EMBO reports

Manuscript EMBOR-2011-34915

CD8αα and αβ isotypes are equally recruited to the immunological synapse through their ability to bind MHC class I

Vasily Rybakin, Jean-Pierre Clamme, Jeanette Ampudia, Pia P Yachi and Nicholas RJ Gascoigne

Corresponding author: Nicholas RJ Gascoigne, The Scripps Research Institute

Review timeline:

Submission date:	23 March 2011
Editorial Decision:	27 April 2011
Revision received:	23 August 2011
Editorial Decision:	09 September 2011
Revision received:	22 September 2011
Accepted:	23 September 2011

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 27 April 2011

Thank you for the submission of your research manuscript to our editorial office. We have now received the evaluation of three expert reviewers on it.

As the reports are pasted below I would prefer not to repeat them here in detail, but to only summarize the main points raised by the referees. You will see that, while the referees agree on the potential interest of the findings, they also feel that additional work is needed to strengthen the conclusions put forward.

It becomes clear from the comments of referee 1 that s/he feels that in some instances the data are not yet fully conclusive and that several technical issues would need to be addressed. S/he remarks that the ratio of CD8 to pMHC in the experimental settings used here needs to be clarified. This referee also feels that apparent contradictions to previously reported results would need to be sorted out and states that it should be clarified whether true immunological synapses or pre-synaptic cell-cell contact sites are investigated in the experiments shown in figure 4. Referee 2 raises a concern about possible oligomer formation and reviewer 3 points out several instances in which further clarifications and technical improvements are needed.

Overall, given the reviewers constructive comments and the potential interest of the study, I would like to give you the opportunity to revise your manuscript, with the understanding that the main concerns of the referees (as outlined above and in their reports) must be addressed, especially with regard to comments 2, 4, and 6 of referee 1. Acceptance of the manuscript will depend on a positive outcome of a second round of review and I should also remind you that it is EMBO reports 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.

I look forward to seeing a revised form of your manuscript when it is ready.

Yours sincerely,

Editor
EMBO reports

REFEREE REPORTS:

Referee #1:

The manuscript by Rybakin et al. investigates the recruitments of CD8aa and CD8ab to the immunological synapse (IS) and concludes that they are essentially determined by intercellular CD8-pMHC I interactions and hence comparable for the two CD8 isoforms. The experimental approach used to address this issue, namely the use of BiFC, is good and overall the results support the conclusion. However, there are some concerns that should be addressed.

Comments

1) Thymus derived TCRab+ CD8+ T cells express CD8ab only, i.e. expression of CD8aa +/- CD8ab is a peculiarity of intraepithelial TCRab+ T cells that exert tissue-specific, specialized functions. This is should be stated to appreciate the wider biological context, namely to distinguish these cells from peripheral CD8+ CTL, which are significantly different. In the light of the present study, co-expression of CD8aa on CTL would be detrimental to their cytolytic function, which requires focussing of lck at the IS.

2) By binding to TCR-associated cognate pMHC CD8 can function as coreceptor and by binding to non-cognate pMHC on APC strengthen cell-cell adhesion and CTL signalling. On peripheral CD8ab+ TCRab+ T cells there are much less CD8ab molecules than pMHC complexes on average APC/target cells. It is not clear what the situation is in the experimental system used in the present study. If the CD8/pMHC ratio were grossly different in this system, namely the number of CD8 comparable or larger to the number of pMHC, the biological significance of the reported findings would be questionable. To appreciate this, it would be useful to add flow cytometric analysis of CD8 and MHC I surface expression of ex vivo CD8+, TCRab+ peripheral T cells to allow comparison with the hybridomas and APC under study.

3) Figures, Supplementary Figures and their legends should be revised for better clarity. For example: i) in Fig. S1B it should be specified what the last lane in the lower panel is; ii) in Fig. S3 the legend for the left hand panels indicates that the same color codes are used as in Fig. 2A; presumably this should read as in Fig. S3B ? In the same panels the x-axis values should be indicated (same thing for Fig. S4); iii) the legend for Fig. S3 contains a panel D, whereas Fig. S3 does not; iv) in Fig 3C it would be good to add a row showing the corresponding transmission images (or labelled contours of the cells) and to use the same colors in A and C; v) in Fig. 4 the y-axis should be labelled.

