We are grateful to the reviewers and the editor for useful suggestions and corrections that have helped us to improve the manuscript. The manuscript has been substantially revised and edited. Our responses to specific points raised can be found below, highlighted in yellow.

Responses to editor’s comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

The manuscript has been proofread to ensure that there are no spelling or grammar issues.

2. Please provide an email address for each author.

This has been provided.

3. Please use SI abbreviations for all units: L, mL, µL, h, min, s, etc.

SI abbreviations are used thought the manuscript text.

4. Lines 165-199, etc.: Please revise the protocol to contain only action items that direct the reader to do something (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” Please include all safety procedures and use of hoods, etc. However, notes should be used sparingly and actions should be described in the imperative tense wherever possible. Please move the discussion about the protocol to the Discussion.

The protocol has been substantially revised, with the descriptive sections 1 and 2 moved to the introduction. Information on safety procedures has been included thought the manuscript.

5. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please simplify the Protocol so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step. Use sub-steps as necessary. Please move the discussion about the protocol to the Discussion.

The protocol has been substantially revised according to these guidelines.

6. Please remove commercial language from the manuscript: Opti-MEM, Cytofix/Cytoperm, etc.

This has been corrected.

7. Line 198: Please provide the composition of the culture media for T-Rex CHO cells.

This has been provided. This section of the protocol has been substantially edited for better clarity.

8. 2.2.3: Please specify what is considered to be high confluence (80%? 90%?). Please describe how to trypsinise the cells and specify the concentration of trypsin and reaction conditions.

This information is provided in the revised version of the manuscript.

9. 2.2.4: Please describe how to perform antibody staining.

This information is provided in the revised version of the manuscript.

10. 4.1.1: Please specify the conditions for culturing CHO cells.

This information is provided in the revised version of the manuscript.

11. 4.1.2: Please split into several substeps.

This section of the protocol has been substantially edited for better clarity.

12. After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

The relevant sections are highlighted in yellow.

13. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense.

14. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

15. Please note that JoVE requires Representative Results and Discussion to be two separate sections. Representative Results sections should explain the results in the context of the technique you have described, e.g., how do these results show the technique, suggestions about how to analyze the outcome, etc. The paragraph text should refer to all of the figures. However for figures showing the experimental set-up, please reference them in the Protocol. Data from both successful and sub-optimal experiments can be included.

The revised version of the manuscript contains Representative Results and Discussion.

16. JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please add a Discussion section to explicitly cover the following in detail in 3-6 paragraphs with citations:

a) Critical steps within the protocol

b) Any modifications and troubleshooting of the technique

c) Any limitations of the technique

d) The significance with respect to existing methods

e) Any future applications of the technique

The revised version of the manuscript contains Discussion.

17. References: Please do not abbreviate journal titles.

The references have been re-formatted.

18. Table of Equipment and Materials: Please provide lot numbers and RRIDs of antibodies, if available. Please sort the items in alphabetical order according to the Name of Material/ Equipment.

The table has been re-formatted.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

\* Are the title and abstract appropriate for this methods article?

Yes

\* Are there any other potential applications for the method/protocol the authors could discuss?

Yes - the authors could mention that linked peptide technology could also be used to measure co-agonism by MHC class II for CD4, and study the potential influence of non-classical MHC class I molecules such as HLA-E or even CD1.

This has been included in the discussion of the revised manuscript.

\* Are all the materials and equipment needed listed in the table? (Please note that any basic lab materials or equipment do not need to be listed, e.g. pipettes.)

Yes

\* Do you think the steps listed in the procedure would lead to the described outcome?

Yes

\* Are the steps listed in the procedure clearly explained?

Yes- note there is an error on line 203, streptomycin is 100µg/ml not 10mg/ml! Also there is a discrepancy between the timing of co-culture in the procedure (4h) and Fig 3 (3h for A, B, and D). Line 355 should be amended to 3-4h to avoid confusion.

Thank you for spotting the mistakes. This has been corrected in the revised manuscript.

\* Are any important steps missing from the procedure?

No

\* Are appropriate controls suggested?

Yes, although one is absent- specifically mutation of scGAG to abolish CD8 binding.

This has been added to the discussion in the revised manuscript.

\* Are all the critical steps highlighted?

Yes

\* Is there any additional information that would be useful to include?

Yes, some alternatives for key reagents/instruments would be useful. eg if no gamma irradiator is available another method for inactivating the PBMCs (such as mitomycin C or UV irradiation) could be given. Similarly, if no specific "TCR-like" antibody is available, an alternative such as using an sc-peptide-HLAB7 construct and mAb BB7.1 could be discussed. Likewise, in section 4.3.1 the use of a non-enzymatic tissue dissociation medium as an alternative to scraping should be discussed. Also, as the CMV promoter is particularly sensitive to methylation dependent inactivation, some indication of how long the clones based on pCDNA3 can be maintained in culture should be given, and/or alternatives such as a constitutive vector with an IRES driven drug selection cassette mentioned. Finally, it should be noted that the CHO cells will still express Hamster MHC class I molecules. Although these appear irrelevant for the system studied, the possibility that other human TCRs might show xenoreactivity should be mentioned, and the use of CRISPR/Cas9 technology to eliminate the hamster beta 2 microglobulin given as an alternative in the event of a background signal is detected.

