nature research

Peer Review Information

Journal: Nature Immunology
Manuscript Title: HLA class I signal peptide polymorphism determines the level of CD94/NKG2:HLA-E-mediated regulation of effector cell responses
Corresponding author name(s): Dr. Mary Carrington

Reviewer Comments & Decisions:

Decision Letter, initial version:

13th Oct 2022

Dear Dr Carrington,

Your Article, "HLA class I signal peptide polymorphism determines the level of CD94/NKG2:HLA-E-mediated regulation of effector cell responses" has now been seen by 3 referees. You will see from their comments copied below that while they find your work of considerable potential interest, they have raised quite substantial concerns that must be addressed. In light of these comments, we cannot accept the manuscript for publication, but would be very interested in considering a revised version that addresses these serious concerns.

We hope you will find the referees' comments useful as you decide how to proceed. If you wish to submit a substantially revised manuscript, please bear in mind that we will be reluctant to approach the referees again in the absence of major revisions. We hope that you will find the prioritised set of referee points to be useful when revising your study. Please do not hesitate to get in touch if you would like to discuss these issues further.

Please ensure to address all of the concerns raised by reviewers, in particular points 1 and 2 raised by R#3.

We are committed to providing a fair and constructive peer-review process. Do not hesitate to contact us if there are specific requests from the reviewers that you believe are technically impossible or unlikely to yield a meaningful outcome.

If revising your manuscript:

* Include a "Response to referees" document detailing, point-by-point, how you addressed each referee comment. If no action was taken to address a point, you must provide a compelling argument.

This response will be sent back to the referees along with the revised manuscript.

* If you have not done so already please begin to revise your manuscript so that it conforms to our Article format instructions at http://www.nature.com/ni/authors/index.html. Refer also to any guidelines provided in this letter.

* Include a revised version of any required reporting checklist. It will be available to referees (and, potentially, statisticians) to aid in their evaluation if the manuscript goes back for peer review. A revised checklist is essential for re-review of the paper.

The Reporting Summary can be found here:
https://www.nature.com/documents/nr-reporting-summary.pdf

When submitting the revised version of your manuscript, please pay close attention to our href="https://www.nature.com/nature-portfolio/editorial-policies/image-integrity">Digital Image Integrity Guidelines. and to the following points below:

-- that unprocessed scans are clearly labelled and match the gels and western blots presented in figures.
-- that control panels for gels and western blots are appropriately described as loading on sample processing controls
-- all images in the paper are checked for duplication of panels and for splicing of gel lanes.

Finally, please ensure that you retain unprocessed data and metadata files after publication, ideally archiving data in perpetuity, as these may be requested during the peer review and production process or after publication if any issues arise.

You may use the link below to submit your revised manuscript and related files:

[REDACTED]

If you wish to submit a suitably revised manuscript we would hope to receive it within 6 months. If you cannot send it within this time, please let us know. We will be happy to consider your revision so long as nothing similar has been accepted for publication at Nature Immunology or published elsewhere.

Nature Immunology is committed to improving transparency in authorship. As part of our efforts in this direction, we are now requesting that all authors identified as 'corresponding author' on published papers create and link their Open Researcher and Contributor Identifier (ORCID) with their account on the Manuscript Tracking System (MTS), prior to acceptance. ORCID helps the scientific community achieve unambiguous attribution of all scholarly contributions. You can create and link your ORCID from the home page of the MTS by clicking on 'Modify my Springer Nature account'. For more information please visit please visit www.springernature.com/orcid.

Please do not hesitate to contact me if you have any questions or would like to discuss the required revisions further.

Thank you for the opportunity to review your work.

Sincerely,

Stephanie Houston
Editor
Nature Immunology

Reviewers' Comments:

Reviewer #1:
Remarks to the Author:
In this manuscript, the amino acid sequence of common HLA class I A, B, C and G allotype signal peptides (SP) was determined to assess the number of existing variants. Of the 16 identified, only 6 were processed in a manner that supported the formation of complexes between 9-mer VL9 epitopes and HLA-E, able to engage the activating receptor NKG2A/CD94. This group of 6 peptides were called functional SPs. One of these functional SPs was derived from HLA-B allotypes with a methionine at position -21 (-21M). Although this peptide stabilized high expression of HLA-E, it conferred poor NKG2A/CD94 recognition. It also competed with other functional SPs for forming complexes with HLA-E leading to an overall reduction in target cell recognition by NKG2A/CD94. Analysis of genetic population data found a positive correlation between frequencies of functional SPs in humans and corresponding cytomegalovirus (CMV) mimics, suggesting viral escape from host responses. The authors discuss the potential impact of their findings on human health and disease.
The authors did a good job presenting a complex topic in a clear manner. The abstract was lucid and appropriate for the work described in this manuscript. The introduction provides relevant background and context. The conclusions follow from the results and provide potentially exciting clinically relevant applications for the findings generated though the application of these results are likely to be in the future.
Overall, the work has a high level of originality. The findings are robust, reliable and interpreted in a fair and balanced manner.
A strength of the manuscript is that the 16 SP variants were identified using sequences derived from HLA allotypes present at frequencies of >0.7% in a large (18,200 persons) dataset that included African, European Caucasian, Chinese and Southeast Asian populations. They also worked with datasets of genotypes in which they were able to assign SP variants to 1266 individuals. The number of subjects studied and their varied ethnicity reflect the importance of this work and its wider global applicability.

