nature portfolio

Peer Review File

Structural characterization of two γδ TCR/CD3 complexes

Corresponding Author: Dr Mohammed Hoque

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
In this paper, the authors present the cryo-EM structures of two γδ TCRs, G115 (Vγ9Vδ2) and 9C2 (Vγ5Vδ1) complexed with Fabs. They observed the same dimeric conformation for 9C2 TCR/CD3 and the cholesterol density in G115 TCR/CD3 as reported by Xin et al. The novelty of this work lies in the Fab-binding structures, particularly the interaction of Fab 3 with 9C2 in a region that is typically inaccessible in αβ TCR/CD3 complexes.

Overall, these structures advance our understanding of the γδ TCR/CD3 complex and its mechanism of action. Therefore, I recommend this manuscript for publication, pending the following revisions:

1. The authors claim to have resolved two “full-length” γδ TCR structures. However, only the extracellular domains (ECD) are resolved in the two 9C2-Fab binding structures, and the ECD region is missing in the G115 structures. The term “full-length” may not be appropriate in this context.

2. In Figure 4B, the authors present the sequence alignment of TCR γ-chain variable regions and highlight the residues involved in the dimer interface. Xin et al. also compared the γ-chain variable sequences in Figure 5d of their work, identifying additional residues contributing to the dimer interface. Could the authors explain the differences in the residues indicated?

3. In the validation report of the G115 γδ TCR/CD3 complex bound by OKT3 Fab, the map in the orthogonal surface view of the raw map (Section 6.5.2) is much smaller than that of the primary map (6.5.1). Please confirm with the EMDB staff if the validation report was generated correctly. Additionally, the unmasked map FSC in Section 8.1 drops below 0.5 at around 10 Å resolution, indicating heterogeneous particles remain in the dataset. Further 3D classification or heterogeneous refinement should be performed to improve cross-correlation in the low-resolution range. CTF refinement should also be considered to correct for any high-order aberrations.

4. Xin et al. also faced challenges in resolving the full-length 9C2 structure. They employed focus masks to resolve the ECD, MPD–TMD, MPDI–TMDI, and MPDII–TMDII regions individually. Have the authors tried a focus mask to the MPDI-TMDI region for the Fab 2/3 bound 9C2 TCR/CD3 complex? Alternatively, have they attempted a single focus map for the Fab, ECD, and MPDI-TMDI regions to resolve the full-length 9C2 bound to Fab?

5. In Figure 5B, the authors demonstrate how Fab 3 would result in steric clash within the αβ TCR/CD3 complex. Have the authors superimposed the Fab3 bound 9C2 structure with PDB 8JCB? Are there any interaction of Fab 3 with the 9C2 CD3 chains?

6. The authors should include a supplementary figure to systematically compare their structures with those reported by Xin et al.

7. For the OKT3 Fab-bound G115 TCR/CD3 structure, the unmasked map resolution in Figure S2B is reported as 3.8 Å. However, the unmasked FSC in the validation report is 6.38 Å. Can the authors explain this discrepancy?

Reviewer #2

(Remarks to the Author)
Hoque et al study two gdTCR-CD3 complexes by cryo-electron microscopy: Vg9Vd2 TCR G115 which binds butyrophilins

and thereby senses elevated intracellular levels of so-called phosphoantigens and Vg5Vd1 TCR 9C2 which binds to CD1d-glycolipid complexes. The main findings are similar interaction between CD3 proteins and the transmembrane regions of abTCR and gdTCR. A high flexibility of the connecting peptide of both gd TCR and an unexpected Vg5 domain interaction leads to dimers of the TCR. The G115 was analyzed in complexes with the CD3e chain--specific Fab of mAb OKT3 for G115 and a Vd2 specific Fab (Fab 1). 9C2 in complexes with two Vd specific Fab (Fab 2 and 3). Fab 3 is bound to a site that is most likely accessible only as a consequence of the highly flexible connecting peptide. This along with the capacity of the intact mAb to induce Vd1 T cell expansion are presented as evidences to support the importance of flexible CP in gd T cell function. The third major finding is a dimerization of Vg5 domain and the TCR.
The same TCRs were analyzed in a much more comprehensive study, published in Nature (Xin et al) during the preparation of the manuscript. The paper of Hoque clearly concentrates on structural aspects and provides only limited functional data. In the structural part, both studies differ in two aspects. First, Xin et al analyze TCR-CD3 in the absence of stabilizing Fab fragments and second 2C9-CD3 preparation co-elute with the monomeric G115-CD3 while Xin et al observe that both 2C9-CD3 elutes earlier and is found with the same micelle while Hoque et al find them in two associated micelles. The authors suggest that the latter difference results from concentration and vitrification of the material used by Xin. If I understood it correctly Hoque et al suggest that two distinct TCR-CD3 complexes associate via interaction of their extracellular domains analogous to the dimers found in the TCR-ligand crystals.

