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Adaptability of the semi-invariant natural killer T cell receptor toward structurally diverse CD1d-restricted ligands

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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 16 July 2009

Thank you for submitting your manuscript to the EMBO Journal. Your study has now been seen by three experts in the field and their comments to the authors are provided below. As you can see there is an interest in the study. While referees #2 and 3 raise relative minor concerns, referee #1 raises more significant ones regarding the experimental design. In particular, this referee finds that further titration studies are needed in order to further support the findings reported. Given these comments, I would like to ask you to submit a suitably revised manuscript that takes into consideration the specific points raised by referee #1 as well as the other issues raised by referees #2 and 3. I should remind you that it is EMBO Journal policy to allow a single round of revision only and that, therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript.

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):

General comments
Although I agree with the authors that we still know little about the structural basis of diverse glycolipid Ag recognition by iNKT TCR, their conclusions about the role played by the TCRb chain rely exclusively on indirect functional observations and therefore, remain quite speculative. This study would be significantly strengthened by more detailed surface plasmon resonance / thermodynamic analyses of TCR/Ag interactions. I am also concerned by the fact that most functional studies have been done at a single antigen concentration : without detailed titration studies and estimation of the EC50 (Ag concentration yielding the half maximal response), I doubt that one can accurately compare the functional avidities of natural and mutant iNKT hybridomas for the set of glycolipid Ag tested here.

Specific comments
1. In Fig.1A, the Vb14 and Vb8.2 hybridomas seem to recognize aGalCer equally well although titration experiments shown Fig.3B indicate an overall lower avidity of Vb14 than Vb8.2 hybridomas for this Ag. This underlines the need for more in depth titration studies throughout the manuscript.
2. It is very difficult to conclude from the data shown in Fig.1C that the hybridomas respond similarly after CD3 crosslinking. There are only two informative points (1 and 10 ug/ml of anti-CD3 Ab). Moreover while some hybridoma yield maximal IL2 response at 1 ug/ml of anti-CD3, several do not which would indicate heterogeneous response patterns. The authors should perform a more detailed titration study (eg 3 fold instead of 10 fold Ab dilutions starting from 0.1 ug/ml to 27 ug/ml of anti-CD3).
3. The ratio of the response to Ag vs anti-CD3 in Fig.1D does not mean so much for the aforementioned reasons, unless the authors calculate the EC50 in each stimulation conditions.
4. How accurate and quantitative is the assay shown in Fig.2 ? The band intensities in the absence of competitor and the % of inhibition are quite heterogeneous from one compound to another (ranging from 60 to 230 AU in the former case and from 15 % to 65 % in the latter case). Can the author really conclude that for instance 3'-N-acetyl derivative, which yields about 15 % inhibition of aGalCer binding at maximal concentration (lower left panel) binds CD1d “quite well” ?

Referee #2 (Remarks to the Author):

The manuscript entitled "Adaptability of the semi-invariant natural killer T cell receptor toward structurally diverse CD1d-restricted ligands" by W. C. Florence et al. describes the TCR requirements for productive interaction with mouse CD1d loaded with different analogs of lipid antigens.

This study make the novel point that the recognition of three different antigens is probably made with similar docking of the TCR with the CD1d-antigen complex and that the relative importance of the CDR loops in the TCR β chain varies according to the antigen.

The manuscript clearly describes the experimental data, the figures appropriately summarize the large amount of work, and the discussion adequately points the relevant aspects of the study. The reference list is adequate.

This referee has the following minor issues. Their consideration may improve the quality of the work.

At page 9 it is stated that "above data suggest that CDR3β together with CDR1β and CDR2β dictate NKTcr's ability to recognize structurally diverse ligands". The data do not consider the importance of other molecules, different from TCR, which might participate in antigen recognition. The results therefore might be influenced also by other molecules which are differently expressed by tested hybridomas. The authors may smooth their statement.

I have a problem with the experiments shown in figure 2 and with the readout of the competition assay. For example, the reduction of binding with 3'-N-acetyl- c -GalCer changes from 145 to 120 x

10-3. This is less than 20%. Is it significant? Another caveat of the assay is that it is difficult to evaluate micelle formation when increased amounts of analog are mixed with fixed amounts of biotinylated α-GalCer. In that case the decreased signal could be due to a reduced availability of biotinylated α-GalCer and not to competitive binding of the analogs.
Since all the molecules stimulate at least one hybridoma, it is clear that all the analogs also bind to CD1d. Therefore, I suggest to eliminate this figure.
If the authors want to carefully investigate the binding of each ligand to CD1d, they should use other assays permitting better quantitation.

