natureresearch

Peer Review File

Manuscript Title: Crystal structure of the SARS-CoV-2 spike receptor-binding domain bound with the ACE2 receptor

Editorial Notes:

Redactions – Mention of other journals

This document only contains reviewer comments, rebuttal and decision letters for versions considered at *Nature*. Mentions of the other journal have been redacted.

Reviewer Comments & Author Rebuttals

Reviewer Reports on the Initial Version:

Referee #1 (Remarks to the Author):

In this manuscript authored by Lan et al., they present the crystal structure of the 2019-nCoV spike receptor-binding domain (RBD) bound with the ACE2 receptor. They observed that 2019-nCoV RBD has almost the same overall structure and binding mode to ACE2 as SARS-CoV RBD. They further identified a few key amino acid residues in 2019-nCoV for the receptor interaction. By comparing the structures of SARS-CoV RBD and 2019-nCoV RBD, they explained why two SARS-CoV antibodies could not neutralize 2019-nCoV, which may shed insight on pan-coronavirus antibody development.
1. In line 143, it is stated that the binding affinities between ACE2 and 2019-nCoV and SARS-CoV is RBDs fall into the same range of (~10-60 nM). Where is the data for these values? Is this just based off the structural similarities or was a binding assay performed?
2. It would be informative if the authors can measure the RBD binding affinity to ACE2 for both 2019-nCoV and SARS-CoV. In addition, if the authors observe any significant difference, key amino residues can be examined to confirm their contribution to the increased/decreased binding affinity.

Referee #2 (Remarks to the Author):

Binding of SARS-CoV to the ACE2 receptor is a critical step to enter target cells. Recent studies pointed to the role of ACE2 in mediating entry of the new coronavirus SARS-CoV-2 as well. To elucidate the SARS-CoV-2 receptor binding domain and ACE2 interaction at a higher resolution, Lan et al., determined the structure of the SARS-CoV-2 RBD bound to ACE2 by X-ray crystallography. The main observation is that the overall ACE2-binding mode of the SARS-CoV-2 RBD is nearly identical to that of the SARS-CoV RBD. Structural analysis identified residues in the RBD critical for ACE2 binding, the majority of which are either highly conserved or shared similar side chain properties with those in the SARS-CoV RBD. Overall this study provides important insights in the interaction of the virus with its receptor. The manuscript is clearly written with detailed information on the critical amino acids from the two RBDs that interact with ACE2.

Recently, the nomenclature for 2019-nCoV has changed and the authors should mention COVID-19 or SARS-CoV-2 were appropriate.

A few additional references from other groups should be included in the introduction section (52-58) regarding the background information on structural interaction of the SARS-CoV spike with ACE2.

The manuscript should be checked for minor spelling mistakes, e.g line 54 (a cascade of events), line 60 (cells expressing ACE2 are susceptible), line 61 (spell out SPR), line 132 (residue) etc…

Referee #3 (Remarks to the Author):

In this manuscript, Lan et al report the crystal structure of the 2019-nCoV RBD in complex with human ACE2 at 2.45Å resolution, thereby allowing accurate analysis of interactions. These results are important as they reveal the molecular basis of receptor binding by the newly emerged 2019-nCoV and show that this virus binds ACE2 efficiently. The work is of high quality although I have several comments and concerns described below.

To avoid future confusion, I encourage the authors to use the designated name of the virus throughout the manuscript (SARS-CoV-2)

Lines 50-51: Homology cannot be quantified. The authors are referring to % identity or similarity.

Line 59: these reference should be added: Hoffmann et al Cell 2020, Walls et al Cell 2020, Letko et al [redacted].

Lines 61-63: The author's statement is incorrect. It has been long known that the binding affinity for ACE2 of the free SARS-CoV RBD is higher than that of SARS-CoV S1 (Wong SK et al JBC 2004)

Line 66: As 2 structures of the 2019-nCoV spike have been solved, these references should be added: Wrapp et al Science 2020 & Walls et al Cell 2020

Lines 84-86: this reference should be added: Li et al Science 2005

Lines 93-95: The author state that 9 Cys residues are found in the RBD. Do they suggest that there is an unpaired cysteine in each protomer then?

Lines 107-108: 1,700Å^2 corresponds to the sum of buried surface areas by each molecule. It should either be clearly stated or the average value should be reported as it is more common. The authors should also compare the buried surface area between the 2019-nCoV RBD + ACE2 and the SARS-CoV RBD + ACE2 complexes.

