

Sideways lipid presentation by the antigen-presenting molecule CD1c

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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 manuscript, Cao et al. used a powerful lipidomics platform developed by the authors to identify endogenous lipids trapped between CD1c and an autoreactive TCR (3C8). Surprisingly, lipids with bulky headgroups were found to be permissive for 3C8 binding, even though the tight interface between 3C8 and CD1c in a previously reported crystal structure of the complex is inconsistent with the upward positioning of headgroups observed in conventional lipid presentation by CD1. To resolve this conundrum, Cao et al. determined structures of CD1c-MAG-GD3, CD1c-MAG-GM3, and CD1c-MPM-GM1 complexes, revealing two lipids in the CD1c binding cleft. Bulky ganglioside headgroups pointed sideways rather than upwards such that they did not interfere with TCR binding. Sideways presentation is probably a general mechanism for CD1c, alongside the standard upright mode. The lipid analyses and structure determinations are technically well done and the results support the authors' conclusions. This study would have been considerably enhanced by determining the structure of TCR 3C8 bound to CD1c-MAG-GD3, CD1c-MAG-GM3, CD1c-MPM-GM1, or other two-lipid CD1c ligand. Indeed, it is rather surprising that such a complex was not reported here and so will likely be published elsewhere. Nevertheless, this work represents an important contribution to our understanding of lipid display by CD1c.

Reviewer #2

(Remarks to the Author)

This manuscript reports an unexpected mode of CD1c-lipid interaction, discovered during characterization of cellular lipids bound by recombinant CD1c expressed in mammalian cells that were permissive for binding to the 3C8 abTCR, previously shown to interact with CD1c with a tight interface inconsistent with large lipid head groups. CD1c molecules interacting with 3C8 TCR exhibited a surprising bimodal GF profile, with small single-side chain monoacyl glycerols and fatty acids in early (strongly-excluded) fractions, and glycolipids and dual side-chain PS, PC, SM in weakly and non-interacting fractions. Structural characterization of CD1c bound to several of the glycolipids revealed another surprise - lipids with a large glycolipid head group bound to CD1c in a site previously found to be occupied with "stuffer lipid" fragments, and projected "sideways" out of the F' pocket through a novel G' portal, where their head groups could potentially interact with TCR in a novel binding mode. This binding occurred in addition to other smaller lipids binding in a more conventional "upright" mode, with headgroups projecting out near the A' pocket. Tetramer staining and single-cell TCRab/gd sequencing revealed many TCR about to bind. Simultaneous presentation of two different antigens bound in different binding sites would seem to be completely novel in the family of MHC and MHC-like proteins. Some effort was made to understand the potential effects of such 2:1 antigen presentation on T cell recognition, but a straightforward overarching picture did not seem to emerge. Positive, negative, or null effects of "sideways" presentation were observed by SPR, tetramer staining, and transfectant activation assays for different TCRs, even for the same lipid combinations. Regardless the results substantially expand our understanding of CD1c lipid presentation.

Some key points to address:

1. I'm having trouble understanding the stoichiometry of TCR interaction with the various CD1c-lipid complexes. How does the reduced 3C8 TCR engagement observed with occupancy of the F' site as measured by SPR (Fig 2b, GD3 vs endo) fit with the lack of an effect on 3C8 engagement measured by tetramer binding (Fig 2a, GD3 vs endo)? Do the lower and higher RUmax levels observed by SPR represent monovalent and divalent engagement of TCR by CD1c? If so can the stoichiometry be validated experimentally? In other MHC-TCR systems the stoichiometry has been characterized by SEC/MALS, ITC, AUC and other methods. For example, a SEC/MALS experiment for the gel permeation chromatography

run shown in Fig 1 should be possible.

2. Is this the same as for DN6 TCR in Fig 4? Here there is reduced binding of GD3 vs PM as measure by SPR, with similar K_d (consistent with Fig 2) but the tetramer binding signal is much reduced or negative for GD3 vs PM (different from Fig 2), and the PM+GM3 response is attenuated vs PM alone (Fig 4b), consistent with reduced response in Jurkat transfectants (Fig 4c). This seems to imply a negative effect on TCR binding for the sideways presented lipids.

3. On the other hand, GD3 or GM3 seems to provide substantially increased tetramer binding for polyclonal ab T cells from donor 2 as compared to endogenous-lipid loaded or mock tetramers (Fig 5b). Is the implication that in this case the recognition of sideways-presented lipids has a positive not negative effect? That would seem to be supported by the results with GL1 and GL4 TCR transductants (Fig 5c), which show increased binding of GD3+PM versus GD3 or PM alone. This behavior seems quite different from that observed for the 3C8 and DN6 TCRs tested in Figs 2 and 4. SPR experiments for TCRs GL1 and GL4 might help to resolve this apparent contradiction.

4. GD3 seems not to be in the eluted lipid pool but is used in many of the experimental tests including crystal structure and TCR binding. It is described only as a "bulky glycolipid" and seems to be considered with the "long-chain / large-headgroup" lipids. What exactly is GD3 and why is chosen? Chemical structure shown in Fig 2d shows only the head group. The manuscript lists Avanti as the source, and their catalog lists "ganglioside GD3", but this apparently a crude preparation from bovine milk that seems to contain a mixture of both head groups and lipid tails and which seems to be an odd choice when synthetic analogs are available.

