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Dissociation of β_2m from MHC Class I triggers formation of noncovalent transient heavy chain dimers

Cindy Dirscherl, Sara Löchte, Zeynep Hein, Janine-Denise Kopicki, Antonia Regina Harders, Noemi Linden, Andreas Karner, Johannes Preiner, Julian Weghuber, Maria Garcia-Alai, Charlotte Uetrecht, Martin Zacharias, Jacob Piehler, Peter Lanzerstorfer and Sebastian Springer
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First decision letter

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MS TITLE: Dissociation of ß$_2$m from MHC Class I Triggers Formation of Noncovalent, Transient Heavy Chain Dimers

AUTHORS: Cindy Dirscherl, Sara Loechte, Zeynep Hein, Janine-Denise Kopicki, Antonia Regina Harders, Noemi Linden, Julian Weghuber, Maria Garcia-Alai, Charlotte Uetrecht, Martin Zacharias, Jacob Piehler, Peter Lanzerstorfer, and Sebastian Springer
ARTICLE TYPE: Research Article

We have now reached a decision on the above manuscript.

To see the reviewers' reports and a copy of this decision letter, please go to: https://submit-jcs.biologists.org and click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

As you will see, all three reviewers found the work to be well performed and were supportive of the study. A few concerns were raised that should be easily addressed in particular those from Reviewer 2 and 3 regarding the heavy chain association. Additionally, I agree with reviewer 1 that the 'biological relevance' of this nice study is lost in the technical details and for readers of a Cell Biology journal it would be beneficial if you could lead the reader along and explain the significance as it pertains to the biology. if you can address their concerns. If you think that you can deal satisfactorily with the criticisms on revision, I would be pleased to see a revised manuscript. We would then return it to the reviewers.

We are aware that you may be experiencing disruption to the normal running of your lab that makes experimental revisions challenging. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then

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provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Please ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion.

I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. Please attend to all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

The authors reveal a transient, non-covalent association between MCH-I heavy chains (following loss of peptide and subsequent dissociation of beta 2m). The apparent stoichiometry points to dimerization (almost no higher order oligomerization is observed), with a short life-time (~ 1s). The importance of the finding is two-fold: 1. we don't really know what happens with the extremely important MHC-I (delivered WITH a peptide to the plasma membrane, to display it to the inspection of T-cells) once it loses its peptide (and it does lose it since the peptide is not covalently bound in the MHC-I groove). Many various scenarios have occupied scientists, including binding of a foreign peptide from outside the cell (leading to a "fooling" of the immune system). It is thus important to understand what precisely occurs once the peptide, and consequently beta 2m are lost. The authors describe a mechanism previously unknown: the remaining heavy chains are dimerizing, apparently via an exposed region in the alpha 3 domain. 2. having established the prevalence of such FHC dimers, many new questions arise, such as the possible functions of these dimers, ranging from endocytosis signaling, to modulation of the immune system. The discovery is opening new research avenues.

Comments for the author

This is a very carefully and elegantly executed study, involving a myriad of techniques (many of them Biophysical), cross validation between techniques, and careful controls. The conclusions are very well substantiated, and seem compelling.
I definitely recommend publication.
It is the feeling of this reviewer, however, that the "biological story" may be lost somewhat in the details of the techniques, by a large portion of the readers.
We would thus suggest helping the less initiated (in the biophysical methods) readers follow the "big picture". Perhaps by explaining in plain words why it is important to reveal the story, perhaps by providing short summaries and conclusions at the end of the technical description of the various experiments.
The discussion section provides, to a certain extent the "big picture", but it may perhaps be too late for those that have dropped along the way?