We are grateful to the reviewer for these excellent suggestions, which have been included in the discussion in the revised manuscript.

\* Are the anticipated results reasonable, and if so, are they useful to readers?

Yes

\* Are any important references missing and are the included references useful?

Yes, at least one reference discussing mutations to the alpha 3 domain that abolish CD8 binding (eg Purbhoo et al J. Biol Chem Vol. 276, pp. 32786-32792, 2001) should be given. Given that the V152E scGAG was still active as a co-agonist (although there was no control using a GAG-specific T cell showing that this mutation was also effective for this pMHC complex- an important point given the author's comments about the ineffectiveness of 7 other mutations on E183) the use of a D227K/T228A mutant scGAG that would be predicted to eliminate/reduce its co-agonism would also be an important control.

This has been added to the discussion in the revised manuscript.

Minor point - I found the yellow circles in Fig 1C difficult to see - I would recommend filling the "cells" with gray to increase the contrast, or substituting with a different color such as cyan.

Thank you for the suggestion. The figure has been re-formatted.

Reviewer #2:

Manuscript Summary:

This article by Brzostek et al. presents the development of an important protocol to develop single chain (sc) MHC class I to investigate molecular interaction in human CD8+T cell activation. The article focuses on sc MHC class I technology to investigate co-agonism during HBV T cell activation. Though not-stimulatory self-peptide MHC (pMHC) complexes do not induce T cell activation and effector functions, it can enhance T cell responses to agonist pMHC. This 'co-agonism' process may be useful in cases where there is only low level of surface MHC-1 presented in the cell. Therefore understanding the mechanism of co-agonism may provide an alternative perspective for immunotherapy, possibly by enhancing the presentation of endogenous pMHC to induce co-agonism-mediated T cell responses. In addition the method developed in this paper can be easily adapted for research on different aspects of CD8+ T cell activation in human T cell systems with known pMHC-I specificity.

Previous research showed that when a fixed amount of agonist pMHC are allowed in the presence or absence of non-stimulatory pMHC, the presence of non- stimulatory pMHC enhanced activation of mouse thymocytes, naïve peripheral CD8+ T cells and CTLs. However, this experimental system does not allow testing the relative contributions of TCR and CD8 coreceptor binding to agonist and co-agonist pMHC in mediating the activation enhancement, as any modifications altering TCR or CD8 binding to MHC class I would affect all MHC molecules, regardless of the presented peptide. To overcome this shortcoming, the authors developed a clever approach by using MHC class I single chain technology (sc MHC I). The scMHC- I consists of peptide, β2-microglobulin and MHC-I heavy chain joined by two flexible linkers. This protocol describes use of human scMHC-I to investigate co-agonism during human CTL clone activation.

They used the tetracycline-regulated expression (T-Rex) CHO cell line to express agonist and co-agonist constructs. The advantage of using this is in the presence of tetracycline repressor, pcDNA5/TO plasmid allows only very low, "leaky" expression of the protein of interest, high expression levels can be induced by addition of tetracycline. This way they precisely control the pMHC presentation in the cell surface to induce T cell activation. This engineered xenogeneic APC system allows presentation of low levels of agonist pMHC in the presence or absence of co-agoinst pMHC. They tested potential CD8+T cell activation in engineered APCs in samples and controls: three negative controls (T cell only, untransfected T-Rex CHO cells and T-Rex CHO cells expressing high levels of co-agonist), two samples (low level of agonist in the absence and in the presence of co-agonist), and one positive control (high level of agonist). Simultaneous quantification of the two major effector functions, cytokine production and cytotoxicity using T cell activation assay showed expression of low level of agonist induced lower levels of IFN- production and degranulation and these outcomes were enhanced by the presence of co- agonist. They also used mutations in TCR to show that TCR binding to co-agonist pMHC is not required for co-agonist pMHC dependent CD8+Tcell activation.

Major Concerns:

I think this is a very exciting, innovative protocol to study general T cell activation. It is clear and well written. The sample and controls were designed with a clear concept and the data presented here validate their claim without ambiguity. The statistical analysis of their data can be improved, as it is not clear how many times each experiment was repeated and what the sample size is for the functional analysis.

This information has been added to the revised version of the manuscript.

It is also not clear from the manuscript the critical amount of co-agonist needed to trigger T cell activation when low level of agonist present in the cell. Is it possible to vary the amount of tetracycline to regulate agonist antigen expression? Or is it possible to vary the amount of leaky agonist for a fixed amount of co-agonist? Can they measure quantitatively the relative contribution of agonist and co-agonist, which trigger T cell activation as in the case of high amount of agonist.

These are great questions, and we have addressed them in the discussion in the revised manuscript.

Minor Concerns:

1. A brief description of why they used the tetracycline-regulated expression?

This information has been added to the introduction in the revised manuscript.

2. The protocol steps 4.1, 4.1.1.and 4.1.2 seem to miss the continuity (in line 312, 315 and 319).

This section of the protocol has been substantially edited to improve clarity.

3. The centrifugation time is missing in line 317, 340 and 345.

Thank you for spotting this. This has been corrected.

4. The axis labels in the flow histograms are small and x-axis values in Fig 1D and 1E are missing.

Thank you for spotting this. This has been corrected.