EMBO reports

Biparatopic sybodies neutralize SARS-CoV-2 variants of concern and mitigate drug resistance

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1st Editorial Decision 22nd Oct 2021

Dear Dr. Seeger,

Thank you for transferring your manuscript from Review Commons to EMBO reports. I now went through your manuscript, the referee reports (attached again below), and your revision plan. All referees acknowledge the high interest of the findings. They have raised a number of suggestions to improve the manuscript, which I see you are able to address during a minor revision of the manuscript.

We would thus like to invite you to revise your manuscript with the understanding that all referee points must be addressed in the revised manuscript or in the detailed point-by-point response (as suggested in your revision plan). Acceptance of your manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

Revised manuscripts should be submitted within three months of a request for revision. Please contact me to discuss the revision should you need additional time.

When submitting your revised manuscript, please also carefully review the instructions that follow below.

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The Expanded View format, which will be displayed in the main HTML of the paper in a collapsible format, has replaced the Supplementary information. You can submit up to 5 images as Expanded View. Please follow the nomenclature Figure EV1, Figure EV2 etc. The figure legend for these should be included in the main manuscript document file in a section called Expanded View Figure Legends after the main Figure Legends section. Additional Supplementary material should be supplied as a single pdf file labeled Appendix. The Appendix should have page numbers and needs to include a table of content on the first page (with page numbers) and legends for all content. Please follow the nomenclature Appendix Figure Sx, Appendix Table Sx etc. throughout the text, and also label the figures and tables according to this nomenclature.
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3) a .docx formatted letter INCLUDING the reviewers' reports and your detailed point-by-point responses to their comments. As part of the EMBO Press transparent editorial process, the point-by-point response is part of the Review Process File (RPF), which will be published alongside your paper.

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5) that primary datasets produced in this study (e.g. RNA-seq, ChIP-seq, structural and array data) are deposited in an appropriate public database. If no primary datasets have been deposited, please also state this a dedicated section (e.g. 'No primary datasets have been generated and deposited'), see below.

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Please remember to provide a reviewer password if the datasets are not yet public.

The accession numbers and database should be listed in a formal "Data Availability " section (placed after Materials & Methods) that follows the model below. This is now mandatory (like the COI statement). Please note that the Data Availability Section is restricted to new primary data that are part of this study.

Data availability

The datasets produced in this study are available in the following databases:

- RNA-Seq data: Gene Expression Omnibus GSE46843 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE46843)
- [data type]: [name of the resource] [accession number/identifier/doi] ([URL or identifiers.org/DATABASE:ACCESSION])

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Moreover, I have these editorial requests:

6) We strongly encourage the publication of original source data with the aim of making primary data more accessible and transparent to the reader. The source data will be published in a separate source data file online along with the accepted manuscript and will be linked to the relevant figure. If you would like to use this opportunity, please submit the source data (for example scans of entire gels or blots, data points of graphs in an excel sheet, additional images, etc.) of your key experiments together with the revised manuscript. If you want to provide source data, please include size markers for scans of entire gels, label the scans with figure and panel number, and send one PDF file per figure.

7) Our journal encourages inclusion of *data citations in the reference list* to directly cite datasets that were re-used and obtained from public databases. Data citations in the article text are distinct from normal bibliographical citations and should directly link to the database records from which the data can be accessed. In the main text, data citations are formatted as follows: "Data ref: Smith et al, 2001" or "Data ref: NCBI Sequence Read Archive PRJNA342805, 2017". In the Reference list, data citations must be labeled with "[DATASET]". A data reference must provide the database name, accession number/identifiers and a resolvable link to the landing page from which the data can be accessed at the end of the reference. Further instructions are available at: http://www.embopress.org/page/journal/14693178/authorguide#referencesformat

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10) Please add up to 5 key words to the title page.

11) Please provide the abstract written in present tense.

12) Please add a conflict-of-interest statement to the manuscript text and order the manuscript sections like this:
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- a short, two-sentence summary of the manuscript (not more than 35 words).
- two to four short bullet points highlighting the key findings of your study.
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I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

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Achim Breiling
Editor
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Referee #1:

Two Sybodies have been selected against RBD. These bind to non-overlapping epitopes. Cryo EM and X-Ray crystallography was used to find the recognition epitopes on the trimeric antigen and to reveal conformational variance. Each anybody was modestly neutralising the virus or viral particles, however, the biparatopic construct were highly neutralising infectivity. Also escape variants were no longer generated when viruses were challenged with the biparatopic construct.

The paper is written in a clear and logical manner.

** Significance **

Data are convincing, all experimental details are disclosed. The conclusions are based on solid data.
Significance (Required) This paper gives us deep-insight in possible strategies to avoid the emergence of SARS-Cov2 mutants, while treating victims of Covid19.

Referees cross-commenting

All three reviewers agree that this is solid work and deserves publication after minor amendments (rephrasing) and without the need of additional experiments. All remarks raised can be easily answered (within oen or two weeks time). The extra explanations that are requested are probably already available in the drawers of the authors (or can be provided after quick consultation of literature to have the digits right).

