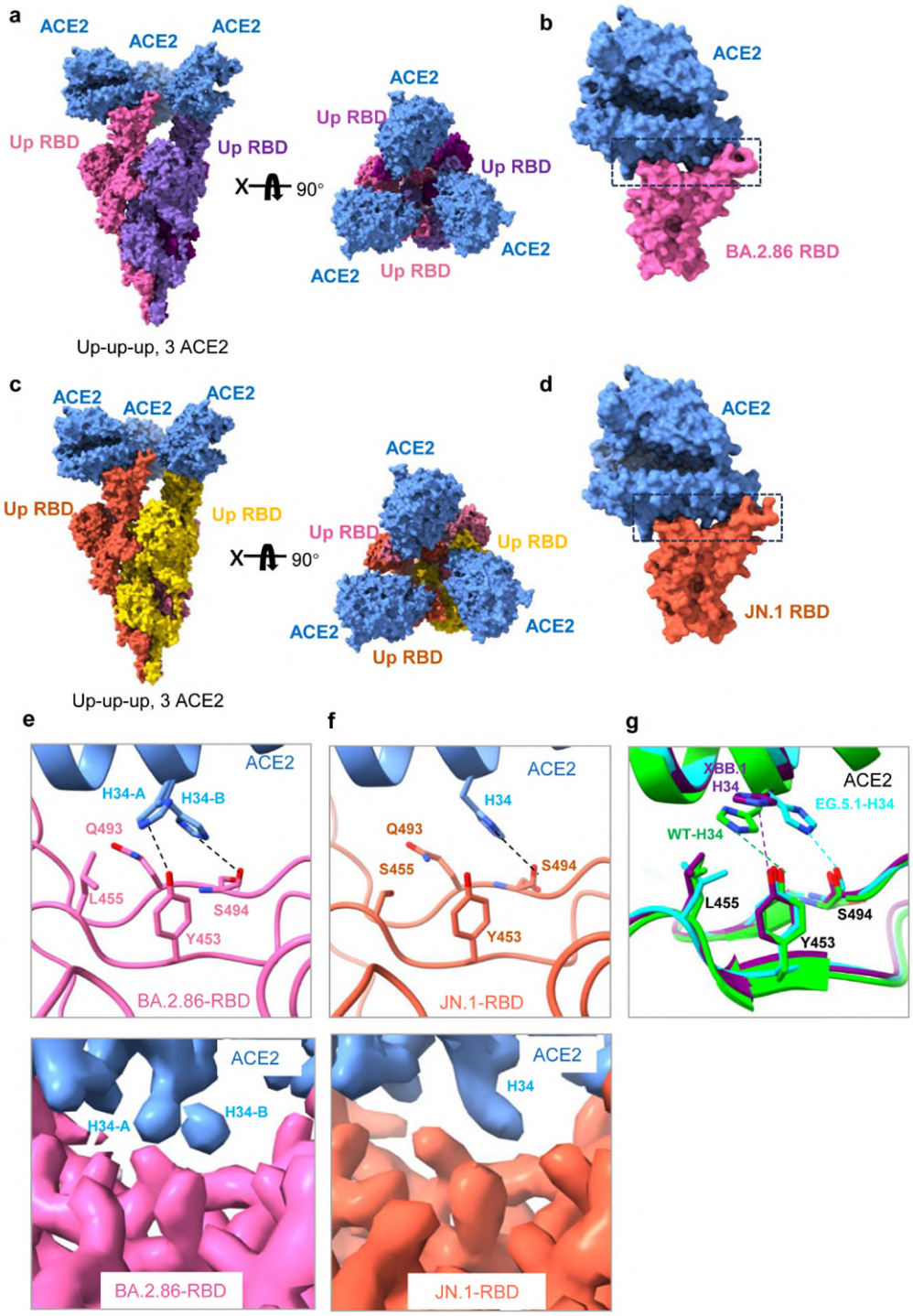


Figure 5. Cryo-EM structures of BA.2.86/JN.1 spikes in complex with ACE2.

-The authors use two panels (e and f) to show the two conformers of His34. I suggest the authors show a single panel where the two orientations of His 34 are shown (like they have in the model) labelled as conformer A and B.

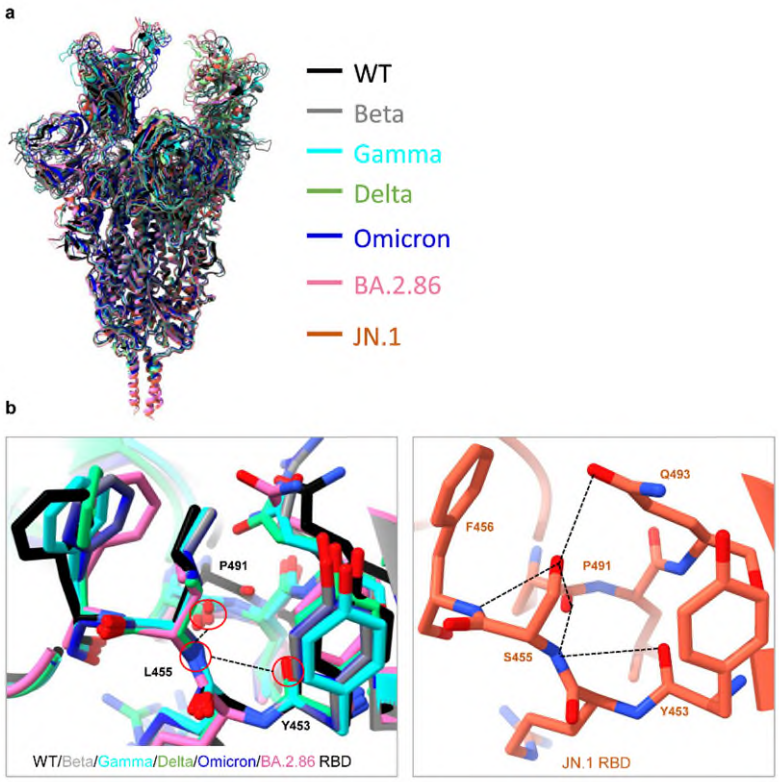
Response: Thank you for the suggestion. The conformers of His34 have been displayed on the same panel and conformers A and B have been marked (**Figure 5e**).