Reviewer 2

Advance summary and potential significance to field

This paper provides unique information on the dynamic oligomerization of plasma membrane proteins while located in the plasma membrane. The protein of interest MHC class I, is central to immune immunosurveillance and neuronal development.
This work is important for understanding MHC function is is likely applicable to other membrane proteins

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Comments for the author

This is a fine study based on a unique assay developed by the Springer laboratory that leverages decades of knowledge regarding class I assembly and partial disassembly. It is an important contribution to the fields of antigen presentation and cell membrane protein dynamics. My only suggestion is that the authors consider confirming their conclusion that dimers assemble via the HC alpha 3 domain using Fc fragment of mAbs specific for alhpa 3 vs alpha 2 domains.
Such Abs are readily available fro ATCC and academic labs.

Reviewer 3

Advance summary and potential significance to field

Discherl, et al. use a suite of biophysical approaches to study the interactions of major histocompatibility complex class I molecules (MHC-1) in the absence of loaded peptides. They show evidence for non-covalent MHC-1 dimers. Overall, the work is comprehensive and compelling.

Comments for the author

Major Comments
1. Features of the micropattern assay need to be clarified:
    i. densities of anti-HA
    ii. the absence of adhesion molecules iii. statistics for data presented; N=3 is in the Methods, but the data sampling in the manuscript is very sparse iv. time points for data and kinetic features of the observations
2. The characterization of the protein-protein interaction (PPI) between FHC monomers needs further refinement and evidence. To clarify dimerization through the extracellular domains, truncated mutants are needed.
3. The implication of the alpha-3 domain in FHC dimerization depends mainly on SEC data and the micropattern assay. Alpha-3 knockout studies should be considered.

Minor Comments
1. What is the interaction percentage for WT HA-Kb?. This is necessary to appreciate the effect of the single cysteine mutation.
2. Figure 2C is illegible

First revision

Author response to reviewers' comments

Response to the Reviewers

Dear Editors,

we are very happy to be able to submit to you a revised manuscript. We thank the referees for their time and for their careful reading of our manuscript and their valuable comments, which helped us to improve the quality of the manuscript. Our point-by-point reply to the referee's comments is summarized below. All changes are marked yellow in the revised manuscript and supporting information.

Reviewer 1: Advance Summary and Potential Significance to Field: *The authors reveal a transient, non-covalent association between MCH-I heavy chains (following loss of peptide and subsequent dissociation of beta 2m). The apparent stoichiometry points to dimerization (almost no higher order oligomerization is observed), with a short lifetime (~ 1s). The importance of the finding is two-fold: 1. we don't really know what happens with the extremely important MHC-I (delivered WITH a peptide to the plasma membrane, to display it to the inspection of T-cells)*

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once it loses its peptide (and it does lose it, since the peptide is not covalently bound in the MHC-I groove). Many various scenarios have occupied scientists, including binding of a foreign peptide from outside the cell (leading to a "fooling" of the immune system). It is thus important to understand what precisely occurs once the peptide, and consequently beta 2m are lost. The authors describe a mechanism previously unknown: the remaining heavy chains are dimerizing, apparently via an exposed region in the alpha 3 domain. 2. having established the prevalence of such FHC dimers, many new questions arise, such as the possible functions of these dimers, ranging from endocytosis signalling to modulation of the immune system. The discovery is opening new research avenues.
R1.0. We thank the reviewer for pointing out the importance of our observations.

Reviewer 1 Comments for the Author: *This is a very carefully and elegantly executed study, involving a myriad of techniques (many of them Biophysical), cross validation between techniques, and careful controls. The conclusions are very well substantiated and seem compelling. I definitely recommend publication. It is the feeling of this reviewer, however, that the "biological story" may be lost somewhat in the details of the techniques, by a large portion of the readers. We would thus suggest helping the less initiated (in the biophysical methods) readers follow the "big picture". Perhaps by explaining in plain words why it is important to reveal the story, perhaps by providing short summaries and conclusions at the end of the technical description of the various experiments. The discussion section provides, to a certain extent the "big picture", but it may perhaps be too late for those that have dropped along the way?*
R1.1. We thank the reviewer for recommending such an interdisciplinary and doubtlessly complex manuscript for publication. We have now carefully re-read the manuscript and inserted additional conclusions and summaries throughout the results section in order to make it easier to understand. This is especially important since the revised version contains even more additional techniques that have been employed as additional controls.