In figures 5 and 6 the CDR1 α L32A mutant shows increased reactivity with all tested analogs. Is this mutant autoreactive? It is not discussed.

In figure 8 red and blue colors indicate "residues that decreased (or increased) binding". It would be more appropriate to write "that facilitate (or reduce) T cell activation", which is the test used to assay the mutants.

In the Results the indication of several mutants is different from that used in the figures. For eample, at page 14, line 9, it is indicated mutant D29A, whereas in the figure there is mutant N29A. Other mutants are indicated in a wrong manner. The authors should make homogenous the text with the figures.

Page 10, line 8, it is indicated Fig. 2A, it should be 3A.

Referee #3 (Remarks to the Author):

invariant NKT cells are the archetype of innate-like T lymphocytes and express a conserved semi-invariant TCR. They play an important role in cancer control, infections and autoimmunity. Their effector functions are elicited upon recognition of very different lipid antigens, including glycosphingo and glycoglycero lipids, presented by CD1d. In this study, Florence and coll have addressed a very relevant issue in the physiology of iNKT cells, namely what are the structural bases for the recognition of a remarkably large series of molecularly distinct lipid ligands by a TCR made of an invariant Va chain paired with a limited set of variable Vb chains.
Authors utilize a balanced blend of cellular assays with a large number of different lipid analogues presented to a panel of iNKT cell hybridomas expressing the invariant Va14-Ja18 chain paired with different CD1d-restricted Vb chains, or of Vb.2-Jb1.1 point mutated hybridomas, to dissect the molecular interactions crucial for the observed antigen recognition and T cell activation. The experimental design and interpretation of the results are supported by accurate molecular modeling, based on elaborations of the known crystal structures of the NKTcr-lipid-CD1d trimeric complex.

The results obtained by Florence et al disclose a novel role for the NKTcr CDR3beta region in determining the fine specificity of recognition of molecularly distinct glycolipid antigens, in addition to confirming the relevance for the NKTcr "hotspots" CDR1alpha, CDR3alpha and CDR2beta in docking onto the lipid-CD1d complex.
This novel information is both relevant and timely, and help moving forward in the understanding of the unique features of iNKT cell physiology.

Minor comments:
1. It would help to state in the main text that the different glycolipid analogues do not induce conformational changes in the groove of CD1d because they bear different hydrophobic moieties (fatty acid and/or sphingosine length and saturation);
2. Results p.10: Fig 2A should be labeled Fig.3A

1st Revision - authors' response 18 August 2009

Point-by-point response to the criticisms of the original EMBOJ-2009-071640 manuscript entitled "Adaptability of the semi-invariant natural killer T cell receptor toward

structurally diverse CD1d-restricted ligands" by WC Florence et al. Our responses are italicized and provided beneath each critique.

Response to Referee #1:

General comments
Although I agree with the authors that we still know little about the structural basis of diverse glycolipid Ag recognition by iNKT TCR, their conclusions about the role played by the TCRb chain rely exclusively on indirect functional observations and therefore, remain quite speculative. This study would be significantly strengthened by more detailed surface plasmon resonance / thermodynamic analyses of TCR/Ag interactions. I am also concerned by the fact that most functional studies have been done at a single antigen concentration : without detailed titration studies and estimation of the EC50 (Ag concentration yielding the half maximal response), I doubt that one can accurately compare the functional avidities of natural and mutant iNKT hybridomas for the set of glycolipid Ag tested here.

We very much appreciate the positive yet critical comments provided by this Reviewer. We have attempted our best to address every one of them systematically.

We fully appreciate the suggestion that "This study would be significantly strengthened by more detailed surface plasmon resonance/thermodynamic analyses of TCR/Ag interactions." Notwithstanding, this would be a major undertaking for a group that has no experience with such analyses because we will first have to generate numerous mutants, if not all those analysed here, in milligram quantities and then master the techniques and methods necessary to accomplish the task. Even if we engage in a collaborative study, SPR and thermodynamic analyses canít be accomplished in a short amount of time. To make public the interesting findings contained in this report, we have performed rigorous titration studies and EC50 values are reported in the revised manuscript new (Figure 1A and D).

We have also noted in the revised manuscript (see page 8) that iGb3 (10 g/ml) and αGalDAG (20 g/ml) could not be titrated below the concentration used in the reported analyses. We did not venture into using higher concentrations because bioavailability due to micelle formation and toxic effects of DMSO would override the outcome of the functional assays. These limitations are not surprising owing to low affinity binding to CD1d and because serious attempts at SPR analyses of iGb3 and GalDAG by one of the co-authors have been futile to date. Therefore, we are restricted to a single concentration in the case of iGb3 and αGalDAG for the reported studies.

