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Lysophospholipid presentation by CD1d and recognition by a human Natural Killer T cell receptor.

Jacinto Lopez-Sagaseta, Leah V Sibener, Jennifer E Kung, Jenny Gumperz and Erin J Adams

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 16 January 2012

Thank you for submitting your manuscript to the EMBO Journal. Your study has now been seen by three referees and their comments are provided below.

As you can see the referees find the study interesting, timely and suitable for publication in the EMBO Journal. They raise a number of specific comments that shouldn't involve too much additional work to resolve. Given the referees' positive recommendations, I would like to invite you to submit a revised version of the manuscript that addresses the raised concerns.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website:
http://www.nature.com/emboj/about/process.html

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1

Adams and co-authors report an interesting series of structures that are centered on the Natural Killer T (NKT) cell antigen receptor. Unlike conventional T-cell receptors (TCRs) that recognize peptide antigens (Ags) bound to the MHC, NKT TCRs specifically recognize lipid-based antigens restricted to CD1d. Whilst we know a reasonable amount regarding TCR-pMHC interactions, our understanding of TCR-CD1d interactions, whilst growing, is still limited. Thus, the report, focusing on NKT TCR recognition of a lyso-Ag is timely, conceptually innovative and interesting. In essence, the report comprises the 1st structure of the NKT TCR recognizing a "single-tail" Ag.

There are a few aspects that require consideration prior to resubmission.

1) Overall, the work is technically well done, although the crystallographic statistics (and electron density quality) is a little concerning. Greater justification of the resolution cut-offs are required and it would be useful to see omit maps wrt the ligand in the binary and complexed state. Fig 6b, as acknowledged by the authors, is unconvincing. Similar arguments extend to the lack of resolution of the CDR3 loops and the comments regarding mobility upon complexation. Conclusions regarding mobility of binding should be tempered given the quality of the crystallographic data.

2) It is curious as to why the A'-pocket would close upon complexation given the docking mode of the NKT TCR above the F-pocket. The authors need to clarify/speculate this. Is it not conceivable that the lyso-Ag can exist in both pockets of Cd1d and that only one form has been selectively crystallized in the binary state?

3) It seems a little strange why the point of reference is continually the a-GC complexes when there is a NKT TCR-CD1d-phosphatidylinositol complex that would serve as a useful comparison, especially considering head-group interactions as the PI headgroup is swung towards the Va chain and appears to make similar interactions to the lyso-Ag reported here.

4) The authors should clarify whether Va-Vb docking observed here is dissimilar to the recently solved NKT TCR complexes (Patel PNAS 2011, Uldrich NI 2011). The impact of the Vb chain on Va conformation is interesting and has resonances with a recent observation by Huseby et al (Immunity 2011).

5) It is unclear whether the mode of NKT TCR-CD1d-lyso-Ag docking is attributable to the Ag per se. Would the same NKT TCR dock onto CD1d-aGC (for example) similarly? The authors need to comment on this.

Referee #2

Jacinto Lopez-Sagaseta's manuscript reports the structures of human CD1d bound to a lyso-phosphatidyl choline (LPC) and a human NKT T cell receptor as well as separate structures of the CD1d-LPC and the unliganded TCR. The manuscript extensively develops comparisons among these three new structures and other published structures. Figures clearly document the comparisons of mouse versus human CD1d, single chain versus dual chain ligands, glycosylated and non-glycosylated ligands, CD1-LPC with and without the TCR. These comparative analyses include by residue analyses and a footprint map with domain by domain analysis of the TCR contacts with ligand and the distal CD1d surface.