Reviewer 2 Advance Summary and Potential Significance to Field: *This paper provides unique information on the dynamic oligomerization of plasma membrane proteins while located in the plasma membrane. The protein of interest, MHC class I, is central to immune immunosurveillance and neuronal development. This work is important for understanding MHC function is is likely applicable to other membrane proteins.*
R2.0. We thank the reviewer for pointing out the significance of our observations.

Reviewer 2 Comments for the Author: *This is a fine study based on a unique assay developed by the Springer laboratory that leverages decades of knowledge regarding class I assembly and partial disassembly. It is an important contribution to the fields of antigen presentation and cell membrane protein dynamics. My only suggestion is that the authors consider confirming their conclusion that dimers assemble via the HC alpha 3 domain using Fc fragment of mAbs specific for alpha 3 vs alpha 2 domains. Such Abs are readily available from ATCC and academic labs.*
R2.1. We thank the reviewer for suggesting this very interesting experiment, i.e., blocking the interaction with externally added antibodies. Indeed, we have tried similar experiments without ever seeing any effects of the antibodies, and our analysis – using fluorescently labeled antibodies – has established that they are unable to diffuse into the gap between the glass coverslip and the bottom surface of the adherent cells, where the interaction is monitored. The Fab fragments (which we think are what the reviewer means) are smaller and might be easier to diffuse, but in the shortness of time we have not conducted that experiment since we also found earlier that even beta-2 microglobulin, which is about half the size of an Fab fragment, does not reach the bottom surface of the cells.

We therefore addressed this issue by micropatterning experiments with a K^b construct lacking the α3 domain, which is also now included in the manuscript. See R.3.5. (Figure R3) for more details.

Reviewer 3 Advance Summary and Potential Significance to Field: *Dischert, et al. use a suite of biophysical approaches to study the interactions of major histocompatibility complex class I molecules (MHC-1) in the absence of loaded peptides. They show evidence for non-covalent MHC-1 dimers. Overall, the work is comprehensive and compelling.*
R3.0. We thank the reviewer for pointing this out.

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Reviewer 3 Comments for the Author:

Major Comments

1. Features of the micropattern assay need to be clarified:

i. densities of anti-HA.

R3.1. We thank the reviewer for this idea, and we thought it an interesting challenge. Based on our (PL) previous publications and similar studies from other labs using protein-patterned interfaces, we acted on the assumption that the applied streptavidin and antibody concentrations lead to saturated conditions within the pattern elements. However, to extract real numbers, or at least a meaningful approximation of the antibody density within the pattern elements, we performed high-speed atomic force microscopy (HS-AFM) (Figure R1 below), capable of resolving individual antibodies on surfaces (Preiner et al. Nat. Comm. 2014, https://doi.org/10.1038/s41467-020-16949-4). Since the antibody coverage after 20 min incubation was too high to discriminate and count individual molecules, we used the images of the shortest incubation time (15 sec) and thus lowest coverage and largest distance between individual molecules to estimate the area coverage per antibody (neglecting potential tip convolution effects). We applied a height threshold of 2.7 nm above the streptavidin layer and detected particle density and mean area per particle (~190 nm^2), which were then used to convert the total area above the threshold in the other images (30 sec – 20 min) to a surface density. The resulting surface density of antibodies as a function of incubation time is reasonably described by a mono- exponential function as typical for a pseudo-first order reaction, i.e. $A^*(1 - e^{-t/\tau})$ with **A = 7646 ± 1828 IgGs/µm^2** (and τ = 77 ± 43 s). The value is equal to an antibody surface coverage of >85%. This information is in line with our presumption and is now included into the revised manuscript, as well as a short description of the HS-AFM measurements. The figure R1 shown below has been included into the manuscript as new **Supplementary Figure 2S1**.