The portion of these SPs that interacts with HLA-E are nine-mer epitope variants called VL9s. The authors identified 10 distinct VL9 epitopes. The number given on page 4, line 84 is 11 distinct VL9 epitopes but I think this is a mistake as only 10 appear in Table 1, Figure 1 and in Extended data Figure 2. This should be checked and corrected as appropriate.

The methodology used to generate results is characterized by multiple experimental approaches that reinforce each other and deepen the impact of the results. Where possible, ex vivo peripheral blood mononuclear cells (PBMC) are used in addition to cell lines. This allowed assessment of the possible

effects of intracellular processing of SPs into VL9 peptides and was done by using lentiviral constructs encoding hybrid SP/HLA molecules containing each of the VL9 variants, HLA-E:01, HLA-E:03 (SPE) or HLA-B:57 (SPB) and a FLAG tag. Antibodies specific for HLA-E and FLAG were used to assess the mean fluorescent intensity (MFI) of HLA-E expression following transduction of these .221/SPE or .221/SPB constructs. The results generated by the two antibodies were well correlated.

A novel cellular construct was generated as a reporter cell system to estimate the effect of SP polymorphisms on CD94/NKG2 binding. The read out used for this was reporter cell activation measured by CD69 cell surface expression by flow cytometry. This reporter cell system expressed either the extracellular portion of NKG2A/CD94 or NKG2C/CD94 with intracellular machinery that conferred activating signals. When this reporter cell system was stimulated by a panel .221-SPE cells in which natural intracellular SP processing was allowed to occur, it revealed the hierarchy of the stimulatory capacity of 7 SP variants (if the one derived from HLA-G is included), which identified them as functional SP variants. Results using the reporter cell system (JURKATNKG2) stimulated with the panel of .221-SPE cells were compared to their stimulation with VL9 peptide pulsed .221 cells. As well, NK cells from PBMC and the NK cell line NKL were used instead of JURKATNKG2 to assess their responses to .221 cells pulsed with VL9 peptides by measuring the frequency of NK cells externalizing CD107a (degranulation) as a read out for function. Despite different read outs the results using these approaches were well and appropriately correlated with each other, thus illustrating the consistent impact of VL9 variants on effector cell activity. However, results of stimulation of primary NK cells and NKL cells following stimulation with .221-SPE cells did not discriminate well between SP variants. The authors propose that this may be due to lower levels of HLA-E on these cells compared to that on VL9 pulsed .221 cells. This appears to be a reasonable supposition.

One question I have here is regarding the testing of NKG2A-NKG2C+ NK cells for their responses to VL9 pulsed .221 cells. Were these NKG2A-NKG2C+ from individuals known to be cytomegalovirus (CMV) infected? What was the frequency of these cells in the samples studied Shah SV et al. (Cell Reports 2018) working with rhesus macaques infected or not with rhesus CMV and/or SIV found that CMV infection was required not only to expand this population of NK cells with adaptive-like properties but also to acquire functionality. Information on CMV serostatus and frequency of NKG2C+NKG2A- NK cells in their study subjects should be provided. If the results generated illuminate this idea further in humans, it should be added to the discussion.

Although one of the functional SP, SP-6B, binds strongly to HLA-E, the SP-6B/HLA-E complex exhibits poor receptor recognition. Increasing copy number for SP-6B decreases while increasing copy number of another SP, SP-1C, increases reporter cell activation. Experiments were designed whose results were interpreted as evidence that there is competition between VL9 variants for binding to HLA-E. The consequence of this is that SP polymorphism are not additive in diploid cells. This raises an interesting idea because CMV encodes UL40, an HLA mimic with VL9 variants having sequences identical to VL9 peptides SP-1C, SP-1A/2C and SP-2A. In the discussion, the authors bring together information that suggests that more common CMV clinical isolates may use these SP mimics as self peptides in a manner that avoids CD8+ T cells recognition and to bind NKG2A/CD94+ NK cells to inhibit NK cell activation. Since CMV derived VL9 is made in greater quantities than HLA derived VL9, avoidance of recognition would be in favor of the virus.

The authors propose ways in which the results presented in this manuscript could be used clinically. For example, inhibition of NKG2A/CD94+ NK and T cells through interactions with HLA-E has been shown to have an impact on anti-tumor immunity. This led to the development of monalizumab, an

antibody that disrupts the interaction between this ligand receptor pair. If HLA class I genotypes predictably regulate the strength of inhibition by NKG2A/CD94, the authors propose that it may be possible to select patients who would benefit most from monolizumab therapy. Other interesting clinical applications of the results presented in this manuscript were proposed in the discussion.

Overall, the methods used in this manuscript are appropriate. As well, they are innovative and state of the art. The results generated using the different experimental approaches generally confirm each other. Controls are in place and details that could affect the interpretation of the results are considered.

In Figure 6 panel A x-axis and Panel B (left panel) y-axis what is the unit for MFI? Does this need to be corrected by multiplying by a certain number? Other figures reporting MFI should also be checked to ensure correct units are being reported.

The manuscript has a strong statistical foundation. This said, the results presented in Figure 1C, Figure 2 and Figure 3A do not show any statistical differences between the SPE constructs and HLA-E MFI. Is there none?