4) According to Fig. S3 CD8aa strengthens tetramer binding more avidly than CD8ab, which is at variance with previous reports, namely those showing that CD8ab, but not CD8aa associates with TCR/CD3. It is not clear why ? Can artefacts related to the gating strategy and fluorochrome usage be excluded ? i.e. are the same findings obtained when using sorted hybridomas are used without the gating ? It would be good to add total surface CD8aa CD8b and TCR expressions for this

experiment. It is noted that while the gating strategy used focuses on Cerulean+ and/or Venus+ cells, why should hybridomas transfected as shown in Fig. 1 not also express other, non-fluorescent CD8 species (e.g. cells that express CD8b-VN+CD8a2-CC could also express CD8a2-CC homodimers). This should be clarified, as for tetramer binding all surface expressed CD8 species are relevant; this is also pertinent to Fig. S4.

5) Data shown in Figs. 3 C, D indicate that the mutant CD8b S101A has a lesser effect on CD8 recruitment to the IS than the CD8a mutation N107A, arguing that the latter mutation stronger impairs pMHC binding than the former. If this were so, why then have these mutations essentially the same effect on tetramer binding (Fig. S4) ?

6) Data shown in Fig. 4 indicate that the presence of TL on APC promotes preferential recruitment of CD8aa to the IS, namely in the absence of cognate Kb/OVA. Since in this case there is no TCR triggering, how then can an IS be formed ? By virtue of non-cognate intercellular CD8-pMHC interactions CD8 molecules are expected to concentrate at pre-synaptic T cell-APC contact sites. This poses the question to what extent are we looking at true IS versus pMHC driven CD8 focusing at pre-synpatic cell-cell contact sites. As it has been shown previously that CD8aa and CD8ab bind pMHC with comparable affinities, in this case one would indeed expect that CD8aa and CD8ab are recruited to the contact site with comparable efficiencies.

7) The authors are encouraged to consider not only the interaction of CD8 isoformes with lck, but also with LAT and TCR/CD3, respectively.

1. The manuscript EMBOR-2011-34915V1 does report a single key finding, namely that CD8ab and CD8aa are recruited to the immunological synapse to comparable extents.

2. The reported finding is of significance and is not confirmatory, i.e. has never been previously reported.

3. The finding is of broad interest to immunologists, namely those interested in T cell immunity.

4. The single major finding is robustly documented by independent lines of experimental evidence.

Referee #2:

The authors use bimolecular complementation to study the role of two different CD8 forms in recruitment to the immunological synapse. The conventional CD8ab construct and the innate recognition associated CD8aa homodimer were differentially labeled with CFP or YFP by attaching the N-terminal half of GFP to CD8a2, the C-terminal half of CFP to CD8a1, and the C-terminal half of YFP to CD8b. In this way the CD8a1-a2 heterdimer is labeled with CFP and the CD8a-b heterodimer is labeled with YFP. The supplemental materials show some biochemistry, but the gels are cut off in a way that higher order oligomers cannot be seen if present. There is no reason the CD8a1a1 and CD8a2a2 homodimers could not form. These forms would be non-fluorescent unless they were to form higher order oligomers with CD8b or the the complementary dimers. Thus, there is a potential for higher order systems to be generated . Based on the typical applications of bimolecule complementation its my impression that these higher order-oligomers might not form unless the CD8 forms had a tendency to cluster, but its not clear since CD8 may be pre-organized in some fashion. Co-capping experiments are consistent with some unexpected interactions, although it seemed that most the dimers might be formed as expected. Since CD8 is a disulfide linked dimer is should be possible to detect the a1a1 and a2a2 homodimers by immunodepletion. If all homodimers at a1a2 then either the Ly2.1 of 2.2 should deplete everything. If a1a1 and a2a2 dimers are present then 25% should be left behind by this depletion. If higher order oligomers exist they could be detected by native electrophoresis. The oligomers could complicate that comparison quite a bit since they may have a higher avidity.

Beyond these issue the results are strongly in favor of the idea that both aa and ab dimers are recruited to the immunological synapse by binding to MHC class I molecules. aa dimers are found to bind better to TL, which is expected. Thus, altough ab binding Lck and promotes TCR signaling most effectively, aa dimer bind MHC class I similarly and this process drives interactions in contact areas.

This is an elegant study of the issue of aa based higher order oligomers can be reasonably ruled out.

The work is of high technical quality, but the they need to go further with the biochemistry to rule out a possible artifact.

Referee #3:

1. The manuscript reports a single key finding: CD8 isoypes are recruited to the immunological synapse by their ability to interact with MHC class I. The strong recruitment of the CD8aa form that acts as a relatively poor co-receptor is unexpected.

2. This work is significant. While it uses new technology to nicely confirm previous reports on the differences between CD8aa and CD8ab it also extends this work and makes a significant contribution.

