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Use of single chain MHC technology to investigate co-agonism in human CD8+ T cell activation
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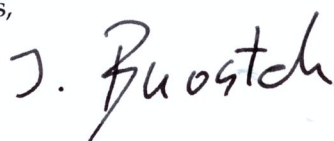
The Editors
Journal of Visualized Experiments

Dear Editors,

We wish to submit an original article entitled "Use of single chain MHC technology to investigate co-agonism in human CD8+ T cell activation" for consideration by Journal of Visualized Experiments.

We have recently shown that non-stimulatory peptide MHC class I complexes can enhance human CD8+ T cell activation though process termed co-agonism (Zhao et al., 2018 Nonstimulatory peptide-MHC enhances human T-cell antigen-specific responses by amplifying proximal TCR signaling. Nature Communications 13:2716. doi: 10.1038/s41467-018-05288-0). Our methods manuscript describes an experimental system to investigate co-agonism during human CD8+ T cell activation by expressing human MHC class I molecules presenting pre-determined peptides as single polypeptides (single chain MHC) in a xenogeneic cell line. The protocol describes: cell line transfection with single chain MHC constructs, generation of stable cell lines, culture of hepatitis B virus-specific human CD8+ T cells and T cell activation experiments simultaneously quantifying cytokine production and degranulation. The presented methods and the single chain MHC class I antigen presentation system can be used for research on different aspects of CD8+ T cell activation in human T cell systems with known peptide MHC specificity.

We confirm that this work is original and has not been published elsewhere, nor is it currently under consideration for publication elsewhere.

Sincerely yours,

J. Brzostek

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1   **TITLE:**
2   Use of Single Chain MHC Technology to Investigate Co-Agonism in Human CD8+ T Cell
3   Activation
4
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KEYWORDS:

SUMMARY:

This protocol describes the use of single chain MHC class I complexes to investigate molecular interactions in human CD8+ T cell activation: generation of engineered antigen presenting cells expressing single chain constructs, culture of human CD8+ T cell clone and T cell activation experiments.

ABSTRACT:

Non-stimulatory self peptide MHC (pMHC) complexes do not induce T cell activation and effector functions, but can enhance T cell responses to agonist pMHC, through a process termed co-agonism. This protocol describes an experimental system to investigate co-agonism during human CD8+ T cell activation by expressing human MHC class I molecules presenting pre-determined peptides as single polypeptides (single chain MHC) in a xenogeneic cell line. We expressed single chain MHCs under conditions where low levels of agonist single chain p-MHC complexes and high levels of non-stimulatory single chain p-MHC complexes were expressed. Use of this experimental system allowed us to compare CD8+ T cell responses to agonist pMHC in the presence or absence of non-stimulatory pMHC. The protocol describes cell line transfection with single chain MHC constructs, generation of stable cell lines, culture of hepatitis B virus-specific human CD8+ T cells and T cell activation experiments simultaneously quantifying cytokine production and degranulation. The presented methods can be used for research on different aspects of CD8+ T cell activation in human T cell systems with known peptide MHC specificity.

INTRODUCTION:

MHC class I (MHC-I) molecules present short (8-10 amino acids) peptides derived from proteins synthesized by each cell. MHC-I molecules on healthy cells present self peptides derived from endogenous proteins. Viral infection leads to presentation of non-self virus-derived peptides on MHC-I, but even infected cells present non-self peptides in the presence of large amounts of self peptide-MHC (pMHC). MHC-I is recognized by the T cell receptor (TCR) and the CD8 co-

89 receptor on T cells. TCR binding affinity to peptide pMHC is highly dependent on the sequence
90 of presented peptides, whereas CD8 binding is peptide-independent. TCR binding to non-self
91 agonist pMHC complex leads to T cell activation and effector functions. Non-stimulatory self
92 pMHC complexes do not induce T cell activation and effector functions, but can enhance T cell
93 responses to agonist pMHC, through a process termed co-agonism[1-3]. Co-agonism has been
94 observed in mouse CD4+ T cells[4-6] as well as in mouse and human CD8+ T cells[7-13]. Under
95 physiological conditions, T cells need to recognize limited quantities of agonist pMHC on
96 antigen presenting cells (APCs) co-presenting high levels of non-stimulatory pMHC complexes,
97 suggesting that co-agonism contributes to optimal T cell responses in vivo. Total cell surface
98 MHC class I levels are frequently downregulated during viral infections[14] and on tumors[15]. This
99 immune evasion strategy decreases agonist pMHC presentation, but also reduces the amount
100 of co-agonist pMHC I available for T cell activation enhancement. Moreover, high cell surface
101 expression levels of the MHC class I molecule HLA-C have been shown to correlate with
102 improved HIV control[16], suggesting a critical role for total MHC class I expression in T cell
103 activation. Despite the physiological significance of co-agonism, its molecular mechanism is still
104 incompletely understood. This is largely due to technical challenges of experimentally
105 controlling the amount of presented co-agonist pMHC while keeping agonist pMHC constant.
106
107 We developed an experimental system to investigate co-agonism during human CD8+ T cell
108 activation by expressing human MHC class I molecules presenting pre-determined peptide as
109 single polypeptide (single chain MHC-I, **Figure 1A**) in a xenogeneic cell line[9,10]. Single chain (sc)
110 agonist pMHC was expressed under control of tetracycline-inducible promoter in the hamster-
111 derived T-REx CHO cell line expressing tetracycline repressor; this allows very low "leaky"
112 expression of sc agonist pMHC in the absence of tetracycline and high sc agonist pMHC
113 expression after addition of tetracycline (**Figure 1B,C**). The sc agonist pMHC-expressing T-REx
114 CHO cells were then supertransfected with non-stimulatory, co-agonist sc pMHC-I under
115 control of a constitutively active promoter (**Figure 1B,C**). Use of this experimental system
116 allowed us to compare CD8+ T cell responses to agonist pMHC in the presence or absence of
117 high levels of non-stimulatory pMHC. Critically, since peptide and MHC-I heavy chain are
118 covalently linked in the scMHC-I format, this allows introductions of mutations into MHC
119 molecules presenting pre-determined peptides. Use of scMHC-I technology thus allowed us to
120 specifically modulate TCR or CD8-binding properties of agonist or co-agonist pMHC.
121
122 Co-agonism in CD8+ T cell activation has been observed using purified MHC-I complexes
123 presenting agonist and co-agonist peptides in the form of heterodimers[11,17] or presented on
124 quantum dots[12,13]. Early work on co-agonism in CD8+ T cell activation in the context of agonist
125 pMHC recognition on APC used TAP2-deficient cell lines as APCs. TAP is required for peptide
126 transport from the cytoplasm to the endoplasmic reticulum, and TAP-deficiency severely
127 reduces the pool of available MHC class I-binding peptides. In the absence of peptides, the
128 nascent complex of MHC class I heavy chain and β_2-microglobulin is unstable, resulting in very
129 low MHC-I cell surface expression. Initially, comparison of T cells stimulated with antigen
130 loaded on TAP-sufficient and -deficient mouse RMA and RMA-S cell lines, respectively, as APCs,
131 did not reveal any evidence for co-agonism during mouse T cell activation[18]. However, use of
132 this experimental system did not allow precise control over the amount of agonist pMHC