-The authors report a change in side-chain interaction, or rather a preferential orientation of a single residue, but a comparison with other variants is missing. I suggest adding panels that show the same RBD:ACE2 interface region in other recent variants, as well as describe differences and similarities in the result section and/or discussion section.

Response: Thank you for your suggestion. We added the RBD-ACE2 interface regions of WT, XBB.1 and EG.5.1 in **Figure 5g**. Structural analysis of the RBD-ACE2 interface regions of BA.2.86, JN.1, WT, XBB.1 and EG.5.1 variants revealed that most variant has only one conformation at $ACE2^{H34}$ and no binding mode similar to BA.2.86, which could explain the strong binding force between BA.2.86. S and ACE2.

-Is there any difference in the trimer structures compared to previous variants? The resolution is very high for the trimer structure, so the authors should be able to point out differences between the new variants reported and the old variants.

Response: Thank you for your suggestion. We compared BA.2.86 and JN.1 S trimer with other variants. BA.2.86 and JN.1 S trimer showed no significant change in trimer structure compared to the previous variants. However, it is noteworthy that a significant difference was observed at the specific mutation site of JN.1 RBD^{L455S}. In this region, RBD^{S455} can form 5 pairs of hydrogen bonds with neighboring F456, Q493, P491 and Y453, but RBD^{L455} in other mutants can only form 2 pairs of hydrogen bonds with P491 and Y453. Based on the structural analysis, it is inferred that JN.1 has more hydrogen bonds and a more stable internal structure. This may explain why JN.1 has a significantly enhanced infectiveness although it only has one more L455S mutation than BA.2.86, which needs to be further verified. These new findings have now been included as **Supplementary Figure 11** of the revised manuscript.



Supplementary Figure 11. Structural comparation of variants at spike residue 455.

Minor issues:

Protein expression and purification: mentions “full-length spike” but the protein is secreted. The authors should clarify whether their construct is the full-length transmembrane trimer or the extracellular portion of the trimer.
Response: Thank you for the comment. The S proteins involved in this study are all spike ectodomain. We have clarified this in the revised manuscript.

Cryo-EM methods: “frozen for 2-s”, do the authors mean blotted for 2 s?
Response: Thank you for the comment. "Frozen for 2-s" means blotted for 2 s. We have revised the text accordingly.

Line 280: replace “frozen” with “vitrified”
Response: Thank you for pointing this out. It has been revised.

Line 380: remove “the”
Response: It was corrected as suggested.

REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

The authors have adequately addressed the points raised by this reviewer.

Reviewer #2 (Remarks to the Author):

The authors have addressed all concerns.

Reviewer #3 (Remarks to the Author):

The authors improved the figures and addressed the concerns raised for the first round of review.

However, their addition of the hydrogen bond analysis for Ser455 in JN.1 is too speculative and there is no evidence of an increase stabilization as a result of increased hydrogen bonding. In fact hydrophobic interactions, such as those possible for Leu455, would be more favorable in that region where there are several aromatics (Phe456 and Tyr453). I recommend removing statements about increased stabilization resulting from increased hydrogen bonds. More hydrogen bonds does not necessarily mean more stable, especially when the mutated residue was able to display more stabilizing hydrophobic interactions. All the authors can say with the current data is that the hydrogen bond network **changes** as a result of the L455S mutation.

Minor issues: Phe456 and Gln493 should be labelled also in the left panel of Supplementary Figure 11b.

Manuscript reference number: NCOMMS-24-22063B

Title: Lineage-specific pathogenicity, immune evasion, and virological features of SARS-CoV-2 BA.2.86/JN.1 and EG.5.1/HK.3

REVIEWER COMMENTS

Reviewer #3 (Remarks to the Author):

The authors improved the figures and addressed the concerns raised for the first round of review.

However, their addition of the hydrogen bond analysis for Ser455 in JN.1 is too speculative and there is no evidence of an increase stabilization as a result of increased hydrogen bonding. In fact hydrophobic interactions, such as those possible for Leu455, would be more favorable in that region where there are several aromatics (Phe456 and Tyr453). I recommend removing statements about increased stabilization resulting from increased hydrogen bonds. More hydrogen bonds does not necessarily mean more stable, especially when the mutated residue was able to display more stabilizing hydrophobic interactions. All the authors can say with the current data is that the hydrogen bond network **changes** as a result of the L455S mutation.

Response: Thank you for the comment. We have revised the description according to your suggestion.

Line 327 "…it is inferred that the hydrogen bond network **changes** as a result of the L455S mutation…"

Minor issues: Phe456 and Gln493 should be labelled also in the left panel of Supplementary Figure 11b.

Response: Thank you for the suggestion. The labels were added as suggested.