Although the field of CD1d crystallography is becoming crowded, most prior studies focus on mono- or tri-glycosyl sphingolipids related to alpha galactosyl ceramide, many of which are artificial superagonists. Here, Lopez-Sagaseta focuses on an endogenous lipid that is generated in cells and so is of high interest because it is plausibly a natural autoantigen that mediates autoreactivity. LPC is structurally distinct from the most extensively studied alpha-galactosyl ceramide compounds because it has only one fatty acyl chain and a smaller zwitterionic head group rather than carbohydrate. Therefore, the CD1-LPC-TCR interaction offers new insights into the mechanism of antigen recognition. Recent studies have emphasized a lock and key model whereby

recognition is mainly determined by a rigid TCR that recognizes alpha- or beta-linked glycolipids with most contacts occurring with CD1d. This paper supports lock and key by showing how the zwitterionic head group can pivot significantly when the TCR binds. Yet against the lock and key model, the altered structure of the TCR itself is a divergent and somewhat flexible structure. Prior structures mostly used structurally related ligands, so the LPC ligand is both a more stringent test of the model than prior studies, and this study reveals some divergence from prior predictions. Also, the observation of an open A' roof that that changes in the TCR docked structure, provides insight suggesting the decreased need for threading interactions during loading. Overall the writing is extremely well done: the manuscript if focused, terse, well illustrated and precise in discussion of the literature. Although somewhat low resolution and subject to protein engineering to achieve what is a challenging ternary structure, the discussion forthrightly acknowledges certain aspects of the structure must be interpreted with care, and the key observations are well supported.

Although water-soluble compared to other lipids, what is the critical micellar concentration of LPC (Fig. 1) ? Unless it is above that of the added concentrations and reported equilibrium value, then the molecule may be aggregated and so deviate in behavior from assumptions used in equilibrium calculations. This is a general issue for the CD1 field.

Citation of 7 largely overlapping review articles on disease importance could be replaced with one review or 7 original papers that prove the point. (p.3)

Referee #3

The article by Jacinto López-Sagaseta et al., describes the crystal structure of the ternary complex consisting of human CD1d-lysoPC-and the human Va24Vb11 TCR of human iNKT cells, as well as unbound hCD1d-LPC and TCR for comparison.

This is the first crystal structure of the human ternary complex in which the lipid does not belong the the family of the prototypical antigen alpha-Galactosyl ceramide. As such, the structure is of high interest. Also, even considering that many structures have been determined using the evolutionary conserved mouse molecules, this is the first case in which a single chain lipid has been crystallized to which the TCR binds.

The authors report that the affinity of the LPC ligand to CD1d is rather low, which might explain their unsuccessful attempts in determining the TCR binding affinity by SPR, however, they have not tried the reversed strategy with immobilizing the TCR on the chip. Also the authors note that in the absence of any TCR, LPC binding to CD1d results in an opening above the A' pocket, due to a lateral shift of the a1-helix by ~2Angstrom. However, as the LPC ligands binds at the opposite side in the F' pocket, the reason for the structural change within the A' pocket is not clear, especially since a spacer lipid is bound inside the A' pocket to presumably stabilize the hydrophobic cavity. However, this structural change adds to the current data also seen for mouse CD1d molecules, that the CD1d binding groove and especially the entrance to the groove is more flexible than previously anticipated.

By determining the crystal structure of LPC-hCD1d with and without bound TCR, the authors set out to explain differences in the presentation of LPC by CD1d vs how LPC is ultimately recognized and bound by the TCR. In that regard, the TCR pushes the ligand into a position amendable for proper binding. However, quite unexpectedly, the TCR does not follow the conserved binding footprint that has been seen so many times using the orthologous mouse molecules. Here the ligands are also moved around by the TCR in a way that allows the TCR to bind in almost identical orientations above CD1d. Along the same line, it is very surprising that not all human iNKT clones respond to the LPC ligand and the reason for that is not fully explained. Also quite remarkable is the fact that CDR3a residues do not contact the LPC in contrast to all the structures obtained using the ortholog mouse molecules.

I think the truly remarkable feature of this structure is the lack of the conserved TCR footprint onto CD1d and if that holds true for other ligands, then this will truly change our perception about the conserved pattern-like recognition of different lipids by a semi-conserved TCR. The downside to

this is that this is only one clone that binds LPC and how representative is that clone in comparison to the natural iNKT cell repertoire?

Overall the ms is well-written and the results support the drawn conclusions.