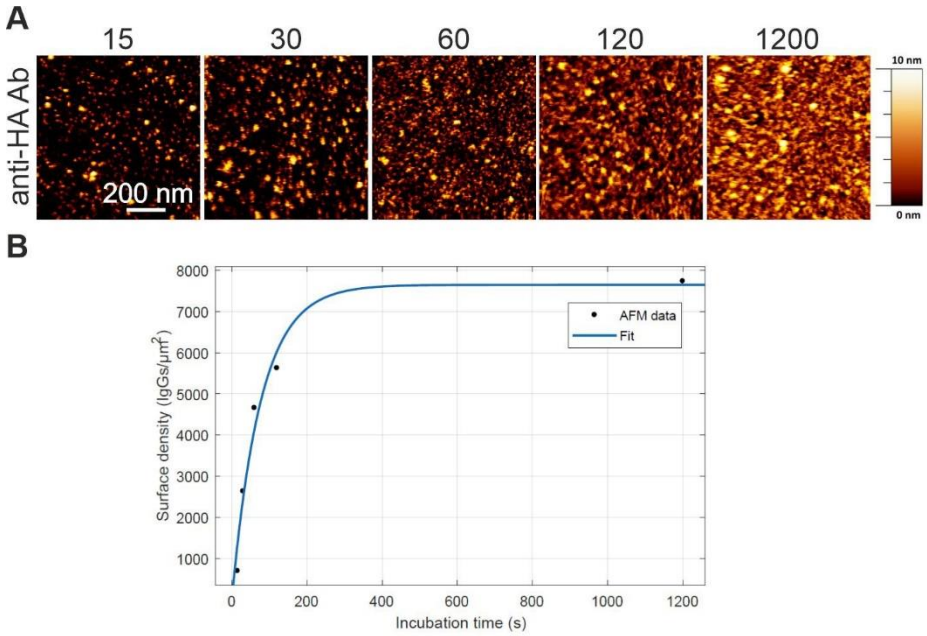

Figure R1 (Figure 2S1 in the manuscript) Estimation of antibody densities in pattern elements by HS-AFM. (A) anti-HA antibodies were incubated on streptavidin coated mica sample disks (50 µg/mL) and subsequently incubated with 10 µg/mL antibody for indicated time periods (s) followed by HS-AFM imaging. (B) Based on respective threshold images, the antibody surface density for each time point was calculated and fitted by an pseudo-first order kinetics.

ii. the absence of adhesion molecules.

R3.2. We thank the reviewer for pointing out this important issue. Cell adhesion is indeed a critical parameter in subcellular micropatterning experiments due to some reasons:

1) Especially in TIRF microscopy, a flat interface between the plasma membrane and the patterned substrate is required to avoid false-positive signals and misinterpretation. One concern is that plasma membrane curvature may be affected by the presence of

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different adhesion molecules inside the pattern elements. We have tested this by uniformly staining the plasma membrane with the lipophilic dye DiD. As depicted in Figure R2 below, DiD (red) shows a homogenous membrane distribution in the central regions of K^b-patterned cells, indicating that the bait micropatterning does not induce a curvature of the membrane or pull it closer to the glass surface in the pattern elements.

2) Another concern is the lateral association of MHC class I proteins with adhesion molecules in the pattern elements. As stated above, protein micropatterning might lead to enrichment of various membrane proteins, including adhesion molecules, which e.g. stabilize the immunological synapse under physiological conditions (Muntjewerff et al. Front Immunol. 2020, https://doi.org/10.3389/fimmu.2020.605958). Consequently, MHC molecules might be co-recruited into the pattern elements partially due to the presence of (or interaction with) such adhesion molecules and not due to specific MHC/MHC dimerization as proposed. It is not a fruitful approach to try to demonstrate the absence of adhesion molecules in the pattern elements, since i) there are many candidates and ii) it is not clear which amount and type of adhesion molecule would be required to recruit class I molecules, especially since iii) such molecules might not recruit class I molecules at all. Instead, we are able to exclude non- specific recruitment of class I molecules by the intrinsic controls that the class I system offers, *i.e.*, the lack of clustering in the presence of peptide or at 25 °C. Those experiments indicate specific dimerization processes independent of adhesion molecules.

