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Galactose modified iNKT cell agonists stabilized by an induced fit of CD1d prevent tumor metastasis

Sandrine Aspeslagh, Yali Li, Esther Dawen Yu, Nora Pauwels, Matthias Trappeniers, Enrico Girardi, Tine Decruy, Katrien Van Beneden, Koen Venken, Michael Drennan, Luc Leybaert, Mrs. Jing Wang, Richard W. Franck, Serge Van Calenbergh, Dirk M. Zajonc and Dirk Elewaut

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Transaction Report:

(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 14 March 2011

Thank you for submitting your manuscript to the EMBO Journal. Three referees have now seen your study and their comments are provided below.

As you can see, the referees find the study interesting and suitable for publication in the EMBO Journal. They raise some issues, mostly minor ones, with the analysis that should not involve too much additional work to address. The most significant one is raised by referee #3 who would like to see the inclusion of KD measurements to look at the binding affinities between glycolipids and mCD1. Given the comments provided, I would like to invite you to submit a suitably revised manuscript for our consideration. 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 initiative, 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 (Remarks to the Author):

Aspeslagh and colleagues present a manuscript describing functional, structural and biophysical approaches to understanding iNKT cell recognition of a set of novel a-GalCer derivatives. These derivatives were designed for enhanced Th1 polarized potency, and as the authors describe, one of these NU-a-GalCer appears to fit the bill. The structural work demonstrates structural flexibility of CD1d which has only been seen in a few cases previously and is thus highly novel. This "induced-fit" of NU-a-GalCer suggests that CD1d can accommodate lipids not just through the binding of their lipid tails, but also through modification of the portals that bind the more polar headgroups.

Overall this is a beautiful paper that is very well-written, easy to follow and the complement of structure/biophysics/function is very satisfying. The results describe a novel candidate compound for treatment of tumor metastasis, and the data is internally consistent. Overall this is certainly worthy of publication in EMBO. This reviewer only has one very small comment: the second sentence of the discussion suggests that affinity between the lipid and CD1d was actually calculated, which it was not. Perhaps this should be qualified to say "the apparent affinity" or "enhances the association of..." just to not confuse true binding measurements with what looks to be obvious. (I'm sure this was just an oversight.)

Referee #2 (Remarks to the Author):

This MS is an extended functional and structural analysis of a 6'-OH substituted analogue of αGalCer previously described by these authors (here referred to as NU-αGalCer). The NU-αGalCer compound induces a "Th1-biased" iNKT response and pronounced anti-tumor activity similar to what has been described for the C-glycoside variant of αGalCer (α-C-GalCer). Most importantly, they present a detailed and in depth analysis of TCR binding and of ternary complex structure by X-ray crystallography. The results are potentially important because they reveal a significant difference between the interactions of NU-αGalCer versus α-C-GalCer with mCD1d. Thus, in spite of the mostly similar biologic properties of these two iNKT cell activators, the analysis of NU-αGalCer reveals a different route to the design of potent analogues that might deliver enhanced anti-tumor responses and other useful activities. Their data also suggest that NU-αGalCer shows more activity with human iNKT cells than α-C-GalCer, which further heightens the potential importance of the current study.
Overall, the MS reports original data that contribute to advancing our understanding of structure-activity relationships for glycolipid antigens recognized by iNKT cells. A substantial amount of data is presented to combine biological and structural insights. This should be of interest to a significant fraction of the journal's readership. The work is well done and comprehensive in most regards. There are no major flaws, but a number of minor issues could be addressed by the authors:

1. Second sentence of Discussion: Referring to the effect of the additional binding pocket for the aromatic group, they state "This in turn enhances the affinity of the glycolipids for CD1d..." A logical conclusion, but this appears to be speculation and should probably be stated as such. There does not appear to be any direct data assessing the stability of the NU-αGalCer or other glycolipids with CD1d in the MS. This is arguably the major deficiency in the data in the MS. If the authors have data addressing this point they might consider adding it as supplemental information to make their study more complete.
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11. Fig. 1a: structures of αGalCer and xylo-αGalCer look identical.
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To understand how the bulky 6' naphthylurea moiety of NU-α-GalCer is accommodated in the interface between the iNKT TCR and mCD1d, the authors determined the crystal structure of the corresponding TCR/NU-α-GalCer/mCD1d complex to high resolution (2.3 Å). Compared to the TCR/α-GalCer/mCD1d complex recently reported by Rossjohn and colleagues, the α1 and α2 helices of mCD1d are opened laterally to accommodate the aromatic NU group, and the Met69α1 side chain undergoes a shift to create a hydrophobic pocket that binds NU as a third anchor, in addition to the two lipid chains. On this basis, the authors ascribe the stronger Th1 response of NU-α-GalCer than of α-GalCer (or the other glycolipids tested) to tighter binding to mCD1. Although this is a reasonable supposition, the authors do not provide biochemical data that actually demonstrate enhanced stability of the NU-α-GalCer/mCD1d complex, which is a serious limitation of this otherwise immunologically significant and technically very well done study. Indeed, the authors state in several places (e.g. abstract, 1st paragraph of discussion) that NU-α-GalCer has enhanced affinity for mCD1. The 2008 JACS paper first reporting NU-α-GalCer and other 6'-derivatized analogs also did not include binding data.