Minor comments:

1) As the affinity value for LPC binding to hCD1d is dependent upon lipid solubility and ability to replace endogenous ligands, it is difficult to compare the obtained KD to values obtained from other labs. It would be good to measure it in parallel also with aGalCer, to see if that is radically different. aGalCer should have a much higher affinity for CD1d but lower solubility.

2) On p.9 first paragraph, the authors stat that the A' shift of the helix has not been observed in any of the mouse CD1d structures. That is not fully true, as it has been observed by Aspeslagh et al. (EMBO J, 2011), even when the TCR is bound. True, the shift was not that dramatic but nevertheless showed that there is flexibility in the A'roof of mouse CD1d, as a direct result of lipid binding. Please include a comment about this.

3) The SPR measurements are rather important, especially since the electron density for LPC in the ternary complex is not that great. The authors argue that failure to see TCR binding in a SPR experiment is due to the weak CD1d-lipid affinity and I agree. However, have the authors considered immobilizing the TCR (through a engineered birA tag or random TCR biotinylation)? This way the authors would prevent washing off the lipid from CD1d and always inject fresh CD1d-lipid complexes. The resulting KD value would be dependent on the lipid loading level but should not differ more than 2-3 fold form a 100% loaded complex.

4) on p. 12, third line from bottom, in addition to Pellicci et al 2011, please include the reference for Yu, ED. et al 2011, J Immunol., which also discusses the additional K69 contact for iGb3.

5) If only certain CDR3b residues allow for binding to the hCD1d-LPC complex, why is there no common sequence motive (maybe it is cryptic). Can it be that CDR3b region affects the orientation of the other CDRs in a way that the TCR can only bind when the conserved footprint cannot be maintained? How does different residues in CDR3b affect the binding orientation of the other CDR loops that are conserved in sequence? Those questions should be addressed more in the discussion.

1st Revision - authors' response 20 January 2012

Referee #1

1) Overall, the work is technically well done, although the crystallographic statistics (and electron density quality) is a little concerning. Greater justification of the resolution cut-offs are required and it would be useful to see omit maps wrt the ligand in the binary and complexed state. Fig 6b, as acknowledged by the authors, is unconvincing. Similar arguments extend to the lack of resolution of the CDR3 loops and the comments regarding mobility upon complexation. Conclusions regarding mobility of binding should be tempered given the quality of the crystallographic data.

We appreciate that the reviewer feels our work was technically well done. Our resolution cut-offs decisions were based on a combination of factors that are usually monitored during data processing and refinement. As with many statistical issues, there is debate as to which should be the key indicator of appropriate cutoff. We have thus tried to balance our cut-offs between I/sI (often argued to be one of the more important statistics, and should be >1) with Rmerge, completeness and redundancy. Our refinement statistics (Rwork/Rfree) are very good for our resolution cutoff and we have experimented with cutting off our data at lower resolution, only to the detriment of our electron density. We don't believe that reporting a structure at 2.8Å versus 2.9Å would affect its ability to be published, nor 3.0 versus 3.1, therefore we chose to include the higher resolution data rather than eliminate data that contributes to the interpretation of our model. In response to the reviewers concerns about the quality of our electron density, we have revised Figure 6b, replacing the figure showing a 2Fo-Fc map with that of a composite omit map (2Fo-Fc). The electron density is very similar to that shown in the 2Fo-Fc. We have also included a supplemental figure (Supplemental

Figure 1) showing omit map density of the LPC ligand and spacer lipids in the binary CD1d/LPC structure. There is strong electron density consistent with placement of the LPC ligand in the F' pocket. In regards to the reviewer's comments about CDR3 loops and mobility, we have changed the discussion to focus more on the CDR3α (which has clear structural differences between the unliganded and liganded states). The lack of electron density for the remaining loops in the unliganded TCRs is not necessarily due to poor quality data as the remaining electron density of the structures are very good. We hypothesized that the disordered regions were a product of loop flexibility, but have modified our statement in the text to indicate that disordered regions can be due to a variety of factors (although we would like to note that many crystallographers interpret this as we do, that disordered regions can be due to flexibility in that region of the structure). As this is typical for many antibody and TCR structures, we are unclear as to why this reviewer feels we cannot make the same suggestion here.