3. This work should be of general biological interest. TCR coreceptors perform a unique role in biology by boosting signaling when an antigen receptor engages its ligand. T-cells have a very difficult role as they are expected to recognize, and respond to, all foreign antigens when they have never seen them before and, are unable to adapt to them. Only the unique function of CD8 and CD4 coreceptors makes this possible.

4. The single major finding is robustly documented.

SUMMARY
The T-cell coreceptors CD8 and CD4 are unique in nature as they 'co-receive' peptide-MHC ligands with the abTCR. While CD8 and CD4 share similar roles in binding to and co-receiving antigen there are several important differences between the two molecules. The most obvious structural difference between CD8 and CD4 is that CD8 consists of two polypeptides at the T-cell surface while CD4 is a single chain. The most obvious difference in terms of function is that CD8 is known to stabilize TCR/peptide-MHC interactions at the cell surface, a function not performed by CD4.

This particular study focuses on the CD8 coreceptor. CD8 performs an important role in recognition of pMHC class I (pMHCI) ligands and has been reported to enhance the recognition sensitivity of exogenously supplied of peptide by up to 1 million fold. The CD8 coreceptor can exist in two main forms - an alpha-alpha homodimer (CD8aa) and an alpha-beta heterodimer (CD8ab). CD8ab acts as ~100-fold better coreceptor than CD8aa. The reasons for the superiority of CD8ab are unclear as both molecules bind equally well to pMHCI. It is established that the dominant role of CD8 during antigen recognition is in phosphorylating intracellular immunoreceptor tyrosine activation motifs on the TCR/CD3 complex. The primary kinase undertaking this role, Lck, is known to associate with the cytoplasmic tail of CD8a. Thus one would expect that both CD8aa and CD8ab could undertake this role. Immanuel Luescher and colleagues proposed that the superiority of CD8ab as a coreceptor stemmed from the fact that CD8b, unlike CD8a, could be palmitoylated. Enrichment of CD8ab and the TCR in lipid rafts was reduced if this palmitoylation was removed. The present study confirms that CD8aa and CD8ab partition into different membrane domains (Figure S2). It also examines the role of CD8aa and CD8ab as coreceptors directly by visualizing their involvement at the immunological synapse directly using Bimolecular Fluorescence Complementation (BiFC). This is the first time that CD8aa and CD8ab have been visually tracked on the surface of the same cell when it engages antigen. I have no expertise with BiFC so I cannot comment on this aspect. However, the BiFC data in the manuscript look convincing to my non-expert eye. The results are wholly unexpected as they indicate that Lck exhibits a heavy preference for association with CD8ab when CD8aa and CD8ab are both present at the cell surface.

MAJOR CONCERNS

1. FIGURE S1B concerns me. It is very difficult to follow as presented.
It would be helpful if more description of the experiment were included in the legend. It appears as if lanes 5-8 in the top blot are just whole cell lysate from the indicated cells immunoblotted with Lck Ab without IP (its not easy to tell from the legend). If so, then why is there considerably less Lck in CD8-ve cells (lane 5) compared to CD8 +ve cells (lanes 6-8). How many times was this experiment repeated? What is the WCL in the bottom blot? Why does this show hundreds of times more Lck than WCL in the top blot? I cannot follow this given the current legend. The preferential association of Lck with CD8ab is unexpected so it is important that the authors get this right. What's the last lane on the bottom blot in Figure S1B? It is not labeled or mentioned in the legend yet it contains some Lck ? Overall, the preference of Lck for association with CD8ab over CD8aa is important so these data should be made more convincing.

MINOR CONCERNS

1. FIGURE 2: How representative are these data of the population? It is unclear exactly what the error bars in C represent. The same is true of Figure 3. It would be helpful to know how representative these data are as presumably the images show the best example in each case.

2. FIGURE S3 also concerns me. The legend mentions Figure S3D and mentions phosphor-Erk. I do not see this in the version I have downloaded. It would also be helpful if the four individual populations studied were clearly marked in the left panel in A. This is done nicely in Figure S4 and makes it much easier to follow. This is an interesting experiment and I like the assay and approach. Once again, the legend could be considerably improved to help the reader. Which cells are which in the right panel of A? The only chance I have of working this out is to take the descriptions of the populations described in the left panel of A and apply the color-coding used in the left panel of figure B. Is that correct?

3. FIGURE S4 Co-expression of MHCI binding mutant CD8a or CD8b does not affect tetramer binding. Does tetramer binding with no CD8 overlay that with CD8aa' or CD8ab' alone? i.e. Have the authors proved that these molecules don't bind to CD8 at the cell surface as they claim? Their system provides a nice way to address this issue.