Suggested minor improvements have been mentioned throughout the body of this review.

The manuscript appropriately references previous work.

Reviewer #2:
Remarks to the Author:

This manuscript reports the first comprehensive study and analysis of the human signal peptides that engage with the CD94:NKG2A receptor of human NK cells. This study provides a major leap forward in our understanding of NK cell functions.

Reviewer #3:
Remarks to the Author:
The CD94-NKG2-HLA-E axis is an important component of NK cell biology, whereby this NK cell receptor recognises MHC-I/ Ib leader sequences presented by the essentially monomorphic HLA-E molecule. The overarching principle, spanning decades, is that recognition of peptide-HLA-E by the NKG2A inhibitory receptor prevents cell lysis, and subsequent downregulation of MHC class I leads (eg viral infection, transformed cell) to reduction in HLA-E cell surface expression, thereby taking the breaks off inhibitory signalling, and cell lysis.

The manuscript by Lin and colleagues aims to refine this view wherein the main contention is that not all MHC-I leader sequences are ‘created equal’ and the ensuing NK cell mediated responses are a function of the leader sequence polymorphisms presented by HLA-E and recognised by CD94-NKG2. This has fundamental implications for NK cell biology and downstream applications centered on immunotherapies and patient stratification. It has been known for quite some time from distinct published snippets that leader sequence polymorphism can impact HLA-E binding and recognition, and

how UL40 sequence variation can impact NK cell and HLA-E-restricted T cell responses – and this could potentially be used to undermine the novelty of the work . However, what is impressive in this study is the systems view, thorough and meticulous investigation of the MHC class I leader sequences and their impact on NK cell function. While the narrative is potentially strong and of general interest, there are nevertheless key issues pertaining to existing data as interpreted and data that would be required to substantiate the main thesis of this study

1) Much of the current data is heavily reliant on observations using reporter cells and indeed the peptide-dependent differences observed with the reporter cell system do not seem as pronounced when using bone-fide NK cells. As such, the real significance of such variation may be its impact on NK cell education, something which is not addressed

2) The assignment of 'functional SPs' needs further justification, as the data reported in Fig. 2c does not seem convincing, and downstream analyses of functional SPs are critically dependent on such assignment. Namely, there is no statistics applied to either the Jurkat reporters or the data on NK cell lines or primary cells, and the number of independent experiments conducted is not stated. There does not appear to be robust differences in responses against many of the leader sequences, thereby at odds with the general narrative. Robust evidence for this functional SP assignment is crucial.

3) Linked to 2) the apparent differences in responses to leader sequences are diminished/lost when using the 221-SPE cells (extended figure 5), and the authors speculate that HLA-E upregulation by IFNg may be required to recapitulate the findings when HLA-E is artificially over-expressed. It is incumbent on the authors to address this experimentally as it speaks to physiological relevance.

4) HLA-E stability and ensuing NKG2A response. While Figure 1a shows 6B/7B upregulating HLA-E above other SPs, extended data figure 2b does not show this, and thus the narrative that 6B/7B are potent HLA-E upregulators (yet non-functional) appears at odds with their data presented. What is also unclear, and not touched upon, is why this polymorphism at position 7 of the SP would impact on NKG2A recognition. The structures of the ternary complexes of CD94-HLA-E-peptide have been solved, and position 7 does not participate in contacts. As such, the mechanism of their observations are unclear.

5) to claim that SP6-B is an antagonist for CD94-NKG2A is incorrect. It is a weak agonist. To suggest that SP6-B would outcompete stronger agonist SPs would require mass spectrometry studies from HLA-E eluted peptide elution studies. Given that the authors have already used mass spec in their experiments, these experiments should not be too onerous.

Other points
The paper is jargon filled and challenging to deconvolute. The authors should try to make the study more accessible to the non NK cell aficionados.

Need to show mass spec data and not just summary table
HLA-E – it is unclear which isoform they are working in in some experiments
There are no affinity measurements between CD94-NKG2 and HLA-E-peptide, so either the authors conduct such experiments, or modify their terminology

Author Rebuttal to Initial comments

Dear Dr. Houston,

Thank you for your willingness to further consider our manuscript entitled "HLA class I signal peptide polymorphism determines the level of CD94/NKG2:HLA-E-mediated regulation of effector cell responses" for publication in *Nature Immunology.* The Reviewers' comments were remarkably helpful, and I think the experiments they suggested have basically transformed the paper, strengthening the work considerably.

Please find a point-by-point response to each comment and the revised manuscript with changes highlighted. All additional figures or altered figures are noted in the response to the Reviewers. We look forward to hearing your thoughts and those of the Reviewers' in due course.

With best wishes,
Mary Carrington

Reviewer #1:
Remarks to the Author:
In this manuscript, the amino acid sequence of common HLA class I A, B, C and G allotype signal peptides (SP) was determined to assess the number of existing variants. Of the 16 identified, only 6 were processed in a manner that supported the formation of complexes between 9-mer VL9 epitopes and HLA-E, able to engage the activating receptor NKG2A/CD94. This group of 6 peptides were called functional SPs. One of these functional SPs was derived from HLA-B allotypes with a methionine at position -21 (-21M). Although this peptide stabilized high expression of HLA-E, it conferred poor NKG2A/CD94 recognition. It also competed with other functional SPs for forming complexes with HLA-E leading to an overall reduction in target cell recognition by NKG2A/CD94. Analysis of genetic population data found a positive correlation between frequencies of functional SPs in humans and corresponding cytomegalovirus (CMV) mimics, suggesting viral escape from host responses. The authors discuss the potential impact of their findings on human health and disease.
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We thank the Reviewer for the supportive comments and the excellent summary of the data presented in our manuscript.