To strengthen their conclusions and improve their manuscript, the authors should carry out KD measurements using methods developed by Teyton and colleagues for α-GalCer and other glycolipids [J. Immunol. 170, 4673 (2003)]. These methods are IEF and/or ITC, which gave equilibrium binding constants that were in general agreement. In this way, the authors would at least be able to rank NU-α-GalCer and the other glycolipids in terms of their relative (if not absolute) affinities for mCD1.

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1st Revision - authors' response 31 March 2011

Point by point responses to referees

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We thank the referee for his appreciation of the paper. As requested we adapted the second sentence of the discussion.

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Panel C of Figure 7 shows the lipid stability assay and a brief method (p. 16), figure legend (p.21) and result section (p. 10) have been incorporated into the manuscript.

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This has been adapted in the revised manuscript

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We have previously assumed that lipid dissociation from CD1d can occur, which would have an effect on TCR dissociation. However, we have previously analyzed the lipid decay from CD1d using a CD1d and α-GalCer specific antibody (from Dr. Porcelli) and concluded, that α-GalCer for example does not come off at all during the course of the experiment and under our experimental conditions. We have unpublished data (native IEF gels using charged lipids) indicating that increased concentration of Tween 20 (0.2% instead of the 0.005% used in the Biacore running buffer) can help unload the lipid from CD1d and, therefore, performed the Biacore analysis in absence of Tween20. However, this observation forms the rational for repeating CD1d-stability assay under increasing Tween 20 detergent conditions.
Also, lipids with different binding kinetics, such as a-GalCer versus a-C-GalCer are not measured in the same experiment, as the TCR comes off after 30 min in the case of a-GalCer, while for a-C-GalCer dissociation takes place in only 3 minutes. Therefore, each SPR experiment is tailored for the particular glycolipid or group of glycolipids that have similar kinetics.
An earlier study (McCarthy et al. J Exp Med. 2007), did directly analyze the lipid decay factor during SPR experiments by injecting the TCR from low to high concentration and also from high to low and saw similar kinetic behavior. The authors concluded that lipid decay has no noticeable effect of the binding kinetics. Taken together, noticeable lipid decay does not occur under our modified experimental conditions used in our SPR studies.

4. Page 9; "Specifically, the 2"-, 3"- and 4"-OH groups of the galactose ring form H-bonds with...respectively". Not clear exactly what "respectively" means here (there are three OH groups by two TCR residues mentioned). This could be rewritten for better clarity.

We agree and have adapted the revised version accordingly.

5. Discussion, page 11: "...where it form..." should be corrected to "...where it forms..."

This has been adapted in the revised manuscript

6. Page 11: the sentence beginning with "Although the structure of OCH does not reveal..." could be rewritten for better clarity. What is being referred to is the structure of the OCH-h CD1d complex.

Rather than "by containing a spacer", it would be more clear as "because of the presence of a spacer".

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As requested, we have included these data in attachment. We show that A20 cells with the tail deleted form of CD1d are also able to present NU-aGC, aGC and xylo-aGC, however to a lesser extent which at least indicates that their presentation is not strictly dependent upon endosomal loading.

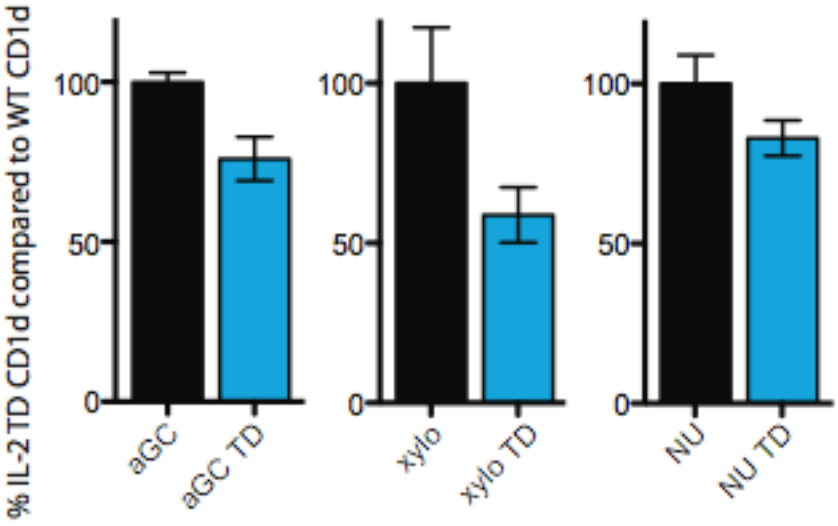

Figure
Results
response to APCs expressing tail deleted (TD) form of CD1d
-A20 lymphoma cells transfected with CD1d or TD CD1d
-loading time with glycolipid: 3 or 5 hours
-IL-2 results were normalized to WT CD1d in order to evaluate role of endosomal loading which is only possible in the presence of the 'tail'
-one experiment representative for 3 experiments
CONCLUSION: all 3 GLs can be presented by the TD form of CD1d

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This was addressed in point 1

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GalCer, which allows continuous replenishment of cell surface complexes.