2) It is curious as to why the A'-pocket would close upon complexation given the docking mode of the NKT TCR above the F-pocket. The authors need to clarify/speculate this. Is it not conceivable that the lyso-Ag can exist in both pockets of Cd1d and that only one form has been selectively crystallized in the binary state?

We have added a statement (Page 10) speculating why the A' pocket closes upon complexation with the TCR. We hypothesize that the movement of the LPC headgroup upon binding of the TCR allows for the establishment of a hydrogen-bond between the phosphate of LPC and His68 of CD1d. This may contribute to the stabilization of CD1d in the "closed" conformation in the absence of lipid ligands with two hydrocarbon chains. It is certainly conceivable that LPC can exist in both pockets (we have no data to support this, however), so we have modified our discussion to raise this possibility (Page 17).

3) It seems a little strange why the point of reference is continually the α-GC complexes when there is a NKT TCR-CD1d-phosphatidylinositol complex that would serve as a useful comparison, especially considering head-group interactions as the PI headgroup is swung towards the Va chain and appears to make similar interactions to the lyso-Ag reported here.

We were careful to compare our structures only to the available human complex structures both for simplicity (a comprehensive comparison with the many mouse complex structures is more suitable for a review) and because the mouse structures show variation in the docking of the Vβ loops due to different Vβ domains being used in mouse iNKT-TCRs. The NKT-TCR-CD1d-PI structure was not only mouse, but this iNKT-TCR was also engineered (in the CDR2β and CDR3β) for high-affinity binding to "empty" CD1d, which in our opinion may not necessarily recapitulate the range of binding of native iNKT-TCRs to CD1d with endogenous ligands and thus is not an ideal comparison for our structure.

4) The authors should clarify whether Vα-Vβ docking observed here is dissimilar to the recently solved NKT TCR complexes (Patel PNAS 2011, Uldrich NI 2011). The impact of the Vβ chain on Vα conformation is interesting and has resonances with a recent observation by Huseby et al (Immunity 2011).

Again, we were careful to compare our structure to only human iNKT-TCR/CD1d complexes because of the heterogeneity of the mouse TCR sequences and that effect on their docking mode (based on variable Vβ domain usage, see point 3 above). The papers cited by the reviewer are mouse iNKT TCRs and one (the "non-invariant") uses a different Vα domain. The structure reported in the Patel paper, when compared to the mouse CD1d/αGalCer/iNKT-TCR structure is essentially identical for the Vα loops (similar to what is see with most, if not all mouse iNKT-TCRs), whereas there are some minor shifts in the Vβ loops. The "non-invariant" TCR, as discussed in detail in the Uldrich paper, is shifted in both the Vα and Vβ loop contacts due to the sequence differences encoded within these loops (importantly this TCR uses a Jα50 segment instead of the canonical Jα18 segment). While the overall orientation is conserved, these TCRs "recreate" the iNKT binding mode despite having such sequence differences. We believe the significance of our shift is because these human TCRs *are essentially identical in their loop sequences* (five out of the six CDR loops are identical), differing only in the CDR3β sequence and, as we point out, in the Jβ segments in the region proximal to the Cβ domain. The recent elegant work from the Huseby lab (Stadinski et al.

2011, Immunity) demonstrating that differential Vα and Vβ chain pairing can influence how TCRs recognize MHCp is a very interesting result, however the human TCRs we compare in our work use the same Vα and Vβ domains (which is why we think our result is particularly interesting for this system). We attribute some of the difference in docking mode to the differential usage of the Jβ segment, which is not mentioned in the Stadinski et al paper. They do cite the use of the Jα50 segment in the "non-invariant" NKT-TCR, however these residues contribute directly to contacting CD1d (and, in the case of their TCRs, MHCp), whereas the differences we cite in the Jβ segment are far from the CDR loops and rather affect how the Vβ domain associates with the Cβ domain. This then affects how the Vα and Vβ domains orient in relation to each other, despite having identical pairing sequences.