Referee #2:

Summary

The manuscript by Walter et al. describes the identification and characterisation of synthetic nanobodies (Sybodies) reactive against the SARS-CoV-2 RBD. Two leading candidates binding non-overlapping epitopes were selected and engineered into first a biparatopic construct, then a "trimer of dimers", with binding affinity for the RBD being increased by each engineering stage. Though for SB#15, neutralisation potency against VSV pseudotyped with the SARS-CoV-2 spike protein was reduced for the Beta variant, the biparatopic construct GS4 and particularly the Trimer of dimers, showed high neutralising potency against VSV pseudotyped with the SARS-CoV-2 Alpha and Beta variant spikes, overcoming the sensitivity to variants of the individual nanobodies.

Major Comments

1.Line 187: The increase in potency seen here for the Biparatopic GS4 construct is described as due to a highly synergistic mechanism, the increase seen with co-administration of SB#15 and SB#68 as individual entities seemed to be additive rather than displaying synergy. How do the authors distinguish increased potency due to avidity effects in GS4 and a "synergistic" mechanism? Could they please describe this or remove the statement.

2.Line 336-338: The authors report here that neutralisation values of the sybodies described is comparable to those isolated from immune libraries using phage display. Nanobodies have been described in several papers from these platforms that neutralise much more potently than those described here, for example 0.1 nM in a PRNT assay (Pymm et al. 2021), 0.022 nM in PRNT and 0.045 nM in pseudovirus assays (Xiang et al. 2020) as compared with >2000 nM for both SB#15 and SB#68 in PRNT and >140 nM in pseudovirus assays.

Of the references used here, Koenig et al also describe neutralisation values in their PRNT assay that range from ~50-150 nM rather than the >2000 nM seen here, and 2-3 fold more potent neutralisation values in their pseudovirus assay. Wagner describes neutralisation values of 7 nM and Hanke report pseudovirus neutralisation at ~50 nM (again approximately 2-3 fold more potent).

Could the authors please discuss the neutralisation potency in the context of these values as they do not seem comparable to many of the nanobodies generated from immune libraries. There are also other examples of sybodies in neutralisation assays for SARS-CoV-2, do the neutralisation values measured in these studies add to the argument that these platforms are comparable?

Minor Comments

1.Line 233: The tripod design shown here appears to contain trimerization domains from parainfluenza virus and T4 bacteriophage. Do the authors anticipate problems with immune reactivity to these domains in therapeutic settings and how could this be mitigated if so? Could the authors please add a line to the discussion addressing this?

2.Line 52-55: Many papers have started to detail the antibody binding landscape for RBD reactive antibodies and nanobodies in SARS-CoV-2 (Barnes, West et al., 2020; Dejnirattisai et al., 2021, Sun, D et al. 2021; Wheatley, AK et al. 2021 and others). Are the percentage of antibodies targeting these two "hotspots" known in-vivo and if so, could this be included to better reflect the diversity of antibodies characterised to date?

3.Line 106-108: Could the authors please include whether they thought the improvement seen here was an additive or synergistic effect if known?

4.Line 171: Could the authors please add a figure call out for S7B here.

5.Line 174: Could the authors please add a figure call out for S7D and E here.

6.Line 176: Figure S7B is called out here, but the text appears to be referring to figure S7C. Please could the authors correct this and alter figure S7 so that the figures are called out in order (as S7D and E have already been described in the text).

7.Line 293: WNb 10 (Pymm et al.) which also binds in this region was additionally shown to cross recognise SARS-CoV-1. Please could the authors mention the cross-recognition of the WNb 10 nanobody with SARS-CoV-1 here.

8.Line 609: For Cryo-EM studies, the complex is described as using a 1:1.3 molar ratio of Spike to sybody, given that each spike contains three RBD moieties that are potential binding sites for the sybody, is the ratio given here correct, or does it refer to the ratio for each RBD?

9.Figure 3: Could the authors please add labels for both D and E to show regions of the spike monomer, e.g. RBD, NTD, S1, S2 etc. and the angular displacement of the up-out RBD conformation from the up-RBD.

10.Figure 5A legend: The colour code described for figure 5A should be reversed (the salmon spheres seem to refer to the global variants, not the adaptation experiments).

11.Figures S5-S7: The RBD labels in these figures are in each case directly over the NTD. Could an arrow be added or positioning altered to improve clarity?

** Significance **

This is a considerable body of high-quality work, though of course is in an extremely rapidly moving field. Nanobodies capable of neutralising SARS-CoV-2 virus in vitro, both alone and as cocktail combinations have been widely described with detailed structural work to define their epitopes and interaction with residues mutated in the variants. Indeed, the affinities of SB#15 and SB#68 for WT SARS-CoV-2 RBD and the mutations contained within the Alpha and Beta variants, as well as the crystal structure for SB#68 have previously been published (Ahmad et al. 2021). The authors acknowledge this through comparison of their nanobodies and the epitopes they target with others in the field, many of which have also demonstrated efficacy in preventing SARS-CoV-2 infection in in vivo models.

The manuscript defines a novel orientation of the RBD within the spike trimer presumably driven by the binding of these nanobodies to both the RBD involved and adjacent RBD's, though it is not clear if this unique orientation has any direct impact on the stability of the spike trimer and mechanism of neutralisation.

The combination of the two sybodies into a biparatopic format, and the incorporation of these biparatopic moieties into a trimeric construct is a novel aspect of the paper, as previous trimers have involved either single nanobodies joined into a trimer, or single nanobodies displayed on a similar "tripod" structure (Güttler et al. 2021). This format demonstrates an ability to compensate for

the sensitivity of component nanobodies to RBD variation and to considerably increase neutralising potency, though the ability of this construct to neutralise SARS-CoV-2 in vivo and to be translated into a viable therapeutic remains to be addressed.