133 presented independently of non-stimulatory self pMHC. Moreover, these two cell lines may
134 also differ in expression of other molecules that modulate T cell activation, such as ligands to T
135 cell co-stimulatory or inhibitory receptors, or adhesion molecules.
136
137 Subsequently, we developed a protocol for loading TAP2-deficient RMA-S cells with exogenous
138 peptides to allow presentation of a fixed amount of agonist pMHC in the presence or absence
139 of non-stimulatory pMHC. This was achieved through the following: incubation of TAP2-
140 deficient RMA-S cells at lower temperatures (28 °C, instead of the usual 37 °C) to stabilize
141 empty MHC class I heavy chain/β_2 microglobulin complexes[19]; incubation at 28 °C with
142 exogenous agonist peptide; incubation at 28 °C in the presence or absence of exogenous non-
143 stimulatory peptide; and incubation at 37 °C to reduce cell surface expression of empty MHC
144 class I complexes[8,9]. Use of this experimental set-up revealed that the presence of non-
145 stimulatory pMHC enhanced activation of mouse thymocytes, naïve peripheral CD8+ T cells and
146 CTLs[8]. Mechanistically, the presence of non-stimulatory pMHC can induce CD8 recruitment to
147 the T cell:APC immune synapse even in the absence of agonist pMHC, and non-stimulatory
148 pMHC can enhance TCR interaction with CD8 coreceptor[7,8]. However, this experimental system
149 does not allow testing the relative contributions of TCR and CD8 coreceptor binding to agonist
150 and co-agonist pMHC in mediating the activation enhancement, as any modifications altering
151 TCR or CD8 binding to MHC class I would affect all MHC molecules, regardless of the presented
152 peptide. We therefore used MHC class I single chain technology to overcome this problem.
153
154 The single chain (sc) MHC class I format has been used before to improve antigenic pMHC
155 presentation for therapeutic strategies, and to answer fundamental questions on mechanisms
156 of TCR and NK cell receptor activation and function[20,21]. scMHC-I consists of peptide, β_2-
157 microglobulin and MHC-I heavy chain joined by two flexible serine/glycine-rich linkers (**Figure
158 1A**), several different linker sequences have been developed and tested[22]. scMHC-I can fold
159 independently of the TAP complex and chaperone proteins required for conventional pMHC
160 complex assembly[20]. scMHC-I have been shown to fold correctly, as determined using
161 conformation-specific antibody staining[22] and by solving sc structure with X-ray
162 crystallography[23]. The basic scMHC-I design has been modified to improve binding of low
163 affinity peptides[24,25].
164
165 This protocol describes the use of human scMHC-I to investigate co-agonism during human CTL
166 clone activation. The protocol has been optimized for human CTL clone specific for an epitope
167 of Hepatitis B virus (HBV). HBV is a severe healthcare burden worldwide, as there are 350
168 million people infected with HBV and chronic HBV infection can lead to development of
169 hepatocellular carcinoma (HCC). HCC cells resulting from HBV infection can usually present
170 HBV-derived epitopes and can be recognized by specific human T cells. HBV and HCC clearance
171 relies on the human T cell responses[26], but HCC patients often suffer from T cell exhaustion and
172 reduced T cell responses[27]. Introduction of HBV-specific TCR into T cells of HBV has been tried
173 as a potential immunotherapy strategy to eliminate chronic HBV infection[28]. However, it is
174 unknown if this method will be effective in a clinical setting, due to the low levels of surface
175 MHC-I in human hepatocytes[29]. Therefore, understanding the mechanism of coagonism may
176 provide alternative perspectives for immunotherapy of HBV, possibly by enhancing the

177 presentation of endogenous pMHC to induce co-agonism-mediated T cell responses. The
178 protocol covers all the necessary steps for research on human co-agonism: transfection of T-
179 REx CHO cells with agonist and co-agonist sc pMHC, generation of stable T-REx CHO cell lines,
180 culture of HBV-specific human CTL CD8+ T cell line and CTL activation experiments quantifying
181 cytokine production and degranulation in response to T-REx CHO cells. Although we focus on
182 using the sc MHC class I technology to investigate co-agonism during HBV T cell activation, it
183 must be noted that the presented methods can easily be adapted for research on other aspects
184 of T cell activation in human T cell systems with known pMHC-I specificity.
185
186 This protocol uses a human CD8+ CTL line specific for HBV-derived peptide E183-91 (FLLTRILTI:
187 "E183")[30] presented on HLA-A2. sc HLA molecules consisting of a signal sequence from human
188 β2-microglobulin, a HLA-A2 binding peptide of interest, a glycine/serine linker, a human β2-
189 microglobulin, a glycine/serine linker and a A2 heavy chain can be commercially synthesized
190 (**Figure 1A**). Plasmids encoding scA2 constructs with agonist E183 peptide, or coagonist HIV
191 GAG (SLYNTVATL)[31] peptides are available from the authors upon request. The peptide
192 sequence can be changed using site directed-mutagenesis. A tetracycline-inducible vector
193 pcDNA5/TO was used to express the agonist single chain HLA-A2 with E183 peptide (sc E183-
194 HLA-A2). In the presence of tetracycline repressor, the pcDNA5/TO plasmid allows only very
195 low, "leaky" expression of the protein of interest; high expression can be induced by the
196 addition of tetracycline (**Figure 1B,C**). Use of a tetracycline-inducible expression system allows
197 control over the amount of cell surface agonist pMHC expression. A constitutive expression
198 plasmid pcDNA3.1 was used to express coagonist scA2 with GAG peptide (sc GAG-HLA-A2). The
199 tetracycline-regulated expression (T-REx) CHO cell line (hamster cell line, no endogenous
200 human MHC) was used to express agonist and co-agonist sc-HLA-A2 constructs. This
201 experimental system allows precise control over pMHC presentation (**Figure 1C**): no pMHC
202 presentation (untransfected T-REx), a high amount of co-agonist pMHC presentation
203 (constitutive sc GAG-HLA-A2 expression), a low amount of agonist pMHC presentation (sc E183-
204 HLA-A2 in the absence of tetracycline), a high amount of agonist pMHC presentation (sc E183-
205 HLA-A2 in the presence of tetracycline), a low amount of agonist pMHC presentation and high
206 expression of co-agonist pMHC (sc E183-HLA-A2 in the absence of tetracycline, and constitutive
207 sc GAG-HLA-A2 expression). Use of this experimental system allows precise control of cell
208 surface amounts of agonist and coagonist pMHC.
209
210 **PROTOCOL:**
211
212 NOTE: All steps involving use of live, non-fixed cells should be performed in a biosafety hood,
213 and any biohazardous waste should be discarded according to the local health and safety
214 regulations.
215
216 **1 CHO cell transfection and generation of stable CHO cell lines.**
217
218 1.1 CHO cell transfection
219