5) It is unclear whether the mode of NKT TCR-CD1d-lyso-Ag docking is attributable to the Ag per se. Would the same NKT TCR dock onto CD1d-αGC (for example) similarly? The authors need to comment on this.

This is an excellent point raised by the reviewer; we have aggressively pursued crystallization of this iNKT-TCR with CD1d/αGalCer to answer this exact question. Unfortunately we have only obtained a 4Å dataset which we felt did not have adequate information to definitively answer this question. We have added a short statement of this point in our discussion as it is indeed an important point. We have measured the affinity of this iNKT-TCR with CD1d-αGalCer, and found similar affinities to CD1d-αGalCer as with other human iNKT-TCRs. We have added this data as a supplement (Supplemental Figure 2) and discussed it in the text (Page 16 and 17). While we cannot comment on the docking mode to CD1d/αGalCer, it is at least not exceptionally divergent in its binding affinity.

Referee #2

Although water-soluble compared to other lipids, what is the critical micellar concentration of LPC (Fig. 1) ? Unless it is above that of the added concentrations and reported equilibrium value, then the molecule may be aggregated and so deviate in behavior from assumptions used in equilibrium calculations. This is a general issue for the CD1 field.

The reviewer raises an excellent point which we tried to address by calling our measurements "apparent" K_D as the affinity we have calculated is dependent not only on the solubility of the lipid (i.e. CMC value) but also by its ability to displace lipids that are already in the CD1d molecule and finally its true affinity for CD1d (See Page 7). We realize that the first two features are difficult to control for, but we feel that this measurement is a good way to assess lipid affinities as it does not require modification of either the lipid or CD1d. We have measured the affinity of CD1d/αGalCer to compare this with other values in the literature and obtained a value (0.11uM) not very different from that of αGalCer with mouse CD1d as measured by SPR (0.34uM). This is presented in the text on Page 7 and in Supplemental Figure 1A. The CMC values for LPC 18:1 which was used in the measurements is ~7uM (see discussion in Stafford et al (1989) Biochemistry 28:5113 and Smith et al (1981) J.Lipid Res. 22:697), so is within the upper range of the concentrations we have surveyed. We still believe this can be a useful technique to look at apparent affinities however.

Citation of 7 largely overlapping review articles on disease importance could be replaced with one review or 7 original papers that prove the point. (p.3)

We have changed the references accordingly.

Referee #3

Minor comments:

1) As the affinity value for LPC binding to hCD1d is dependent upon lipid solubility and ability to replace endogenous ligands, it is difficult to compare the obtained KD to values obtained from other labs. It would be good to measure it in parallel also with αGalCer, to see if that is radically

different. αGalCer should have a much higher affinity for CD1d but lower solubility.

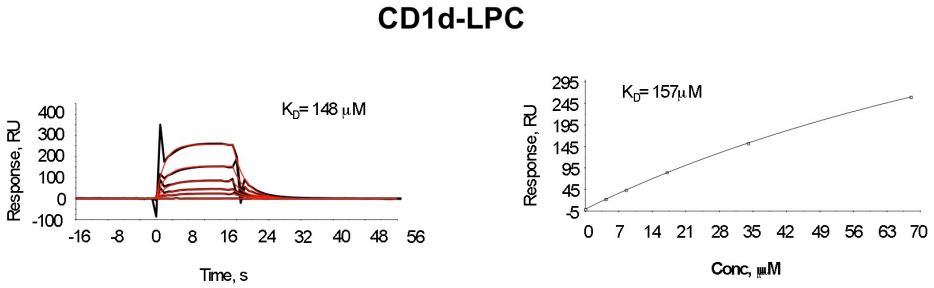
This is an important point, please see our response to Reviewer #2 above. We have presented this data in Supplemental Figure 1A and discussed it in the text on Page 7.

