nature portfolio

Peer Review File

Rapid affinity optimization of an anti-TREM2 clinical lead antibody by cross-lineage immune repertoire mining

REVIEWER COMMENTS

Reviewer #1 antibody engineering (Remarks to the Author):

Hsiao et al. propose the mining of "convergent" and "parallel" lineages for antibody affinity maturation. Specifically though an impressive amount of work the authors isolated antibodies to anti-triggering receptor on myeloid cells 2 (TREM2) with picomolar affinity. The authors subsequently performed extensive structural mutagenesis studies and MD studies to delineate the energetics and kinetics of binding to the epitope and also report on functional assays in vitro. Overall this is an impressive amount of work and there are several aspects that are of significant interest to a broader audience. Thus in principle the paper is suitable for publication in Nature Communications. However having said that the authors need to address several issue primarily in order to clarify the overarching idea to improve readability but also in a few instances for scientific accuracy.

1) An important concern is that the definition of convergent and parallel lineages seems arbitrary. Egg, line 123- : "Two of the clones, 3.10A7 and 3.22B9, appear to be part of the same clonal lineage as assessed by V and J segment use in both chains and CDR H3 length 11 (CDR definition and antibody residue numbering in the IMGT® system43,44 used throughout except where noted) with high (>67%) amino acid sequence identity, Why 67% was chosen? Also why limit the lineage to a precise CDRH3 length? AID activity can lead to insertions/deletions in CDRH3 within a PHYLOGENETICALLY defined lineage, for example with members sharing key mutations in the framework or other CDRs. These points require careful explanation and justification.

2) A minor point along the lines above, in the methods (line 585-7) clonotypes is defined slightly differently "...length lass than 70% identity".

3) Line 138:'A total of 67 VH reads with VH10-5 germline segments with CDR H3 length 11, 12 and 13 from different clonotypes (defined as less than 68% CDR H3 amino acid identity to any other selected read or clone with the same CDR H3 length) were selected... How were these 67 reads selected? Likely there were many more reads of this family given the sequencing depth, no? How many of these sequences could be considered clonal related when the entire sequence data set is taken into account so that phylogenetic trees constructed? Are all these truly different lineages from a B cell development

perspective, i.e do not trace to the same UCA when all the data is taken into account.
4) "A total of 29 VH reads from 24 different clonotypes with the same VH6-8 germline segment as antibody 3.10C2 and CDR H3 lengths 5, 6 and 7, were selected from the datasets of the 3 immunized rats". Again the authors need to state whether some or many of these fall within the same lineage when all the data is used and trees are constructed. Also please explain the criteria for how these 29 reads were selected.
5) Why was only the CDRH3 length range 5-7 aa investigated. The argument presented on line 208 is not persuasive, I do not see why a longer length would not be expected to be compatible with the binding grove. Also, why do the authors expect pairing with a VL with a long CDR3>.
6) How were 3.10C2, 3.27H7... were selected? Presumably since the authors specify that these are anti-TREM2 they must have been isolated by screening? Clarifications needed.
7) For the most abundant gene families there are thousands of V genes with CDRH3 within a certain range, say 5-7 aa as above. There has to be some additional criterion for identifying the subset of these that have the same epitope specificity. Is this a consequence of mining hyperimmunized animals? Still I assume that somehow the authors must exclude gremlin VH genes which likely are encoded by naive cells.

Other:
1) The complete sequences of at least some of the IgG genes should be presented, at least for 3.10A7, 3.10C2 and a few other Mabs that are critical for the story

Reviewer #2 antibody structure (Remarks to the Author):
In this work, Hsiao et al. present a workflow for identifying therapeutic antibody candidates by using the parallel lineage framework to guide mining of bulk deep sequencing datasets. Typical repertoire mining is performed within the confines of clonotype lineages and is therefore restricted in the sequence space explored in comparison to the sequence diversity otherwise available to antibodies. Contrastingly, the authors expand the sequence space explored, notably for CDR H3, by mining bulk deep sequencing datasets and identifying antibodies which share the same VH germline segments. The rationale behind this approach is to exploit an antibody convergence class referred to as parallel lineage – clonotypically

independent antibodies which share germline segments and recognize the same epitope with the same binding mode irrespective of the CDR H3 regions. Using this methodology, the authors identify two parallel lineages which recognize TREM2 epitopes. These VH based parallel lineages are comprised of 67 and 29 anti-TREM2 heavy chain sequences respectively. The authors then focus on an anti-TREM2 antibody, 3.10C2, and a related parallel lineage clone Para.09 – studies of these two antibodies is focused on structural interpretation, molecular dynamics investigation, mutational studies and energy analysis. Altogether the authors conclude this methodology provides a platform for rapid affinity optimization of therapeutic antibody candidates with expanded sequence space in the CDR H3.

My primary concern with this methodology is how it is being presented as a platform optimizing the affinity of a therapeutic antibody candidate. As written, the overall goal/conclusion of the paper can be summarized by line 392, “Mining of deep sequencing repertoires can readily identify VH segments from different clonotypes with the expected parallel lineage binding properties can be used to rapidly and effectively optimize the affinity of an antibody with already high affinity while preserving key functional properties relevant for clinical applications in a predictable manner”. That said, incorporating portions of the sequence space explored from a parallel lineage clonotype into an already high affinity antibody doesn’t necessarily optimize its affinity – this is demonstrated in the section which incorporates “mutations” from the Para.09 CDR H3 mutation into the parent 3.10C2. Any single “mutation” incorporated into the parent 3.10C2 results in significantly faster binding off-rates. It’s only when mutations are incorporated in tandem do you see an increase in affinity compared to the parent 3.10C2, essentially swapping out the CDR H3 and turning it into the parallel lineage antibody. Why then, would one not simply pursue further studies with the parallel lineage antibody? It’s potentially even more problematic when considering antibodies with even longer CDR H3s. The exploration of 3.10C2 parallel lineages was restricted to those with CDR H3 lengths between 5-7. Contrastingly the 3.10A7 parallel lineage antibodies had CDR H3 lengths between 11-13; how many in tandem mutation permutations need to be explored to end up with a higher affinity antibody? The authors demonstrate how this methodology is extremely strong in identifying alternative antibody candidates, highlighted by the fact that Para.09 binds with an approximately 10x

higher affinity than 3.10C2 – why not frame/focus the manuscript in that context compared to how it is currently presented as an affinity optimization tool?

Larger Comments:

Supplementary Fig 3: It would be particularly interesting to see the differences in the consensus sequences between the top 22 heavy chain sequences further investigated for light chain pairing compared to the bottom 45.

Lines 281-285: In regards to differential recognition of H154A between C.10C2 and Para.09; The authors suggest that a larger groove in Para.09 can accommodate either a His or an Ala, whereas the 3.10C2 groove seems to be more specific for His in that position. Nothing in the structure suggests that 3.10C2 cannot accommodate an alanine in this position – mutating it in the structure doesn't lead to any clashes. Additionally, one would intuitively think that a deeper groove would be more specific for a particular residue vs more permissive. Further, the 2BIE in supplementary table 3 suggests this mutation to be particularly deleterious for both antibodies, and does not support the epitope specificity profile in Fig 2A. The authors should expand their analysis and/or structural/energetic interpretation for a more convincing argument for this opposite behavior.

Lines 348-355: The authors suggest that the superior binding kinetics of Para.09 is due to the reduced conformational selectivity of TREM2 binding (vs preorganization of CDR loops) which minimizes the entropic penalty of binding the antigen. I am not convinced of this argument at all. The increased fluctuations of TREM2 are localized to residues 162-165, however these residues lie outside the epitope boundary as indicated by supplementary figure 1. Structural investigation would have demonstrated this region as particularly dynamic to begin with; these residues are modeled in an alternate conformation with missing residues in the structure when comparing the two molecules in the asymmetric unit. These same issues also extend to the particularly dynamic 149-150. If one were to have simulated these systems with just bound 151-161 epitope, this argument likely would not have been put forward. Given the data presented in the paper, these residues aren't responsible for binding (nor bound at all), and as such I'm not sold on their fluctuations being responsible for the 10-fold increase in affinity. I would suggest further analysis of the data for more potentially subtle mechanisms that may be driving this drastic difference in affinity.

Lines 376-378: The authors suggest that the differences in recognition of S158A is likely attributed to entropic vs enthalpic contributions. Molecular dynamics simulations of this mutant (and the other alanine mutants / H154Y) could be particularly useful in strengthening this argument.

Smaller Comments:
Lines 118 and 184 – context should be provided as to how/why these clones were selected
Line 206 – The authors should clarify what a 'potentially unstable residue' is – how much does their exclusion impact the number of clonotypes identified?
Line 214 – This sentence should be clarified to reflect that these are parallel lineage clones which exclusively bound the same TREM2 peptides as 3.10C2. As written it's unclear why other parallel lineage clones weren't chosen
Structural investigation: Hydrogen bond distance and Donor-DonorH – Acceptor angle should be provided either in text or in the figures
Figures 1 and 3: The blue gradients chosen make it hard to differentiate read counts – either revise the color scheme or rescale the colors with larger indices

Reviewer #3 antibody structure – supervisor for reviewer #2
No additional comments

Reviewer #4 antibody repertoire (Remarks to the Author):
In their manuscript "Rapid affinity optimization of an anti-TREM2 clinical lead antibody by cross-lineage immune repertoire mining" Dr. Hsiao et al. argue in favor of using heavy chain variants clonally independent antigen-specific VH regions with diversity in CDRH3 as a source of diversity to evolve already high affinity antibodies. They pursued this effort using stereotyped/public responses, in this case to human TREM2 after immunization of Sprague Dawley rats and generation of hybridomas, several of which showed evidence of stereotyped sequence features. Such responses are not uncommon and particularly easy to identify among clonotypes with short CDR3 of the heavy chain. The authors have carried out

an impressive study with large sets of data to support their findings. Yet, there are a number of issues that I would like to see described in larger detail.