Lines 114-116: Although the authors detected 17 hydrogen bonds and 2 salt bridges at the 2019-nCoV+ACE2 interface, this reviewer found only 13 hydrogen bonds and 2 salt bridges using the pisa server and the model provided. For SARS-CoV+ACE2 interface (pdb-2ajf), 13 hydrogen bonds and 3 salt bridges were identified with pisa (which also differs from the number reported by the authors).

Lines 140-142: 2019-nCoV and SARS-CoV are both lineage B β-CoVs, they are not part of distinct lineages strictly speaking (although they do not cluster together within SARS-CoV-like viruses).

Lines 154-156: Polyclonal antibodies (i.e. serum) are likely to target multiple sites on the CoV spike. This underscores the conservation between SARS-CoV and 2019-nCoV spikes not solely among RBDs.

Based on the positive Fourier difference map this reviewer could observe around Zn 901, this ion has likely not been correctly identified.
Analysis of the maps suggest several water molecules and alternate side chain conformations could be (and should be) modeled in density.

Also the Ramachandran statistics reported in Table S1 are poorer than they actually are for the model provided by the author. This should be fixed.

It also appears that the ACE2 glycan at position N90 was not fully modeled. Does this N-linked oligosaccharide contact the RBM. This should be discussed in the text and compared to SARS-CoV.

In the current manuscript, the analysis of the interface between the RBD and ACE2 is limited to listing similar and different residues. It lacks details of the interactions formed and a thorough analysis of the differences between the two viruses along with their putative relationships to binding affinities.

Superimposition of the structure determined by these authors with the SARS-CoV+RBD structure (pdb-2ajf) shows there is a conformational difference around residues 270-290 (2019-nCoV numbering). The authors should discuss it and its potential implications for binding to ACE2.

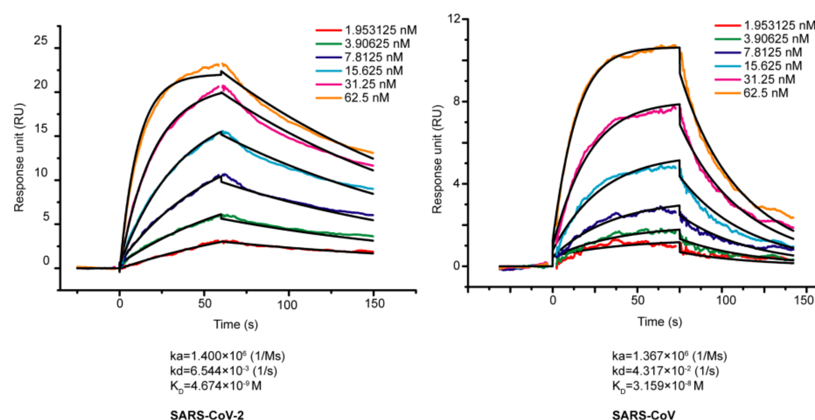
Author Rebuttals to Initial Comments:

Reviewer #1

In this manuscript authored by Lan et al., they present the crystal structure of the 2019-nCoV spike receptor-binding domain (RBD) bound with the ACE2 receptor. They observed that 2019-nCoV RBD has almost the same overall structure and binding mode to ACE2 as SARS-CoV RBD. They further identified a few key amino acid residues in 2019-nCoV for the receptor interaction. By comparing the structures of SARS-CoV RBD and 2019-nCoV RBD, they explained why two SARS-CoV antibodies could not neutralize 2019-nCoV, which may shed insight on pan-coronavirus antibody development.

1. **In line 143, it is stated that the binding affinities between ACE2 and 2019-nCoV and SARS-CoV is RBDs fall into the same range of (~10-60 nM). Where is the data for these values? Is this just based off the structural similarities or was a binding assay performed?**
2. **It would be informative if the authors can measure the RBD binding affinity to ACE2 for both 2019-nCoV and SARS-CoV. In addition, if the authors observe any significant difference, key amino residues can be examined to confirm their contribution to the increased/decreased binding affinity.**