As for antigen titration against the different mutant hybridomas, J Scott-Browne and colleagues had performed such an analysis for their manuscript (Nat Immunol 8: 1105ó1113; 2007). Because their data clearly demonstrated that the mutant hybridomas responded similarly to αGalCer, we did not deem it necessary to repeat the titration studies here again. Notwithstanding, we have noted this fact within the revised manuscript on pages 13 and 32.

Specific comments

1. In Fig.1A, the Vb14 and Vb8.2 hybridomas seem to recognize aGalCer equally well although titration experiments shown Fig.3B indicate an overall lower avidity of Vb14 than Vb8.2 hybridomas for this Ag. This underlines the need for more in depth titration studies throughout the manuscript.

*We agree with this comment because it was our oversight that the EC_{50} for the various NKTcr antigen interactions were not determined for the original report. As stated in the response to the general comments above, we have now performed rigorous titration analyses of αGalCer. The new data shown in Figure 1 provide EC_{50} of GalCer for activating Vβ8.2, Vβ7 and Vβ**14***

positive NKT cell hybridomas. Neither the half maximal response nor the EC_{50} values are too very different between Vβ8.2, Vβ7 and Vβ14 positive NKT cell hybridomas (see Figure 1A).

2. It is very difficult to conclude from the data shown in Fig.1C that the hybridomas respond similarly after CD3 crosslinking. There are only two informative points (1 and 10 ug/ml of anti-CD3 Ab). Moreover while some hybridoma yield maximal IL2 response at 1 ug/ml of anti-CD3, several do not which would indicate heterogeneous response patterns. The authors should perform a more detailed titration study (eg 3 fold instead of 10 fold Ab dilutions starting from 0.1 ug/ml to 27 ug/ml of anti-CD3).

We have performed such a titration study as shown in revised Figure 1D. The data clearly demonstrate that the hybridomas have very similar sensitivity to antigen-independent stimulation. Therefore, the differences in response to different antigens and their analogues most likely owe to the extend of antigen recognition.

3. The ratio of the response to Ag vs anti-CD3 in Fig.1D does not mean so much for the aforementioned reasons, unless the authors calculate the EC50 in each stimulation conditions.

In the revised Figure 1, we have removed this form of analysis but instead clearly show that the EC50 for antigen-independent stimulation of all hybridomas irrespective of Vβ usage is similar (Figure 1D).

4. How accurate and quantitative is the assay shown in Fig.2 ? The band intensities in the absence of competitor and the % of inhibition are quite heterogeneous from one compound to another (ranging from 60 to 230 AU in the former case and from 15 % to 65 % in the latter case). Can the author really conclude that for instance 3'-N-acetyl derivative, which yields about 15 % inhibition of aGalCer binding at maximal concentration (lower left panel) binds CD1d « quite well » ?

As suggested by Reviewer 2, we have dropped this aspect of the original manuscript because of the inherent difficulty with estimating bioavailability of the lipid antigens especially at high concentrations. Additionally, also as Reviewer 2 pointed out, "Since all the molecules stimulate at least one hybridoma, it is clear that all the analogs also bind to CD1d". Therefore, the point made in the original manuscript regarding antigen binding to CD1d would be redundant.

Response to Referee #2:

The manuscript entitled "Adaptability of the semi-invariant natural killer T cell receptor toward structurally diverse CD1d-restricted ligands" by W. C. Florence et al. describes the TCR requirements for productive interaction with mouse CD1d loaded with different analogs of lipid antigens.

This study make the novel point that the recognition of three different antigens is probably made with similar docking of the TCR with the CD1d-antigen complex and that the relative importance of the CDR loops in the TCR β chain varies according to the antigen.

The manuscript clearly describes the experimental data, the figures appropriately summarize the large amount of work, and the discussion adequately points the relevant aspects of the study. The reference list is adequate.

We very much appreciate the positive, encouraging remarks of this Reviewer and thank him for finding our work important and worth publishing after careful revision.

This referee has the following minor issues. Their consideration may improve the quality of the work.

1. At page 9 it is stated that "above data suggest that CDR3β together with CDR1β and CDR2β dictate NKTcr's ability to recognize structurally diverse ligands". The data do not consider the importance of other molecules, different from TCR, which might participate in antigen recognition. The results therefore might be influenced also by other molecules which are differently expressed by tested hybridomas. The authors may smooth their statement.

We agree that other cell surface molecule(s) could be at play in modulating antigen recognition. We have now tempered our conclusion in the revised manuscript as indicated on page 9: "Alternatively, although less likely, other cell surface molecules differentially expressed by the hybridomas could result in the observed differences in responses."