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We appreciate the close attention the Reviewer gave to the numbers stated in the paper and have fixed this error in the revised manuscript.

Lines 95-97: "There are ten distinct VL9 peptide sequences among the common classical HLA-A, -B, and -C and non-classical HLA-G (HLA-E and -F contain deletions within the canonical VL9 sequence; see Table 1)."

The methodology used to generate results is characterized by multiple experimental approaches that reinforce each other and deepen the impact of the results. Where possible, ex vivo peripheral blood mononuclear cells (PBMC) are used in addition to cell lines. This allowed assessment of the possible effects of intracellular processing of SPs into VL9 peptides and was done by using lentiviral constructs encoding hybrid SP/HLA molecules containing each of the VL9 variants, HLA-E:01, HLA-E:03 (SPE) or HLA-B:57 (SPB) and a FLAG tag. Antibodies specific for HLA-E and FLAG were used to assess the mean fluorescent intensity (MFI) of HLA-E expression following transduction of these .221/SPE or .221/SPB constructs. The results generated by the two antibodies were well correlated.

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machinery that conferred activating signals. When this reporter cell system was stimulated by a panel .221-SPE cells in which natural intracellular SP processing was allowed to occur, it revealed the hierarchy of the stimulatory capacity of 7 SP variants (if the one derived from HLA-G is included), which identified them as functional SP variants. Results using the reporter cell system (JURKAT^{NKG2}) stimulated with the panel of .221-SPE cells were compared to their stimulation with VL9 peptide pulsed .221 cells. As well, NK cells from PBMC and the NK cell line NKL were used instead of JURKAT^{NKG2} to assess their responses to .221 cells pulsed with VL9 peptides by measuring the frequency of NK cells externalizing CD107a (degranulation) as a read out for function. Despite different read outs the results using these approaches were well and appropriately correlated with each other, thus illustrating the consistent impact of VL9 variants on effector cell activity. However, results of stimulation of primary NK cells and NKL cells following stimulation with .221-SPE cells did not discriminate well between SP variants. The authors propose that this may be due to lower levels of HLA-E on these cells compared to that on VL9 pulsed .221 cells. This appears to be a reasonable supposition.

We have now used SPE and SPB constructs that do not include the gene encoding ZsGreen-P2A upstream of the SPE/SPB gene, as *ZsGreen-P2A located upstream of a second gene in the vector artifactually decreases expression of the second gene. The new vectors resulted in higher expression of HLA-E on the .221 cell surface, which in turn, resulted in very good discrimination of SP variants by both primary NK cells and NKL cells. Please also see our response to the first point made by Reviewer 3.*

One question I have here is regarding the testing of NKG2A-NKG2C+ NK cells for their responses to VL9 pulsed .221 cells. Were these NKG2A-NKG2C+ from individuals known to be cytomegalovirus (CMV) infected? What was the frequency of these cells in the samples studied? Shah SV et al. (Cell Reports 2018) working with rhesus macaques infected or not with rhesus CMV and/or SIV found that CMV infection was required not only to expand this population of NK cells with adaptive-like properties but also to acquire functionality. Information on CMV serostatus and frequency of NKG2C+NKG2A- NK cells in their study subjects should be provided. If the results generated illuminate this idea further in humans, it should be added to the discussion.

The Reviewer is correct, and we have now made clear that all donors in our study were HCMV-seropositive and displayed relatively large NKG2C+ NK cell populations, likely representing adaptive NK cells. We provide the range of NKG2C+ cell frequencies observed, and in the Discussion, we note that the differential effect of SP polymorphism observed in our experiments may reflect responses of adaptive NKG2C+ NK cells in vivo, as the Reviewer suggests.

Lines 208-210: "All donors were HCMV-seropositive and displayed a relatively large NKG2C+ NK population (10-40% of total NK cells), which likely represent adaptive NK cells that develop in response to HCMV infection[23]."

Lines 318-321: "Differential reporter activities were validated in blood NKG2A+ and NKG2C+ NK cells as well as NKL cells, suggesting that these data are applicable to NK cells populations in general, including tissue-resident NK cells[28] that express high levels of NKG2A, as well as HCMV-induced adaptive NKG2C+ NK cells[23]."

Although one of the functional SP, SP-6B, binds strongly to HLA-E, the SP-6B/HLA-E complex exhibits poor receptor recognition. Increasing copy number for SP-6B decreases while increasing copy number of another SP, SP-1C, increases reporter cell activation. Experiments were designed whose results were interpreted as evidence that there is competition between VL9 variants for binding to HLA-E. The consequence of this is that SP polymorphism are not additive in diploid cells. This raises an interesting idea because CMV encodes UL40, an HLA mimic with VL9 variants having sequences identical to VL9 peptides SP-1C, SP-1A/2C and SP-2A. In the discussion, the authors bring together information that suggests that more common CMV clinical isolates may use these SP mimics as self peptides in a manner that avoids CD8+ T cells recognition and to bind NKG2A/CD94+ NK cells to inhibit NK cell activation. Since CMV derived VL9 is made in greater quantities than HLA derived VL9, avoidance of recognition would be in favor of the virus.