1. In particular the statement that “we propose that the superior binding kinetics of huPara.09 is not due to preorganization of its CDR loops, but instead due to reduced conformational selectivity of TREM2 binding poses” is particularly novel and suggests a new path for antibody evolution but this matter ought to be discussed in the context of https://doi.org/10.3389/fimmu.2018.00413.

2. Stereotyped antibodies may develop with very different levels of similarity. Here the authors identify and characterize those that have relatively short CDR3, certainly shorten than most human antibodies but also, it seems shorter than many rat CDRH3. There is, compared to human and mouse, relatively limited knowledge of rat antibody repertoires. A paper by Goldstein et al. (https://doi.org/10.1038/s42003-019-0551-y), referred to in the paper but somewhat surprisingly not extensively discussed in the context of this study, holds such data. Here CDRH3 has an average of about 12 aa (shorter than the length of a typical human CDRH3). The rat IGH locus carries many D segments, several of which are long. Consequently some/several/all of the investigated rearrangements may have been created with incorporation of very few N/P nucleotides. Thus, the relevant rearrangements may be more common than one might expect. This is discussed in the literature, for instance in the works of Victor Greiff et al. As the nucleotide sequences of the genes are not available this matter is difficult to assess. Such data should be provided, and a discussion of the matter would be highly appreciated in particular if the findings of this study are restricted to antibodies with relatively short CDRH3s as their diversity is so much more limited both in terms of the low number of residues and the limited contribution of untemplated N nucleotides to diversity. Although challenging, an attempt should be made to determine the nature of the rearrangement including assignment of IGHD gene. The role of CDR3 length for antigen specificity has been reported in the past (https://doi.org/10.1002/jmr.679) and study that long before the development of high throughput and single cell sequencing concluded that “CDRH3 length restrictions may be encountered in specific immune responses and that CDRH3 length diversity and restriction is a factor to consider when

designing optimal libraries for molecular evolution of antigen-specific paratopes.”, somewhat in line with the present study, although here applied in a different context based on the technologies that were available at the time.

3. On page 5 clones 3.10A7 and 3.22B9 are considered to be part of the same original clonotype based on gene usage and CDR3 amino acid sequence similarity. This is better done at nucleotide level as the clone’s origin is determined at that level. Please provide such information. Also sequence similarity >67% sequence identity is considered high. Again this is better defined at the nucleotide level and typically sequence identity at 80-90% is used. The exact numbers can of course be discussed but the herein used identity level seems unusually low.

4. Supplementary Figure 3 seems to suggest a dominance of residues encoded by the 3’-end of IGHV and three codons upstream of W118 encoded by IGHJ. It is difficult to interpret the rest of the diversity as it is shown as summary information. It would be very beneficial to have each sequence (and the accompanying nucleotide sequence). Of note, unimmunized rats may provide a similar pattern (illustrated by rIGHV10-5 with 11 aa long CDRH3 as derived from supplementary information of Goldstein et al. (enclosed as pdf))

In all, are the investigated sequences dominated by sequences with specificity for TREM2 or just sequences derived from clones of other specificity? This would not invalidate the approach but suggests that evolution may use related heavy chains irrespective of their original specificity (at least in cases when much of the specificity is encoded by the IGHV gene and not by the precise rearrangement).

5. Supplementary Figure 5 and Figure 6 suggest that high amounts of antibody had been captured on the sensorchip. This may gravely affect binding kinetics as the binding and dissociation might be very limited by dilution. It is suggested that the experiment is repeated with substantially lower amounts of bound antibody.

6. In the supplementary data of Goldstein et al. multiple CDR3 from unimmunized rats with similar sequence as those shown in Fig 3 (similar to CDR3 of the 3.10C2 lineage) can be

identified. It seems that the authors suggest that the sequences they find are the result of an immune response to TREM2. This may not at all be the case as highly similar sequences are seen also in nonimmunized animals (see also comment 4 above). It is suggested that the authors amend their text accordingly.

7. The epitopes targeted by the herein described antibody clonotypes appears to reside in a disorganized part of the antigen. Is this so? If so, are the results limited to such antibodies? A, in some respects, highly similar stereotyped human immune response targets the similarly likely disorganized AD-2 epitope of human cytomegalovirus gB (see for instance reference 36 and https://doi.org/10.1016/j.molimm.2014.03.015, https://doi.org/10.4161/mabs.27760). Can the herein defined results be discussed in the context of another peptide specific immune response like this one.

8. As the study has been conducted in part on peptides and in parts on proteins it is difficult to know if the former fully capture the nature of the interaction with the intact target. Is the affinity for the peptide similar to that for the protein? If not, one cannot draw some of the conclusions that are made as there might be additional contacts offered by the intact protein and/or that their and structural diversity in the peptides that despite the disorganized nature of this part of TREM2 might be different from that of different peptides (i.e. will the peptide reflect the structure space of this part of the protein?). Please discuss. It would be of value to determine the thermodynamic properties of the antibody-antigen interaction using either SPR or ITC to support the findings and the discussion.

9. It seems to me that many critical interactions of 3.10C2and its modified relatives are dependent on residues that are not commonly in contact with the antigen e.g. residue 2, 4, 28 and 117. 3.10C2 and Para.09 might thus not represent typical antibodies and the role of CDR3 might be different in this specificity in comparison to many (protein) specific antibodies. This implicates that the findings of this study relate to a minor subset of all specific antibodies and might not be generally applicable. Please discuss.

10. It would be very beneficial to obtain the PDB coordinates of the structures as part of the review process. These have been submitted to PDB but are not yet released into the public

domain.

11. The results section starts with a description of the biology of TREM2. This is not appropriate in the context in which the text is located. Please remove or move elsewhere.

Reviewer #5 antibody repertoire, computational (Remarks to the Author):

The authors describe a strategy to mine repertoire sequencing data to identify high-affinity antibodies by a combinatory approach of non-clonally related sequences from same or across several subjects (animals) that target the same epitope. They term this strategy "parallel lineage" antibody mining. With this strategy, the authors identify antibody candidates with increased affinity to the TREM2 target and agonistic effects.

Although the method sounds promising and has proven to lead to improve candidates, there are some open questions to the methodology and definitions being used:

Q1. The authors define parallel lineages as convergent antibodies that bind the same epitope with the same geometry or binding mode, additionally sharing the VH and VL germline sequences but having CDRH3 regions that differ significantly in sequence and even length. However, the strategy to mine repertoires according to "parallel lineages" was limited to an increase in the CDRH3 length of 1 to 2 amino acids. This potentially does not exclude antibodies belonging to the same clonal lineage when retrieved from the same animal, as affinity maturation has been described to potentially introduce insertions and deletions (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3449029/, https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2199186/). A more robust definition of parallel lineages would be those with shared VH and VL germline sequences but identified in different individuals / animals. Additionally, the tested antibodies had swapped light chains so the VL sequence was not conserved. Therefore, the definition of the method employed and the actual tested sequences seems contradictory.

Q2. The authors consider antibodies from the same animal to belong to the same clonal lineage as sharing the IGHV and IGHJ genes, same CDRH3 length and amino acid identity of >67% percent. The identity threshold sounds somehow arbitrary. How was this specific

threshold chosen?

Q3. The similarity among CDR3 is only reported among sequences of the same length. For the reasons mentioned above, that indels can be introduced during SHM, it would be necessary to report sequence identity when additionally considering gaps (e.g. Levenshtein distances) across sequences from parallel lineages.

Q4. What was the criteria used to select the 4 initially produced MAb antibodies?

Q5 The fact that there are 3 immunized rats should be mentioned on line 116. Do R18/R19/R20 read counts correspond to the read counts in each of the immunized rats? If so, this should be specified on the figure caption.

Q6. The mouse inhibin binding experiments in Fig 1 lack a positive control.

Q7. What do the read counts in Fig 1 and 3 represent? It is not clear from the explanation. What do the annotations a,b,c,d in Fig 3 mean? It's not specified on the caption. Additionally, the %similarity should also be reported in this figure like in Fig 1.

Q8. A second round of optimization is described as utilizing affinity optimization by exclusively parallel lineage mining (page 7 line 189-183). Reporting the animal from which each of the sequences was retrieved and the J gene for each of them would better support the hypothesis that all the sequences come from parallel clones.

Q9. In line 468 (page 15) the term clonotype is introduced in comparison to clone. What is the difference in definition between the two terms?

We thank the Reviewers for their detailed and thoughtful comments and queries. These are addressed individually below, referring to other queries when overlapping.

REVIEWER COMMENTS

Reviewer #1 antibody engineering (Remarks to the Author):

Hsiao et al. propose the mining of "convergent" and "parallel" lineages for antibody affinity maturation. Specifically though an impressive amount of work the authors isolated antibodies to anti-triggering receptor on myeloid cells 2 (TREM2) with picomolar affinity. The authors subsequently performed extensive structural mutagenesis studies and MD studies to delineate the energetics and kinetics of binding to the epitope and also report on functional assays in vitro. Overall this is an impressive amount of work and there are several aspects that are of significant interest to a broader audience. Thus in principle the paper is suitable for publication in Nature Communications. However having said that the authors need to address several issue primarily in order to clarify the overarching idea to improve readability but also in a few instances for scientific accuracy.