5. An outstanding question is whether particular lipids are required for TCR binding to CD1c, or whether some lipids are inhibitory for interaction but many different permissive lipids are generally recognized similarly. Answers to these questions are important for understanding possible roles in self-lipid recognition and homeostasis versus a potential role for particular lipids in autoimmunity or pathogen recognition. With this context, the statement from the discussion "Accordingly, while the sideways presentation of gangliosides can lead to the formation of TCR recognition determinants, the major consequence of such presentation is to not impede the broader CD1c-restricted autoreactive TCR repertoire" (lines 264-267) seems pretty definitive given the variety of experimental data presented for different TCRs. What is rationale for prioritizing the neutral effects of sideways lipid presentation in the big picture over the clear positive (GL1, GL4, F10) or negative (3C8, DN6, and SM 34:1 blocking reported in ref 3) effects seen with individual clones?

6. Neither the method used for TCR sequencing nor the actual sequences identified were provided for the data shown in Suppl Fig S8 and used for experiments in Fig 5. Sequences for both tetramer-sorted ab and gdTCR should be provided.

Minor issues:

1- Fig 2a, are the differences in tetramer binding for the various CD1c complexes significant? Data from only single experiment are shown.

2- The experimental data in Fig 5b are not described clearly in the text (lines 233-235). What specific alterations are being referenced here, and are the effects positive or negative (and statistically significant?)

3- Sup Fig S1 is missing from my pdf copy of the extended data.

4- Sup Fig S8c also missing, Fig S8d only has axes and labels

5- SCARB1-negative HEK cells are used for TCR interaction studies— what is SCARB1 and what is the significance of deleting it?

6- Is the effect of open and closed CD1c conformers clear? The change in interhelical distance and orientation is described as critical for TCR interaction (lines 217-221), with DN6 and 22.5 TCRs favoring the open conformer and GD3 promoting the closed, but what is the actual evidence for this phenomenon as compared the TCR recognition differences being due to direct TCR-lipid interactions instead of indirect effects on CD1c conformation?

7- Would TCR engagement of lipid presented by "sideways" binding mode need to be substantially different from previously observed "skewed" or "tilted" binding modes suggested to be important for autoreactive TCRab-MHCII binding? Does modelling show that that a two TCR interaction is even sterically possible, or is there a steric block on 2:1 TCR:CD1c binding reflecting the 2:1 lipid:CD1c presentation mode? Would this require a non-CDR3-centric binding mode?

Reviewer #3

(Remarks to the Author)

In this manuscript, Cao et al describe an interesting observation – the ability for CD1c to present lipid molecules at a second site that is only partially overlapping with the canonical site. This site has a wider degree of lipids that can be presented while retaining recognition of T cells, due to the site being not centered upon the TCR's typical recognition interface. The work here is well presented, comprehensive around lipidomics, structure, and some function, and interesting – the biggest questions revolve around whether these results amount to an interesting corner case in terms of the biology or whether it carries an important immunomodulatory function. The paper would be strengthened by the authors commenting more upon this.

Some specific comments:

- The authors use a mass spec fractionation approach to identify novel lipids, including this class of gangliosides that can be presented 'sideways' in the antigen binding groove. While the data that these lipids can be present in CD1c is convincing, it is difficult to contextualize exactly how common or uncommon these lipids are in terms of a cell's repertoire of CD1c. Are the bulky lipids found on a large portion of the CD1c on a cell surface (and across cell types), or is it more of a rare occurrence, with the large majority of CD1c solely expressing previously characterized lipids? Is there a way to quantify this? This would seem to help determine how big of an effect presenting bulkier lipids may have, especially if it's proposed to be a blocking mechanism.

- The authors make some reasonable speculation about the potential functions of the second lipid being presented, and the evolutionary conservation of the G' portal could suggest that this is functionally important. However, it's also possible that this is an interesting curiosity that doesn't carry much immunological impact - instead, it could be a neutral effect of a

secondary lipid binding site that is interesting to note without having much immunological impact. This doesn't detract from the observation here and I wouldn't expect the authors to go find a phenotype, but I'd encourage at least some moderating language to this effect added to the discussion section somewhere.

- The reduction of tetramer staining and SPR R_{max} are interesting results, and the proposed rationale (an accessible and an inaccessible conformation of the lipid) makes sense. Would the reduction in R_{max} suggest that the authors would guess that the occupancy in each conformation is ~50%, and perhaps kinetically trapped to not readily interconvert?

Minor:

- Line 205, there was an extra GD3.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The authors have responded satisfactorily to the previous critiques.

Reviewer #2

(Remarks to the Author)

The revised manuscript includes clarifications that address ambiguities in the original manuscript, and the new supplemental movies help the reader visualize the stoichiometry issues and conversion between proposed open and closed confirmations. All other issues brought up in the original manuscript were addressed.

Reviewer #3

(Remarks to the Author)

My comments have been addressed and I support publication in this form

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