Altogether, the paper is largely confirmatory and concentrates on the structural aspect. In my eyes such independent conformation has its value of evidence but only if overlaps and differences to published work are more clearly stated and discussed. E.g. , whether the contact residues between the Vd5 are the same or different from those of Xin et al. Also relevant for parts of the discussion could be exploring the work in comparison with the paper of Gully et al. which came out most recently in Nature.

Finally, there are some important errors and questionable citations with respect to the activation of Vg9Vd2 T cells which need to be corrected.
Line 38 and 81. Vg9Vd2 T cells recognize the accumulation of intermediates of isoprenoid synthesis with di- or pyrophosphates also dubbed as phosphoantigens and not phosphonates. For the fundamental difference between phosphonates see https://en.wikipedia.org/wiki/Phosphonate. Some substances which modulate phosphoantigen synthesis are phosphonates but these do not interact with butyrophilins and act by inhibiting enzymes of isoprenoid synthesis such as the amino bisphosphonate zoledronate or the antibiotic fosmidomycin.

The reference that phosphor(n)ate accumulation is sensed by butyrophilins (Ref 7) is not correct. This excellent review dates from 2007. while the critical role of the butyrophilin 3A(1) in activation of Vg9Vd2 T cells by phosphoantigens was published 5 years later (Harly C, et al. Blood. 2012. PMID: 22767497). Not correct is also line 81 stating that butyrophilins are upregulated upon accumulation of intracellular “phosphonate” species. Intracellular phosphoantigens bind to BTN3A1 and induces formation of activating butyrophilin complexes (Yan et al 2023 Nature. 2023 Sep;621(7980):840-848. PMID: 37674084) and Ref 34-36. Ref. 36 cited as preprint has now been published Nat Immunol. 2024 Aug;25(8):1355-1366 PMID: 39014161. Recognition of phosphoantigens is a very dynamic field. An update on the recent literature can be found in a very recent review which may be cited if found to be helpful, Herrmann, T. Karunakaran, M.M. Phosphoantigen recognition by Vγ9Vδ2 T cells. Eur J Immunol. 2024 Aug 15:e2451068. PMID: 39148158 .

Reviewer #3

(Remarks to the Author)
Hoque and colleagues describe cryoEM structures of gd T cell receptor [TCR] complexes. The manuscript is notable as the authors were essentially "scooped" earlier this year by a competing group reporting very similar structures (and results) in Nature. Nonetheless, the results are interesting on their own, and while obviously not of the same impact, provide important points of comparison, reproducibility, etc. The manuscript is exceptionally well written and in this reviewer's opinion the results stand on their own.

The primary observation is the lack of density for the extracellular components of the TCR gamma and delta chains, suggesting a less rigid complex than seen with alpha/beta complexes. This is an important observation that the authors suggest may be related to the types of ligands the gd vs ab TCRs must engage. A secondary observation is the result of the dimeric form of the Vg5/Vd1 TCR.

Although I feel this is a strong manuscript, I have suggestions for a revised form:

1) Back in the 1990s / early 2000s, salt bridges between TCR transmembrane helices were discussed as the "glue" that holds the TCR/CD3 complex together. The results here provide an opportunity for the authors to discuss this - with these results in hand, is it possible that in the alpha/beta TCRs the extracellular TCR-CD3 contacts don't really contribute much to complex stability? That is, is the rigidity of the alpha/beta complex more due to connecting peptides, angles of presentation, etc., or are there interactions between the alpha/beta constant domains and CD3 components that are responsible for rigidity? Or are the constant domains of gd TCRs "repulsive" for the extracellular CD3 chains in some way? (see also point 4 below)

I am not asking for experiments here (unless the authors have some in mind), but this is something that should be discussed and considered in the discussion.