Response: Thank the reviewer for the suggestions and here we answer the above two questions together. In the previous manuscript, we stated that the binding affinities between ACE2 and SARS-CoV-2 and SARS-CoV RBDs fall into the same range of (~10-60 nM) based on reported binding results (Tian et al, Emerging Microbes & Infections, 2020; Sui et al, Journal of Virology, 2014). As suggested by the reviewer, we conducted additional SPR experiments to compare the binding affinities between ACE2 and SARS-CoV-2 and SARS-CoV RBDs. Our measured K_D value between ACE2 and SARS-CoV-2 RBD was 4.7 nM, and that between ACE2 and SARS-CoV RBD was 31 nM. A recent Cell paper also reported the BLI-measured averaged K_D value of ~1.2 nM between ACE2 and SARS-CoV-2 RBD and of ~5.0 nM between ACE2 and SARS-CoV RBD (Walls et al, Cell, 2020). These independent studies showed that the binding affinities of ACE2 to SARS-CoV-2



and SARS-CoV RBDs fall into a similar range. We edited our manuscript to reflect those changes (page 4, lines 71-74) and added a new Fig S5.

Fig S5. SPR sensorgrams showing the binding of immobilized human ACE2 with SARS-CoV-2 RBD (left) and SARS-CoV RBD (right). Data are shown as different color lines and the best fit of the data to a 1:1 binding model is shown in black.

Reviewer #2

Binding of SARS-CoV to the ACE2 receptor is a critical step to enter target cells. Recent studies pointed to the role of ACE2 in mediating entry of the new coronavirus SARS-CoV-2 as well. To elucidate the SARS-CoV-2 receptor binding domain and ACE2 interaction at a higher resolution, Lan et al., determined the structure of the SARS-CoV-2 RBD bound to ACE2 by X-ray crystallography. The main observation is that the overall ACE2-binding mode of the SARS-CoV-2 RBD is nearly identical to that of the SARS-CoV RBD. Structural analysis identified residues in the RBD critical for ACE2 binding, the majority of which are either highly conserved or shared similar side chain properties with those in the SARS-CoV RBD. Overall this study provides important insights in the interaction of the virus with its receptor. The manuscript is clearly written with detailed information on the critical amino acids from the two RBDs that interact with ACE2.

1. **Recently, the nomenclature for 2019-nCoV has changed and the authors should mention COVID-19 or SARS-CoV-2 were appropriate.**
   Response: We agreed with the suggestion and replaced 2019-nCoV with SARS-CoV-2 throughout the manuscript.

2. **A few additional references from other groups should be included in the introduction section (52-58) regarding the background information on structural interaction of the SARS-CoV spike with ACE2.**
   Response: Thank the reviewer for your suggestions. Here we added two other references related to the study of SARS-CoV spike and its interaction with ACE2 (page 4 lines 65-67).

   Kirchdoerfer, R. N. et al. Stabilized coronavirus spikes are resistant to conformational changes induced by receptor recognition or proteolysis. Sci Rep 8, 15701, doi:10.1038/s41598-018-34171-7 (2018).

   Yuan, Y. et al. Cryo-EM structures of MERS-CoV and SARS-CoV spike glycoproteins reveal the dynamic receptor binding domains. Nat Commun 8, 15092, doi:10.1038/ncomms15092 (2017).

3. **The manuscript should be checked for minor spelling mistakes, e.g line 54 (a cascade of events), line 60 (cells expressing ACE2 are susceptible), line 61 (spell out SPR), line 132 (residue) etc...**
   Response: Thank the reviewer for the careful examination of our manuscript. We have corrected the spelling mistakes and rechecked the spelling of the revised manuscript.

<u>Reviewer #3</u>

In this manuscript, Lan et al report the crystal structure of the 2019-nCoV RBD in complex with human ACE2 at 2.45Å resolution, thereby allowing accurate analysis of interactions. These results are important as they reveal the molecular basis of receptor binding by the newly emerged 2019-nCoV and show that this virus binds ACE2 efficiently. The work is of high quality although I have several comments and concerns described below.

1. **To avoid future confusion, I encourage the authors to use the designated name of the virus throughout the manuscript (SARS-CoV-2)**
   Response: Thank the reviewer for the suggestion. We have replaced 2019-nCoV with SARS-CoV-2 throughout the manuscript.

2. **Lines 50-51: Homology cannot be quantified. The authors are referring to % identity or similarity.**
   Response: We agree with the reviewer and replaced the homology with sequence identity in the revised manuscript. The sentence in the revised manuscript is "Bat coronavirus RaTG13 appears to be the closest relative of the 2019-nCoV sharing over 93.1% sequence identity in the spike (S) gene. SARS-CoV and other SARSr-CoVs however are rather distinct with less than 80% sequence identity." (page 3 lines 57-59).