220 NOTE: This section describes CHO cell transfection using polyethylenimine (PEI). However,
221 alternative methods can be used, as CHO cells are relatively easy to transfect using
222 commercially available lipid transfection reagents.
223
224 1.1.1 Culture CHO cells in complete F12 (cF12, F12 supplemented with 5% FBS, 100 U/mL
225 penicillin, 100 µg/mL streptomycin and 10 µg/mL blasticidin to maintain expression of the
226 tetracycline repressor). Passage the cells every 3-4 days at a 1:10 ratio.
227
228 1.1.2 One day before transfection, prepare the CHO cell suspension. Wash CHO cells in the flask
229 using PBS (10 mL for PBS for a T75 flask, 5 mL PBS for a T25 flask). Add 0.05% Trypsin/0.02%
230 EDTA in PBS (2 mL for a T75 flask, 1 mL for a T25 flask) to the flask and incubate for 5-10 min at
231 room temperature (RT). Using a light microscope, check that the cells have detached, and stop
232 trypsinization by adding cF12 to the flask (8 mL for a T75 flask, 4 mL for a T25 flask).
233
234 1.1.3 Transfer the CHO cell suspension to a 15 mL tube, and centrifuge at 300-400 x g for 5 min
235 at 4 °C or RT. Remove supernatant, re-suspend in cF12 (10 mL for a T75 flask, 5 mL for a T25
236 flask) and count using a hemocytometer.
237
238 1.1.4 Adjust cell concentration to 60,000-100,000 CHO cells/mL. Add 5 mL of CHO cell
239 suspension (300,000-500,000 CHO cells) per well in a 6 well plate. Incubate overnight at 37 °C,
240 5% CO_2.
241
242 1.1.5 On the day of the transfection, add 400 µL of serum-free F12 media or low serum media
243 to a 1.5 mL tube. Add 3 µg of plasmid to 400 µL of media. Mix by pipetting. Add 6 µg of PEI (6 µL
244 of 1 mg/mL PEI solution) to the media/DNA mix, and vortex immediately for 10 s.
245
246 1.1.3 Incubate the media/DNA/PEI mix at RT for 10-15 min. During this incubation, replace CHO
247 cell media in the 6 well plate with 5 mL of fresh cF12 media.
248
249 1.1.4 Add the media/DNA/PEI solution to CHO cells. Add the solution dropwise and evenly over
250 the well. Incubate overnight at 37 °C, 5% CO_2.
251
252 1.2 Generation of stable CHO cell lines
253
254 1.2.1 One day after transfection, remove media from the wells and add 5 mL of cF12 containing
255 the appropriate selection antibiotics to each well. Use 0.3 mg/mL hygromycin for pcDNA5TO-
256 based plasmids, and 1 mg/mL geneticin for pcDNA3-based plasmids.
257
258 1.2.2 If the cells reach more than 90% confluency at 24 h post transfection, trypsinize the cells.
259 Wash wells with 1 mL of PBS per well, add 1 mL of 0.05% trypsin/0.02% EDTA in PBS and
260 incubate for 5-10 min at RT. Using a light microscope, check that the cells have detached, and
261 stop trypsinization by adding 3-4 mL of cF12 per well.
262

263 1.2.3 Transfer the CHO cell suspension to a 15 mL tube, and centrifuge at 300-400 x g for 5 min
264 at 4 °C or RT. Remove the supernatant and re-suspend in 10 mL of cF12. Add 5 mL of cell
265 suspension per well in 6 well plate. Use two wells to maximize yield of the transfected cells.
266
267 1.2.4 Incubate the 6 well plates at 37 °C, 5% CO_2. Monitor cell death and the growth of resistant
268 colonies using a light microscope. Remove media and replace it with fresh media containing the
269 appropriate antibiotics after 4-5 days, or when significant cell death is observed.
270
271 1.2.5 Once the resistant cells reach more than 70% confluence, trypsinize the cells as described
272 in step 1.2.2 and transfer to a T25 flask for expansion. The antibiotic selection takes 1-2 weeks.
273
274 1.2.6 Once the antibiotic-resistant CHO cells in the T25 flask reach 70-80% confluence, perform
275 antibody staining to verify cell surface expression of the constructs of interest. One day before
276 antibody staining, trypsinize the cells as described in step 1.2.2 and adjust the cell
277 concentration to 200,000 cells/mL, and add 1 mL of cell suspension to 12 well plate. For testing
278 of tetracycline-inducible constructs, add 50-60 ng/mL of tetracycline. Incubate overnight at 37
279 °C, 5% CO_2.
280
281 1.2.7 Use cell scraper to detach CHO cells from bottom of the well. This method is preferable to
282 trypsinization, as it reduces the risk of epitope damage due to proteolytic cleavage.
283
284 1.2.8 Transfer the 1 mL of media containing the CHO cells to a FACS tube. Spin down at 300-400
285 x g, 4 °C for 5 min. Re-suspend in 100 μL of anti-HLA antibody diluted in FWB. Incubate 30 min
286 at 4 °C on ice. Perform this staining with anti-A2 antibody to detect total A2 cell surface
287 expression levels, and TCR-like antibody specific for A2 in complex with E183 peptide to detect
288 agonist pMHC-I cell surface expression levels.
289
290 1.2.9 After verifying MHC expression, FACS-sort the positive cells, and maintain the sorted cells
291 in media with antibiotics to reduce transgene loss. For CHO cells expressing agonist pMHC,
292 single cell sorting is recommended, to ensure comparable agonist pMHC expression with and
293 without supertransfection with co-agonist pMHC. 2-3 weeks are required for CHO cell growth
294 following the single cell sorting.
295
296 **2) HBV-specific CTL culture**
297
298 2.1 Preparation of PBMCs as feeders for HBV-specific human CTL re-stimulation
299
300 NOTE: For the A2-E183-specific CTL clone, re-stimulation with freshly isolated PBMCs, rather
301 than thawed PBMCs, gives the best results.
302
303 CAUTION: Obtain all the necessary ethical approvals before recruiting volunteers for blood
304 donations. For the purpose of this work, PBMC were collected from healthy volunteers under
305 the protocol approved by NUS IRB. Informed consent was obtained from all donors. Follow all

306   the necessary precautions when working with human blood to reduce risk of transmission of
307   blood borne diseases.

309   2.1.1 Before starting, set the centrifuge temperature at 19-20 °C and pre-warm the density
310   gradient (e.g., Ficoll) to RT.

312   2.1.2 Add 15 mL of room temperature density gradient to 50 mL tube. Centrifuge at 400 x *g* for
313   5 min at 19-20 °C. This is to ensure there is no density gradient on the side of the tube.

315   2.1.3 Collect 20 mL of blood from a healthy volunteer donor through venipuncture. Add 15 mL
316   of PBS to 20 mL of blood in a 50 mL tube. Mix by pipetting up and down.