Reviewer field of expertise: Structural biology, infection and immunity.

Referee #3:

Summary:

The manuscript 'Biparatopic sybody constructs neutralize SARS-CoV-2 variants of concern and mitigate emergence of drug resistance' by Walter et al. describes and characterizes a pair of synergistic SARS-CoV-2 neutralizing sybodies. Sybodies are proteins derived from synthetic libraries designed based on variable domains of camelid heavy chain-only antibodies. The authors characterize binding properties of the two sybodies Sb#15 and Sb#68 and quantify their neutralization potential against SARS-CoV-2 spike-pseudotyped VSV and authentic virus. They revealed synergy between both sybodies and systematically followed up the improvement of neutralizing activity by biparatopic fusions (Sb#15-linker-Sb#68), Fc fusions leading to homodimerization (Sb#15-Fc; Sb#68-Fc), as well as an entirely new hexameric configuration realized by a trimerization domain fused to bi-paratopic Sb#68-linker-Sb#15. The latter arrangement ultimately improved neutralization by more than 1000-fold and thus may serve a general blueprint for the improvement of antiviral sybodies or nanobodies.

The authors also determined the structural basis of neutralization and found that only the combination of both sybodies stabilized the RBD 3-up conformation of spike, which may block Ace2 binding and induce the pre-mature rearrangement (and thus inactivation) of spike as postulated previously. A novel configuration with one RBD up, one RBD up-and-out, and one RBD down (a sofar not described configuration) was found in the presence of both sybodies as well as Sb#15 alone.

The authors further confirm by in vitro evolution experiments that the combination of sybodies targeting two epitopes prevents the emergence of escape variants, while experiments with single nanobodies revealed possible escape variants that had in part already been identified in patients. In line with the multiple epitopes targeted, the bi- and multi-valent sybodies retained the capacity to neutralize emerging SARS-CoV-2 variants.

Major comments

The data of this manuscript is of high quality. The claims are supported by solid data and no further experiments are required to back up the claims. The data is presented in a transparent matter and replicates and statistical analyses are appropriate for all experiments.

Minor suggestions for data presentation:

As neutralization experiments and the resulting IC50 values differ from system to system and lab to lab, it would be helpful to include on additional reagent for neutralization that is described in other publications and would help to compare values. This could be (commercially available) ACE2-Fc or any other nanobody or sybody with published neutralization potential.
While all the IC50 values of neutralization are described in table 3, it may be helpful to also show them in the respective graphs themselves.

** Significance **

This is the latest in a long series of antibodies, nanobodies, and sybodies neutralizing SARS-CoV-2 by binding to the RBD of spike ((Güttler et al., 2021; Hanke et al., 2020; Huo et al., 2020; Koenig et al., 2021; Lv et al., 2020; Schoof et al., 2020; Wrapp et al., 2020; Xiang et al., 2020). In fact, one of the first (and fastest) publications on SARS-CoV-2 specific sybodies is from the authors of this manuscript themselves, although this first description of SARS sybodies is only published on BioRxiv (Walter et al., 2020). Now the authors picked up one pair of particularly interesting synergistic sybodies and analyzed them in detail. Few other publications have characterized nanobodies in that degree of functional, evolutionary, structural, and mechanistic detail. It turns out that the epitopes of both nanobodies as well as the likely mechanism of action is shared with a similar pair of nanobodies derived from immunized camelids, and likely also with more combinations of nanobodies binding to these dominant epitopes (Koenig et al., 2021). While the study does not provide novel mechanistic insight per se, in particular the structural information will be helpful to deduce common mechanisms of synergistic neutralization. Importantly, the authors have developed an entirely novel format to trimerize bi-paratopic nanobodies, which improves neutralization even more and has the potential to be applied for neutralizing nanobody against this and other viruses (or other receptors).

To reveal the structural basis of synergistic neutralization, and likely gain insights into coronavirus fusion itself, it will require a number of structures as the one described here. In particular the cryo EM structures of both nanobodies bound to the ectodomain of spike as well as the confirmed stabilization of the RBD 3-up configuration will thus be of value for the field. Few studies determine sybody- or nanobody-specific escape variants as described here, and the data will therefore also be of value for more systematic assessments of antigenic escape.

The study itself is interesting for a broader audience interested in virology, antibody responses (and evolution), nanobody technology and translational aspect of sybodies and other biologics derived from antibodies.

My expertise is based on long standing research in virology and nanobody development. While I can interpret the structures of nanobody-target complexes on a functional level, my expertise is not sufficient to judge the technical aspects of the solution of electron microscopy structures themselves.

Güttler, T., Aksu, M., Dickmanns, A., Stegmann, K. M., Gregor, K., Rees, R., Taxer, W., Rymarenko, O., Schünemann, J., Dienemann, C., Gunkel, P., Mussil, B., Krull, J., Teichmann, U., Groß, U., Cordes, V. C., Dobbelstein, M., & Görlich, D. (2021). Neutralization of SARS-CoV-2 by highly potent, hyperthermostable, and mutation-tolerant nanobodies. The EMBO Journal, e107985. https://doi.org/10.15252/EMBJ.2021107985
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Referees cross-commenting

I concur with reviewer 1. This solid piece of work is ready for publication after minor text changes and does not require further experiments. The authors should discuss their claim of synergy and comment on the potency of neutralization (ideally with some data on a molecule whose potency was also quantified by other systems).