The writing is clear. If the Figure S1B issue is address then I do not believe that the manuscript is overselling its claims. The presentation of the some of the figures could be improved as indicated. In particular, the legends need to include further information aimed at telling the reader exactly what is being shown.

1st Revision - authors' response 23 August 2011

Point-by-point response to reviewers. (Reviewer's comments in italics).

We thank the reviewers for their criticisms and comments. We have amended the paper in many respects in the light of these comments, and believe that it is now substantially improved.

Reviewer 1

1) Thymus derived TCR$\alpha\beta^+$ CD8$^+$ T cells express CD8$\alpha\beta$ only, i.e. expression of CD8$\alpha\alpha$ +/- CD8$\alpha\beta$ is a peculiarity of intraepithelial TCR$\alpha\beta^+$ T cells that exert tissue-specific, specialized functions. This is should be stated to appreciate the wider biological context, namely to distinguish these cells from peripheral CD8$^+$ CTL, which are significantly different. In the light of the present study, co-expression of CD8$\alpha\alpha$ on CTL would be detrimental to their cytolytic function, which requires focussing of lck at the IS.

We have re-written the relevant section in the Introduction,

2) By binding to TCR-associated cognate pMHC CD8 can function as coreceptor and by binding to non-cognate pMHC on APC strengthen cell-cell adhesion and CTL signalling. On peripheral CD8$\alpha\beta^+$ TCR$\alpha\beta^+$ T cells there are much less CD8$\alpha\beta$ molecules than pMHC complexes on average APC/target cells. It is not clear what the situation is in the experimental system used in the present study. If the CD8/pMHC ratio were grossly different in this system, namely the number of CD8 comparable or larger to the number of pMHC, the biological significance of the reported findings would be questionable. To appreciate this, it would be useful to add flow cytometric analysis of CD8 and MHC I surface expression of ex vivo CD8$^+$, TCR$\alpha\beta^+$ peripheral T cells to allow comparison with the hybridomas and APC under study.

We now present these data in Fig. S1. Anti-CD8 staining of the hybridomas showed that they had similar brightness to each other, and that this was somewhat brighter staining than for the lymph node-derived CD8+ cells. Staining of hybridomas was 1.7x brighter with anti-CD8β (a directly labeled fluorescent Ab), and 3.3x brighter with anti-CD8α1 (biotinylated + fluorescent streptavidin). The anti-CD8α2 was an IgM, and was labeled using a secondary Ab. Because of the mutivalency of the IgM, as well as the secondary Ab, we don't think it is safe to compare staining of cells with different surface areas and possibly different concentrations of the molecules under study. The biotin-avidin system may also have inaccuracies due to crosslinking/multivalency, making the anti-CD8β the safest Ab for comparison of the difference between the hybridomas and the primary cells. Whether we rely on the anti-CD8β or the anti-CD8α1, the difference in brightness is somewhere in the range of ~2–3-fold higher for the hybridomas. However, when we calculated the relative cell-surface CD8 concentrations based on the estimated surface area of each cell type, the concentration of CD8 molecules was lower in the hybridomas: 28% (CD8β) or 54% (CD8α1) of the concentration on the primary cells. This is described in the legend to S1. The cell diameter measurements and calculations can be presented if requested.

As for the amount of MHC class I antigen presenting cells, we have previously calculated that RMA-S cells loaded with peptide under our conditions have around 50,000 H2K^b molecules per cell (Yachi et al, 2007, *J Exp Med* **204:** 2747). RMA and EL4 have around 100,000 molecules per cell, as do transfected L-K^b cells (Porgador et al., 1997, *Immunity* **6:** 715). Thus the APC used here, peptide-loaded RMA-S and EL4, are comparable to each other. EL4 is a standard "good" APC and target for CD8 T cells. These data indicate that it is unlikely that the results are due to there being too much CD8 for the amount of available MHC class I, as raised by the reviewer.

3) Figures, Supplementary Figures and their legends should be revised for better clarity. For example: i) in Fig. S1B it should be specified what the last lane in the lower panel is; ii) in Fig. S3 the legend for the left hand panels indicates that the same color codes are used as in Fig. 2A; presumably this should read as in Fig. S3B ? In the same panels the x-axis values should be indicated (same thing for Fig. S4); iii) the legend for Fig. S3 contains a panel D, whereas Fig. S3 does not; iv) in Fig 3C it would be good to add a row showing the corresponding transmission images (or labelled contours of the cells) and to use the same colors in A and C; v) in Fig. 4 the y-axis should be labelled.

We have revised the legends for clarity, and added the axis values/labels as requested. Fig S1B has been replaced by a new Fig S4. Fig 3A and C now use the same color scheme, and we have added labels identifying the T cells and APCs in 3C (outlining the cell contours looked horrible).