318   2.1.4 Slowly, add the 35 mL of blood diluted in PBS on top of the 15 mL of density gradient from
319   step 2.1.2. Make sure that the blood and the density gradient do not mix. Centrifuge the tubes
320   at 19 °C, 1,200 x *g* for 20 min with the brakes off.

322   2.1.5 Remove approximately 70% of the plasma layer. Carefully aspirate the layer containing
323   the PBMC (the cloudy layer on top of the clear density gradient layer) using a Pasteur pipette
324   and place it in a new 50 mL tube.

326   2.1.6 Add PBS to PBMCs to obtain a 50 mL total volume. Centrifuge tube at 4 °C, 300-400 x *g* for
327   10 min. Carefully remove the supernatant using the vacuum aspirator and sterile glass Pasteur
328   pipette, or by decanting.

330   2.1.7 Re-suspend the cells in 50 mL of PBS. Centrifuge tube at 4 °C, 300 x *g* for 10 min. Carefully
331   remove the supernatant using the vacuum aspirator and sterile glass Pasteur pipette, or by
332   decanting.

334   2.1.8 Re-suspend cells in 2 mL of complete human T cell media (serum free media
335   supplemented with 2% human serum). Count the cells using hemocytometer. This protocol
336   gives on average 1-2 x 10^6 PBMCs per 1 mL of blood used.

338   2.2 CTL re-stimulation using PBMCs as feeder cells

340   2.2.1 Irradiate PBMCs at 30 Gy to inhibit proliferation in response to subsequent stimulation.

342   CAUTION: Ensure adequate training and compliance with the laboratory safety requirements
343   when using the irradiator.

345   2.2.2 Re-suspend the irradiated PBMCs in complete human T cell media at 2 x 10^6 cells/mL. Add
346   cytokines and lectin from *Phaseolus vulgaris* (PHA) for the CTL re-stimulation at 2x
347   concentration into the PBMC suspension. The final cytokine and PHA concentrations are: 20
348   U/mL recombinant human IL-2, 10 ng/mL recombinant IL-7, 10 ng/mL recombinant human IL-
349   15 and 1.5 μg/mL PHA.

350

351 2.2.3 Thaw the frozen CTL vial in a 37 °C water bath. Transfer the thawed cells into a 15 mL tube
352 with 10 mL of complete human T cell media. Spin down at 300-400 x g at 4 °C for 5 min.
353 Carefully remove the supernatant using the vacuum aspirator and sterile glass Pasteur pipette,
354 or by decanting.

355

356 2.2.4 Re-suspend CTLs in 2 mL of complete human T cell media. Count the cells using
357 hemocytometer. Add complete human T cell media to obtain cell concentration of 10^6 cells/mL.

358

359 2.2.5 In a 24 well plate, add 0.5 mL of irradiated PBMCs (10^6 cells) with 2x cytokines and PHA
360 (step 2.2.2), and 0.5 mL of CTLs (0.5 x 10^6 cells) per well. The number of wells used depends on
361 the total number of CTLs or PBMCs available. Incubate at 37 °C, 5% CO_2.

362

363 2.2.7 After 4-5 days, when CTL blasts are visible and the media becomes yellow, transfer the
364 cultures into 6 well plates, and supplement the initial 1 mL volume with 4 mL of complete
365 human T cell media with 20 U/mL IL-2, 10 ng/mL IL-7 and 10 ng/mL IL-15 (no PHA).

366

367 2.2.8 After 2-3 days, transfer the cultures into a T75 flask, add 40 mL of complete human T cell
368 media with cytokines (step 2.2.2, no PHA) and culture in an upright position.

369

370 2.2.9 Maintain CTLs at a concentration of 1-2 x 10^6 cells/mL by adding fresh media
371 supplemented with cytokines (step 2.2.2, no PHA) at a volume equal to that of the existing
372 culture volume at every other day. CTLs can be used for experiments 7-14 days after re-
373 stimulation.

374

375 **3) T cell activation experiments**

376

377 NOTE: A typical experiment requires several different T-RExCHO lines: untransfected T-REx CHO
378 cells (negative control), T-REx CHO cells expressing non-stimulatory pMHC only (scA2-GAG;
379 negative control), T-REx CHO cells expressing low amounts of agonist pMHC-I (scA2-ENV
380 without tetracycline, experimental sample), T-REx CHO cells expressing low amounts of agonist
381 pMHC and high amounts of non-stimulatory co-agonist pMHC (scA2-ENV and scA2-GAG,
382 without tetracycline, experimental sample), and T-REx CHO cells expressing high amounts of
383 agonist pMHC-I scA2-ENV with tetracycline, positive control), as shown on **Figure 1C,D**.

384

385 3.1 Plating CHO cells as antigen presenting cells

386

387 3.1.1 Culture CHO cells in T75 flask, as described in step 1.1.1. Use cells at 70-90% confluence
388 for best results. One day before the T cell activation experiment, trypsinize the cells as
389 described in section 1.1.2. After trypsinization, transfer the cell suspension into a 15 mL tube,
390 centrifuge at 400 x g for 5 min at 4 °C or RT, remove supernatant by pipetting or decanting.

391

392 3.1.2 Re-suspend the cell pellet in 5 mL of cF12. Count CHO cells using a hemocytometer.

393

3.1.3 Adjust the cell concentration to 2×10^5/mL using cF12. Add 50-60 ng/mL of tetracycline to the agonist only samples to induce high amounts of agonist pMHC-I expression as positive control (**Figure 1C**). CHO cells will settle down in 15 mL tubes, so make sure to mix them by pipetting or vortexing before plating.

3.1.4 For T cell activation assay, add 100 μL of the CHO cell suspension per well to U-bottom 96 well plate, and use triplicates per CHO cell line. For CHO cell anti-MHC staining, add 1 mL of cell suspension to 12 well plate, and use triplicates per cell line. Incubate overnight at 37 °C, 5% CO_2.

3.2 T cell activation assay

NOTE: This T cell activation assay allows simultaneous quantification of T cell degranulation (CD107a staining, a measure of T cell cytotoxicity) and cytokine production.

3.2.1 The day before the experiment, centrifuge CTLs cells at 400 x g at 4 °C for 5 min, count using hemocytometer and re-suspend the cells at 10^6 cells/mL in complete human T cell media without cytokines or PHA. Incubate at 37 °C, 5% CO_2. The purpose of this rest step is to reduce CTL sensitivity, as we have observed maximum activation in response to low amounts of antigen without this step.

3.2.2 On the day of the experiment, centrifuge T cells at 400 x g at 4 °C for 5 min, remove the supernatant by decanting or pipetting, and re-suspend the cells in 2 mL complete serum-free media (without cytokines or PHA). Count live T cells using a hemocytometer, and adjust T cell concentration to 10^6 live cells/mL using complete human T cell media (without cytokines or PHA).