Point-by-point answer to the reviewer comments

We thank the reviewers for their constructive feedback and we have revised the manuscript accordingly.

Reviewer #1 (Evidence, reproducibility and clarity (Required)):

Two Sybodies have been selected against RBD. These bind to non-overlapping epitopes. Cryo EM and X-Ray crystallography was used to find the recognition epitopes on the trimeric antigen and to reveal conformational variance. Each anybody was modestly neutralising the virus or viral particles, however, the biparatopic construct were highly neutralising infectivity. Also escape variants were no longer generated when viruses were challenged with the biparatopic construct.
The paper is written in a clear and logical manner.
Data are convincing, all experimental details are disclosed. The conclusions are based on solid data.

Reviewer #1 (Significance (Required)):

This paper gives us deep-insight in possible strategies to avoid the emergence of SARS-Cov2 mutants, while treating victims of Covid19.

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All three reviewers agree that this is solid work and deserves publication after minor amendments (rephrasing) and without the need of additional experiments.
All remarks raised can be easily answered (within oen or two weeks time). The extra explanations that are requested are probably already available in the drawers of the authors (or can be provided after quick consultation of literature to have the digits right).
Reviewer 1 (Serge Muyldermans).

We thank Reviewer 1 for this positive assessment of our manuscript.

Reviewer #2 (Evidence, reproducibility and clarity (Required)):

Summary

The manuscript by Walter et al. describes the identification and characterisation of synthetic nanobodies (Sybodies) reactive against the SARS-CoV-2 RBD. Two leading candidates binding non-overlapping epitopes were selected and engineered into first a biparatopic construct, then a "trimer of dimers", with binding affinity for the RBD being increased by each engineering stage. Though for SB#15, neutralisation potency against VSV pseudotyped with the SARS-CoV-2 spike protein was reduced for the Beta variant, the biparatopic construct GS4 and particularly the Trimer of dimers, showed high neutralising potency against VSV pseudotyped with the SARS-CoV-2 Alpha and Beta variant spikes, overcoming the sensitivity to variants of the individual nanobodies.

Major Comments

1.Line 187: The increase in potency seen here for the Biparatopic GS4 construct is described as due to a highly synergistic mechanism, the increase seen with co-administration of SB#15 and SB#68 as individual entities seemed to be additive rather than displaying synergy. How do the authors distinguish increased potency due to avidity effects in GS4 and a "synergistic" mechanism? Could they please describe this or remove the statement.
We agree with the reviewer's concern. Indeed, our data do not support synergy of Sb#15 and Sb#68, because (as the reviewer rightly points out) the increase of potency is minimal when these sybodies are added as individual entities. Rather, our data support a mechanism in which the increased potency of GS4 is solely attributed to the avidity effect of the construct. We have thus removed this statement in line 187 and throughout the rest of the manuscript.

2.Line 336-338: The authors report here that neutralisation values of the sybodies described is comparable to those isolated from immune libraries using phage display. Nanobodies have been described in several papers from these platforms that neutralise much more potently than those described here, for example 0.1 nM in a PRNT assay (Pymm et al. 2021), 0.022 nM in PRNT and 0.045 nM in pseudovirus assays (Xiang et al. 2020) as compared with >2000 nM for both SB#15 and SB#68 in PRNT and >140 nM in pseudovirus assays.

We believe that reviewer#2 misunderstood/misinterpreted our statement.

In line 336-338, we state:

"In contrast to a number of synthetic or naïve SARS-CoV-2 nanobodies from other libraries that required a post-selection maturation process to reach satisfactory affinities [28, 40-42], sybodies selected by us and by other labs [36, 43] exhibited affinities in the single and double digit nM range and where thus of similar affinity as nanobodies isolated from immune libraries using classical phage display [11, 37, 44]."

Hence, our statement relates to binder affinities (and not neutralization values, which appear to differ quite a lot among different studies). In addition, we explicitly mention that the affinities of the sybodies (single digit nM/low double digit nM) are in a similar range as nanobodies isolated from immune library using classical phage display (i.e. with a similar standard-screening approach via ELISA), as can be read/looked up in the cited papers [11, 37, 44].
Although it is correct that the affinities of the nanobodies described in Pymm et al. 2021 and Xiang et al. 2020 were found to be stronger than sybodies (i.e. a good number of picomolar binders identified), one has to take into consideration that the immunzation/screening regime was quite extensive for Pymm et al. and that in Xiang et al., a massive deep-screen using NGS and mass spectrometry was performed to identify highly affine nanobodies.

If selection and screening efforts on par with Pymm et al. and Xiang et al. had been performed with sybodies, we are convinced that picomolar binders would have been identified as well.

For these reasons, we consider our original statement as adequate.

Of the references used here, Koenig et al also describe neutralisation values in their PRNT assay that range from ~50-150 nM rather than the >2000 nM seen here, and 2-3 fold more potent neutralisation values in their pseudovirus assay. Wagner describes neutralisation values of 7 nM and Hanke report pseudovirus neutralisation at ~50 nM (again approximately 2-3 fold more potent).