4) According to Fig. S3 CD8$\alpha\alpha$ strengthens tetramer binding more avidly than CD8$\alpha\beta$, which is at variance with previous reports, namely those showing that CD8$\alpha\beta$, but not CD8$\alpha\alpha$ associates with TCR/CD3. It is not clear why? Can artefacts related to the gating strategy and fluorochrome usage be excluded? i.e. are the same findings obtained when using sorted hybridomas are used without the gating? It would be good to add total surface CD8$\alpha\alpha$ CD8β and TCR expressions for this experiment. It is noted that while the gating strategy used focuses on Cerulean+ and/or Venus+ cells, why should hybridomas transfected as shown in Fig. 1 not also express other, non-fluorescent CD8 species (e.g. cells that express CD8β-VN+CD8α2-CC could also express CD8α2-CC homodimers). This should be clarified, as for tetramer binding all surface expressed CD8 species are relevant; this is also pertinent to Fig. S4.

The point of this figure is simply to show that the fluorescent CD8 molecules worked to enhance tetramer binding to the TCR, which they did. The difference in tetramer binding between CD8αα and CD8αβ was surprising but consistent in several experiments, and was also seen when cell lines were analyzed individually rather than in the mixed sample. It was not due to a compensation artifact as there is no possibility of bleed-through from the cerulean channel to the PE channel, as these were excited by different lasers. The venus channel could potentially bleed into the PE channel, but as we have higher tetramer-PE staining with the αα (cerulean), this could not have affected the results. Also, these experiments used the LSR2 which has auto-compensation so eliminating operator errors in compensation. Compensation was done using single fluorescent cells and included "fluorescence-minus-one" controls. All compensation was done using the actual fluorophore.

It is true that non-fluorescent CD8 molecules can also be formed from the BiFC constructs (stated in the legend to Fig. 1), which may affect tetramer binding. The amount of CD8 expression on the cell surface is shown in Fig. S1. There was about 1.3-fold more CD8α2 present on the surface of the CD8αα-only cells than in the CD8αβ-only cells (which can also have non-fluorescent CD8αα). This could potentially account for some of the increased tetramer binding to the CD8αα-only cells. We have added another analysis in Fig. S3D, where the CD8αα+CD8αβ+ cells from S3C were sub-gated for increasing ratio of one CD8 species to the other. Tetramer binding increased as the proportion of CD8αα increased relative to CD8αβ. Fig S4 is now S5, and has been changed as suggested.

5) Data shown in Figs. 3C, D indicate that the mutant CD8β S101A has a lesser effect on CD8 recruitment to the IS than the CD8α mutation N107A, arguing that the latter mutation stronger impairs pMHC binding than the former. If this were so, why then have these mutations essentially the same effect on tetramer binding (Fig. S4)?

We don't think that the type of experiment shown in Fig. 3 can be considered as quantitative as the FACS analysis of Fig S4 (now Fig S5). We would not dare to make such a conclusion from these data.

6) Data shown in Fig. 4 indicate that the presence of TL on APC promotes preferential recruitment of CD8αα to the IS, namely in the absence of cognate Kb/OVA. Since in this case there is no TCR triggering, how then can an IS be formed? By virtue of non-cognate intercellular CD8-pMHC interactions CD8 molecules are expected to concentrate at pre-synaptic T cell-APC contact sites. This poses the question to what extent are we looking at true IS versus pMHC driven CD8 focusing at pre-synpatic cell-cell contact sites. As it has been shown previously that CD8αα and CD8αβ bind pMHC with comparable affinities, in this case one would indeed expect that CD8αα and CD8αβ are recruited to the contact site with comparable efficiencies.

In our previous publications we have used a broad definition of the IS, to mean the interface between the T cell and the APC, rather than the "doughnut" shape originally defined by Kupfer and Dustin. However, the referee's point is well taken, and we have therefore clarified the situation in the TL experiments. We believe that the concentration of CD8 to the T-APC interface in antigen-stimulated T hybridomas in the experiments reported here is not due simply to the non-cognate CD8-MHC interaction. We previously showed that CD8 recruitment occurs faster in the presence of antigen (accompanied by concentration of TCR) (Yachi et al., 2005. *Nat Immunol* **6:** 785). Without antigen, there are relatively few T cell-APC conjugates, although CD8 concentration does occur.

7) The authors are encouraged to consider not only the interaction of CD8 isoformes with lck, but also with LAT and TCR/CD3, respectively.

We have added notes on these points to the text.