2) Since the publication of cryoEM structures of alpha/beta TCRs were published, there has been some discussion about signal transduction mechanisms, and that, with TCR alpha/beta extracellular domains directly stuck to CD3 extracellular domains somehow TCR binding information is communicated from one to the other, either via some allosteric mechanism involving changes in dynamics or subtle structural shifts, or via force-induced modifications. This is something else the authors could touch on in the discussion - what do these results say about gamma/delta TCR signaling mechanisms? Obviously what works for g/d does not have to be the same for a/b, but given that we still argue quite a bit about TCR signaling mechanisms, this deserves some commentary.

3) With dimerization, Xin et al. Nature (2024) provided more comprehensive biochemical/functional characterization of the dimer interface. Are the details here compatible with that (i.e., the same specific mutations should do the same thing?). This should be commented on.

4) On lines 169-172, it would be helpful to show the differences between the g/d and a/b constant domains mapped to the structures, particularly with regard to the TCR a/b CD3 structure to emphasize the kinds of contacts that might be disrupted (or repulsions introduced) by g/d vs. a/b TCR domains (this is related to point 1 above).

5) I found Fig. S8 is quite interesting. I suggest the authors consider moving this to a main figure rather than having it in supplemental information.

Minor points:

a) Gaussian should be capitalized throughout.
b) Introduction line 35 - MHC-independent manner. This is correct, but only if we consider classical MHC proteins. CD1, etc. are non-classical molecules but still maintain the overall MHC fold. The authors could write this more clearly to reflect the range of targets and architectures that gd TCRs are believed to recognize, compared to the much more restricted nature of ab TCRs.

Reviewer #4

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
All my concerns have been addressed. I have no further comments at this time.

Reviewer #2

(Remarks to the Author)
My questions have been reasonably well addressed.

Reviewer #3

(Remarks to the Author)
The authors have addressed my concerns.

Reviewer #4

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Editor notes:

Dear Mohammed,

Thank you again for submitting your manuscript "Structural characterization of two full length γδ TCR/CD3 complexes" to Nature Communications. We have now received reports from 4 reviewers and, based on their comments, we have decided to invite a revision of your work. Your revision should address all the points raised by our reviewers (see their reports below). In particular, please do compare your work thoroughly with the findings by Xin et al. and Gully et al. (both at Nature), as suggested by the reviewers. If you would like to discuss the extent of your revisions, please do not hesitate to get in touch.

When resubmitting, you must provide a point-by-point response to the reviewers' comments. Please show all changes in the manuscript text file with track changes or colour highlighting. If you are unable to address specific reviewer requests or find any points invalid, please explain why in the point-by-point response.

Author responses are highlighted yellow.

Reviewer #1 (Remarks to the Author):

In this paper, the authors present the cryo-EM structures of two γδ TCRs, G115 (Vγ9Vδ2) and 9C2 (Vγ5Vδ1) complexed with Fabs. They observed the same dimeric conformation for 9C2 TCR/CD3 and the cholesterol density in G115 TCR/CD3 as reported by Xin et al. The novelty of this work lies in the Fab-binding structures, particularly the interaction of Fab 3 with 9C2 in a region that is typically inaccessible in αβ TCR/CD3 complexes.

Overall, these structures advance our understanding of the γδ TCR/CD3 complex and its mechanism of action. Therefore, I recommend this manuscript for publication, pending the following revisions:

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We agree with the reviewer that the structures aren't "full-length", although the protein reagents examined by cryoEM indeed represent the full γδ TCR/CD3 complex. Nonetheless to avoid confusion we have removed "full-length" as a descriptor in the title and abstract and elsewhere in the manuscript.

2. In Figure 4B, the authors present the sequence alignment of TCR γ-chain variable regions and highlight the residues involved in the dimer interface. Xin et al. also compared the γ-chain variable sequences in Figure 5d of their work, identifying additional residues

contributing to the dimer interface. Could the authors explain the differences in the residues indicated?