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We thank the Reviewer for accurately summarizing the salient points described in the manuscript.

Overall, the methods used in this manuscript are appropriate. As well, they are innovative and state of the art. The results generated using the different experimental approaches generally

confirm each other. Controls are in place and details that could affect the interpretation of the results are considered.

In Figure 6 panel A x-axis and Panel B (left panel) y-axis what is the unit for MFI? Does this need to be corrected by multiplying by a certain number? Other figures reporting MFI should also be checked to ensure correct units are being reported.

Figures 6, 7 and Extended Data Figure 7 of the original manuscript showed normalized MFI values of HLA-E expression, as opposed to other figures in the paper, and we apologize for that inconsistency. As the Reviewer suggested, we adjusted these normalized values, multiplying them by a factor to make the data comparable to others throughout the paper. This factor was generated by taking ratios of MFI values for samples that were repeated between the experiments (e.g. five BLCLs in Extended Data Fig. 4 and Fig. 6, 7). Thus, MFI values in Figures 6, 7, and Extended Data Figure 10 of the revised manuscript are now comparable to data shown in Figure 1 and Extended Fig. 4. Please note that the new data on monocyte-derived macrophages in the Extended Data Figure 6 of the revised manuscript was derived using a different flow cytometer, so the MFI values are not comparable to the other Figures.

We have added the following statement in the Methods section of the revised manuscript: Lines 513-516: "MFI values were normalized to the average MFI across samples to adjust for daily instrumental variation. In order to compare MFI data across different experiments, reference sets of samples repeated between the experiments were used for further adjustment of MFI values."

The manuscript has a strong statistical foundation. This said, the results presented in Figure 1C, Figure 2 and Figure 3A do not show any statistical differences between the SPE constructs and HLA-E MFI. Is there none?

*We agree with the Reviewer and now provide p values for comparisons of the various effector cell responses to proper negative controls in Figures 2 and 3. Statistics in Figure 3 were used to define functional SPs and discriminate them from non-functional SPs, so these p values are meaningful indeed. We also provide statistics for correlations between HLA-E expression levels on corresponding SP transductants for respective pairs of SPE*01:01, SPE*01:03, and SPB*57:01 transduced .221 cells (see Extended Date Figure 3c and 3d), indicating remarkable consistency of HLA-E expression level among these three sets of transduced .221 cells. Figure 1c is meant to show relative patterns of HLA-E expression levels, and we did not want to emphasize significant*

*differences in their expression levels, as we know that even cells transduced with nonfunctional SPs, such as SP-3A and SP-4A, can express low levels of HLA-E, and thus may be significantly higher than empty-vector controls. However, unlike HLA-E expressed by cells transduced with functional SP, eluates of peptides from HLA-E expressed by cells transduced with non-functional SP are devoid of VL9 peptides (as determined by mass spec of peptides eluted from HLA-E on cells transduced with the non-functional SP-3A and SP-4A). The purpose of Figure 1c is to show that some SPs elicit greater HLA-E expression levels than do others and that the expression pattern across SPs is consistent between SPE*01:01, SPE*01:03 and SPB*57:01 (as formalized with statistics in Extended Data Fig. 3c,d). It is a subtle point, but we think this message could be misconstrued by including p values in Figure 1c. We hope the Reviewer will agree.*

Suggested minor improvements have been mentioned throughout the body of this review.

The manuscript appropriately references previous work.

Once again, we very much appreciate your thorough perusal and review of the paper, and terrific summary of the work.

Reviewer #2:
Remarks to the Author:

This manuscript reports the first comprehensive study and analysis of the human signal peptides that engage with the CD94:NKG2A receptor of human NK cells. This study provides a major leap forward in our understanding of NK cell functions.

Many thanks for those kind, encouraging words. We are delighted that the Reviewer finds the work to be consequential.

Reviewer #3:
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The CD94-NKG2-HLA-E axis is an important component of NK cell biology, whereby this NK cell receptor recognises MHC-I/ Ib leader sequences presented by the essentially monomorphic HLA-E molecule. The overarching principle, spanning decades, is that recognition of peptide-

HLA-E by the NKG2A inhibitory receptor prevents cell lysis, and subsequent downregulation of MHC class I leads (eg viral infection, transformed cell) to reduction in HLA-E cell surface expression, thereby taking the breaks off inhibitory signalling, and cell lysis.

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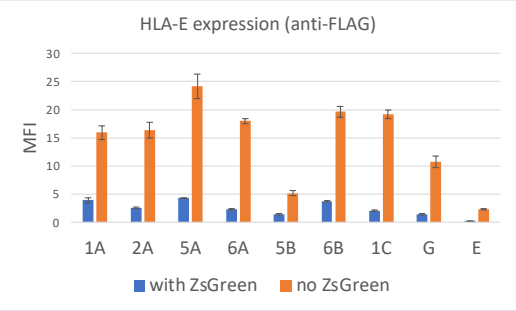
1) Much of the current data is heavily reliant on observations using reporter cells and indeed the peptide-dependent differences observed with the reporter cell system do not seem as pronounced when using bone-fide NK cells. As such, the real significance of such variation may be its impact on NK cell education, something which is not addressed

We agree with the Reviewer that the differential effect of SP variants seen with reporter cells was muted in primary NK cells. We suspected that the low levels of HLA-E expression on .221-SPE might explain this observation. Our original lentiviral constructs contained ZsGreen linked to SPE via a P2A self-cleaving peptide (i.e. ZsGreen-P2A-SPE). To increase SPE expression, we removed ZsGreen-P2A from all constructs and were able increase MFI levels for HLA-E by a factor of ~ 4 times or more as measured by anti-FLAG staining (see Figure below). This increase concurs with data from Liu et al. (Sci. Rep. 7, 2017) who reported ~ 70% lower expression of a protein downstream P2A compared to the upstream protein.