Reviewer 2

1) The authors use bimolecular complementation to study the role of two different CD8 forms in recruitment to the immunological synapse. The conventional CD8αβ construct and the innate recognition associated CD8αα homodimer were differentially labeled with CFP or YFP by attaching

the N-terminal half of GFP to CD8α2, the C-terminal half of CFP to CD8α1, and the C-terminal half of YFP to CD8β. In this way the CD8α1-α2 heterdimer is labeled with CFP and the CD8α–β heterodimer is labeled with YFP. The supplemental materials show some biochemistry, but the gels are cut off in a way that higher order oligomers cannot be seen if present. There is no reason the CD8α1α1 and CD8α2α2 homodimers could not form. These forms would be non-fluorescent unless they were to form higher order oligomers with CD8β or the complementary dimers.

CD8α1α1 and CD8α2α2 homodimers, as well as CD8α1–CD8α2 heterodimers could form and be expressed at the cell surface (see legend to Fig 1). The slight increase in CD8α2 on the surface of the CD8αα-only cells compared to CD8αβ-only cells could be due to such non-fluorescent CD8αα (Fig. S1, see response to Reviewer 1, #4).

2) Thus, there is a potential for higher order systems to be generated. Based on the typical applications of bimolecule complementation its my impression that these higher order-oligomers might not form unless the CD8 forms had a tendency to cluster, but its not clear since CD8 may be pre-organized in some fashion. Co-capping experiments are consistent with some unexpected interactions, although it seemed that most the dimers might be formed as expected. Since CD8 is a disulfide linked dimer is should be possible to detect the α1α1 and α2α2 homodimers by immunodepletion. If all homodimers at α1α2 then either the Ly2.1 of 2.2 should deplete everything. If α1α1 and α2α2 dimers are present then 25% should be left behind by this depletion. If higher order oligomers exist they could be detected by native electrophoresis. The oligomers could complicate that comparison quite a bit since they may have a higher avidity.

We do not think that there are higher order oligomers in our cells, as suggested by the reviewer. As far as we are aware, there are no published data showing such higher-order dimers of CD8 – if they exist, then they would cause the same problem of interpretation in all previous studies on CD8, as there is no reason why they should form any more in our expression system than with expression of native CD8 subunits. We also have data arguing against any such higher-order structures. The experiment shown in Fig 3C,D demonstrates that CD8αα is not associated in any higher-order structure with CD8αβ: the two species were only recruited to the synapse if both could bind to MHC class I. If either the CD8αα or the CD8αβ was compromised in its ability to bind MHC-I, the compromised species was not recruited. Although the suggested immunodepletion experiments could be done in principle, we do not think that they would add much to the content of the paper.

3) Beyond these issue the results are strongly in favor of the idea that both αα and αβ dimers are recruited to the immunological synapse by binding to MHC class I molecules. αα dimers are found to bind better to TL, which is expected. Thus, altough αβ binding Lck and promotes TCR signaling most effectively, αα dimer bind MHC class I similarly and this process drives interactions in contact areas.
This is an elegant study of the issue of αα based higher order oligomers can be reasonably ruled out. The work is of high technical quality, but the they need to go further with the biochemistry to rule out a possible artifact.

We are very grateful for these positive comments on the work, but as stated above, we strongly disagree that there is any reason to suggest that CD8 forms higher-order structures in our system, any more than in other CD8-expression experiments.

Reviewer 3
MAJOR CONCERNS
1) (referring to original Fig S1B, which is now replaced with new Fig. S4)
The results are wholly unexpected as they indicate that Lck exhibits a heavy preference for association with CD8αβ when CD8αα and CD8αβ are both present at the cell surface……
Fig S1B concerns me. It is very difficult to follow as presented. It would be helpful if more description of the experiment were included in the legend. It appears as if lanes 5-8 in the top blot are just whole cell lysate from the indicated cells immunoblotted with Lck Ab without IP (its not easy to tell from the legend). If so, then why is there considerably less Lck in CD8-ve cells (lane 5) compared to CD8 +ve cells (lanes 6-8). How many times was this experiment repeated? What is the WCL in the bottom blot? Why does this show hundreds of times more Lck than WCL in the top blot? I cannot follow this given the current legend. The preferential association of Lck with CD8αβ is unexpected so it is important that the authors get this right. What's the last lane on the bottom blot in Figure S1B? It is not labeled or mentioned in the legend yet it contains some Lck ? Overall,

the preference of Lck for association with CD8αβ over CD8αα is important so these data should be made more convincing.