Could the authors please discuss the neutralisation potency in the context of these values as they do not seem comparable to many of the nanobodies generated from immune libraries. There are also other examples of sybodies in neutralisation assays for SARS-CoV-2, do the neutralisation values measured in these studies add to the argument that these platforms are comparable?

We agree with reviewer#2 that the gap between PRNT and VSV neutralization assays were rather large in our case. Therefore, we repeated all our PRNT assays and part of the VSV neutralization assays. Further, we attempted to include the well-characterized antibody EY6A as well as the sybody MR3 (https://www.nature.com/articles/s41467-021-24905-z) as control.
In the repeated experiments of the PRNT assay, the potency of our sybodies and sybody constructs are indeed better, and the respective neutralization values between PRNT and VSV are now less apart (around 3-6 fold).

To explain this discrepancy, we added the following sentence to the text (line 118):
The approximately 3 to 6-fold discrepancy in neutralization efficacies, measured using either live SARS-CoV-2 virus or pseudotyped VSV, may reflect slight differences in viral physiology (variation of incorporated spikes per viral particle) or could owe to the different assay methods (luciferase emission versus plaque reduction determination).

Further, we were able to determine the neutralization values for the control sybody MR3, which was found to be identical to a value published in a previous study for the VSV assay.
Unfortunately, a commercially acquired EY6A antibody was found to be inactive in our PRNT assay for unclear reasons.

Nevertheless, we feel that we have sufficiently validated our neutralization assays, because their main purpose was not to exactly determine absolute neutralization values, but rather relative values among the different sybodies and their fusion constructs.

Minor Comments

1.Line 233: The tripod design shown here appears to contain trimerization domains from parainfluenza virus and T4 bacteriophage. Do the authors anticipate problems with immune reactivity to these domains in therapeutic settings and how could this be mitigated if so? Could the authors please add a line to the discussion addressing this?

The rationale behind the Tripod design was to investigate whether multivalency could further improve the potency of the biparatopic sybody construct. Our data indeed revealed that Tripod reached high neutralization efficiency against all tested SARS-CoV-2 variant of concerns (picomolar

range). We wish to note that even against pseudoviruses harboring spikes with mutations that disturbed single sybody's efficacy (*e.g.* Sb#15), IC_{50}s for the Tripod construct were still highly potent, thereby spotlighting the power of combining multivalency with the biparatopic strategy.

To facilitate trimerization of the biparatopic construct, we fused the T4 bacteriophage-derived foldon sequence as well as a mutated peptide derived from the trimeric canine distemper virus fusion (CDV-F) protein, a strategy that proved to be very effective.

We nevertheless fully agree with this reviewer's comment that such domains may affect drug's efficacy *in vivo* by, for instance, triggering undesired anti-drug immunity.

We added the following cautionary statement to the discussion (line 344):
"While our own Tripod-GS4r construct may cause problems with immune reactivity in a therapeutic setting due to the viral origin of the utilized trimerization domain, trimerization domains of human origin can be used instead to overcome this potential issue (Guttler et al, 2021)."

For *in vivo* experiments and potential future clinical treatments in humans, it would indeed be wise to exchange the CDV-F peptide and the foldon motif by a well-characterized trimerization domain derived from a human protein. As recently demonstrated (https://doi.org/10.15252/embj.2021107985), the NC1 trimerization motif of collagen XVIII is an excellent candidate (10.1016/j.jmb.2009.07.057).
We have added a sentence to the discussion addressing this important point.

As pointed out by reviewer #3, such a strategy may act as a general blueprint for the improvement of antiviral sybodies/nanobodies in the future.

2.Line 52-55: Many papers have started to detail the antibody binding landscape for RBD reactive antibodies and nanobodies in SARS-CoV-2 (Barnes, West et al., 2020; Dejnirattisai et al., 2021, Sun, D et al. 2021; Wheatley, AK et al. 2021 and others). Are the percentage of antibodies targeting these two "hotspots" known in-vivo and if so, could this be included to better reflect the diversity of antibodies characterised to date?

To date, most characterized antibodies were selected based on their ability to bind the RBD/NTD and neutralize SARS-CoV-2 infection. This is reflected in studies which aim to systematically categorize panels of antibodies/nanobodies against SARS-CoV-2 (KM Hastie, Science (2021): https://www.science.org/doi/10.1126/science.abh2315; D Sun, et al, Bioarxiv (2021): https://www.biorxiv.org/content/10.1101/2021.03.09.434592v1.full;
Y Wu, et al, Cell Host & Microb. (2020): https://www.cell.com/cell-host-microbe/fulltext/S1931-3128(20)30250-X;L Liu et al, Nature (2020): https://www.nature.com/articles/s41586-020-2571-7). Due to this selection bias, it is difficult to assess the true overall "natural" percentage of antibodies targeting the two RBD "hotspots" in vivo. Available surveys of patient immune responses (E Shrock, et al, Science (2020): https://www.science.org/doi/10.1126/science.abd4250; AK Wheatley, Cell Reports (2021): https://www.sciencedirect.com/science/article/pii/S2211124721012869; AS Heffron, et al, Plos Biol (2021):

https://journals.plos.org/plosbiology/article?id=10.1371/journal.pbio.3001265) tend to focus on a qualitative description of the entire epitope landscape, giving a perspective of overall epitope diversity yet still not enabling reliable quantification of percentages of antibodies targeting particular hotspots. With these points in mind, a recent report characterizing 179 anti-RBD monoclonal antibodies found that 83% (148/179) directly competed with ACE2 for RBD binding, whereas 17% (31/179) either did not compete with ACE2 or were found to be below the assay threshold to qualify as ACE2 blockers (KM Hastie 2021).