1) An important concern is that the definition of convergent and parallel lineages seems arbitrary. Egg, line 123- : "Two of the clones, 3.10A7 and 3.22B9, appear to be part of the same clonal lineage as assessed by V and J segment use in both chains and CDR H3 length 11 (CDR definition and antibody residue numbering in the IMGT® system[43,44] used throughout except where noted) with high (>67%) amino acid sequence identity, Why 67% was chosen? Also why limit the lineage to a precise CDRH3 length? AID activity can lead to insertions/deletions in CDRH3 within a PHYLOGENETICALLY defined lineage, for example with members sharing key mutations in the framework or other CDRs. These points require careful explanation and justification.

A new paragraph was introduced in the results to expand on lineage/clonotype definitions, which in turn define parallel lineages (starting on line 136). In that paragraph two different lineage/clonotype definitions are introduced. The first one is “biological clonotyping”, based on mechanisms of VDJ recombination, somatic mutation and clonal expansion, as well as animal origin. For this definition there is usually a threshold of nucleotide/amino acid identity that needs to be met for inclusion of two clones in the same lineage. This threshold is also somewhat arbitrary in the literature but usually set at 80% sequence identity. It is also explained that because indels due to somatic mutations cannot be effectively distinguished from distinct VDJ recombination events in natural repertoires, a notion supported in previous publications (see below), clones must have the same CDR H3 length for inclusion in the same lineage/clonotype.

For engineering purposes the biological definition is insufficient because two biologically distinct clonotypes can have very similar sequences, including from different animals. For that reason the 67% (2/3) threshold is set and other criteria are dropped, to group sequences in the same clonotype that, while clonally independent by the biological clonotype definition, are similar enough to have limited engineering value. This helps

maximize sequence space search for engineering. Therefore, we agree the 67% threshold is arbitrary and could be another value, but it was chosen to yield sufficiently distinct CDR H3 sequences compared to what the traditionally used 80% threshold might yield. We now call this the “working definition” of clonotype in the manuscript, to distinguish it from the more traditional biological clonotype definition described above. In summary, the “working definition” was used for read selection to maximize CDR H3 sequence diversity in tested clones whereas the relatively more stringent “biological definition” was used to assess their biological uniqueness. This is now made clear in the manuscript.

We would emphasize that insertions and deletions have been well-documented in antibodies during somatic mutation but, as mentioned above, in CDR H3 this is effectively indistinguishable from differential VDJ recombination in natural repertoires. New references 46 (and a number of references therein) and 47 highlight this point, with this limitation explicitly stated. The only documented case of insertions/deletions in CDR H3 during somatic mutation of which we are aware is in reference 47. However, in that case, that was based on an in vitro system with a single initial clone undergoing somatic mutation with recombinantly expressed AID, allowing direct comparison of original and in vitro-modified clones. Even in that report the authors refrain from analyzing CDR H3 indels in natural repertoires. Finally, to our knowledge no other repertoire studies in the literature considers indels in CDR H3 for clonotyping and in fact state that CDR H3 indels are not allowed within clonal groups when those are defined, one example being https://doi.org/10.1371/journal. pcbi.1007977.

Finally, the use of other framework or CDR somatic mutations by themselves are not suitable for definition of clonotypes to identify insertions and deletions in CDR H3. In an example where somatic mutations are used for clonal group definition (Nouri and Kleinstein, https://doi.org/10.1371/journal. pcbi.1007977), these are used in conjunction with CDR H3 similarity for better clonal group definition, where CDR H3 is not allowed to vary in length within the clonal group. Somatic mutations, especially those positively impacting binding, may conceivably converge in parallel lineages due to similar binding mode. An example is given in this manuscript with 3.10C2 and Para.09. These two clones are from distinct lineages as assessed by animal origin and germline use and now by more detailed junctional sequence analysis (new Supplementary Figure 3). These two clones nonetheless share 4 somatic mutations in VH CDRs 1 and 2 (Fig. 5a), as might expected from convergent clones with the same binding mode. The high prevalence of parallel lineages means somatic mutations are probably of limited utility, on their own (independently of CDR H3-based definitions), as markers to define clonal groups to subsequently identify SHM-induced CDR H3 indels.

2) A minor point along the lines above, in the methods (line 585-7) clonotypes is defined slightly differently "...length lass than 70% identity".

This is now changed to “same length and less than 68% amino acid identity” for consistency. However, for the CDR H3 lengths analyzed, less than 70% or 68% leads to

the same clonotype groupings. Therefore no changes are required in the rest of the manuscript.

3) Line 138:'A total of 67 VH reads with VH10-5 germline segments with CDR H3 length 11, 12 and 13 from different clonotypes (defined as less than 68% CDR H3 amino acid identity to any other selected read or clone with the same CDR H3 length) were selected... How were these 67 reads selected? Likely there were many more reads of this family given the sequencing depth, no? How many of these sequences could be considered clonal related when the entire sequence data set is taken into account so that phylogenetic trees constructed? Are all these truly different lineages from a B cell development perspective, i.e do not trace to the same UCA when all the data is taken into account.

The clones were selected randomly from the set of clonotypes as defined by same VH germline segment, CDR H3 length and <68% sequence identity by prioritizing both high sequence count and high somatic mutation load. That was described in the methods section of the original manuscript. It is now reiterated in the results section (lines 184-195). The results section states that the “67 selected clonotypes [represent] about half of the 157 V_H10-5 working definition clonotypes with CDR H3 length 11 to 13 in the bulk sequencing dataset from the three rats” (lines 187-194). This addresses the relative sampling depth for these clonotypes.

Phylogenetic trees are not generally helpful for robust clonotype definition as it gives more weight to the longer VH sequence than the shorter, but more significant, CDR H3 region in defining clonotypes and also does not consider the potential convergence of somatic mutations in parallel lineages. To address the independent clonal origin of the antibodies tested here we did a detailed VH junctional sequence analysis. This analysis indicates that most, if not all, selected clones that bound TREM2 are from different biological lineages and do not share a UCA. The paragraph describing these results includes a passage (lines 196-203) describing this. The section concludes that “When allowing gapped comparisons of these junctional sequences within an immunized rat, only two clones, R18-11-08 and R18-12-22 derived from rat 18, could be aligned by introducing 3 nucleotides (81% gapped nucleotide sequence identity), while only 5 clones had another clone from any rat sharing more than 67% CDR H3 amino acid sequence identity by gapped alignment (Fig. 1), confirming the independent clonal origin of most or all selected clones and high CDR H3 sequence diversity.”

4) "A total of 29 VH reads from 24 different clonotypes with the same VH6-8 germline segment as antibody 3.10C2 and CDR H3 lengths 5, 6 and 7, were selected from the datasets of the 3 immunized rats". Again the authors need to state whether some or many of these fall within the same lineage when all the data is used and trees are constructed. Also please explain the criteria for how these 29 reads were selected.

We are including detailed junctional analyses to determine biological clonality (Supplementary Figure 3) rather than phylogenetic trees as stated above. Clones were randomly selected, except that clonotypes with certain potentially unstable residues

were excluded for manufacturing purposes. This is now expanded upon in the results (lines 277-287) and methods sections (lines 762 to 770). The exclusion of sequences with potential manufacturing liabilities is mentioned to highlight that the selected sequences are less likely to include Trp, Met and free Cys residues and a few selected amino acid motifs, unrelated to any method limitations (it is in fact a strength of the method, as it allows these liabilities to be excluded early and avoids follow-up with problematic clones). There were no other criteria used for clone selection when attempting to select clonotypes with truly distinct CDR H3 sequences other than the working clonotype definition used to differentiate these CDR H3 sequences and the relatively arbitrary CDR H3 length range of the clonotypes to limit sampling and potentially maximize hit rate. “Manually selected” was changed to “randomly selected” in methods line 762 to better reflect this.

5) Why was only the CDRH3 length range 5-7 aa investigated. The argument presented on line 208 is not persuasive, I do not see why a longer length would not be expected to be compatible with the binding grove. Also, why do the authors expect pairing with a VL with a long CDR3>.

That is correct, there is no reason for why longer CDR H3 lengths would not be compatible with a groove. However, we stated that shorter lengths would be *more likely* (as in the original manuscript, now on line 279) to retain a groove, not that longer lengths would not allow a groove. Therefore we maintain the statement for the rationale about selecting short CDR H3 reads made in the original manuscript and expanded it to mention that the 3.10C2 VL has a long CDR L1 (line 281), which was not made clear in the original manuscript, to support the expectation of a grooved binding site (we assume Reviewer #1 is asking about VL with a long CDR1, not 3). This groove and the expected role of the long CDR L1 and short CDR H3 in forming the groove were confirmed in the subsequent structural analyses.

In summary, the restriction in CDR H3 lengths explored was a practical decision based on the above assumption rather than a limitation imposed by the parallel lineage framework. The parallel lineage framework does not impose a limit on CDR H3 lengths that could be explored as stated in the manuscript. The question of whether useful clones would be found outside the selected range for this parallel lineage remains open but it is moot in this case given the successful results with Para.09. Exhaustive search across the range of CDR H3 lengths is beyond the scope of this engineering study. If Para.09 had not been identified then additional sampling within and outside the CDR H3 length range would have been the next logical step.