3.2.3 Add anti-human CD107a antibody at a 1:100 dilution (final concentration of 2.5 μg/mL), add brefeldin A at a 1:1000 dilution (final concentration of 1 μg/mL).

3.2.4 Remove media from the CHO cells in the 96 well plate by aspirating with glass Pasteur pipette or by flicking the plate. Per well, add 200 μL of the T cell suspension (0.2 x 10^6 cells) containing anti-CD107a and brefeldin A. Co-culture T cells with CHO cells for 3-4 h.

3.2.5 At the end of the co-culture, perform T cell surface antigen staining. Spin down the 96 well plate at 300-400 x g, 5 min at 4 °C, and remove the supernatant by aspirating or flicking. Add 2.5 μL of CD3 and 2.5 μL of CD8 antibodies in 50 μL of 0.5% BSA in PBS (flow wash buffer, FWB) per well for cell surface antigen staining. Incubate samples on ice for 30 min in the dark.

3.2.6 Wash samples by adding 150 μL of FWB per well. Spin down the 96 well plate at 300-400 x g, 5 min at 4 °C, and remove the supernatant by aspirating or flicking.

436 3.2.7 Perform fixation/permeabilization steps using a fixation/permeabilization kit. Add 100 μL
437 of fixation/permeabilization solution (from the fixation/permeabilization kit) per well, mix by
438 pipetting. Incubate on ice for 20 min.

440 3.2.8 Centrifuge at 300-400 x g and 4 °C for 5 min, and remove the supernatant by aspirating or
441 flicking. Add 200 μL of 1x perm/wash buffer (dilute the 10x perm/wash stock buffer from the kit
442 prior to use) per well. Repeat this step.

444 3.2.9 Re-suspend the cell pellet in 50 μL of 1x perm/wash buffer containing anti-IFN-γ antibody
445 at 0.3 μg/mL. Incubate on ice for 30 min in dark.

447 3.2.10 Add 150 μL of 1x perm/wash buffer. Centrifuge at 300-400 x g for at 4 °C for 5 min, and
448 remove the supernatant by aspirating or flicking. Add 200 μL of 1x perm/wash buffer,
449 centrifuge 300-400 x g for at 4 °C for 5 min, and remove the supernatant by aspirating or
450 flicking.

452 3.2.11 Re-suspend each sample in 200 μL of FWB. Proceed with flow cytometric analysis.

454 3.3 CHO cell MHC class I staining

456 NOTE: It is critical to verify scMHC-I cell surface levels on cells used on each experiment, as cell
457 lines may lose transgene expression. When preparing 96 well plate for T cell stimulation, set up
458 12 well plate for CHO cell staining, as outlined in Section 3.1.4.

460 3.3.1 Use a cell scraper to detach CHO cells from the bottom of the well. This method is
461 preferable to trypsinization, as it reduces the risk of epitope damage due to proteolytic
462 cleavage.

464 3.3.2 Transfer the 1 mL of media containing the CHO cells to the FACS tube. Spin down at 300-
465 400 x g at 4 °C for 5 min. Re-suspend in 100 μL of anti-HLA antibody diluted in FWB. Incubate
466 for 30 min at 4 °C on ice.

468 3.3.2.1. Perform this staining with anti-A2 antibody to detect total A2 cell surface expression
469 levels, and TCR-like antibody specific for A2 in complex with E183 peptide[32] to detect agonist
470 pMHC-I cell surface expression levels.

472 3.3.3 Add 1 mL of FWB to each CHO sample, spin down at 300-400 x g, 4 °C, 5 min, and then re-
473 suspend the sample in 0.3 mL of FWB. Proceed with flow cytometry analysis.

475 **REPRESENTATIVE RESULTS:**
476 The sc MHC class I technology used here allows precise control of cell surface expression of
477 MHC class I with known, covalently linked peptides, to induce T cell activation. We have
478 generated an engineered xenogeneic APC system (**Figure 1A,B,C**) that allows presentation of
479 low levels of agonist pMHC in the presence or absence of co-agonist pMHC (**Figure 1D,E**).

480   Critically, we have used a TCR-like antibody specific for HLA-A2 presenting E183 peptide to
481   show that presentation of the agonist sc E183-HLA-A2 is not altered by co-expression of co-
482   agonist sc GAG-HLA-A2 (**Figure 1E**). However, it must be noted that the E183-HLA-A2 specific
483   TCR-like antibody shows some binding to HLA-A2 presenting GAG peptide (**Figure 1E**). Similar
484   engineered antigen presenting cell systems can be constructed using different peptides or MHC
485   molecules to investigate molecular requirements for TCR and/or coreceptor interactions in
486   human T cells with different specificities.

488   We used these engineered APCs to stimulate an E183-HLA-A2-specific human CD8+ T cell clone.
489   Three negative controls were used: T cells only, untransfected T-REx CHO cells, and T-REx CHO
490   cells expressing high amounts of co-agonist sc GAG-HLA-A2 to test for potential T cell activation
491   by any molecules expressed on T-REx CHO cells (untransfected T-REx CHO cells as compared to
492   T cell only), and for T cell activation by sc-GAG-HLA-A2 (T-REx CHO cells expressing high levels of
493   sc-GAG-HLA-A2 as compared to untransfected T-REx CHO cells). Untransfected T-REx CHO cells
494   or T-REx CHO cells expressing sc-GAG-HLA-A2 did not induce activation of E183-HLA-A2-specific
495   CD8+ CTL clone, as determined by quantifying IFN-γ production and expression of
496   degranulation marker CD107a as a measure of T cell cytotoxicity (**Figure 2A,B**). Expression of
497   high levels of agonist sc E183-HLA-A2, used as positive control, induced very efficient IFN-γ
498   production and degranulation (**Figure 2A,B**), demonstrating that the sc HLA-A2 construct can be
499   recognized by a specific TCR to activate T cell effector functions. Expression of low levels of sc
500   E183-HLA-A2 induced lower levels of IFN-γ production and degranulation, and this was
501   enhanced by presence of co-agonist sc GAG-HLA-A2 (**Figure 2A,B**). It must be noted that very
502   high percentages of CD107a+ T cells were observed even in response to low levels of antigenic
503   pMHC (**Figure 2B**), consistent with previous reports of relatively low requirements for the
504   amount of agonist pMHC for induction of T cell cytotoxicity[33]. The CD107a MFI, indicating the
505   amount of degranulation on a single cell basis, provides a better dynamic range (**Figure 2B**). The
506   T cell activation assay used allows simultaneous quantification of two major effector functions:
507   cytokine production and cytotoxicity, and provides very sensitive readout with good dynamic
508   range.