In summary, we feel that the question of "hotspots" is a very complicated one, which cannot be addressed with our own data. Therefore, we feel that it does not make sense to comment on this issue/topic in the discussion of our paper.

3.Line 106-108: Could the authors please include whether they thought the improvement seen here was an additive or synergistic effect if known?

As pointed out above, we have removed our statement regarding synergy, because our data does not support a synergistic action of the two sybodies.

4.Line 171: Could the authors please add a figure call out for S7B here.

We have re-organized Figure S7, as suggested.

5.Line 174: Could the authors please add a figure call out for S7D and E here.
We have re-organized Figure S7, as suggested.

6.Line 176: Figure S7B is called out here, but the text appears to be referring to figure S7C. Please could the authors correct this and alter figure S7 so that the figures are called out in order (as S7D and E have already been described in the text).
We have re-organized Figure S7, as suggested.

7.Line 293: WNb 10 (Pymm et al.) which also binds in this region was additionally shown to cross recognise SARS-CoV-1. Please could the authors mention the cross-recognition of the WNb 10 nanobody with SARS-CoV-1 here.

The mentioned paper (Pymm et al.) does not contain data supporting the cross-reactivity of WNb 10 with SARS-CoV-1. But its epitope (akin to Sb#68) overlaps with the one of VHH72 (originally selected against SARS-CoV-1) and therefore, WNb 10 likely cross-reacts.
Just one sentence down, we take reference to Pymm et al. Further, we show WNb 10 in the context of Figure 7. Hence, we feel that this nanobody is appropriately mentioned and referenced.

8.Line 609: For Cryo-EM studies, the complex is described as using a 1:1.3 molar ratio of Spike to sybody, given that each spike contains three RBD moieties that are potential binding sites for the sybody, is the ratio given here correct, or does it refer to the ratio for each RBD?

We thank the reviewer for having spotted this. The 1.3-fold molar excess of sybody was added relative to the spike monomer concentration, so this refers to the ratio for each RBD. We have changed the text accordingly.

9.Figure 3: Could the authors please add labels for both D and E to show regions of the spike monomer, e.g. RBD, NTD, S1, S2 etc. and the angular displacement of the up-out RBD conformation from the up-RBD.
This was changed accordingly.

10.Figure 5A legend: The colour code described for figure 5A should be reversed (the salmon spheres seem to refer to the global variants, not the adaptation experiments).
Thanks a lot for spotting this error, which we in the meantime corrected.

11.Figures S5-S7: The RBD labels in these figures are in each case directly over the NTD. Could an arrow be added or positioning altered to improve clarity?
This was changed accordingly.

Reviewer #2 (Significance (Required)):

This is a considerable body of high-quality work, though of course is in an extremely rapidly moving field. Nanobodies capable of neutralising SARS-CoV-2 virus in vitro, both alone and as cocktail combinations have been widely described with detailed structural work to define their epitopes and interaction with residues mutated in the variants. Indeed, the affinities of SB#15 and SB#68 for WT SARS-CoV-2 RBD and the mutations contained within the Alpha and Beta variants, as well as the crystal structure for SB#68 have previously been published (Ahmad et al. 2021).

It is worth mentioning that we were among the first groups making nanobody sequences freely available to the science community. The team around Ahmad et al. gained access to the sybodies via Addgene and independently characterized them at the structural level. Importantly, these confirmatory analyses (published recently in JBC) in fact re-inforce our findings and add an additional layer of validation. Finally, it should be added that we have determined here cryo-EM structures (not performed in Ahmad et al) and we made the bi-paratopic and tripod constructs and characterized them in terms of neutralization potency and viral escape mutations.

The authors acknowledge this through comparison of their nanobodies and the epitopes they target with others in the field, many of which have also demonstrated efficacy in preventing SARS-CoV-2 infection in in vivo models.

The manuscript defines a novel orientation of the RBD within the spike trimer presumably driven by the binding of these nanobodies to both the RBD involved and adjacent RBD's, though it is not clear if this unique orientation has any direct impact on the stability of the spike trimer and mechanism of neutralisation.

The combination of the two sybodies into a biparatopic format, and the incorporation of these biparatopic moieties into a trimeric construct is a novel aspect of the paper, as previous trimers have

involved either single nanobodies joined into a trimer, or single nanobodies displayed on a similar "tripod" structure (Güttler et al. 2021). This format demonstrates an ability to compensate for the sensitivity of component nanobodies to RBD variation and to considerably increase neutralising potency, though the ability of this construct to neutralise SARS-CoV-2 in vivo and to be translated into a viable therapeutic remains to be addressed.

Reviewer field of expertise: Structural biology, infection and immunity.