*The removal of ZsGreen-P2A not only increased HLA-E expression levels, but also improved consistency of the expression patterns across SPE*01:01, SPE*01:03 and SPB*57:01-transduced cells (Fig. 1c and Extended Data Fig. 3b,c). Reporter cells recognized .221-SPE cells to a greater extent, especially Jurkat^{NKG2C} reporters (Fig. 2c) that had shown very low activity previously when ZsGreen-P2A was present in the vector. Importantly, higher HLA-E expression levels allowed us to detect differential recognition of naturally processed HLA-E/VL9 on .221-SPE target cells by primary NK cells and NKL (Fig. 3), which, as the Reviewer points out, was not clear when using the previous versions of .221-SPE transduced with vectors containing ZsGreen-P2A. The newly detected NKL/primary NK cell responses correlated well with reporter cell responses to the same targets expressing naturally processed HLA-E/VL9 variants. We have updated the manuscript accordingly and replaced all data that had previously involved ZsGreen-P2A-containing vectors to those in which ZsGreen-P2A is absent.*

It is entirely possible that SP polymorphism may impact NK cell education of NKG2A^{+} NK cells. Testing this experimentally will be an extensive undertaking and while we agree that this is an important question, we would like to approach it thoroughly over the next couple of years.

2) The assignment of 'functional SPs' needs further justification, as the data reported in Fig. 2c does not seem convincing, and downstream analyses of functional SPs are critically dependent on such assignment. Namely, there is no statistics applied to either the Jurkat reporters or the data on NK cell lines or primary cells, and the number of independent experiments conducted is not stated. There does not appear to be robust differences in responses against many of the leader sequences, thereby at odds with the general narrative. Robust evidence for this functional SP assignment is crucial.

*We agree with the Reviewer that we should have included statistics in the original submission. As described above, we have updated Figure 2c to reflect .221-SPE cells that express higher HLA-E levels, resulting in greater sensitivity in the assays. Further, the new data using the Jurkat^{NKG2A} and Jurkat^{NKG2C} reporter cells, as well as target cells expressing SPE*01:01 and SPE*01:03, are*

*highly correlated, allowing better justification for assignment of functional SPs. These experiments were repeated three times and we have stated this in the revised legend. We deem SPs to be functional if corresponding reporter cell responses involving Jurkat^{NKG2C} and Jurkat^{NKG2A} and targets involving SPE*01:01 and SPE*01:03 were significantly higher than background ($p<0.05$; unpaired t-test) across all four sets of experiments shown in Figure 2c. We think the consistent effect across experiments provides a reasonable basis for defining the functional SPs. Further, the new dataset shows robust discrimination between functional and non-functional SPs in experiments with primary NK cells and NKL cells (Fig. 3a,c,e), where t-tests were used to determine significant differences between each functional SP as compared to the non-functional SP-5B. As indicated in legend of Fig. 3 of the revised manuscript, NKL cell experiments were repeated three times and primary NK cell data represent 8 donors.*

3) Linked to 2) the apparent differences in responses to leader sequences are diminished/lost when using the 221-SPE cells (extended figure 5), and the authors speculate that HLA-E upregulation by IFNg may be required to recapitulate the findings when HLA-E is artificially over-expressed. It is incumbent on the authors to address this experimentally as it speaks to physiological relevance.

Our initial hypothesis that increased HLA-E expression would provide better differential recognition of target cells by NK cells has been supported by the data obtained using the new expression system, which showed consistent results across Jurkat^{NKG2}, NKL, and primary effector cells (Fig. 2,3). HLA-E expression levels in the updated SPE system are higher than in PBMCs, but close to BLCLs (Extended Data Fig. 4a). In the revised manuscript, we show that IFN-γ treatment of PBMCs as well as monocyte-derived macrophages results in increased HLA-E expression levels (Extended Data Fig. 4b and 6), and we propose that HLA-E expression levels of .221-SPE cells may reflect, to some extent, in vivo inflammatory conditions.

*Lines 144-149: "The .221-SPE*01:03 cells expressed HLA-E at lower levels than VL9 pulsed .221 cells, at higher levels than peripheral blood cells, and at similar levels as BLCLs (Extended Data Fig. 4a). Peripheral blood cells show an increase in HLA-E surface expression upon treatment with interferon-γ (IFN-γ; Extended Data Fig. 4b), in agreement with previous observations[20]. Thus, our .221-SPE cellular model may reflect HLA-E expression levels under inflammatory conditions."*
Lines 167-170: "Activity of Jurkat^{NKG2A} cells was also tested against monocyte-derived macrophages that were incubated with IFN-γ and/or SP-G-derived VL9 (VL9^{G}; Extended Data Fig. 6). As expected, IFN-γ treatment enhanced HLA-E expression and increased reporter cell activity for both unpulsed and VL9^{G}-pulsed target cells."