We briefly discuss the above-mentioned points in the revised manuscript, and the figure R2 shown below is now included in the manuscript as new Supplementary **Figure 2S2**.

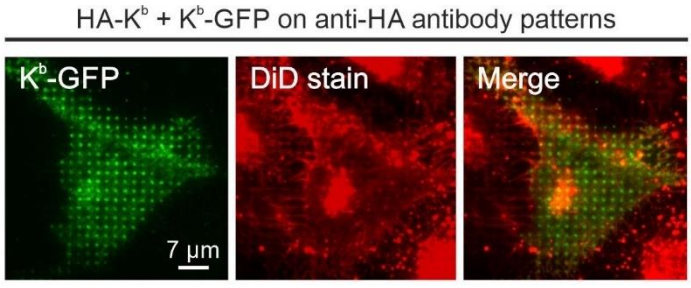

Figure R2 (= Figure 2S2 in the manuscript). DiD membrane stain to visualize membrane-substrate interface. STF1 cells expressing HA-K^b and K^b-GFP were grown on anti-HA antibody patterned surfaces and cell membrane was uniformly labelled by the lipophilic tracer DiD. Homogenous signal confirms sufficient cell attachment of the cells to the functionalized surface.

iii. statistics for data presented; N=3 is in the Methods, but the data sampling in the manuscript is very sparse.
R3.3. We thank the reviewer for this important comment. The sample size is now included in each figure legend where appropriate, or else referenced in the Methods section.

iv. time points for data and kinetic features of the observations.
R3.4. It is not clear to us what the reviewer is specifically referring to in this comment. We have checked again to make sure that

1) all time points of any observations are clearly given in the respective figures or in the Methods section;
2) a detailed description of the experimental procedures of the FRAP experiments (including an in-depth explanation of data processing and calculation of kinetic parameters) are given in the manuscript.

2. The characterization of the protein-protein interaction (PPI) between FHC monomers needs further refinement and evidence. To clarify dimerization through the extracellular domains, truncated mutants are needed. The implication of the alpha-3 domain in FHC dimerization depends mainly on SEC data and the micropattern assay. Alpha-3 knockout studies should be

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considered.

R3.5. We thank the reviewer for this thoughtful suggestion. We managed to design an HA-tagged H- $2K^b$ construct that contained no α3 domain, but was still expressed in cells and transported to the cell surface. That construct shows a dramatic and highly significant (but not complete) loss of patterning with the wild type protein (Figure R3 below), suggesting that the α3 domain is important in the interaction but not the only factor. We think that in addition to the α3-α3 interaction, the α3 domain may also bind to the α1/α2 domain of the other protein (which is still possible in the experimental setup shown in Figure R3), or that even the α1/α2 domains of the two proteins may interact with each other. We cannot currently distinguish between these possibilities.
The Figure R3 below is now included in the manuscript as **Supplementary Figure 4S2** and discussed as appropriate.
With these data, we now have a deletion of the α1/α2 domain (*i.e.*, the α3 domain only, Figure 4AB), and also a deletion of the α3 domain (Supplementary Figure 4S2). Smaller truncations or deletions are not practical, since

a) the α3 domain constitutes one complete immunoglobulin constant (IgC)-type domain, and deletions within it would most likely compromise the structural integrity;
b) the α1/α2 domain, *i.e.*, the peptide binding domain, is one structural entity and also cannot be truncated without endangering its folding.

We believe that further structural elucidation requires protein crystallography and perhaps mutational studies based on its results, which is clearly beyond the scope of this study.