Reviewer #3 (Evidence, reproducibility and clarity (Required)):

Summary:

The manuscript 'Biparatopic sybody constructs neutralize SARS-CoV-2 variants of concern and mitigate emergence of drug resistance' by Walter et al. describes and characterizes a pair of synergistic SARS-CoV-2 neutralizing sybodies. Sybodies are proteins derived from synthetic libraries designed based on variable domains of camelid heavy chain-only antibodies. The authors characterize binding properties of the two sybodies Sb#15 and Sb#68 and quantify their neutralization potential against SARS-CoV-2 spike-pseudotyped VSV and authentic virus. They revealed synergy between both sybodies and systematically followed up the improvement of neutralizing activity by biparatopic fusions (Sb#15-linker-Sb#68), Fc fusions leading to homodimerization (Sb#15-Fc; Sb#68-Fc), as well as an entirely new hexameric configuration realized by a trimerization domain fused to bi-paratopic Sb#68-linker-Sb#15. The latter arrangement ultimately improved neutralization by more than 1000-fold and thus may serve a general blueprint for the improvement of antiviral sybodies or nanobodies.

The authors also determined the structural basis of neutralization and found that only the combination of both sybodies stabilized the RBD 3-up conformation of spike, which may block Ace2 binding and induce the pre-mature rearrangement (and thus inactivation) of spike as postulated previously. A novel configuration with one RBD up, one RBD up-and-out, and one RBD down (a sofar not described configuration) was found in the presence of both sybodies as well as Sb#15 alone.

The authors further confirm by in vitro evolution experiments that the combination of sybodies targeting two epitopes prevents the emergence of escape variants, while experiments with single nanobodies revealed possible escape variants that had in part already been identified in patients. In line with the multiple epitopes targeted, the bi- and multi-valent sybodies retained the capacity to neutralize emerging SARS-CoV-2 variants.

Major comments

The data of this manuscript is of high quality. The claims are supported by solid data and no further experiments are required to back up the claims. The data is presented in a transparent matter and replicates and statistical analyses are appropriate for all experiments.

We thank reviewer#3 for this very positive assessment.

Minor suggestions for data presentation:

As neutralization experiments and the resulting IC50 values differ from system to system and lab to lab, it would be helpful to include on additional reagent for neutralization that is described in other publications and would help to compare values. This could be (commercially available) ACE2-Fc or any other nanobody or sybody with published neutralization potential.

As pointed out above in our response to reviewer #2, we addressed this concern with the following experiments:
We repeated all our PRNT assays and part of the VSV neutralization assays. Further, we attempted to include the well-characterized antibody EY6A as well as the sybody MR3 (https://www.nature.com/articles/s41467-021-24905-z) as control.
In the repeated experiments of the PRNT assay, the potency of our sybodies and sybody constructs are indeed better, and the respective neutralization values between PRNT and VSV are now less apart (around 3-6 fold).

To explain this discrepancy, we added the following sentence to the text (line 118):
The approximately 3 to 6-fold discrepancy in neutralization efficacies, measured using either live SARS-CoV-2 virus or pseudotyped VSV, may reflect slight differences in viral physiology (variation of incorporated spikes per viral particle) or could owe to the different assay methods (luciferase emission versus plaque reduction determination).

Further, we were able to determine the neutralization values for the control sybody MR3, which was found to be identical to a value published in a previous study for the VSV assay.
Unfortunately, a commercially acquired EY6A antibody was found to be inactive in our PRNT assay for unclear reasons.

Nevertheless, we feel that we have sufficiently validated our neutralization assays, because their main purpose was not to exactly determine absolute neutralization values, but rather relative values among the different sybodies and their fusion constructs.

While all the IC50 values of neutralization are described in table 3, it may be helpful to also show them in the respective graphs themselves.

We tried to include these values directly in the graphs, but this looked confusing and was difficult to read. Therefore, we kept it as it was.

Reviewer #3 (Significance (Required)):

This is the latest in a long series of antibodies, nanobodies, and sybodies neutralizing SARS-CoV-2 by binding to the RBD of spike ((Güttler et al., 2021; Hanke et al., 2020; Huo et al., 2020; Koenig et al., 2021; Lv et al., 2020; Schoof et al., 2020; Wrapp et al., 2020; Xiang et al., 2020). In fact, one of the first (and fastest) publications on SARS-CoV-2 specific sybodies is from the authors of this manuscript themselves, although this first description of SARS sybodies is only published on BioRxiv (Walter et

al., 2020). Now the authors picked up one pair of particularly interesting synergistic sybodies and analyzed them in detail. Few other publications have characterized nanobodies in that degree of functional, evolutionary, structural, and mechanistic detail. It turns out that the epitopes of both nanobodies as well as the likely mechanism of action is shared with a similar pair of nanobodies derived from immunized camelids, and likely also with more combinations of nanobodies binding to these dominant epitopes (Koenig et al., 2021). While the study does not provide novel mechanistic insight per se, in particular the structural information will be helpful to deduce common mechanisms of synergistic neutralization. Importantly, the authors have developed an entirely novel format to trimerize bi-paratopic nanobodies, which improves neutralization even more and has the potential to be applied for neutralizing nanobody against this and other viruses (or other receptors).

To reveal the structural basis of synergistic neutralization, and likely gain insights into coronavirus fusion itself, it will require a number of structures as the one described here. In particular the cryo EM structures of both nanobodies bound to the ectodomain of spike as well as the confirmed stabilization of the RBD 3-up configuration will thus be of value for the field. Few studies determine sybody- or nanobody-specific escape variants as described here, and the data will therefore also be of value for more systematic assessments of antigenic escape.