Our structure of the γ5-mediated TCR ECD dimer is very similar to the structure reported by Xin et al (see our newly added Figure S9G), and as such the dimer interface is essentially the same. The difference in "interface residues" arises from how they were assigned, which can be subjective and depend on minute differences in coordinates resulting from building and refining structures in the ~3Å resolution range. We defined the interface residues using the "find H-bond" and "find contacts" commands in ChimeraX after highlighting the dimeric interface. To our knowledge, Xin et al did not explicitly state how they defined their dimer interface residues. Nonetheless, it appears that Xin et al were slightly more liberal in how they defined the interface residues. For a more in-depth discussion of the dimeric interface and the residues that mediate it, see comments made to reviewer 3 point 3.

3. In the validation report of the G115 γδ TCR/CD3 complex bound by OKT3 Fab, the map in the orthogonal surface view of the raw map (Section 6.5.2) is much smaller than that of the primary map (6.5.1). Please confirm with the EMDB staff if the validation report was generated correctly. Additionally, the unmasked map FSC in Section 8.1 drops below 0.5 at around 10 Å resolution, indicating heterogeneous particles remain in the dataset. Further 3D classification or heterogeneous refinement should be performed to improve cross-correlation in the low-resolution range. CTF refinement should also be considered to correct for any high-order aberrations.

We thank the reviewer for carefully examining our cryoEM data and validation reports.

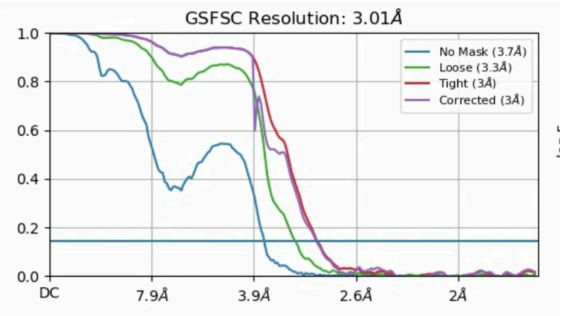
We have contacted the EMDB staff, and they have indicated that the size difference between the raw map and the primary map is likely due to some level of noise that is present in the raw map. Please see the response from the EMDB staff below:

"The change in zoom level is because the raw map will naturally have more noise than the primary map. The protocol for taking the images is:
- open map
- set contour level
- align camera on axis such that the whole visible surface is in view
- take picture
It is likely that there is a very small piece of noise visible somewhere in the raw map image (that is hard to see in those images) that causes the camera to zoom out in order to fit the whole visible surface in."

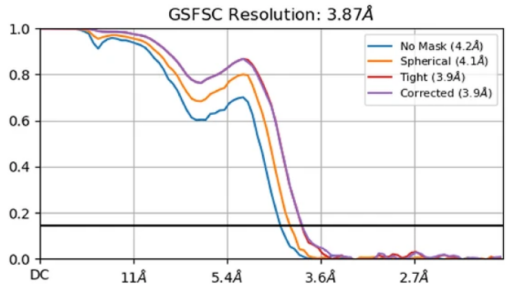
We are unsure what is causing the drop in the FSC curve, but this seems to be a common feature of membrane protein structures refined in cryoSPARC. Furthermore, the G115 TCR map generated by Xin et al. and the G83 TCR map generated by Gully et al. appears to have a similar drop in the FSC (see below). Our attempts to further classify our particles of OKT3 Fab-bound G115 TCR/CD3 using ab initio reconstruction (class similarity 0 and 1) did not

improve the quality of the dataset. CTF refinement also did not yield an improved map, possibly because we used EPU/Aberration-free image shift (AFIS), which corrects for aberrations such as coma and astigmatism during data collection.

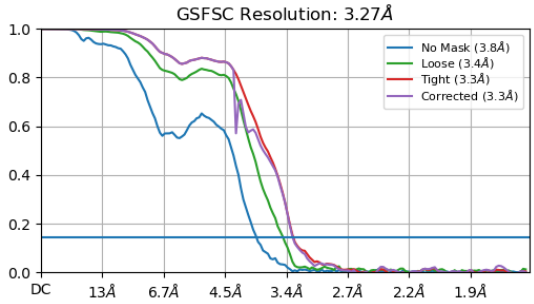
Gully et al. (Extended Data Figure 2)



Xin et al. (Extended Data Figure 2H):



Hoque et al. (Figure S2b):



4. Xin et al. also faced challenges in resolving the full-length 9C2 structure. They employed focus masks to resolve the ECD, MPD–TMD, MPDI–TMDI, and MPDII–TMDII regions individually. Have the authors tried a focus mask to the MPDI-TMDI region for the Fab 2/3 bound 9C2 TCR/CD3 complex? Alternatively, have they attempted a single focus map for the Fab, ECD, and MPDI-TMDI regions to resolve the full-length 9C2 bound to Fab?