2) On p.9 first paragraph, the authors stat that the A' shift of the helix has not been observed in any of the mouse CD1d structures. That is not fully true, as it has been observed by Aspeslagh et al. (EMBO J, 2011), even when the TCR is bound. True, the shift was not that dramatic but nevertheless showed that there is flexibility in the A'roof of mouse CD1d, as a direct result of lipid binding. Please include a comment about this.

We thank the reviewer for pointing out our oversight, we have modified the text to include discussion about the flexibility of the A' roof of mouse CD1d. (Page 9)

3) The SPR measurements are rather important, especially since the electron density for LPC in the ternary complex is not that great. The authors argue that failure to see TCR binding in a SPR experiment is due to the weak CD1d-lipid affinity and I agree. However, have the authors considered immobilizing the TCR (through a engineered birA tag or random TCR biotinylation)? This way the authors would prevent washing off the lipid from CD1d and always inject fresh CD1d-lipid complexes. The resulting KD value would be dependent on the lipid loading level but should not differ more than 2-3 fold form a 100% loaded complex.

We have devoted considerable energy and resources to binding experiments with CD1d-LPC and the iNKT-TCR; we hope the reviewer can understand our frustration in not being able to produce definitive data in this regard. We have tried all possible immobilization strategies (including the one the reviewer proposed, which is a standard approach we use in our laboratory for SPR). Most were fraught with background binding issues. We have semi-successfully used a Nickel-NTA Biacore chip whereby we immobilize the TCR with C-terminal His tags, block remaining Ni sites with His-tag labeled Fc molecules, then flow CD1d-LPC as analyte. Our preliminary results to this end have generated an estimated binding affinity of the iNKT-TCR to CD1d-LPC (flowed as analyte) to be ~150uM. This value ranges (as the reviewer notes) from experiment to experiment between 2-3 fold (often toward the side of weaker affinity), and we deal with considerable background issues when flowing the CD1d-LPC as analyte (likely due to soluble LPC in the running buffer) so we did not feel this data was suitable for publication quality and chose not to include it in our manuscript. We have attached a figure below of our binding traces and equilibrium calculations, while it looks quite good (this is reference subtracted from an Fc-coated only flowcell) it is just not highly reproducible due to the efficiency of loading (and batch to batch variation of the LPC we obtain from our source), immobilization issues and background binding. We continue to try to optimize this method but are currently trying to pursue other interaction methods that can provide a more reproducible result.



4) on p. 12, third line from bottom, in addition to Pellicci et al 2011, please include the reference for Yu, ED. et al 2011, J Immunol., which also discusses the additional K69 contact for iGb3.

We apologize for the oversight, we've included the J Immunol reference.

5) If only certain CDR3β residues allow for binding to the hCD1d-LPC complex, why is there no common sequence motive (maybe it is cryptic). Can it be that CDR3β region affects the orientation of the other CDRs in a way that the TCR can only bind when the conserved footprint cannot be maintained? How does different residues in CDR3β affect the binding orientation of the other CDR

loops that are conserved in sequence? Those questions should be addressed more in the discussion.

We have modified the discussion to expand upon the questions the reviewer raised (See page 21 of discussion). We hypothesize that the CDR3β loop sequence and the variation imposed through the use of different Jβ segments results in the shifted docking pattern. However we do not think that there is a particular CDR3β motif that endows iNKT-TCRs with the ability to recognize LPC (see discussion on page 20 and 21), rather we believe different iNKT-TCRs utilize a convergent recognition mechanism (different "cryptic" motifs as the reviewer notes).

2nd Editorial Decision 02 February 2012

Thank you for submitting your revised manuscript to the EMBO Journal. Your revision has now been seen by referee #1 and the comments are provided below. As you can see the referee appreciates the added changes and support publication here. The referee has a few minor text suggestions that I would like you to take into consideration in a final revision. Once we receive the revision, we will proceed with its acceptance for publication here.

Thank you for submitting your interesting study to the EMBO Journal.