6) How were 3.10C2, 3.27H7... were selected? Presumably since the authors specify that these are anti-TREM2 they must have been isolated by screening? Clarifications needed.

The details of the initial screening, done by standard screening strategies common in antibody discovery, are beyond the scope of this manuscript. The paragraph starting on line 250 of results describes the biochemical and biological properties of this clonal

group that make them relevant for potential therapeutic applications. The first sentence of the paragraph introducing the clonal group was extended to clarify this point: “A second group of 4 clonally related anti-TREM2 antibodies *with biological properties relevant to potential therapeutic application (see below)*, 3.10C2, 3.27H7, 3.18E5 and 3.50G1, was selected *for further engineering*”. This addressed the question of why these clones where selected. The antibody screening work is not fully relevant to the main points of the manuscript and therefore not addressed in detail here for space reasons beyond the fact that these antibodies have the desirable biological properties that need to be maintained. In fact, these antibodies could have been sourced from publications and the entire strategy might still in principle work because parallel lineages transcend individual animals immunized with a given antigen.

7) For the most abundant gene families there are thousands of V genes with CDRH3 within a certain range, say 5-7 aa as above. There has to be some additional criterion for identifying the subset of these that have the same epitope specificity. Is this a consequence of mining hyperimmunized animals? Still I assume that somehow the authors must exclude gremlin VH genes which likely are encoded by naive cells.

The repertoires were amplified with primers specific for isotype-switched, and consequently antigen experienced, transcripts. This was briefly mentioned in the discussion in the original manuscript but we failed to make it explicit in the methods section. This is now mentioned in the methods (lines 748-749) and results (lines 182-184) sections. The constant region primers were added to the list in Supplementary File 2. This amplification strategy excludes the naïve clonotypes, as correctly presumed by Reviewer #1. Other than that there is no procedure to specifically enrich for epitope specificity. The fact that the number of epitope-specific clones is unexpectedly high was discussed in detail in the discussion section.

Other:

1) The complete sequences of at least some of the IgG genes should be presented, at least for 3.10A7, 3.10C2 and a few other Mabs that are critical for the story

We fully agree with this. The original submission included a Supplementary xlsx file with the entire variable region amino acid sequences of all the clones tested and mentioned in the manuscript (Supplementary File 1) as well as the primers used for repertoire amplification (Supplementary File 2). Isotype information for the recombinantly produced antibodies is provided in the methods section (human IgG1, Kappa) and the sequences of these constant regions are widely known. The isotype information for the NGS reads is not available in the data due to amplification method. The isotype information for the original rat Mabs before cloning beyond generic IgG is no longer available. However, neither piece of information is relevant to the points of the manuscript other than repertoire amplification used rat pan-IgG/IgA and kappa primers for the constant regions.

Reviewer #2 antibody structure (Remarks to the Author):

In this work, Hsiao et al. present a workflow for identifying therapeutic antibody candidates by using the parallel lineage framework to guide mining of bulk deep sequencing datasets. Typical repertoire mining is performed within the confines of clonotype lineages and is therefore restricted in the sequence space explored in comparison to the sequence diversity otherwise available to antibodies. Contrastingly, the authors expand the sequence space explored, notably for CDR H3, by mining bulk deep sequencing datasets and identifying antibodies which share the same VH germline segments. The rationale behind this approach is to exploit an antibody convergence class referred to as parallel lineage – clonotypically independent antibodies which share germline segments and recognize the same epitope with the same binding mode irrespective of the CDR H3 regions. Using this methodology, the authors identify two parallel lineages which recognize TREM2 epitopes. These VH based parallel lineages are comprised of 67 and 29 anti-TREM2 heavy chain sequences respectively. The authors then focus on an anti-TREM2 antibody, 3.10C2, and a related parallel lineage clone Para.09 – studies of these two antibodies is focused on structural interpretation, molecular dynamics investigation, mutational studies and energy analysis. Altogether the authors conclude this methodology provides a platform for rapid affinity optimization of therapeutic antibody candidates with expanded sequence space in the CDR H3.

My primary concern with this methodology is how it is being presented as a platform optimizing the affinity of a therapeutic antibody candidate. As written, the overall goal/conclusion of the paper can be summarized by line 392, “Mining of deep sequencing repertoires can readily identify VH segments from different clonotypes with the expected parallel lineage binding properties can be used to rapidly and effectively optimize the affinity of an antibody with already high affinity while preserving key functional properties relevant for clinical applications in a predictable manner”. That said, incorporating portions of the sequence space explored from a parallel lineage clonotype into an already high affinity antibody doesn’t necessarily optimize its affinity – this is demonstrated in the section which incorporates “mutations” from the Para.09 CDR H3 mutation into the parent 3.10C2. Any single “mutation” incorporated into the parent 3.10C2 results in significantly faster binding off-rates. It’s only when mutations are incorporated in tandem do you see an increase in affinity compared to the parent 3.10C2, essentially swapping out the CDR H3 and turning it into the parallel lineage antibody. Why then, would one not simply pursue further studies with the parallel lineage antibody? It’s potentially even more problematic when considering antibodies with even longer CDR H3s. The exploration of 3.10C2 parallel lineages was restricted to those with CDR H3 lengths between 5-7. Contrastingly the 3.10A7 parallel lineage antibodies had CDR H3 lengths between 11-13; how many in tandem mutation permutations need to be explored to end up with a higher affinity antibody? The authors demonstrate how this methodology is extremely strong in identifying alternative antibody candidates, highlighted by the fact that Para.09 binds with an approximately 10x higher affinity than 3.10C2 – why not frame/focus the manuscript in that context compared to how it is currently presented as an affinity optimization tool?

Reviewer #2 is correct in that incorporating individual CDR H3 “mutations” from clone in parallel lineages would be inefficient at best as a method for affinity optimization, especially for longer CDR H3 sequences. However, the method was not described as a source of “mutations” to be used singly or in combination for traditional affinity engineering by point mutations. Instead it is presented as a way to rapidly identify alternative VH variant reads in a parallel lineage to optimize an antibody while retaining other biological properties because the parallel lineage variants are expected to have the same binding mode, a major determinant of biological activity in antibodies, while varying in other properties such as affinity, as suggested by Reviewer #2. The only place in the manuscript where individual CDR H3 residue changes are tested was in Figure 6. These were tested to determine their individual and combined effect on affinity, not as a path for optimization. To address this concern we edited the section pointed out by Reviewer #2 (now on lines 542-543) to state that the method “can be used to rapidly and effectively *identify alternative clones with higher affinity* than an antibody with already high affinity while preserving key functional properties relevant for clinical applications in a predictable manner” rather than simply “*affinity optimize*” as before.

Larger Comments:

Supplementary Fig 3: It would be particularly interesting to see the differences in the consensus sequences between the top 22 heavy chain sequences further investigated for light chain pairing compared to the bottom 45.

The now Supplementary Fig. 4 was expanded to incorporate these comparisons. Panels a-c have the original CDR H3 logo figures for all binders; panels d-f have the logos for the CDR H3 from non-binding binding clones; panels g-h have the CDR H3 logos for the top 22. Rather than combining non-binders with weaker binders we are contrasting strong binders from non-binders. Supplementary File 1 has all CDR H3 sequences listed.

Lines 281-285: In regards to differential recognition of H154A between C.10C2 and Para.09; The authors suggest that a larger groove in Para.09 can accommodate either a His or an Ala, whereas the 3.10C2 groove seems to be more specific for His in that position. Nothing in the structure suggests that 3.10C2 cannot accommodate an alanine in this position – mutating it in the structure doesn’t lead to any clashes. Additionally, one would intuitively think that a deeper groove would be more specific for a particular residue vs more permissive. Further, the 2BIE in supplementary table 3 suggests this mutation to be particularly deleterious for both antibodies, and does not support the epitope specificity profile in Fig 2A. The authors should expand their analysis and/or structural/energetic interpretation for a more convincing argument for this opposite behavior.

We thank the reviewer for their critical inspection of the 2BIE calculation. We discovered a small bug in how the differences were calculated (the raw 2BIE numbers presented in (now) Supplementary Table 3 and within the text are unaffected) and also modified the mutational sampling strategy to explore more repacking and minimization. We also

noticed that the mutagenesis strategy was accidentally omitted from the Methods upon submission, and the modified approach has now been added (lines 849-854). The updated Δ2BIE values have been placed into (now) Supplementary Table 4. We note that the overall trends are nearly identical to before, though we have better agreement with some of the mutations and the specificity profile in Fig. 2A, in particular for mutations S149A, F150A, A153A, and S160A, though we obtain somewhat lower magnitude (though still destabilizing) predictions for H157A. The slight change from equally negligible, to equally slightly negative, energies for S158A, has been acknowledged on lines 510-518 of the main text, and does not affect the conclusions that this indicates a negligible enthalpic difference when bound by the two antibodies and that S158 likely plays a structural role that is borne out in the dynamics of the bound peptide.

To address the reviewer's concerns, we note that while H154A *is* deleterious for both antibodies, it is less so for huPara.09 (consistent with previous numbers as well). We propose that this is due to improved packing against the beta carbon of H154(A) afforded by the insertion of I107h, as now noted on lines 522-523 of the main text. We further characterized these variants by MD simulations, which revealed a stronger tendency to unbind for hu3.10C2 than for huPara.09, shown in Supplementary Fig. 17 and discussed on lines 517-521. Thus, independent approaches with their own force fields both indicate reduced stability of binding to hu3.10C2 than huPara.09, where again dynamics may be at play that are less well-captured by Rosetta mutagenesis on fixed backbones.