This is indeed correct, we have added your comment to the text.

11. Fig. 1a: structures of αGalCer and xylo-αGalCer look identical.

We agree with the referee that the font was too small. We have adapted this figure therefore. xylo – aGC is a stereoisomer of aGC (OH at position 3 of the sphingosine chain).

12. Figs. 1 and 2: the cartoons showing Petri dishes of cells, mice and syringes are unnecessary and could (should?) be eliminated.

They have been eliminated from the revised manuscript.

13. Figure S1 does not show what page 5 says it should (3 lines up from bottom of page) - i.e., the IL-12 response to NU-a-GalCer. It looks like an error, substituting S1 for S2.

This is indeed correct. We apologize for the mistake, which has been corrected.

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As requested they have been adapted.

15. Fig. S3: why is the %tet positive cells so low in liver at day 1? Baseline levels are generally much higher. Does this reflect TCR downmodulation and inability to detect iNKT cells with tetramers at earlier time points?

According to our data also with GSL-loaded BMDCs there is already TCR down modulation after 24h. DMSO-BMDCs do not induce TCR internalization, nor iNKT expansion. We only showed day 5 results for DMSO loaded BMDC in order to have a clear graphical representation of the data. However this result is comparable at each of the measured timepoints. To make the figure more clear, we have adapted the original figures (figure 3 and S3).

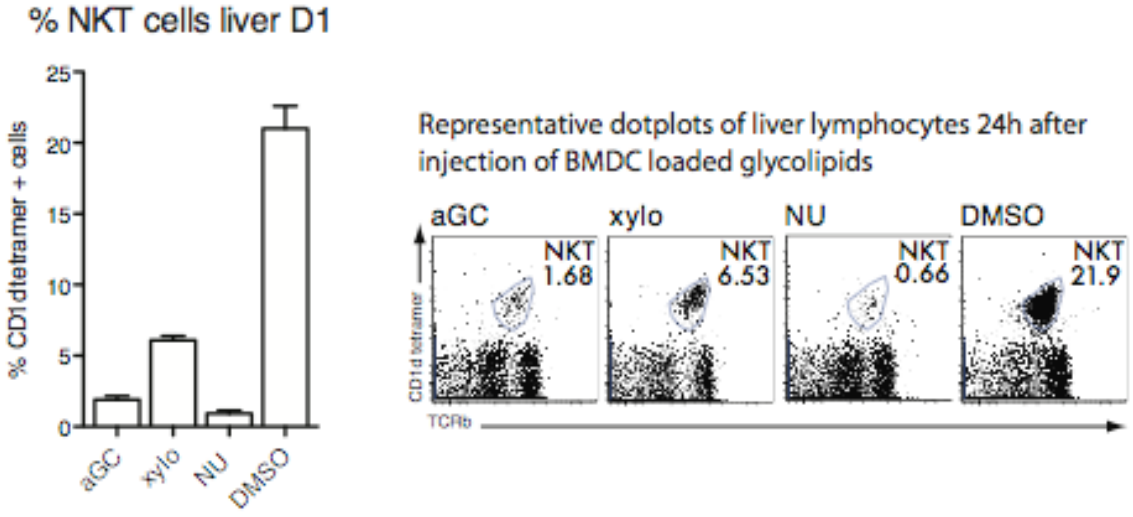

Figure: Percentage iNKT cells 24hours after injection of BMDCs loaded with glycolipids, the 3 glycolipids clearly induce disappearance of iNKT cells in contrast to DMSO

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(see also point 1 referee 2)
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A panel showing the overall CD1d-NU-αGalCer-TCR structure has now been incorporated in Fig. 6 as panel a.

2nd Editorial Decision 13 April 2011

Thank you for submitting your revised manuscript to the EMBO Journal. I asked referee #3 to review the revised version and I have now received the comments. As you can see below, the referee appreciates the introduced changes and supports publication here. I am therefore pleased to accept the paper for publication here. You will receive the formal acceptance letter shortly

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

Editor
The EMBO Journal

REFEREE REPORT

Referee #3:

The authors have responded satisfactorily to the reviewers' concerns, especially with regard to measuring relative affinities of CD1d ligands.