510   We used the single chain technology to test whether TCR binding to co-agonist pMHC is
511   required for co-agonist pMHC-dependent activation enhancement. We mutated valine at
512   position 152 at the edge of the HLA-A2 peptide binding groove (**Figure 3B**) to glutamic acid to
513   create V152E mutant, as this mutation has been reported to abolish TCR binding to HLA-A2[34].
514   We then tested if the V152E mutation can abolish TCR binding for the E183-HLA-A2 CTL clone
515   used, by introducing this mutation into agonist sc E183-HLA-A2 and expressing the mutant
516   agonist sc construct in T-REx CHO cells. Wild type, but not V152E, sc E183-HLA-A2 induced
517   activation of the E183-HLA-A2-specific CTL clone used (**Figure 3A**). It must be noted that any
518   TCR-binding mutation on MHC class I needs to be separately tested for each individual TCR/T
519   cell clone used, as the effect of mutation will depend on the precise molecular interactions
520   between TCR and pMHC complex, and these will differ between different TCRs. For example,
521   we tested eight mutations, either previously reported TCR-binding mutations or mutations
522   predicted to disrupt TCR binding without abrogating peptide binding into HLA-A2. Of these,
523   V152E was the only mutation that abolished activation of the E183-specific T cell clone used[10].

524 We then introduced the V152E mutation into the co-agonist sc GAG-HLA-A2, and co-expressed
525 WT or V152E sc GAG-HLA-A2 with low levels of agonist sc E183-HLA-A2. Both WT and V152E sc
526 GAG-HLA-A2 enhanced IFN-γ production and degranulation (**Figure 3C,D**), suggesting that TCR
527 binding to co-agonist pMHC is not required for co-agonist pMHC-dependent CD8+ T cell
528 activation enhancement. Single chain MHC class I technology, as presented here, can be used to
529 modify TCR and/or coreceptor binding to MHC class I with known peptide to investigate the
530 molecular requirements for T cell antigen recognition and activation.
531
532 **FIGURE AND TABLE LEGENDS:**
533
534 **Figure 1. Single chain HLA-A2 constructs used to investigate co-agonist in human CD8+ T cell**
535 **activation.**
536 (**A**) Schematic diagram of a scHLA-A2 construct, consisting of covalently signal sequence from
537 fused human β_2 microglobulin, peptide of choice, flexible glycine-serine linker (GGGGSGGGGS
538 GGGGS), human β_2 microglobulin, flexible glycine-serine linker and HLA-A2 heavy chain.
539 (**B**) Schematic diagram of the plasmids used to generate engineered antigen presenting cells to
540 investigate co-agonism in human CD8+ T cell activation: tetracycline repressor, agonist sc E183-
541 HLA-A2 under control of tetracycline-inducible promoter, non-stimulatory co-agonist sc GAG-
542 HLA-A2 under control of constitutively active promoter.
543 (**C**) Schematic diagram of the engineered antigen presenting cells used to investigate co-
544 agonism: T-REx CHO cells (no human MHC expression), T-REx CHO cells expressing
545 constitutively high levels of sc GAG-HLA-A2 (non-stimulatory co-agonist pMHC), T-REx CHO cells
546 expressing low "leaky" levels of sc E183-HLA-A2 in the absence of tetracycline (low level of
547 agonist peptide), T-REx CHO cells expressing high levels of sc E183-HLA-A2 in the presence of
548 tetracycline (high level of agonist peptide), T-REx CHO cells expressing low levels of sc E183-
549 HLA-A2 and high levels of sc GAG-HLA-A2 (low level of agonist peptide and high level of co-
550 agonist peptide).
551 (**D**) Cell surface staining of the engineered antigen presenting cell lines using anti-HLA-A2
552 antibody. The numbers shown denote MFI values for MHC stain in the live cell gate. Data
553 representative of 5 independent experiments (**E**) Cell surface staining of the engineered antigen
554 presenting cell lines using TCR-like antibody specific against HLA-A2 presenting E183 peptide.
555 The numbers shown denote MFI values for MHC stain in the live cell gate. Data representative
556 of 5 independent experiments.
557
558 **Figure 2. Presence of co-agonist pMHC enhanced cytokine production and degranulation of**
559 **E183-HLA-A2-specific human CD8+ CTL clone.**
560 (**A**) Intracellular IFN-γ staining of HLA-A2-specific human CD8+ CTL clone after 3h stimulation
561 with the indicated engineered antigen presenting cells. The numbers shown denote the
562 percentage positive for IFN- γ staining. Data representative of 3 independent experiments, each
563 performed with technical triplicates.
564 (**B**) Cell surface CD107a staining of HLA-A2-specific human CD8+ CTL clone after 3h stimulation
565 with the indicated engineered antigen presenting cells. The numbers shown denote the
566 percentage positive for CD107a staining or the CD107a MFI for the CD8+ T cell population. Data
567 representative of 3 independent experiments, each performed with technical triplicates.

568
569 **Figure 3. Co-agonist – mediated T cell activation enhancement does not require TCR binding**
570 **to the co-agonist pMHC complex.**
571 (**A**) V152E mutation in sc E183-HLA-A2 abolishes activation of the E183-HLA-A2-specific human
572 CD8+ CTL clone. T-REx CHO cells expressing high levels (tetracycline induction) of the WT or
573 V152E mutant sc E183-HLA-A2 were used to stimulate E183-HLA-A2-specific human CD8+ CTL
574 clone for 3h. T cell activation was quantified using intracellular IFN-γ staining. The numbers
575 shown denote the percentage positive for IFN- γ staining. Data representative of 3 independent
576 experiments, each performed with technical triplicates.
577 (**B**) Location of V152E mutation within the peptide binding groove of HLA-A2.
578 (**C**) V152E mutation on sc GAG-HLA-A2 co-agonist pMHC complex does not abolish co-agonist-
579 dependent activation enhancement. Intracellular IFN-γ staining of HLA-A2-specific human CD8+
580 CTL clone after 4h stimulation with the indicated engineered antigen presenting cells. The
581 numbers shown denote the percentage positive for IFN- γ staining. Data representative of 3
582 independent experiments, each performed with technical triplicates.
583 (**D**) V152E mutation on sc GAG-HLA-A2 co-agonist pMHC complex does not abolish co-agonist-
584 dependent activation enhancement. Cell surface CD107a staining of HLA-A2-specific human
585 CD8+ CTL clone after 3h stimulation with the indicated engineered antigen presenting cells. The
586 numbers shown denote the percentage positive for CD107a staining or the CD107a MFI for the
587 CD8+ T cell population. Data representative of 3 independent experiments, each performed
588 with technical triplicates.
589
590 **DISCUSSION**
591 The presented protocol provides a robust tool for investigation of molecular interactions during
592 human CD8+ T cell activation. A critical step for investigation of co-agonism during human T cell
593 activation is control over agonist pMHC cell surface expression to ensure equal agonist pMHC
594 presentation on the APCs with or without co-agonist pMHC expression. In our experimental
595 system, this is achieved by using a single cell clone expressing low amounts of agonist pMHC,
596 followed by supertransfection with a co-agonist pMHC construct and agonist pMHC
597 quantification using TCR-like antibody[10,32]. As agonist sc pMHC expression can change over
598 time, it is critical to quantify agonist pMHC surface expression on APCs used in each experiment
599 using TCR-like antibodies. If no specific TCR-like antibody is available, agonist scMHC-I can be
600 expressed as fluorescent protein fusion, and its cell surface expression can then be quantified
601 using a sensitive microscopy method, such as total internal reflection fluorescence
602 microscopy[35,36]. Moreover, cell surface expression of co-agonist pMHC decreases over time,
603 due to methylation-dependent and –independent silencing of the CMV promoter[37], and should
604 be monitored using antibody staining and flow cytometry analysis, with repeated FACS-sorting
605 when needed. We have cultured CHO cells for up to 5 weeks with relatively limited loss of sc
606 pMHC expression. We highly recommend freezing the CHO cells soon after selection/sorting to
607 ensure a reliable stock of cells with known sc MHC expression.
608
609 The several steps of the presented protocols can be modified, depending on the availability of
610 equipment, or depending on the specific research aims. For example, PBMC proliferation
611 potential can be inhibited (Step 3.2.1) using alternative methods that do not require a gamma