We are grateful to the reviewer for taking such note of this figure, as it has ensured that we did not go forward with incorrect data. We carried out additional control experiments and could not verify strict isoform specificity in immunoprecipitation assays, of the current batches of commercial antibodies used in the original experiments. Because strict isoform specificity of all antibodies was validated in flow cytometry and capping experiments (Figs. 2 and S1), we instead tested the ability of Lck to associate with CD8 by analyzing co-capping of Lck after anti-CD8α1 or anti-CD8β-induced capping of CD8 (new Fig S4). This showed that Lck associated with CD8αβ and CD8αα in the cells expressing either or both CD8 species. Please note that the original data and interpretation were not a major point of this work.

MINOR CONCERNS
1) FIGURE 2: How representative are these data of the population? It is unclear exactly what the error bars in C represent. The same is true of Figure 3. It would be helpful to know how representative these data are as presumably the images show the best example in each case.

The images shown are representative of n=16 cells for anti-CD8α1 capping and n=30 for anti-CD8β. Error bars are standard error. This is now stated in the legend. These points are similar for Fig 3A,B, where n was at least 20 cells. In Fig 3D n=17, 18 and 15.

2. FIGURE S3 also concerns me. The legend mentions Figure S3D and mentions phosphor-Erk. I do not see this in the version I have downloaded. It would also be helpful if the four individual populations studied were clearly marked in the left panel in A. This is done nicely in Figure S4 and makes it much easier to follow. This is an interesting experiment and I like the assay and approach. Once again, the legend could be considerably improved to help the reader. Which cells are which in the right panel of A? The only chance I have of working this out is to take the descriptions of the populations described in the left panel of A and apply the color-coding used in the left panel of figure B. Is that correct?

The errors in the original legend have been corrected, and it has been rewritten for clarity. Suitable labels have been added as suggested (the reviewer was correct about the color coding). We have added another analysis as S3D which compares binding of tetramer in cells with different ratios of CD8αα to αβ expression. This demonstrates that tetramer binding increases when there is a higher proportion of CD8αα compared to CD8αβ.

3. FIGURE S4 Co-expression of MHCI binding mutant CD8α or CD8β does not affect tetramer binding. Does tetramer binding with no CD8 overlay that with CD8αα' or CD8αβ' alone? i.e. Have the authors proved that these molecules don't bind to CD8 at the cell surface as they claim? Their system provides a nice way to address this issue.

This is now Fig S5. We have added analysis of cells that express only the mutant fluorescent CD8, and found that they bound significantly less tetramer than if the non-mutant CD8 was also present. However, this binding was higher than that of the CD8- cells. This probably indicates the presence of non-fluorescent CD8α2-CC155 homodimers which, having only non-mutated CD8α, were capable of binding to the tetramer.

2nd Editorial Decision 09 September 2011

Thank you for the submission of your revised manuscript to EMBO reports. As you will see below, the three referees now support publication of the study, pending a few minor modifications. Thus, once all of these small issues have been addressed, I will be happy to accept your study for publication in the next available issue of EMBO reports.

Browsing through the manuscript myself, I have realized that the legend to figure 4 still does not include information on the number of independent experiments performed (n) and the type of error bars calculated. Please include this information during your final revision.

I look forward to seeing a final version of your manuscript as soon as possible.

Yours sincerely,

Editor
EMBO Reports

REFEREE REPORTS:

Referee #1:

The report by Rybakin et al. provides for the first time a comparative assessment of CD8aa and CD8ab to the immunological synapse (IS) and concludes that recruitment of both forms of CD8 to the IS essentially driven by intercellular CD8-pMHC interactions and is slightly higher for CD8ab than for CD8aa. To corroborate this, the authors show that this difference disappears when using APC that co-express H-2 TL, which preferentially binds to CD8aa. While thymus derived TCRab+ CD8+ T cells generally express only CD8ab, CD8aa can be expressed on IEL and reportedly also on peripheral T cells under certain circumstances. This is the first time this issue was analyzed and the use of BiFC to this end is elegant and to the point. The information obtained is novel and increases current knowledge on the biological functions of the two isoforms of CD8.