We have also adjusted the wording in the sentence on lines 383-386 to better reflect the how the improved packing enables support for Ala in huPara.09 that does not work for hu3.10C2, including swapping the word "deeper" for "shallower" and "with more effective packing" which may have introduced confusion previously. As is clear from Fig 5a, the insertion for CDR H3 of huPara.09 indeed pushes the bottom of the groove outwards towards the surface, whereas the previous verbiage of "deeper" evoked the idea of "improved binding" (as would typically be expected of hydrophobic interactions) yet was insufficiently precise.

Lines 348-355: The authors suggest that the superior binding kinetics of Para.09 is due to the reduced conformational selectivity of TREM2 binding (vs preorganization of CDR loops) which minimizes the entropic penalty of binding the antigen. I am not convinced of this argument at all. The increased fluctuations of TREM2 are localized to residues 162-165, however these residues lie outside the epitope boundary as indicated by supplementary figure 1. Structural investigation would have demonstrated this region as particularly dynamic to begin with; these residues are modeled in an alternate conformation with missing residues in the structure when comparing the two molecules in the asymmetric unit. These same issues also extend to the particularly dynamic 149-150. If one were to have simulated these systems with just bound 151-161 epitope, this argument likely would not have been put forward. Given the data presented in the paper, these residues aren't responsible for binding (nor bound at all), and as such I'm not sold on their fluctuations being responsible for the 10-fold increase in affinity. I

would suggest further analysis of the data for more potentially subtle mechanisms that may be driving this drastic difference in affinity.

There are multiple elements of this critique, which we will address in turn. The most fundamental is a misunderstanding about the nature of entropy. The thermodynamic equations for entropy indicate that it is proportional to the *multiplicity* (the number of microstates consistent with a macrostate) of the system of interest. All else being equal, it is the *total* accessible microstates of the antibody-antigen complex that contributes to the entropy of the assembly, not the accessible microstates of the *bound residues* within the complex. Thus, the assertion that entropy does not play a role in the thermodynamics of binding because the majority of the dynamism of the bound peptide is outside of the region defined in Supplementary Figure 1 is false. Taking another perspective, when the antibody is bound to full-length TREM2, the region between the epitope and the rest of the protein is *more structured* when bound to hu3.10C2 and more *unstructured* when bound to huPara.09, indicating that the number of thermally accessible microscopic configurations of the target protein is indeed larger when bound to the latter antibody. As we previously noted in the main text (lines 456-459 and now Supplementary Figure 13) that many of the new regions of Ramachandran space accessed by residues in the huPara.09-bound state match those of the unbound TREM2 peptide, that indicates that the reduction in accessible configurational phase space is smaller when bound to huPara.09 than when bound to hu3.10C2, making the entropic penalty smaller when bound to the former and thus thermodynamically more favored. This has now been noted explicitly on lines 471-474 in the main text.

We further note that, in contrast to the reviewer's assertion, additional diversity can be observed for residues S160 and R161 (part of the bound epitope in Supplementary Figure 1) in Supplementary Figure 13. However, to explore this further, we carried out MD simulations of the truncated 149-161 peptide used for the mutagenesis binding assays and observed identical CDR H3 dynamics (Supplementary Figure 14) with still some additional dynamics (resembling the unbound conformations) in residues I159 and S160 (Supplementary Figure 15), though S160 is significantly more dynamic due to it being close to the peptide terminus. Thus, the criticism that residues within the boundary are not more dynamic is also not applicable. We added lines 459-461 to the text to address this concern directly. We also describe the results of MD simulations of the truncated peptide on lines 468-471.

The reviewer pointed out that residues 149-150 are also particularly dynamic, implying that they are equivalent to the residues 159-163 in our analyses. However, the key difference is that residues F150 and E151 are similarly dynamic whether bound by either antibody or totally unbound (Supplementary Figure 13). Thus, there is no difference in accessible conformations in the bound state, and the contribution to a change in entropy is negligible. We have now directly stated this on lines 461-466 in the main text.

Finally, the reviewer suggested that the dynamism observed in the bound TREM2 peptide is predictable from the multiple structure copies in the asymmetric unit of each

structure. The reviewer is correct that the termini of the bound peptides are in alternative conformations, and some are missing density, so we have added a statement acknowledging this on lines 466-468 in the main text. However, with the exception of residues 149 in hu3.10C2, and that residues 149 and 165 are unresolved in one copy of the huPara.09 structure, we found that the average alpha-carbon RMSD is actually extremely low between both copies of the asymmetric unit when aligning chains A/B/E onto chains C/D/F, with all positions having <1 Å RMSD and average RMSDs for positions 150-164 of <0.17 Å. Plots showing this are depicted below, as we do not believe they add significantly to the manuscript, but the conclusion from this analysis is that the large degree of flexibility that is present in the MD simulations (Figure 5 and Supplementary Figure 13) is by no means obvious from the crystal structure alone.

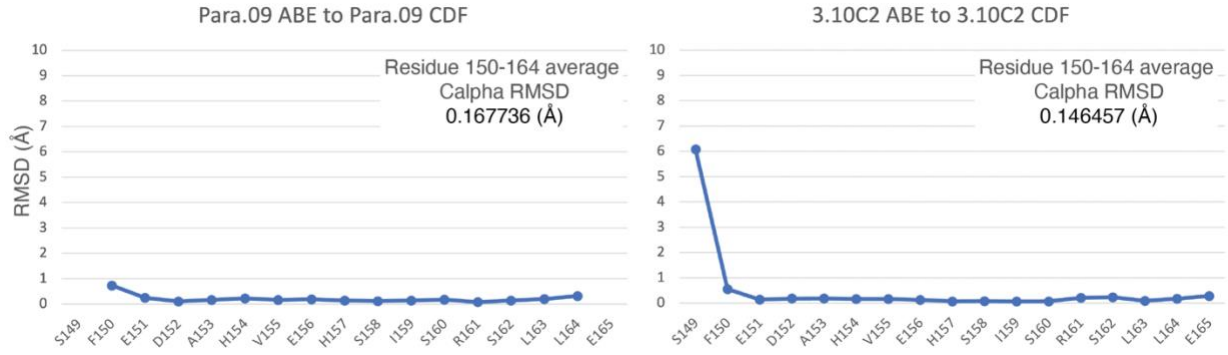

Figure: Per-residue alpha-carbon distances for the two copies of each antibody in their respective asymmetric units. Residues 149 and 165 are unresolved in chain E of the huPara.09 structure and thus cannot be included in the left-most plot.

Lines 376-378: The authors suggest that the differences in recognition of S158A is likely attributed to entropic vs enthalpic contributions. Molecular dynamics simulations of this mutant (and the other alanine mutants / H154Y) could be particularly useful in strengthening this argument.

As noted above, we confirmed that even with the updated Δ2BIE values in (now) Supplementary Table 4, the Δ2BIE of the S158A mutation for huPara.09 and hu3.10C2 is both nearly identical and also slightly negative. This is in line with structural inspection that shows no direct interaction between the S158 sidechain and any antibody contact (the closest is L115$_h$ in huPara.09). Thus, the dramatic change in affinity is likely to be related to the role that S158 plays in stabilizing the kinked conformation of the bound state. We have elaborated on this hypothesis on lines 510-513 in the main text for clarification. We did carry out MD simulations of S158A on the truncated 149-161 peptide (as used for alanine scanning experiments) and reported on the results on lines 513-517 in the main text, and added Supplementary Figures 15 and 16. In short, the differences in dynamics are more subtle and hard to definitively discriminate, as the conformations largely remain stabilized by the antibody contacts, consistent with the Δ2BIE being very small. Under the hypothesis that huPara.09 has improved binding affinity due to reduced conformational selectivity of the unbound peptide, it is possible

that S158A reduces the prevalence of the kinked conformation in solution that could affect binding to hu3.10C2, although simulations are unable to show this conclusively. We did not carry out additional simulations of alanine mutants as most effects are consistent with the Rosetta Δ2BIE calculations. H157Y is also consistent with the experimental observations that it has only a slight increase (lines 263-269) or no change (lines 324-326) in binding off-rate compared to wildtype, as the slightly negative Δ2BIE when mutated to Y indicates comparable binding enthalpies.

Smaller Comments:

Lines 118 and 184 – context should be provided as to how/why these clones were selected

The rationale for the selection of the 3.10A7 group was given in the original manuscript (now lines 126-127: “to determine whether additional V_H parallel lineage clonotypes could be identified by repertoire mining”). We added the fact that binding an epitope defined by a relatively short peptide sequence also facilitates the precise mapping of the epitope of the selected binders (line 132). In addition, the discussion section (now lines 588-590) explicitly stated in the original manuscript that this group was selected based on the fact that the parallel lineage is already evident in the hybridoma panel (“The 3.10A7 parallel lineage is already evident in the relatively small scale hybridoma panel and it was selected as a test case for parallel lineage mining for this reason”). The screening details for this set clones are beyond the scope of this manuscript (please see Reviewer #1, Q6).

The rationale for the 3.10C2 group selection is given in the paragraph of the line cited by the reviewer (now starting on line 250), based on all the biological properties of this clonal group. That is, this group was selected based on potential therapeutic needs rather than clonal sequence properties or epitope mapping properties.