We were unable to resolve a buildable map for the MPD/TMD of 9C2; the only region that we could resolve at reasonable resolution was the dimeric ECD, due to the inherent flexibility between the ECD and the MPD/TMD. At low thresholds of Gaussian filtered 9C2 ECD/Fab maps, the MPD/TMD can be observed as poorly resolved blobs, as shown in Fig. 3B, C.
We aren't sure what the reviewer means by "single focus map". Xin et al were able to generate a composite map of the "full-length" 9C2 TCR by docking in higher resolution maps of the individual portions that were obtained by separate focused refinements into a lower resolution map of the full complex. Since we were unable to resolve the MPD/TMD of the 9C2 TCR at high resolution, we are unable to generate a composite map of the ECD+MPD/TMD.

5. In Figure 5B, the authors demonstrate how Fab 3 would result in steric clash within the αβ TCR/CD3 complex. Have the authors superimposed the Fab3 bound 9C2 structure with

PDB 8JCB? Are there any interaction of Fab 3 with the 9C2 CD3 chains?

When we align our structure to 8JCB, we observe a minor clash between Fab 3 and the CD3ε chain of PDB 8JCB. We have now indicated this in Fig. S9H and mentioned it in the discussion section. However, this clash is unlikely to prevent the antibody from binding 9C2 γδ TCR (as shown in Fig. 5C) due to the flexibility of the TCR ECD.

6. The authors should include a supplementary figure to systematically compare their structures with those reported by Xin et al.

We have now added a supplementary figure comparing our structures to both Xin et al. and Gully et al. (See Fig. S9) and added text to the Discussion section (lines 358 to 377).

7. For the OKT3 Fab-bound G115 TCR/CD3 structure, the unmasked map resolution in Figure S2B is reported as 3.8 Å. However, the unmasked FSC in the validation report is 6.38 Å. Can the authors explain this discrepancy?

We believe that the reviewer may be mixing up the validation reports. In the OKT3 Fab-bound G115 TCR/CD3 validation report (Section 8.2, see below), the unmasked FSC at 0.143 is 3.82 Å, in line with the resolution reported by cryoSPARC. The reviewer may be referring to the 9C2 TCR ECD/Fab 2 complex validation report where the unmasked FSC is 6.38 Å, which is consistent with the unmasked FSC of 6.4 Å reported by cryoSPARC (Fig. S6B). Nonetheless, we thank the reviewer for carefully examining the validation reports for any notable issues.

OKT3 bound G115 TCR/CD3:

8.2 Resolution estimates

Resolution estimate (Å)	Estimation criterion (FSC cut-off)		
	0.143	0.5	Half-bit
Reported by author	3.27	-	-
Author-provided FSC curve	-	-	-
Unmasked-calculated*	3.82	4.31	3.89

Fab 2 bound 9C2 TCR ECD complex:

8.2 Resolution estimates

Resolution estimate (Å)	Estimation criterion (FSC cut-off)		
	0.143	0.5	Half-bit
Reported by author	3.45	-	-
Author-provided FSC curve	-	-	-
Unmasked-calculated*	6.38	9.43	6.95

Reviewer #2 (Remarks to the Author):