4) HLA-E stability and ensuing NKG2A response. While Figure 1a shows 6B/7B upregulating HLA-E above other SPs, extended data figure 2b does not show this, and thus the narrative that 6B/7B are potent HLA-E upregulators (yet non-functional) appears at odds with their data presented.

We agree that data obtained using ELISA-based peptide binding and thermal stability assays (Extended Data Fig. 2b) do not replicate the hierarchy in the binding profiles obtained with peptide pulsing data shown in Fig. 1a and Extended Data Fig. 2a. Rather, the ELISA and thermal stability assays crudely distinguish VL9 binders from nonbinders. We think that these methods are not sensitive in detecting the differences in binding affinity that can be seen in peptide-pulsing experiments (see statement in Lines 107-111). We have generated new data based on mass-spectrometry analysis of VL9 peptides eluted from HLA class I expressed in two BLCLs showing that VL9^{6B} outcompetes other VL9 peptides in occupying the HLA-E peptide binding groove, likely due to its higher affinity for HLA-E (Fig. 6d). Also, the positive correlation between higher HLA-E expression levels and increasing copy number of each given SP variant in BLCLs is the strongest and most significant for SP-6B relative to the other functional SPs, supporting the assertion of higher affinity of VL9^{6B} for HLA-E compared to other VL9 variants (Fig. 7a,b and Extended Fig. 8).

Lines 107-111: "Binding of VL9 variants to HLA-E was also assessed using an ELISA-based HLA-E peptide binding assay[18, 19] and thermal stability analysis of HLA-E/VL9 complexes[19] (Extended Data Fig. 2b). Both methods showed somewhat similar patterns to the peptide pulsing experiments but were less sensitive in detecting differences in binding affinity across VL9 peptides."

What is also unclear, and not touched upon, is why this polymorphism at position 7 of the SP would impact on NKG2A recognition. The structures of the ternary complexes of CD94-HLA-E-peptide have been solved, and position 7 does not participate in contacts. As such, the mechanism of their observations are unclear.

In order to address the Reviewer's pertinent comment, we generated mutant swap constructs, in which the Val/Leu at P7 of VL9 was exchanged between SP-1A and SP-6B (Fig. 6a). These data show that valine at P7, as found in SP-6B and the mutant SP-1A^{V}, is responsible for lower recognition by CD94/NKG2A. The role of P7 is also supported by lower reporter cell recognition of exogenously loaded VL9^{6B} compared to VL9^{1A}, which differ only at P7 (Fig. 2) despite higher

HLA-E expression induced by VL9^{6B} (Fig. 1a). Moreover, newly generated SPR data show a higher K_D value for HLA-E/VL9^{6B} binding to both CD94/NKG2A and CD94/NKG2C as compared to HLA-E/VL9^{1A} (Extended Data Table 1). To understand the mechanism of P7 impact on the stability of the receptor:ligand complex, we performed molecular dynamics (MD) simulations, which showed a higher level of motion in the presence of VL9^{6B} relative to the presence of VL9^{1A} (Extended Data Fig. 6b) and suggested that P7 may influence interactions of other residues in the VL9 peptide, such as arginine at P5, with the receptor.

Lines 189-204: "Constructs encoding swap mutations between SP-1A and SP-6B were generated, and showed that valine at P7 of VL9 is responsible for the decreased reporter cell recognition (Extended data Fig. 7a). The structure of the CD94/NKG2A:HLA-E complex with VL9^{G} loaded onto HLA-E implicated P5, P6, and P8, but not P7 of VL9^{G} in direct interactions with the CD94/NKG2A receptor [22]. Thus, the impact of P7 based on the mutant SP experiments (Extended data Fig. 7a) indicates that this position may affect CD94/NKG2A recognition of HLA-E/VL9 indirectly. Indeed, a 5 μs-long molecular dynamics (MD) simulation analysis of the CD94/NKG2A:HLA-E complex with VL9^{1A} versus VL9^{6B} loaded onto HLA-E indicated that the complex displays a higher level of motion and receptor distortion in the presence of VL9^{6B} as compared to VL9^{1A} (Extended data Fig. 7b), indicating decreased stability of the receptor-ligand complex in the presence of VL9^{6B}. HLA-E/VL9^{1A} engaged 6 to 15 (10.8 on average) amino acid residues in hydrogen-bonded interactions with the receptor, while HLA-E/VL9^{6B} engaged 5 to 10 (7.3 on average). During the simulations, P5-arginine in VL9^{1A} was hydrogen bonded to the receptor for a longer time compared to P5-arginine in VL9^{6B}, supporting an influence of P7 on the interaction between other residues of the VL9 peptide and the CD94/NKG2A receptor."

5) to claim that SP6-B is an antagonist for CD94-NKG2A is incorrect. It is a weak agonist. To suggest that SP6-B would outcompete stronger agonist SPs would require mass spectrometry studies from HLA-E eluted peptide elution studies. Given that the authors have already used mass spec in their experiments, these experiments should not be too onerous.