Line 206 – The authors should clarify what a ‘potentially unstable residue’ is – how much does their exclusion impact the number of clonotypes identified?

The potentially unstable sites are residues that may transform chemically in antibodies during long-term storage or present challenges in manufacturing, an important consideration in clinical applications and a standard consideration in the industry. These include Trp and Met oxidation, Asn deamidation and Asp isomerization and free Cys residues. These are specifically cited on lines 283-285 in results and again on lines 762 to 768 in methods. The total number of clonotypes, clonotypes excluded due to sequence liabilities and final number of selected clones are now more explicitly stated on lines 281-287.

Line 214 – This sentence should be clarified to reflect that these are parallel lineage clones which exclusively bound the same TREM2 peptides as 3.10C2. As written it’s unclear why other parallel lineage clones weren’t chosen

The sentence has been expanded to clarify the point (lines 290-293): "*Two clones, Para.03 and Para.09, specifically bound the peptide with the entire TREM2 stalk region or TREM2 peptide 149-168 with the 3.10C2 epitope and not the control peptide 159-175 that does not include the 3.10C2 epitope. Other clones either did not bind peptide 149-168 with the 3.10C2 epitope or bound both TREM2 peptides or non-specifically to all peptides tested (Fig. 3).*"

Structural investigation: Hydrogen bond distance and Donor-DonorH – Acceptor angle should be provided either in text or in the figures

A table with H-Bonds between antigen and antibody is given now in new Supplementary Table 2.

Figures 1 and 3: The blue gradients chosen make it hard to differentiate read counts – either revise the color scheme or rescale the colors with larger indices

Both Figures have changed, including the percent value within cells with values of 0.5% or above, as now indicated in the figure legends.

Reviewer #3 antibody structure – supervisor for reviewer #2
No additional comments

Reviewer #4 antibody repertoire (Remarks to the Author):

In their manuscript "Rapid affinity optimization of an anti-TREM2 clinical lead antibody by cross-lineage immune repertoire mining" Dr. Hsiao et al. argue in favor of using heavy chain variants clonally independent antigen-specific VH regions with diversity in CDRH3 as a source of diversity to evolve already high affinity antibodies. They pursued this effort using stereotyped/public responses, in this case to human TREM2 after immunization of Sprague Dawley rats and generation of hybridomas, several of which showed evidence of stereotyped sequence features. Such responses are not uncommon and particularly easy to identify among clonotypes with short CDR3 of the heavy chain. The authors have carried out an impressive study with large sets of data to support their findings. Yet, there are a number of issues that I would like to see described in larger detail.

1. In particular the statement that "we propose that the superior binding kinetics of huPara.09 is not due to preorganization of its CDR loops, but instead due to reduced conformational selectivity of TREM2 binding poses" is particularly novel and suggests a new path for antibody evolution but this matter ought to be discussed in the context of https://doi.org/10.3389/fimmu.2018.00413.

Our findings are fully compatible with the findings by Jeliazkov et al., now cited in reference 50. The work by Jeliazkov et al. analyzes affinity improvement during immune responses in B cell lineages during somatic mutation and clonal expansion. Our work

looks at antibodies in different lineages and therefore does not relate to antibody evolution per se, as analyzed by Jeliazkov et al., but rather different binding solutions evolved independently from different VDJ recombination events. We have added a short discussion contextualizing our results within the conclusions of the Jeliazkov et al. paper on lines 483-487 and adjusted our wording about the mechanism slightly on lines 479-483. This section now reads:

"This minimizes the entropic penalty of binding via the antigen, not rigidification of the antibody, as has commonly been attributed to matured, high-affinity binders, yet inconsistently confirmed.[50] This is in line with previous conclusions that there are numerous pathways to affinity maturation via biological selective pressures, with paratope rigidification only comprising one aspect of binding (entropy loss upon complexation) while presumably leaving other recognized aspects (e.g., enthalpy, buried surface area, shape complementarity, solvent entropy) largely unaffected.[50] Our interpretation that the conformational diversity of the bound antigen is also among the many contributing factors to affinity is complementary to the concept of paratope rigidification. We note, however, that these previous bioinformatic analyses[50] were presented in the context of affinity maturation of a single lineage, which is a distinct process from the parallel lineage mining described herein."

2. Stereotyped antibodies may develop with very different levels of similarity. Here the authors identify and characterize those that have relatively short CDR3, certainly shorten than most human antibodies but also, it seems shorter than many rat CDRH3. There is, compared to human and mouse, relatively limited knowledge of rat antibody repertoires. A paper by Goldstein et al. (https://doi.org/10.1038/s42003-019-0551-y), referred to in the paper but somewhat surprisingly not extensively discussed in the context of this study, holds such data. Here CDRH3 has an average of about 12 aa (shorter than the length of a typical human CDRH3). The rat IGH locus carries many D segments, several of which are long. Consequently some/several/all of the investigated rearrangements may have been created with incorporation of very few N/P nucleotides. Thus, the relevant rearrangements may be more common than one might expect. This is discussed in the literature, for instance in the works of Victor Greiff et al. As the nucleotide sequences of the genes are not available this matter is difficult to assess. Such data should be provided, and a discussion of the matter would be highly appreciated in particular if the findings of this study are restricted to antibodies with relatively short CDRH3s as their diversity is so much more limited both in terms of the low number of residues and the limited contribution of untemplated N nucleotides to diversity. Although challenging, an attempt should be made to determine the nature of the rearrangement including assignment of IGHD gene. The role of CDR3 length for antigen specificity has been reported in the past (https://doi.org/10.1002/jmr.679) and study that long before the development of high throughput and single cell sequencing concluded that "CDRH3 length restrictions may be encountered in specific immune responses and that CDRH3 length diversity and restriction is a factor to consider when designing optimal libraries for molecular evolution of antigen-specific paratopes.", somewhat in line with the present study, although here applied in a different context based on the technologies that were available at the time.

Reviewer #4 brings up good points. We now include the detailed analysis of junctional sequences in new Supplementary Fig. 3. We refrained to do so in the initial submission due to the known limitations in junctional analyses as alluded to by Reviewer #4 here. But we agree these are helpful in addressing the clonal origin of different variants. Similar junctional analyses were included in the paper describing the pervasive nature of parallel lineages in immunizations (reference 37, Hsiao et al., Suppl. Fig. 3). These analyses did not support any special constraints of junctional diversity on parallel lineage CDR H3 junctional diversity. Parallel lineages can have relatively long N nucleotide insertions. Parallel lineages do not depend on CDR H3 sequence similarities. They do not arise due to recurring VDJ recombination events. Supplementary Figure 3 with the junctional sequence analysis of binders in detail illustrates this with the clones tested here, within the limits of junctional sequence analyses.

Crucially, parallel lineages are not biased to short CDR H3 sequences, also discussed in Hsiao et al. (and shown in Supplementary Figure 15 of that paper). For brevity we quote from the discussion in that paper: "*The CDR H3 lengths of the parallel lineage clones we characterized largely reflect the CDR H3 lengths of clones in the anti-OVA repertoire, which are slightly longer on average than the CDR H3 length of naïve rat IgG repertoires (Suppl. Fig. 15). Similarly, CDR H3 lengths of previously described human and mouse antibodies with convergent parallel lineages range from 15 to 18 and 9 to 17 residues, respectively,[6 references] slightly longer than the average CDR H3 length of each species.[1 reference] Thus, parallel lineage convergences do not seem to be generally associated with readily apparent CDR H3 length biases within species.*" The reason for the CDR H3 lengths in parallel lineages being slightly longer than the average is that clones with shorter CDR H3 sequences tend to be classified in the same rather than different clonotypes (by the working definition) simply because their sequences tend to be dominated by the more constant contributions from the VH and JH germline segments, inflating the sequence similarity of shorter CDR H3 sequences among disparate clonotypes. Longer CDR H3 clones can be part of parallel lineages, addressed by the experiment in Figure 1 with clones with CDR H3 length 11-13. A brief review of reference 37 to address the size of parallel lineages in repertoires was included in the discussion, starting on line 581. The CDR H3 sequence length question is now addressed in the discussion, paragraph starting on line 611.

3. On page 5 clones 3.10A7 and 3.22B9 are considered to be part of the same original clonotype based on gene usage and CDR3 amino acid sequence similarity. This is better done at nucleotide level as the clone's origin is determined at that level. Please provide such information. Also sequence similarity >67% sequence identity is considered high. Again this is better defined at the nucleotide level and typically sequence identity at 80-90% is used. The exact numbers can of course be discussed but the herein used identity level seems unusually low.

The independent clonal origin of the 3.10A7 group of antibodies is now addressed in a detailed junctional sequence analysis in new Supplementary Fig. 3. All 4 clones were confirmed to be from different clonotypes by this analysis. The reference to clones 3.10A7 and 3.22B9 being part of the same original clonotype was modified just to point

out that, while being from different clonotypes by the “biological clonotyping” definition, these have similar CDR H3 sequences (lines 174-174).

We extend the description of clonotype definitions in the results with a new paragraph starting on line 136. The two definitions are discussed in detail for Reviewer #1, Q1. The working definition used in this paper sets 67% on purpose as the sequence identity threshold to favor inclusion of relatively similar clones in the same clonotype. That is, this definition is more inclusive than other definitions such as the one referred to by Reviewer #4. The reason for this, as now more explicitly explained in the manuscript, was to ensure the clones selected in mining are as different as possible in CDR H3 to maximize sequence space search and avoid clones with relatively similar CDR H3 sequences even if from different biological clonotypes. We use the definition that Reviewer #4 refers to, and junctional nucleotide analysis, just to address questions of biological origin for the clones.