612 (or X-) irradiator, such as treatment with mitomycin C[38]. A non-enzymatic cell dissociation
613 buffers containing metal chelators[39] can be used instead of cell scraping in step 3.3.1.
614 Moreover, the antigen presenting system can be modified to make it more suitable for human
615 CTL clones expressing different TCRs. CHO cells express hamster MHC class I molecules, and
616 although these are irrelevant for the CTL line studied here (**Figure 2A** and **Figure 3A**, we
617 observed no T cell responses to untransfected CHO cells), there is a possibility that other
618 human TCRs may show some xenoreactivity. In that case, hamster MHC class I expression can
619 be reduced by knocking out hamster β2-microglubulin or TAP using CRISPR/Cas9; or a human
620 APC line can be used after CRISPR/Cas9-mediated β2-microglubulin or TAP knock out.

622 The experimental system presented here allows precise control of TCR and CD8 interactions
623 with MHC molecules presenting pre-defined peptides. For example, we have previously shown
624 that mutations abolishing CD8 binding[40] to coagonist pMHC eliminated coagonist-mediated
625 activation enhancement in murine and human CD8+ T cells[9,10], whereas abrogating TCR
626 interaction with co-agonist pMHC had no effect on coagonist-mediated activation
627 enhancement[10]. However, the use of single chain MHC constructs has several limitations. For
628 example, it is time and labor-consuming to test multiple co-agonist peptide sequences using
629 this experimental system, as this involves generation of multiple plasmids encoding sc MHC
630 with different peptides, and subsequent transfections and cell sorting. Previously, we used the
631 murine TAP-deficient cell line RMA-S to test multiple co-agonist peptides in mouse T cell
632 activation[8,9], but this approach was not successful with the TAP-deficient human T2 cell line[10],
633 most likely due to presentation of TAP-independent peptides[41]. Another limitation of our
634 experimental system is that is does not provide a fast method for altering the amount of co-
635 agonist pMHC molecules for the purpose of determining the critical amount of co-agonist
636 pMHC required to trigger T cell activation. Moreover, the tetracycline-inducible system used
637 does not allow titration of agonist pMHC quantity at the single cell level by changing the
638 tetracycline concentration, as varying tetracycline concentration alters the proportion of HLA-
639 A2 positive cells, rather than HLA-A2 levels on per cell basis. An alternative approach that we
640 are currently investigating is to use supported lipid bilayers[42], previously used to investigate co-
641 agonism during murine CD4+ T cell activation[4] or beads presenting fixed amount of agonist
642 pMHC in the presence of varying amounts of co-agonist pMHC. When used in conjunction with
643 UV-cleavable peptide technology[4,43], these alternative experimental systems should allow fast
644 testing of multiple co-agonist peptide sequences as well as different amounts of co-agonist
645 pMHC.

647 The sc MHC technology can be also applicable to investigation of co-agonism in human or
648 murine CD4 T cells, as single chain MHC class II molecules presenting pre-determined peptides
649 have been successfully expressed on mammalian cell surfaces[44]. Moreover, the use of sc MHC
650 technology can be extended to investigation of non-classical MHC molecules such as HLA-E. Sc
651 HLA-E has been described before, and its expression has been shown to inhibit human T cell
652 activation[45]. Another non-classical MHC molecule of interest is CD1, which presents lipids
653 instead of peptides[46]. Sc constructs consisting of CD1 heavy chain and β2-microglobulin have
654 been shown to be expressed on the cell surface and to bind relevant lipid ligands[47]. Single chain

655 MHC technology has great potential as a research tool for investigating molecular interactions
656 during T and NK cell activation.
657
666
667 **DISCLOSURES:**
668 The authors declare no competing interests.
669
670 **REFERENCES:**
671 1 Gascoigne, N. R. Do T cells need endogenous peptides for activation? *Nature Reviews.*
672 *Immunology.* **8** (11), 895-900, doi:10.1038/nri2431 (2008).
673 2 Gascoigne, N. R., Zal, T., Yachi, P. P. & Hoerter, J. A. Co-receptors and recognition of self
674 at the immunological synapse. *Current Topics in Microbiology and Immunology.* **340**
675 171-189, doi:10.1007/978-3-642-03858-7_9 (2010).
676 3 Krogsgaard, M., Juang, J. & Davis, M. M. A role for "self" in T-cell activation. *Seminars in*
677 *Immunology.* **19** (4), 236-244, doi:10.1016/j.smim.2007.04.003 (2007).
678 4 Krogsgaard, M. et al. Agonist/endogenous peptide-MHC heterodimers drive T cell
679 activation and sensitivity. *Nature.* **434** (7030), 238-243, doi:10.1038/nature03391
680 (2005).
681 5 Ebert, P. J., Jiang, S., Xie, J., Li, Q. J. & Davis, M. M. An endogenous positively selecting
682 peptide enhances mature T cell responses and becomes an autoantigen in the absence
683 of microRNA miR-181a. *Nature Immunology.* **10** (11), 1162-1169, doi:10.1038/ni.1797
684 (2009).
685 6 Lo, W. L. et al. An endogenous peptide positively selects and augments the activation
686 and survival of peripheral CD4+ T cells. *Nature Immunology.* **10** (11), 1155-1161,
687 doi:10.1038/ni.1796 (2009).
688 7 Yachi, P. P., Ampudia, J., Gascoigne, N. R. & Zal, T. Nonstimulatory peptides contribute
689 to antigen-induced CD8-T cell receptor interaction at the immunological synapse.
690 *Nature Immunology.* **6** (8), 785-792, doi:10.1038/ni1220 (2005).
691 8 Yachi, P. P., Lotz, C., Ampudia, J. & Gascoigne, N. R. T cell activation enhancement by
692 endogenous pMHC acts for both weak and strong agonists but varies with
693 differentiation state. *The Journal of Experimental Medicine.* **204** (11), 2747-2757,
694 doi:10.1084/jem.20062610 (2007).
695 9 Hoerter, J. A. et al. Coreceptor affinity for MHC defines peptide specificity requirements
696 for TCR interaction with coagonist peptide-MHC. *The Journal of Experimental Medicine.*
697 **210** (9), 1807-1821, doi:10.1084/jem.20122528 (2013).
698 10 Zhao, X. et al. Nonstimulatory peptide-MHC enhances human T-cell antigen-specific

699 responses by amplifying proximal TCR signaling. *Nature Communications.* **9** (1), 2716,
700 doi:10.1038/s41467-018-05288-0 (2018).