Hoque et al study two gdTCR-CD3 complexes by cryo-electron microscopy: Vg9Vd2 TCR G115 which binds butyrophilins and thereby senses elevated intracellular levels of so-called phosphoantigens and Vg5Vd1 TCR 9C2 which binds to CD1d-glycolipid complexes. The main findings are similar interaction between CD3 proteins and the transmembrane regions of abTCR and gdTCR. A high flexibility of the connecting peptide of both gd TCR and an unexpected Vg5 domain interaction leads to dimers of the TCR. The G115 was analyzed in complexes with the CD3e chain--specific Fab of mAb OKT3 for G115 and a Vd2 specific Fab (Fab 1). 9C2 in complexes with two Vd specific Fab (Fab 2 and 3). Fab 3 is bound to a site that is most likely accessible only as a consequence of the highly flexible connecting peptide. This along with the capacity of the intact mAb to induce Vd1 T cell expansion are presented as evidences to support the importance of flexible CP in gd T cell function. The third major finding is a dimerization of Vg5 domain and the TCR.
The same TCRs were analyzed in a much more comprehensive study, published in Nature (Xin et al) during the preparation of the manuscript. The paper of Hoque clearly concentrates on structural aspects and provides only limited functional data.
In the structural part, both studies differ in two aspects. First, Xin et al analyze TCR-CD3 in the absence of stabilizing Fab fragments and second 2C9-CD3 preparation co-elute with the monomeric G115-CD3 while Xin et al observe that both 2C9-CD3 elutes earlier and is found with the same micelle while Hoque et al find them in two associated micelles. The authors suggest that the latter difference results from concentration and vitrification of the material used by Xin. If I understood it correctly Hoque et al suggest that two distinct TCR-CD3 complexes associate via interaction of their extracellular domains analogous to the dimers found in the TCR-ligand crystals.

Altogether, the paper is largely confirmatory and concentrates on the structural aspect. In my eyes such independent conformation has its value of evidence but only if overlaps and differences to published work are more clearly stated and discussed. E.g. , whether the contact residues between the Vd5 are the same or different from those of Xin et al. Also relevant for parts of the discussion could be exploring the work in comparison with the paper of Gully et al. which came out most recently in Nature.

We thank the reviewer for their careful reading of our manuscript and sharing their expertise with us. We have now added an additional section to our discussion section directly comparing our data to that generated by Xin et al. and Gully et al. Furthermore, we discuss our finding of the γ5 chain dimer in the context of the data generated by Xin et al. (See Fig. S9 and associated text in the Discussion section, paragraph 6).

Finally, there are some important errors and questionable citations with respect to the activation of Vg9Vd2 T cells which need to be corrected.
Line 38 and 81. Vg9Vd2 T cells recognize the accumulation of intermediates of isoprenoid synthesis with di- or pyrophosphates also dubbed as phosphoantigens and not phosphonates. For the fundamental difference between phosphonates see https://en.wikipedia.org/wiki/Phosphonate. Some substances which modulate phosphoantigen synthesis are phosphonates but these do not interact with butyrophilins and act by inhibiting enzymes of isoprenoid synthesis such as the amino bisphosphonate zoledronate or the antibiotic fosmidomycin.

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We apologize for these errors and appreciate the reviewer for providing the appropriate citations. We have replaced ref 7 with Harly et al. (ref 7). Ref 36 has now been updated to reflect the publication in Nat. Immunol. We have also added the review suggested by the author (Herrmann and Karunakaran) as ref 10.

Reviewer #3 (Remarks to the Author):

Hoque and colleagues describe cryoEM structures of gd T cell receptor [TCR] complexes. The manuscript is notable as the authors were essentially "scooped" earlier this year by a competing group reporting very similar structures (and results) in Nature. Nonetheless, the results are interesting on their own, and while obviously not of the same impact, provide important points of comparison, reproducibility, etc. The manuscript is exceptionally well written and in this reviewer's opinion the results stand on their own.

The primary observation is the lack of density for the extracellular components of the TCR gamma and delta chains, suggesting a less rigid complex than seen with alpha/beta complexes. This is an important observation that the authors suggest may be related to the types of ligands the gd vs ab TCRs must engage. A secondary observation is the result of the dimeric form of the Vg5/Vd1 TCR.

Although I feel this is a strong manuscript, I have suggestions for a revised form:

1) Back in the 1990s / early 2000s, salt bridges between TCR transmembrane helices were discussed as the "glue" that holds the TCR/CD3 complex together. The results here provide an opportunity for the authors to discuss this - with these results in hand, is it possible that in the alpha/beta TCRs the extracellular TCR-CD3 contacts don't really contribute much to complex stability? That is, is the rigidity of the alpha/beta complex more due to connecting peptides, angles of presentation, etc., or are there interactions between the alpha/beta constant domains and CD3 components that are responsible for rigidity? Or are the constant domains of gd TCRs "repulsive" for the extracellular CD3 chains in some way? (see also point 4 below)

I am not asking for experiments here (unless the authors have some in mind), but this is something that should be discussed and considered in the discussion.