The study itself is interesting for a broader audience interested in virology, antibody responses (and evolution), nanobody technology and translational aspect of sybodies and other biologics derived from antibodies.

My expertise is based on long standing research in virology and nanobody development. While I can interpret the structures of nanobody-target complexes on a functional level, my expertise is not sufficient to judge the technical aspects of the solution of electron microscopy structures themselves.

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Referees cross-commenting

I concur with reviewer 1. This solid piece of work is ready for publication after minor text changes and does not require further experiments. The authors should discuss their claim of synergy and comment on the potency of neutralization (ideally with some data on a molecule whose potency was also quantified by other systems).

1st Revision - Editorial Decision 19th Jan 2022

Dear Dr. Seeger

Thank you for the submission of your revised manuscript to our editorial offices. I now went through your detailed p-b-p-response letter, and I consider the concerns by the referees as adequately addressed.

Before we can proceed with formal acceptance, I have these editorial requests I ask you to address in a final revised manuscript:

- Please shorten the title to not more than 100 characters (including spaces).

- Per journal policy, we do not allow 'data not shown', which is stated in the manuscript (page 14). All data referred to in the paper should be displayed in the main or Expanded View figures, or an Appendix. Thus, please add these data (or change the text accordingly if these data are not central to the study). See: http://embor.embopress.org/authorguide#unpublisheddata

- Please name the 'Data availability statement' 'Data availability section'.

- Please order the manuscript sections like this (using these names):
Title page - Abstract - Introduction - Results - Discussion - Materials and Methods - Data availability section - Acknowledgements - Author contributions - Conflict of interest statement - References - Figure legends - Expanded View Figure legends - Tables with legends

- Please add links to directly access the datasets mentioned in the 'Data availability section'.

- The data deposited at the ENA databank under ID PRJEB49553 cannot be found there. Please make sure the dataset is deposited, that the accession ID is correct, and that the data is public (latest upon publication of the manuscript).

- Please make sure that all figure panels and tables (also those shown in the Appendix) are called out and that they are called out sequentially. Presently, Fig 4D is called out after 6B, and there seem to be no callouts for Fig. EV1B, the separate panels of Fig. EV3 and for panels A, B and D of Fig. EV5. Also callouts for Appendix items are missing. Please check and in case change the order of the panels in the figures.

- Please add a full table of contents including page numbers to the Appendix file. It is also not necessary to repeat here the full title page of the paper.

- It seems that Lea Hürlimann, Imre Gonda, Gianmarco Meier, Sille Remm, Sujani Thavarasah, Geert van Geest and Remy Bruggmann are missing from the author contributions. Please check.

- Thanks for providing the synopsis image. Could you provide this with fonts in bold/filled? Presently, the writing looks a bit fuzzy and weak.

Finally, We updated our journal's competing interests policy in January 2022 and request authors to consider both actual and perceived competing interests. Please review the policy (see: https://www.embopress.org/competing-interests) and update your competing interests if necessary.

In addition, I would need from you:
- a short, two-sentence summary of the manuscript (not more than 35 words).
- two to four short (2 lines) bullet points highlighting the key findings of your study.

I look forward to seeing the final revised version of your manuscript when it is ready. Please let me know if you have questions regarding the revision.

Best,

Achim Breiling
Senior Editor
EMBO Reports

The authors have addressed all minor editorial requests.

2nd Revision - Editorial Decision 31st Jan 2022

Markus Seeger
Institute of Medical Microbiology, University of Zurich
Switzerland

Dear Dr. Seeger,

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

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Editor
EMBO Reports

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Corresponding Author Name: Markus A. Seeger
Journal Submitted to: EMBO Reports
Manuscript Number: EMBOR-2021-54199V1

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 χ^2 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?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - 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.

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repor
http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title
http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tumo
http://datadryad.org
http://figshare.com
http://www.ncbi.nlm.nih.gov/gap
http://www.ebi.ac.uk/ega
http://biomodels.net/
http://biomodels.net/miriam/
http://jjj.biochem.sun.ac.za
https://osp.od.nih.gov/biosafety-biosecurity-and-emerging-biotechnology/
http://www.selectagents.gov/

B- Statistics and general methods

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

Question	Answer
1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	NA
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	NA
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No data were excluced from analysis.
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.	NA
For animal studies, include a statement about randomization even if no randomization was used.	NA
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.	The experiments were not conducted in a blinded manner.
4.b. For animal studies, include a statement about blinding even if no blinding was done	NA
5. For every figure, are statistical tests justified as appropriate?	Confirmed.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	NA
Is there an estimate of variation within each group of data?	NA

Is the variance similar between the groups that are being statistically compared?	NA

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	No antibodies were used in this study.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	African green monkey kidney (VeroE6) cell line was kindly provided by Doreen Muth, Marcel Müller, and Christian Drosten, Charité, Berlin, Germany. The HEK-293T cells were received from ATCC CRL-11268. The BHK-21 cells were received from ATCC BHK-21 [C-13] CCL-10.

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	NA
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	NA
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	NA

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
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.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

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	We have acquired NGS data (escape mutations) and Macromolecular structures (cryo-EM maps). The corresponding data were deposited at respective repositories as indicated in the Data availability section.
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	NA
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	NA