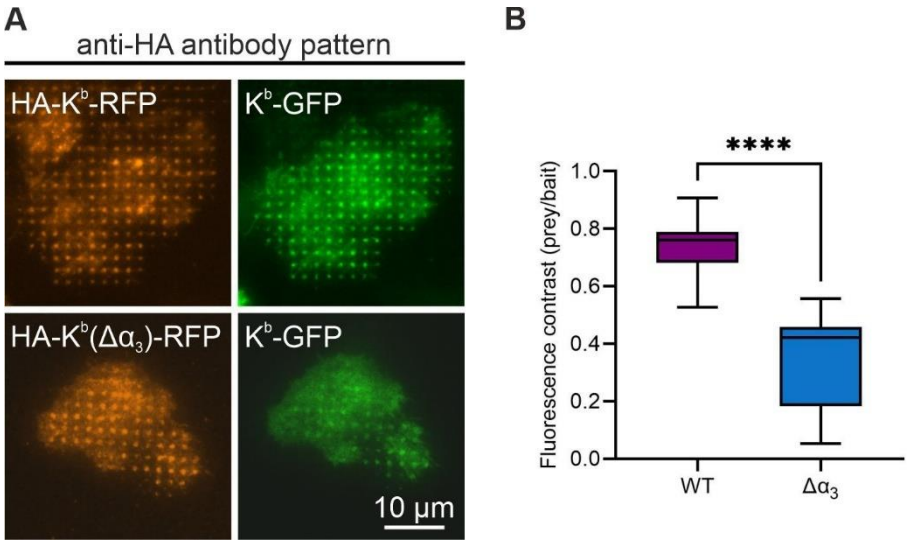

*Figure R3 (=Figure 4S2 in the manuscript): Impact of α3 domain on FHC dimerization. The α3 domain of H-$2K^b$ was truncated and ability of FHC dimerization was compared to WT H-$2K^b$. (A) Representative TIRF microscopy images of STF1 cells transiently transfected with HA- K^b-RFP (WT or Δα3-mutant) and H-$2K^b$-GFP and grown on anti-HA micropatterns. (B) Boxplots show quantitation of bait-normalized prey fluorescence contrast of at least 10 analyzed cells. ****, $p < 0.001$ by unpaired t-test.*

Minor Comments

1. What is the interaction percentage for WT HA-Kb?. This is necessary to appreciate the effect of the single cysteine mutation.

R3.7. We thank the reviewer for pointing out this omission. The interaction percentages for K^b(C332S)- GFP and for the wild type K^b-GFP are identical. This is now mentioned in the manuscript.

2. Figure 2C is illegible

R3.8. We thank the reviewer for careful reading. Figures are now supplied as single high-resolution files for resubmission of the revised manuscript.

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In addition to the points raised by the reviewers, small changes were made to the text, without changing the meaning, to improve readability in some places.

We trust that the manuscript is now acceptable for publication but of course remain available should further questions arise.

Second decision letter

MS ID#: JOCES/2021/259498

MS TITLE: Dissociation of ß$_2$m from MHC Class I Triggers Formation of Noncovalent, Transient Heavy Chain Dimers

AUTHORS: Cindy Dirscherl, Sara Loechte, Zeynep Hein, Janine-Denise Kopicki, Antonia Regina Harders, Noemi Linden, Andreas Karner, Johannes Preiner, Julian Weghuber, Maria Garcia-Alai, Charlotte Uetrecht, Martin Zacharias, Jacob Piehler, Peter Lanzerstorfer, and Sebastian Springer
ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Journal of Cell Science, pending standard ethics checks.

Reviewer 1

Advance summary and potential significance to field

As in the original submission

Comments for the author

The changes made by the authors are satisfactory, thusI recommend the publication of this revised version.

Reviewer 2

Advance summary and potential significance to field

xxx

Comments for the author

Superb response to the reviewer's comments.

Reviewer 3

Advance summary and potential significance to field

Thank you for your excellent and comprehensive review. The study is very well crafted and more complete. I highly recommend the paper for acceptance and publication.

Comments for the author

None

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