This is an excellent point brought up by the reviewer. It is apparent from our structural data that TM domains are chiefly responsible for γδ TCR/CD3 association. In the case of αβ TCR/CD3, there are indeed contacts between the TCR and CD3 ECDs that likely enforce its architectural rigidity, but may not be required for overall complex stability. We have now highlighted this in our discussion section (Discussion paragraph 1, see also Fig S4).

2) Since the publication of cryoEM structures of alpha/beta TCRs were published, there has been some discussion about signal transduction mechanisms, and that, with TCR alpha/beta extracellular domains directly stuck to CD3 extracellular domains somehow TCR binding information is communicated from one to the other, either via some allosteric mechanism involving changes in dynamics or subtle structural shifts, or via force-induced modifications. This is something else the authors could touch on in the discussion - what do these results say about gamma/delta TCR signaling mechanisms? Obviously what works for g/d does not have to be the same for a/b,but given that we still argue quite a bit about TCR signaling mechanisms, this deserves some commentary.

We have expanded on how the γδ TCR/CD3 complex signaling mechanisms compare with αβ TCR/CD3 in the discussion (see Discussion, paragraph 4). Broadly speaking, it appears that the γδ TCR may not act as a mechanosensor or become activated via allosteric means, due to how the TCR ECD is flexibly coupled to the TM portion of the molecule. Experiments

investigating the function of chimeric TCR complexes have provided some insight into the contributions of the TCR constant domains. A chimeric TCR that contains a γδ V domains and an αβ C domain is able to function as a mechanosensor, however the inverse chimera is unable to activate the T-cell. Interestingly, a recent publication demonstrated that γδ T-cell activation may be driven by the kinetic segregation model, where negative regulators of T-cell activation are sterically excluded from the immune synapse leading to activation. However, whether this is the main driver of activation or whether there is a novel mechanism that does is yet to be determined.

3) With dimerization, Xin et al. Nature (2024) provided more comprehensive biochemical/functional characterization of the dimer interface. Are the details here compatible with that (i.e., the same specific mutations should do the same thing?). This should be commented on.

We believe our results align with the biochemical data generated by Xin et al. Both groups report that the Y72 π–π interaction and a salt bridge between R86 and D60 are critical residues in this interface. To highlight the importance of these residues, Xin et al mutated Y72 to a histidine and R86 to a glutamic acid and found that this abolished dimer formation, as assayed by FRET. These points have now been highlighted in the discussion (see Discussion section, paragraph 6).

4) On lines 169-172, it would be helpful to show the differences between the g/d and a/b constant domains mapped to the structures, particularly with regard to the TCR a/b CD3 structure to emphasize the kinds of contacts that might be disrupted (or repulsions introduced) by g/d vs. a/b TCR domains (this is related to point 1 above).

We have now added a panel comparing the structures of αβ and γδ TCR ECDs with a focus on the regions that contact the CD3 ECDs (Figure S4). Poor sequence conservation in the contact regions suggests that the contacts are completely disrupted. Furthermore, the variability of the connecting peptide (CP) sequences makes it difficult for us to conclude whether any repulsions are introduced because the γδ TCR CPs may not permit an "αβ-like" position of the ECD.

5) I found Fig. S8 is quite interesting. I suggest the authors consider moving this to a main figure rather than having it in supplemental information.

Figure S8 has now been moved to Figure 6.

Minor points:

a) Gaussian should be capitalized throughout.

All instances of "gaussian" have been changed to "Gaussian".

b) Introduction line 35 - MHC-independent manner. This is correct, but only if we consider classical MHC proteins. CD1, etc. are non-classical molecules but still maintain the overall MHC fold. The authors could write this more clearly to reflect the range of targets and architectures that gd TCRs are believed to recognize, compared to the much more restricted nature of ab TCRs.

We apologize for any confusion this may have caused. We have revised this portion of the introduction to differentiate between the ligand types of γδ and αβ TCRs more clearly.

Reviewer #4 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.