3. **Line 59: these reference should be added: Hoffmann et al Cell 2020, Walls et al Cell 2020, Letko et al [redacted].**
   Response: We have added these references in the revised manuscript (page 4 line 70).

4. **Lines 61-63: The author's statement is incorrect. It has been long known that the binding affinity for ACE2 of the free SARS-CoV RBD is higher than that of SARS-CoV S1 (Wong SK et al JBC 2004)**
   Response: Thank the reviewer for the suggestion. We have edited this part in the revised manuscript (page 4 lines 71-74). The edited statement is "In vitro binding measurements also showed that the SARS-CoV-2 RBD binds to ACE2 with an affinity in the low nM range, indicating that the RBD is the key functional component within the S1 subunit responsible for binding of SARS-CoV-2 to ACE2." We try to emphasize here that the SARS-CoV-2 RBD has been shown to be able to bind to the receptor ACE2 with high affinity.

5. **Line 66: As 2 structures of the 2019-nCoV spike have been solved, these references should be added: Wrapp et al Science 2020 & Walls et al Cell 2020**
   Response: We have added these references in the manuscript (page 4 line 77).

6. **Lines 84-86: this reference should be added: Li et al Science 2005**
   Response: Thank the reviewer for the suggestion and we have added it into the manuscript (page 5 line 98).

7. **Lines 93-95: The author state that 9 Cys residues are found in the RBD. Do they suggest that there is an unpaired cysteine in each protomer then?**
   Response: In the RBD monomer, four disulfide bonds are formed. The supplementary figure 1 also showed disulfide-bond linked RBD dimer band in the non-reducing SDS-PGE gel, indicating that Cys538 is unpaired in the RBD monomer and Cys538 residues from two monomers form an inter-molecular disulfide bond. The unpaired Cys538 is at the C-terminus of the RBD, which is not resolved in the final structure model.

8. **Lines 107-108: 1,700Å^2 corresponds to the sum of buried surface areas by each molecule. It should either be clearly stated or the average value should be reported as it is more common.**
   Response: The reviewer is right and the 1,700 $Å^2$ is the sum of buried surface areas by each molecule. We have edited the sentence to make it clear in the revised manuscript (page 6 line 126).

9. **The authors should also compare the buried surface area between the 2019-nCoV RBD + ACE2 and the SARS-CoV RBD + ACE2 complexes.**
   Response: We calculated the buried surface area at the SARS-CoV RBD/ACE interface, which is nearly the same with that at the SARS-CoV RBD/ACE interface. We have added the calculated area in the revised manuscript (page 6 line 128).

10. **Lines 114-116: Although the authors detected 17 hydrogen bonds and 2 salt bridges at the 2019-nCoV+ACE2 interface, this reviewer found only 13 hydrogen bonds and 2 salt bridges using the pisa server and the model provided. For SARS-CoV+ACE2 interface (pdb-2ajf), 13 hydrogen bonds and 3 salt bridges were identified with pisa (which also differs from the number reported by the authors).**
    Response: We appreciate the careful examination of our previously provided coordinates by the reviewer. After the initial submission, we further refined the model a couple of more rounds, which provided better refinement statistics (Table S1). Previously we analyzed the interface using the Qt-PISA in the CCP4 suite, and we did notice that there are subtle differences between results calculated by Qt-PISA and PDBePISA server. For example, 12 hydrogen bonds were identified by Qt-PISA and 13 were identified by PDBePISA at the SARS-CoV RBD/ACE2 interface (PDB: 2AJF). We do not know the reasons for this subtle difference. It may due to running PISA program in different environments. As suggested by the reviewer, in the revised manuscript we re-analyzed the SARS-CoV-2 RBD/ACE2 and SARS-CoV RBD/ACE2 interfaces using the PDBePISA server and updated the Table 1 in the revised manuscript (page 14).

11. **Lines 140-142: 2019-nCoV and SARS-CoV are both lineage B-CoVs, they are not part of distinct lineages strictly speaking (although they do not cluster together within SARS-CoV-**

like viruses).
Response: As suggested by the reviewer, we have edited this in the abstract (page 2 lines 33-34) and main text (page 8 lines 186-189) of the revised manuscript (although the SARS-CoV-2 does not cluster within SARS and SARS-related coronaviruses).