4. Supplementary Figure 3 seems to suggest a dominance of residues encoded by the 3’-end of IGHV and three codons upstream of W118 encoded by IGHJ. It is difficult to interpret the rest of the diversity as it is shown as summary information. It would be very beneficial to have each sequence (and the accompanying nucleotide sequence). Of note, unimmunized rats may provide a similar pattern (illustrated by rIGHV10-5 with 11 aa long CDRH3 as derived from supplementary information of Goldstein et al. (enclosed as pdf))

In all, are the investigated sequences dominated by sequences with specificity for TREM2 or just sequences derived from clones of other specificity? This would not invalidate the approach but suggests that evolution may use related heavy chains irrespective of their original specificity (at least in cases when much of the specificity is encoded by the IGHV gene and not by the precise rearrangement).

The sequences of all clones, including the CDR H3 sequences separately were included in Supplementary File 1. We now include the nucleotide sequences as well. We also include now the junctional sequence analysis of binding clones in new Supplementary Fig. 3.

The second part of the question from Reviewer #4 can be summarized as whether VH sequences from antibodies that were not necessarily TREM2-specific, paired to the right VL, result in the observed TREM2 specificity. The fact that many CDR H3 sequences are compatible with a given specificity and binding mode does not mean CDR H3 is not important for binding, only that there are potentially many different CDR H3 sequences that can provide the necessary binding energy for that binding mode. This was previously addressed in reference 37. In one experiment by in vitro selection in that paper, two very well characterized high-affinity anti-Her2 antibodies making key contacts through CDR H3 had their CDR H3 loops completely redesigned while still retaining high affinity binding to their cognate epitopes, changing residues that were repeatedly shown to be essential for binding by single-site mutagenesis. Thus, there can be many CDR H3 structural solutions to the same binding problem. While the

selected NGS reads in binding clones are presumed to be originally from anti-TREM2 clones, that is ultimately unanswerable given the type of sequence data. However, the parallel lineage framework predicts, based on previous work, that such clones exist in repertoires, confirmed here through the experiments shown.

5. Supplementary Figure 5 and Figure 6 suggest that high amounts of antibody had been captured on the sensorchip. This may gravely affect binding kinetics as the binding and dissociation might be very limited by dilution. It is suggested that the experiment is repeated with substantially lower amounts of bound antibody.

The original SPR experiments shown in Supplementary Figure 5 (now Supplementary Figure 6) were performed at a high flow rate, which minimizes issues such a mass transport limits. However, this being an important piece of data in the manuscript, we took the time to re-expressed the main antibodies in the manuscript, 3.10C2 and Para.09 and the humanized variants, and purified the antibodies and ran SPR experiments twice in different days with lower IgG capture levels. The results are essentially the same and were added to the means in Table 1 as additional repeats. Supplementary Figure 6 (old Suppl. Fig. 5) was updated to include the traces for one of these new repeats and the kinetic values for each panel and corresponding repeat not shown indicated in the figure.

Old Supplementary Figure 6 (now Suppl. Fig. 7) is a BLI experiment with capture levels within limits to show reliable off-rate values.

The other SPR traces shown in Supplementary Figures 2 and especially Supplementary Figure 5 (old Suppl. Fig. 4) had lower IgG capture levels. These are less critical to the overall work presented, which focuses on 3.10C2 and Para.09, and were not repeated again. However, note the similarity of the 3.10C2 kinetics in now Supplementary Figures 5 and 6 and Table 1, within margins of error.

6. In the supplementary data of Goldstein et al. multiple CDR3 from unimmunized rats with similar sequence as those shown in Fig 3 (similar to CDR3 of the 3.10C2 lineage) can be identified. It seems that the authors suggest that the sequences they find are the result of an immune response to TREM2. This may not at all be the case as highly similar sequences are seen also in nonimmunized animals (see also comment 4 above). It is suggested that the authors amend their text accordingly.

It is not surprising that similar CDR H3 sequences are found in non-immunized repertoires and perhaps in other immunized repertoires, given the short sequences. However, the rest of VH and VL are also critical for binding, as confirmed here by structural analysis. We do find 302 matches for the CDR H3 sequences in Fig. 3 to the non-immunized dataset of Goldstein et al. if allowing up to 1 amino acid difference (at least 4/5, 5/6 or 6/7 matches). Of these, only 15 have VH6-8 as in Figure 3 and, of these, only 3 have any VK2, and none V_K2S11 as the 3.10C2 parallel lineage. More relevant, if looking only at the binding clones 3.10C2, weakly binding Para.03 and the strongly binding Para.09, there are no matches for Para.09 CDR H3 at all in that dataset

even allowing 1 mismatch, 22 matches for 3.10C2 CDR H3, all with 1 aa (out of only 5) difference, and 1 for the Para.03 clone, also with 1 aa mismatch. Of the partial 3.10C2 and Para.03 CDR H3 matches only 2 clones have VH6-8, neither with VK2. It is not surprising that the VH associated with these CDR H3 sequences are associated with VH6-8 given that this germline segment has an unusual Thr at position 1 of CDR H3 and thus recurs in many VH6-8 clones. Ignoring the almost constant Tyr residue given by JH in the last position of CDR H3, these CDR H3 sequences with VH6-8 have only 2 matching residues not given by the VH and JH germline segments. Thus, the partially matching CDRH3 sequences in the non-immunized repertoire related CDR H3 in non-immunized rats lack statistical and probably biological significance and it is not addressed in the manuscript.

7. The epitopes targeted by the herein described antibody clonotypes appears to reside in a disorganized part of the antigen. Is this so? If so, are the results limited to such antibodies? A, in some respects, highly similar stereotyped human immune response targets the similarly likely disorganized AD-2 epitope of human cytomegalovirus gB (see for instance reference 36 and https://doi.org/10.1016/j.molimm.2014.03.015, https://doi.org/10.4161/mabs.27760). Can the herein defined results be discussed in the context of another peptide specific immune response like this one.

Yes, the epitopes in this study are “linear”. The 3.10C2 has some secondary structure elements within it in the complex structure, though definitely not a globular domain. The 3.10A7 group was chosen for analysis due to the linear epitope but simply to facilitate physical epitope determination for a large number of clones using peptides. The biologically active 3.10C2 just happens to be in a similar region of the protein. The role of epitope class is briefly discussed at the end of the text in the context of our energetics findings (now starting on line 646). Reference 37 (Hsiao et al.) and structures of antibodies with convergent VH/VL (parallel lineages) show that parallel lineages are widespread in folded globular antigens (ovalbumin, lysozyme). Thus, parallel lineage mining should apply to globular domain binders.

8. As the study has been conducted in part on peptides and in parts on proteins it is difficult to know if the former fully capture the nature of the interaction with the intact target. Is the affinity for the peptide similar to that for the protein? If not, one cannot draw some of the conclusions that are made as there might be additional contacts offered by the intact protein and/or that their and structural diversity in the peptides that despite the disorganized nature of this part of TREM2 might be different from that of different peptides (i.e. will the peptide reflect the structure space of this part of the protein?). Please discuss. It would be of value to determine the thermodynamic properties of the antibody-antigen interaction using either SPR or ITC to support the findings and the discussion.

The antigen used experimentally for immunization (lines 123-125) and affinity determinations (line 786, methods) was the full extracellular domain (ECD) including both the IgV domain and the entire stalk region. A note was added to Table 1 with the

kinetics data to re-iterate this. Only the epitope mapping and structural determination were done with peptides as this was impractical or not possible with the full ECD. As described in the results when introducing TREM2 as an antigen, now expanded to mention that TREM2 has a "linear stalk region", the stalk region is linear, non-globular. We added reference 41, which describes TREM2 structurally. Therefore, the peptides were not extracted from a globular domain but rather are themselves peptide-like in their native state. The peptide in the structure reflects one bound state, which is probably one of several that the epitope region can adopt natively and illustrated by the molecular dynamics simulation in Figure 7a-c.

We considered determination of binding by ITC. The affinities in the 3.10C2 and especially Para.09 are too high to determine binding properties by calorimetry reliably. The amount of peptide and consequently antibody that can be tested in the assay is limited by KD, meaning in this case that only very small amounts of reagents can be used to cover the concentrations spanning the binding affinity, leading to minimal heat release readings and unreliable data. It is doubtful SPR would have any more resolution for meaningful conclusions as the affinity of Para.09 is already at the limit of detection of the SPR system even at 37C.

9. It seems to me that many critical interactions of 3.10C2and its modified relatives are dependent on residues that are not commonly in contact with the antigen e.g. residue 2, 4, 28 and 117. 3.10C2 and Para.09 might thus not represent typical antibodies and the role of CDR3 might be different in this specificity in comparison to many (protein) specific antibodies. This implicates that the findings of this study relate to a minor subset of all specific antibodies and might not be generally applicable. Please discuss.

No changes were introduced in the relevant framework region in Para.09 relative to 3.10C2 that explain the affinity differences in this parallel lineage. As for the question if only antibodies that make these types of contacts form parallel lineages, this has been indirectly addressed in previous work by us and others. A few parallel lineage antibody complex structures with mouse and human antibodies were present in the PDB when the Hsiao et al. study describing the widespread prevalence of parallel lineages was published, none of which had these interactions. As Reviewer #4 states, these framework interactions are not common and therefore do not account for the high prevalence of parallel lineages or convergences more generally. Therefore, the framework interaction in 3.10C2 and Para.09 is an epiphenomenon not related to or required for parallel lineages.