2. I have a problem with the experiments shown in figure 2 and with the readout of the competition assay. For example, the reduction of binding with 3'-N-acetyl-α-GalCer changes from 145 to 120 x 10-3. This is less than 20%. Is it significant? Another caveat of the assay is that it is difficult to evaluate micelle formation when increased amounts of analog are mixed with fixed amounts of biotinylated α-GalCer. In that case the decreased signal could be due to a reduced availability of biotinylated α-GalCer and not to competitive binding of the analogs.
Since all the molecules stimulate at least one hybridoma, it is clear that all the analogs also bind to CD1d. Therefore, I suggest to eliminate this figure.
If the authors want to carefully investigate the binding of each ligand to CD1d, they should use other assays permitting better quantitation.

We agree with the suggestion to eliminate the data contained in original Figure 2 because of the inherent difficulty with estimating bioavailability of the lipid antigens at high concentrations. Hence, the revised manuscript does not discuss the CD1d lipid antigen binding data.

3. In figures 5 and 6 the CDR1α L32A mutant shows increased reactivity with all tested analogs. Is this mutant autoreactive? It is not discussed.

No; the L32A mutant is not auto-reactive to bone marrow derived DC, the lipid antigen presenting cells used in all the assays described in our report. We have now mentioned this in the revised manuscript on page 12.

4. In figure 8 red and blue colors indicate "residues that decreased (or increased) binding". It would be more appropriate to write "that facilitate (or reduce) T cell activation", which is the test used to assay the mutants.

We agree with this suggestion and, hence, the revised Figure 7 (original Figure 8) has been changed accordingly.

5. In the Results the indication of several mutants is different from that used in the figures. For eample, at page 14, line 9, it is indicated mutant D29A, whereas in the figure there is mutant N29A. Other mutants are indicated in a wrong manner. The authors should make homogenous the text with the figures.

These oversights on pages 14 and 15 have been corrected in the revised manuscript.
6. Page 10, line 8, it is indicated Fig. 2A, it should be 3A.

Based on this Reviewer's suggestion, we have deleted Figure 2 from the revised manuscript and, hence, the new text now matches with the old figure number.

Response to Referee #3:

Invariant NKT cells are the archetype of innate-like T lymphocytes and express a conserved semi-invariant TCR. They play an important role in cancer control, infections and autoimmunity. Their effector functions are elicited upon recognition of very different lipid antigens, including glycosphingo and glycoglycero lipids, presented by CD1d. In this study, Florence and coll have addressed a very relevant issue in the physiology of iNKT cells, namely what are the structural bases for the recognition of a remarkably large series of molecularly distinct lipid ligands by a TCR made of an invariant Va chain paired with a limited set of variable Vb chains.
Authors utilize a balanced blend of cellular assays with a large number of different lipid analogues presented to a panel of iNKT cell hybridomas expressing the invariant Va14-Ja18 chain paired with different CD1d-restricted Vb chains, or of Vb.2-Jb1.1 point mutated hybridomas, to dissect the molecular interactions crucial for the observed antigen recognition and T cell activation. The experimental design and interpretation of the results are supported by accurate molecular modeling, based on elaborations of the known crystal structures of the NKTcr-lipid-CD1d trimeric complex.

The results obtained by Florence et al disclose a novel role for the NKTcr CDR3beta region in determining the fine specificity of recognition of molecularly distinct glycolipid antigens, in addition to confirming the relevance for the NKTcr "hotspots" CDR1alpha, CDR3alpha and CDR2beta in docking onto the lipid-CD1d complex.
This novel information is both relevant and timely, and help moving forward in the understanding of the unique features of iNKT cell physiology.

We very much appreciate the positive feedback from this Reviewer regarding the original manuscript. She/he had few minor comments, which are addressed in the ensuing:

1. It would help to state in the main text that the different glycolipid analogues do not induce conformational changes in the groove of CD1d because they bear different hydrophobic moieties (fatty acid and/or sphingosine length and saturation);

We agree, and have noted this on page 9 and included references to support this statement.

2. Results p.10: Fig 2A should be labeled Fig.3A

Based on Reviewer 2's suggestion, we have deleted Figure 2 from the revised manuscript and, hence, the new text matches with the old figure number.

2nd Editorial Decision 03 September 2009

Thank you for submitting your revised manuscript to the EMBO Journal. I asked the original referee # 2 to evaluate the revised manuscript and as you can see below this referee finds that the revised version has satisfactorily addressed the original raised concerns and supports publication in the EMBO Journal. I am therefore very

pleased to proceed with the acceptance of the manuscript for publication here

Editor
The EMBO Journal

REFEREE REPORT

Referee #2:

The authors have adequate modifications to the manuscript.