12. **Lines 154-156: Polyclonal antibodies (i.e. serum) are likely to target multiple sites on the CoV spike. This underscores the conservation between SARS-CoV and 2019-nCoV spikes not solely among RBDs.**
Response: We agree with the reviewer. We edited this part in the revised manuscript (page 9 lines 223-231). This part is "The cross-neutralization of SARS-CoV-2 by horse anti-SARS-CoV serum and serum/plasm from recovered SARS patients indicates a great potential in identifying antibodies with cross-reactivity between these two coronaviruses[1,14]. The highly sequence conserved non-RBD regions in the spike such as the NTD in the S1 subunit and the S2 subunit are the potential targets for cross-reactive antibodies. Although the RBD has less sequence identity, conserved residues between SARS-CoV-2 and SARS-nCoV RBD indeed exist, even in the more variable RBM (Fig. 4). Considering that the RBD is the critical region for receptor binding, antibodies targeting the conserved epitopes in the RBD will also present a great promise for developing highly potent cross-reactive therapeutic agents toward diverse coronavirus species including SARS-CoV-2."

13. **Based on the positive Fourier difference map this reviewer could observe around Zn 901, this ion has likely not been correctly identified.**
Response: Previous determined ACE2 structures such as 2ajf, 1r4l, 3sci, 3doi, 6vw1 all have one zinc ion at this position, indicating that this is a conserved position for zinc ion. There is a positive peak at this position in the Fo-Fc map without the Zn901 in the model. Therefore, we added one zinc ion (Zn 901) in the final model.

14. **Analysis of the maps suggest several water molecules and alternate side chain conformations could be (and should be) modeled in density.**
Response: We further refined the model, which has better refinement statistics (Table S1). We checked the residues one-by-one and added two obvious alternate side chain conformations at N228 in the ACE2 and Q493 in the RBD. The number of water molecules in the final model were increased to 80.

15. **Also the Ramachandran statistics reported in Table S1 are poorer than they actually are for the model provided by the author. This should be fixed.**
Response: Thank the reviewer for the careful examination. We have updated the Table S1 in the revised manuscript (in supplementary file).

16. **It also appears that the ACE2 glycan at position N90 was not fully modeled. Does this N-linked oligosaccharide contact the RBM. This should be discussed in the text and compared to SARS-CoV.**

Response: Thank the reviewer for the careful examination. In the previously determined SARS-CoV RBD-ACE2 structure, three glycans NAG-NAG-BMA linked to Asn90 of the ACE2 were modeled and the BMA has contact with the SARS-CoV RBM. In our density map, we could only model one NAG linked to Asn90 and this NAG does not have interaction with the SARS-CoV-2 RBM. However, we could not exclude the possibility that the glycans following the first NAG could have interactions with the SARS-CoV-2 RBM, such as in the SARS-CoV RBD-ACE2 structure. We have added the discussions in the revised manuscript (pages 7-8 lines 175-183).

17. **In the current manuscript, the analysis of the interface between the RBD and ACE2 is limited to listing similar and different residues. It lacks details of the interactions formed and a thorough analysis of the differences between the two viruses along with their putative relationships to binding affinities.**
    Response: We appreciate the reviewer's suggestion. In the revised manuscript, we added details of the interactions and the comparisons between two RBD/ACE2 interfaces (page 7 lines 149-157). Four positions in the RBM that are important for binding of SARS-CoV RBD to ACE2 were analyzed, and the Lys417 position outside the RBD was also analyzed. The analysis and description were also coupled with a new Fig. 3 in the revised manuscript.

18. **Superimposition of the structure determined by these authors with the SARS-CoV+RBD structure (pdb-2ajf) shows there is a conformational difference around residues 270-290 (2019-nCoV numbering). The authors should discuss it and its potential implications for binding to ACE2.**
    Response: The structure of SARS-CoV-2 does not include the region of residues 270-290. The reviewer may refer to the distal loop region in the RBM (residues 475-486), which has a different conformation compared with that in the SARS-CoV RBD structure. Residue Ala475 Ala at the beginning and Phe486 at the end of the SARS-CoV-2 loop still have similar positions with respective Pro462 and Leu472 in the SARS-CoV loop. SARS-CoV-2 RBD residues between Ala475 and Phe486 have more significant conformational changes with their respective residues in the SARS-CoV RBD. However, these positions are not involved in ACE2 binding in both RBDs, so we did not focus on the discussion of them in the revised manuscript.