10. It would be very beneficial to obtain the PDB coordinates of the structures as part of the review process. These have been submitted to PDB but are not yet released into the public domain.

The original submission included PDB coordinate files for both complexes. They were changed to txt files automatically by the submission system for unknown reasons and we apologize for that. The files were however easily accessible by changing suffix back to pdb.

11. The results section starts with a description of the biology of TREM2. This is not appropriate in the context in which the text is located. Please remove or move elsewhere.

The description of TREM2 biology is limited to the basic information needed to understand the relevance of the engineering performed here. That is, the example shown is not a low-bar situation with a high degree of flexibility for choice of clones, but rather one constrained by very specific requirements in a biomedically important context. Also, the main audience may not be immediately familiar with TREM2 and its general structure IgV/linear stalk structure and may benefit from some context to understand experiments on the leads. Moving the single paragraph to the introduction might distract the reader from the main point of the work performed and removing it results in the loss of important context to evaluate the work.

Reviewer #5 antibody repertoire, computational (Remarks to the Author):

The authors describe a strategy to mine repertoire sequencing data to identify high-affinity antibodies by a combinatory approach of non-clonally related sequences from same or across several subjects (animals) that target the same epitope. They term this strategy “parallel lineage” antibody mining. With this strategy, the authors identify antibody candidates with increased affinity to the TREM2 target and agonistic effects.

Although the method sounds promising and has proven to lead to improve candidates, there are some open questions to the methodology and definitions being used:

Q1. The authors define parallel lineages as convergent antibodies that bind the same epitope with the same geometry or binding mode, additionally sharing the VH and VL germline sequences but having CDRH3 regions that differ significantly in sequence and even length. However, the strategy to mine repertoires according to “parallel lineages” was limited to an increase in the CDRH3 length of 1 to 2 amino acids. This potentially does not exclude antibodies belonging to the same clonal lineage when retrieved from the same animal, as affinity maturation has been described to potentially introduce insertions and deletions (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3449029/, https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2199186/). A more robust definition of parallel lineages would be those with shared VH and VL germline sequences but identified in different individuals / animals. Additionally, the tested antibodies had swapped light chains so the VL sequence was not conserved. Therefore, the definition of the method employed and the actual tested sequences seems contradictory.

It is true that somatic mutation can introduce insertions and deletions (indels). However, please note that in the cited reference those indels are not in CDR H3 but rather in the framework regions, where it is straightforward to demonstrate those indels from flanking similarities. Other work shows occasional indels in CDRs 1 and 2 of the heavy chain but, to our knowledge, never in CDR H3 in the context of natural repertoires. The main

reason for this is that, while in principle indels could also occur in CDR H3, it would be difficult to show that a given indel is the product of SHM within a clonotype/lineage rather than VDJ recombination in different clonotypes/lineages even if the rest of the sequence is identical. Therefore, clonotype/lineages are defined in the literature, by and large, by the same CDR H3 length besides other similarity/identity parameters, including germline segment. This is especially true in large repertoire studies, given the computational complexity of allowing variable CDR H3 lengths within clonotypes and the fact that indels are relatively infrequent.

However, although not explicitly discussed in the manuscript, the possibility of indels in the same clonotype were implicitly acknowledged. We pointed out in the original manuscript (now lines 296-299, edited for emphasis) that besides the increase of 1 aa in CDR H3, clone Para.09 is derived from a different animal, in agreement with Reviewer #5, and has a different JH segment from antibody 3.10C2. In addition, as shown in the rest of the manuscript, seemingly minor CDR H3 changes (+1 length, 2 aa changes) can have major functional implications. There is no reason the technique has to be limited to +/-1 length differences. Previous work (reference 37) has shown that CDR H3 lengths can differ significantly within parallel lineages.

Q2. The authors consider antibodies from the same animal to belong to the same clonal lineage as sharing the IGHV and IGHJ genes, same CDRH3 length and amino acid identity of >67% percent. The identity threshold sounds somehow arbitrary. How was this specific threshold chosen?

The 67% corresponds to 2/3. It is arbitrary and lower than the usually used 80% threshold, which is also arbitrary and not biologically defined. The 67% threshold was used to ensure as much as possible that the CDR H3 sequences of clones with the same CDR H3 length are as distinct as possible to maximize sequence space coverage in mining. We do use two clonotype definitions in the revised manuscript, explaining the definitions in a new paragraph in results (line 136). The 67% threshold is used in the “working definition” for the purpose stated above. The more biologically relevant “biological definition” of clonotype setting the threshold to 80% and including other germline and animal origin constraints is used to assess likely shared clonal origin among clones.

Q3. The similarity among CDR3 is only reported among sequences of the same length. For the reasons mentioned above, that indels can be introduced during SHM, it would be necessary to report sequence identity when additionally considering gaps (e.g. Levenshtein distances) across sequences from parallel lineages.

The similarities allowing contiguous indels are now shown in Figure 1, far-right column. Only contiguous indels are allowed as it is unlikely SHM-induced indels would be introduced twice in different CDR H3 spots.

Q4. What was the criteria used to select the 4 initially produced MAb antibodies?

This is addressed above for reviewer 2, smaller comment 1.

Q5 The fact that there are 3 immunized rats should be mentioned on line 116. Do R18/R19/R20 read counts correspond to the read counts in each of the immunized rats? If so, this should be specified on the figure caption.

That is now clarified on line 123. The Figure 1 caption has been edited to note this.

Q6. The mouse inhibin binding experiments in Fig 1 lack a positive control.

That is true, there is no positive control for that particular negative control. No such reagent existed at the time and it was simply a long peptide that was available in the lab. However the true negative control in Figure 1 is the TREM2 peptide not including the 3.10A7 epitope (TREM2 149-168), which does have a positive control. Therefore the mouse inhibin peptide control is redundant in that figure and was removed. However, it is an important control in Figure 3, with some clones showing non-specific binding to it and was therefore retained in Figure 3.

Q7. What do the read counts in Fig 1 and 3 represent? It is not clear from the explanation. What do the annotations a,b,c,d in Fig 3 mean? It's not specified on the caption. Additionally, the %similarity should also be reported in this figure like in Fig 1.

The read counts columns are relative counts for clones with identical VH germline segment and CDR H3 identical to the one in the test antibody, used to identify rat origin of each clone. Absolute counts could not be used to the disparity of total counts between clonotypes. Therefore these have been normalized by the total number of reads for the reads of interest across all 3 rats, stating the percentage of total in each rat. The figure legends have been edited to clarify this. The a-d labels in Fig. 3 indicate clones with CDR H3 sequences with more than 67% amino acid identity and therefore part of the same clonotype by the working definition. This is now in the figure legend. The amino acid identities, with and without contiguous gaps were added to the figure.

Q8. A second round of optimization is described as utilizing affinity optimization by exclusively parallel lineage mining (page 7 line 189-183). Reporting the animal from which each of the sequences was retrieved and the J gene for each of them would better support the hypothesis that all the sequences come from parallel clones.

The animal from which each sequence was retrieved is shown in Figure 3, in the blue shading. The fact that the 3.10C2 lineage and Para.09, the only strong binder from that experiment, come from different rats was stated in the results section (now lines 296-299). The different JH genes for the 3.10C2 lineage and clone Para.09 were stated in the results section (now line 298). In addition, the complete junctional sequence analysis is added to new Supplementary Fig. 3. Of note, only confirmed binders were included in Supplementary Fig. 3.

Q9. In line 468 (page 15) the term clonotype is introduced in comparison to clone. What is the difference in definition between the two terms?

That sentence was edited for clarity. Clones here refer to unique NGS reads whereas clonotypes include all clones presumed to share a common ancestor or that fit the working clonotype definition used here. In the original sentence what was meant is that reads (or antibodies) are selected for testing, not clonotypes (or lineages), which is unnecessarily confusing. It is now simply stated that “combining parallel lineage mining with other tools that allow identification of clonotypes *with similar binding modes* based on predicted similar paratope structures…” (lines 639-641). How clonotypes relate to B cell lineages is described in lines 54 to 58 in the second paragraph of the introduction.

REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

I appreciate the authors very thorough response and edits to the initial submission. SI Figure 3 is nice addition to the paper. The paper is now appropriate for NC.

Reviewer #2 (Remarks to the Author):

I would like to express my sincere appreciation to the authors for their thorough response to my review. I feel that through both their additions to the manuscript and clarifications in their response, my critiques/concerns have been properly addressed.

Editorial note: Reviewer #4 comments were considered by internal editors to be sufficiently addressed.

Reviewer #5 (Remarks to the Author):

The authors have answered all my questions satisfactorily and I do not have any further questions, comments or concerns, and I recommend to publish this manuscript at this stage.

However, I would like to note to the reply of Q2, that even though it is true that 80% is a threshold that is widely and somewhat arbitrarily used in the literature, there are quantitative methods that exist that allow to determine an appropriate clonal threshold given the BCR CDRH3 sequences, which are based on the pairwise Hamming distance distribution of all the antibody sequences in a repertoire. An example method is implemented in the Immcantation suite Shazam R package (https://shazam.readthedocs.io/en/stable/topics/findThreshold/#:~:text=findThreshold automatically determines an optimal,method="density").

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We thank Reviewer #1 for reviewing the manuscript and for the thoughtful feedback.

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