We have avoided use of "antagonist" when describing SP-6B in the revised manuscript. As indicated above, the mass spectrometry analysis of VL9 peptides eluted from HLA class I expressed in two BLCLs has now been performed. The data shows that in the presence of SP-6B, the VL9^{2A} and VL9^{1C} peptides are suppressed (Fig. 6d).

Lines 268-277: "If the molar concentration of HLA-E in the ER is limited, competition between distinct peptides for loading onto HLA-E will ensue, and VL9s with high affinity for HLA-E, such as VL9^{6B}, would outcompete those with lower affinity for HLA-E. We tested this model using mass spectrometry analysis of HLA class I-associated peptides in two BLCLs, each carrying three

functional SPs (Fig. 6d): BLCL1 carrying SP-2A, SP-1C, and SP2C, and BLCL2 carrying SP-2A, SP-1C and SP-6B. As expected, $VL9^{2C}$ and $VL9^{6B}$ were identified only from cells carrying SP-2C and SP-6B, respectively. Strikingly, however, the presence of SP-6B (BLCL2) associated with substantially lower amounts of both $VL9^{2A}$ and $VL9^{1C}$ relative to that observed in the presence of SP-2C (BLCL1), supporting a model in which VL9 from SP-6B can successfully compete against other VL9 variants for binding to HLA-E (Fig. 6d)."

Other points
The paper is jargon filled and challenging to deconvolute. The authors should try to make the study more accessible to the non NK cell aficionados.

The Reviewer is correct, and we have tried to decrease the complexity in this regard. It is a challenge, however, given the nature of the components (various SPs, VL9s, effectors and targets). We hope it is improved overall, but would be happy to incorporate further suggestions.

Need to show mass spec data and not just summary table

*The mass spec data in the original manuscript were generated using the original .221-SPE*01:03 cells expressing ZsGreen-P2A, which we do not present in the revised manuscript. Given the limited space and addition of more critical data, we removed the mass spectrometry analysis of SP-3A and SP-4A that was presented in the original manuscript. The data showed the absence of VL9 peptides among peptides eluted from HLA class I in the corresponding .221 SPE transductants. Given that expression of SP-3A and SP-4A do not elicit reporter cell activities, and are therefore classified as non-functional, we believe presenting the original mass spectrometry data is not critical for the manuscript.*

HLA-E – it is unclear which isoform they are working in in some experiments

*We apologize for not being clear in the original manuscript. We specified clearly in the new version which allotype was used in each experiment, E*01:01 or E*01:03.*

There are no affinity measurements between CD94-NKG2 and HLA-E-peptide, so either the authors conduct such experiments, or modify their terminology.

We now provide SPR data in the Extended Data Table 1 of the revised manuscript. The K_D values obtained in this analysis strongly correlated with the reporter cell activities against VL9-pulsed cells (Extended Data 5c).

Lines 162-166: "Indeed, surface plasmon resonance (SPR) data demonstrated variable K_D values for HLA-E/VL9 binding to CD94/NKG2 as a function of the VL9 peptide involved (Extended Data Table 1), and these K_D values strongly correlated with both Jurkat^{NKG2A} and Jurkat^{NKG2C} reporter activities in response to target cells expressing the corresponding HLA-E/VL9 complexes (Extended Data Fig. 5c)."

Thank you for the many valuable comments, which in our opinion, led to a radical improvement of the paper.

Decision Letter, first revision:

21st Mar 2023

Dear Dr. Carrington,

Thank you for submitting your revised manuscript "HLA class I signal peptide polymorphism determines the level of CD94/NKG2:HLA-E-mediated regulation of effector cell responses" (NI-A34572A). It has now been seen by the original referees and their comments are below. The reviewers find that the paper has improved in revision, and therefore we'll be happy in principle to publish it in Nature Immunology, pending minor revisions to comply with our editorial and formatting guidelines.

We will now perform detailed checks on your paper and will send you a checklist detailing our editorial and formatting requirements in about a week or so. Please do not upload the final materials and make any revisions until you receive this additional information from us.

If you had not uploaded a Word file for the current version of the manuscript, we will need one before beginning the editing process; please email that to immunology@us.nature.com at your earliest convenience.

Thank you again for your interest in Nature Immunology Please do not hesitate to contact me if you have any questions.

Sincerely,

Jamie D K Wilson, D.Phil
Chief Editor
For:

Stephanie Houston
Editor
Nature Immunology

Reviewer #1 (Remarks to the Author):

The revised manuscript submitted by Lin et al. has addressed all the comments I made to the original submission to Nature Immunology. The revised manuscript has a high level of technical merit. In my opinion the results are important and have the potential to be of interest to a broad audience.

Reviewer #3 (Remarks to the Author):

The authors have professionally and comprehensively addressed the major critiques raised regarding the original submission, leading to a much improved paper. Congratulations to the authors on a fine and impactful study!

Final Decision Letter:

Dear Dr. Carrington,

I am delighted to accept your manuscript entitled "HLA class I signal peptide polymorphism determines the level of CD94/NKG2:HLA-E-mediated regulation of effector cell responses" for publication in an upcoming issue of Nature Immunology.

Over the next few weeks, your paper will be copyedited to ensure that it conforms to Nature Immunology style. Once your paper is typeset, you will receive an email with a link to choose the appropriate publishing options for your paper and our Author Services team will be in touch regarding any additional information that may be required.

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Sincerely,

Stephanie Houston
Editor
Nature Immunology