Yours sincerely

Editor
The EMBO Journal

REFEREE REPORT

Referee #1

The revised paper by Adams and co-authors is fine, although they should consider the following points prior to publication

1) Given the poor quality of the data collection statistics (and the authors preferred maintenance of the current resolution limits), the Rfree/work values in the nominal highest resolution bin should be cited. I think it is also a little strong to state "the ed for the LPC headgroup is unambiguous (page 10)." The accompanying figure indicates that the ed was interpretable.

2) Given the corresponding a-GC complex has not been solved, the statement at the end of the introduction that LPC docking requires substantial shifts need to be tempered. Firstly, the term substantial is an exaggeration, and more importantly, it is unclear whether this docking mode is specific for LPC

3) The statement (page 12) that the CDR3a loop has been invariably observed to contact the lipid Ag in all other complexes is incorrect. In the PI complex (Mallevaey et al Immunity 20011), the CDR3a loop did not directly contact the Ag - it was water-mediated only (and the authors cannot reliably comment on water-mediated contacts at this resolution for their lyso complex).

4) While the NKT TCR-CD1d-aGC affinity values reported by Gadola (JEM 2006) were circa 5-6uM, other human iNKT TCR affinity values for CD1d-aGC were much higher (eg 0.5uM, Wun JEM 2008), so it is a little misleading to cherry-pick the affinity values that are consistent with values reported here.

5) BSA values quoted are too accurate - should round up/down accordingly to the nearest ten

I would like to congratulate the authors on a fine and important study

2nd Revision - authors' response 02 February 2012

Referee #1

The revised paper by Adams and co-authors is fine, although they should consider the following points prior to publication

1) Given the poor quality of the data collection statistics (and the authors preferred maintenance of the current resolution limits), the Rfree/work values in the nominal highest resolution bin should be cited. I think it is also a little strong to state "the ed for the LPC headgroup is unambiguous (page 10)." The accompanying figure indicates that the ed was interpretable.

We have included the high resolution Rwork and Rfree values in Table 1. We have modified the quoted sentence, replacing “unambiguous” with “apparent” in reference to the electron density for the LPC headgroup.

2) Given the corresponding a-GC complex has not been solved, the statement at the end of the introduction that LPC docking requires substantial shifts need to be tempered. Firstly, the term substantial is an exaggeration, and more importantly, it is unclear whether this docking mode is specific for LPC

We believe the reviewer might have misunderstood the phrasing we used: The term substantial refers to movement of the CD1d alpha 1 helix (2.3Å) and the LPC headgroup (~7.5Å) upon ligation of the TCR, not the docking mode of the TCR. These shifts are not seen in the uncomplexed/complexed aGalCer structures. Instead we used the phrase “subtle shifts” in the next sentence when referring to the differences in TCR docking mode, as we agree that “substantial” would be too strong for referring to the differences in TCR docking.

3) The statement (page 12) that the CDR3a loop has been invariably observed to contact the lipid Ag in all other complexes is incorrect. In the PI complex (Mallevaey et al Immunity 20011), the CDR3a loop did not directly contact the Ag - it was water-mediated only (and the authors cannot reliably comment on water-mediated contacts at this resolution for their lyso complex).

The CDR1a and CDR2a essentially block the CDR3a from contacting LPC even by water mediated contacts (this wasn’t clear in our text). Unfortunately Mallevaey et al. have not released the coordinates for the PI complex, so it is impossible to analyze in detail their structure but we have modified the text on Page 12 to include discussion of water mediated contacts and to clarify that the CDR1a and CDR2a loops block CDR3a contact.

4) While the NKT TCR-CD1d-aGC affinity values reported by Gadola (JEM 2006) were circa 5-6uM, other human iNKT TCR affinity values for CD1d-aGC were much higher (eg 0.5uM, Wun JEM 2008), so it is a little misleading to cherry-pick the affinity values that are consistent with values reported here.

We have modified the text to include the term “some” so as to make it more clear to the reader that some, but not all, iNKT-TCRs have affinities close to that what we measured in our study.

5) BSA values quoted are too accurate - should round up/down accordingly to the nearest ten

We have modified the BSA values accordingly.

I would like to congratulate the authors on a fine and important study