There are some issues that should be considered when evaluating this study: First, transfecting T cell hybridomas with CD8a and CD8b invariably results in co-expression of CD8ab and CD8aa; therefore strictly speaking the designation CD8aa; CD8ab and CD8ab + CD8aa expressing cells is a priori misleading. Second, the strategy of using BiFC in this study gives rise to non-fluorescent CD8 species that escape fluorescence based observations, yet are biologically active, i.e. participate in other assays, which compromise stringent correlations between the distribution of CD8 species and their functional attributes. However, this is inherent to this approach and experimental alternatives are not evident. Third, there seems to be inconsistencies of data and previously published findings. According to Figure S3 the tetramer binding is stronger increased by CD8aa than by CD8ab; e.g. in Figure S3A the increase in tetramer binding on CD8ab is marginally higher than on CD8- cells and dramatically lower compared to CD8aa+ cells. This is in contradiction to several reports showing the inverse (e.g. the cited Arcaro et al. 2001 and Doucey et al. 2003). These and other reports concur with the data shown in Figure S5, top panel, right side, which it at variance with Figure S3A right side. This should be rectified and put in proper perspective (second section last paragraph) all the more because this issue impacts on the central message of the paper.

In addition:

- On second page of the Introduction, it should read CD8b also mediates interaction...

- In Figure S4 it would be useful to add in the legend what the green and red colours designate and what the respective stainings were (in A) and what N/S designates (in B).

Referee #2:

While there would seem to be a formal possibility that the Bifc probe could have some background interaction in membranes, I have not been able to find any evidence for this in a quick search. So I accept the authors strong conviction that this doesn't happen. If it is necessary to do another revision for other reasons, the authors may also consider expressing the N and C halves on non-dimerizing membrane protein like CD86 at the same density to show what the background is low or nill. I just have some mild concern still that in a 2D setting the very weak interaction of the half domains could lead to some issues.

Referee #3:

The revised manuscript is considerably improved. It is fortunate I questioned the data in the original Figure S1B. This figure has now been removed. Addition of further figure labeling has helped.

The Y-axis of Figure 4 is not labeled.

2nd Revision - authors' response 22 September 2011

Point-by-point response to reviewers. (Reviewer's comments in italics).

Browsing through the manuscript myself, I have realized that the legend to figure 4 still does not include information on the number of independent experiments performed (n) and the type of error bars calculated. Please include this information during your final revision.

We have included this information (n=25. Error bars represent SD).

"…transfecting T cell hybridomas with CD8a and CD8b invariably results in co-expression of CD8ab and CD8aa; therefore strictly speaking the designation CD8aa; CD8ab and CD8ab + CD8aa expressing cells is a priori misleading"

I thought we had already made clear that we were aware of this. However, we have now altered the text and figure legends in several places to specify that we are referring only to the fluorescent species.

"According to Figure S3 the tetramer binding is stronger increased by CD8aa than by CD8ab; e.g. in Figure S3A the increase in tetramer binding on CD8ab is marginally higher than on CD8- cells and dramatically lower compared to CD8aa+ cells. This is in contradiction to several reports showing the inverse (e.g. the cited Arcaro et al. 2001 and Doucey et al. 2003). These and other reports concur with the data shown in Figure S5, top panel, right side, which it at variance with Figure S3A right side. This should be rectified and put in proper perspective (second section last paragraph) all the more because this issue impacts on the central message of the paper."

Fig. S4 shows WT CD8, and Fig. S5 shows mutant CD8, so S5 is not really relevant to this discussion. Other than that, we agree that there is a discrepancy in tetramer binding between the published data and our system. We had provided an explanation based on other data in the literature (Montixi et al, 1998). However, we have now addressed the discrepancy between our results and those of Arcaro et al. 2001 and Doucey et al. 2003 in more detail as follows (from p7):

> This can be explained because like CD8αα, unstimulated TCRs are not raft-associated (Montixi et al, 1998). Earlier data showing increased binding of multimeric MHCp to CD8αβ compared to CD8αα used a T hybridoma where CD8αβ was reported to be constitutively associated with TCR in lipid rafts (Arcaro et al, 2001; Doucey et al, 2003). This is not always the case (Yachi et al, 2005), which may explain the discrepancy in the results.

We honestly don't think that the data on tetramer binding are as compelling or relevant as data on T cells interacting with antigen presented on cells, so we have not altered the final paragraph.

"On second page of the Introduction, it should read CD8b also mediates interaction..."

We think this is incorrect. The cited reference suggests that LAT binding is abolished when the cytosolic tail of CD8α is replaced by the tail of CD8β (Fig. 2B, lane 3 vs 4). Anti-CD8α clone 53-6.7 used for IP recognizes an extracellular epitope which was not affected by the

domain swap.

"In Figure S4 it would be useful to add in the legend what the green and red colours designate and what the respective stainings were (in A) and what N/S designates (in B)."

Done. We have also labeled the axis of Fig. 4 as requested by Referee 3.

3rd Editorial Decision 23 September 2011

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports.

Thank you for your contribution to EMBO reports and congratulations on a successful publication. Please consider us again in the future for your most exciting work.

Yours sincerely,

Editor
EMBO Reports