701 11 Juang, J. et al. Peptide-MHC heterodimers show that thymic positive selection requires a
702 more restricted set of self-peptides than negative selection. *The Journal of Experimental*
703 *Medicine.* **207** (6), 1223-1234, doi:10.1084/jem.20092170 (2010).

704 12 Anikeeva, N. et al. Quantum dot/peptide-MHC biosensors reveal strong CD8-dependent
705 cooperation between self and viral antigens that augment the T cell response.
706 *Proceedings of the National Academy of Sciences of the United States of America.* **103**
707 (45), 16846-16851, doi:10.1073/pnas.0607771103 (2006).

708 13 Anikeeva, N., Gakamsky, D., Scholler, J. & Sykulev, Y. Evidence that the density of self
709 peptide-MHC ligands regulates T-cell receptor signaling. *PloS One.* **7** (8), e41466,
710 doi:10.1371/journal.pone.0041466 (2012).

711 14 Schuren, A. B., Costa, A. I. & Wiertz, E. J. Recent advances in viral evasion of the MHC
712 Class I processing pathway. *Current Opinion in Immunology.* **40** 43-50,
713 doi:10.1016/j.coi.2016.02.007 (2016).

714 15 Bubenik, J. MHC class I down regulation, tumour escape from immune surveillance and
715 design of therapeutic strategies. *Folia Biologica.* **51** (1), 1-2 (2005).

716 16 Apps, R. et al. Influence of HLA-C expression level on HIV control. *Science (New York,*
717 *N.Y.).* **340** (6128), 87-91, doi:10.1126/science.1232685 (2013).

718 17 Cebecauer, M. et al. CD8+ cytotoxic T lymphocyte activation by soluble major
719 histocompatibility complex-peptide dimers. *The Journal of Biological Chemistry.* **280**
720 (25), 23820-23828, doi:10.1074/jbc.M500654200 (2005).

721 18 Sporri, R. & Reis e Sousa, C. Self peptide/MHC class I complexes have a negligible effect
722 on the response of some CD8+ T cells to foreign antigen. *European Journal of*
723 *Immunology.* **32** (11), 3161-3170, doi:10.1002/1521-4141(200211)32:11<3161::aid-
724 immu3161>3.0.co;2-6 (2002).

725 19 Ljunggren, H. G. et al. Empty MHC class I molecules come out in the cold. *Nature.* **346**
726 (6283), 476-480, doi:10.1038/346476a0 (1990).

727 20 Kotsiou, E., Brzostek, J. & Gould, K. G. Properties and applications of single-chain major
728 histocompatibility complex class I molecules. *Antioxidants & Redox Signaling.* **15** (3),
729 645-655, doi:10.1089/ars.2010.3694 (2011).

730 21 Hansen, T. H., Connolly, J. M., Gould, K. G. & Fremont, D. H. Basic and translational
731 applications of engineered MHC class I proteins. *Trends in Immunology.* **31** (10), 363-
732 369, doi:10.1016/j.it.2010.07.003 (2010).

733 22 Yu, Y. Y., Netuschil, N., Lybarger, L., Connolly, J. M. & Hansen, T. H. Cutting edge: single-
734 chain trimers of MHC class I molecules form stable structures that potently stimulate
735 antigen-specific T cells and B cells. *Journal of Immunology (Baltimore, Md. : 1950).* **168**
736 (7), 3145-3149 (2002).

737 23 Mitaksov, V. et al. Structural engineering of pMHC reagents for T cell vaccines and
738 diagnostics. *Chemistry & Biology.* **14** (8), 909-922, doi:10.1016/j.chembiol.2007.07.010
739 (2007).

740 24 Truscott, S. M. et al. Disulfide bond engineering to trap peptides in the MHC class I
741 binding groove. *Journal of Immunology (Baltimore, Md. : 1950).* **178** (10), 6280-6289
742 (2007).

743   25   Truscott, S. M. et al. Human major histocompatibility complex (MHC) class I molecules
744   with disulfide traps secure disease-related antigenic peptides and exclude competitor
745   peptides. *The Journal of Biological Chemistry.* **283** (12), 7480-7490,
746   doi:10.1074/jbc.M709935200 (2008).
747   26   Bertoletti, A. & Ferrari, C. Innate and adaptive immune responses in chronic hepatitis B
748   virus infections: towards restoration of immune control of viral infection. *Postgraduate*
749   *Medical Journal.* **89** (1051), 294-304, doi:10.1136/postgradmedj-2011-301073rep
750   (2013).
751   27   Boni, C. et al. Characterization of hepatitis B virus (HBV)-specific T-cell dysfunction in
752   chronic HBV infection. *Journal of Virology.* **81** (8), 4215-4225, doi:10.1128/jvi.02844-06
753   (2007).
754   28   Gehring, A. J. et al. Engineering virus-specific T cells that target HBV infected
755   hepatocytes and hepatocellular carcinoma cell lines. *Journal of Hepatology.* **55** (1), 103-
756   110, doi:10.1016/j.jhep.2010.10.025 (2011).
757   29   Gehring, A. J. et al. The level of viral antigen presented by hepatocytes influences CD8 T-
758   cell function. *Journal of Virology.* **81** (6), 2940-2949, doi:10.1128/jvi.02415-06 (2007).
759   30   Bertoni, R. et al. Human histocompatibility leukocyte antigen-binding supermotifs
760   predict broadly cross-reactive cytotoxic T lymphocyte responses in patients with acute
761   hepatitis. *The Journal of Clinical Investigation.* **100** (3), 503-513, doi:10.1172/jci119559
762   (1997).
763   31   Goulder, P. J. et al. Patterns of immunodominance in HIV-1-specific cytotoxic T
764   lymphocyte responses in two human histocompatibility leukocyte antigens (HLA)-
765   identical siblings with HLA-A*0201 are influenced by epitope mutation. *The Journal of*
766   *Experimental Medicine.* **185** (8), 1423-1433 (1997).
767   32   Sastry, K. S. et al. Targeting hepatitis B virus-infected cells with a T-cell receptor-like
768   antibody. *Journal of Virology.* **85** (5), 1935-1942, doi:10.1128/jvi.01990-10 (2011).
769   33   van den Berg, H. A. et al. Cellular-level versus receptor-level response threshold
770   hierarchies in T-cell activation. *Frontiers in Immunology.* **4** 250,
771   doi:10.3389/fimmu.2013.00250 (2013).
772   34   Moots, R. J., Matsui, M., Pazmany, L., McMichael, A. J. & Frelinger, J. A. A cluster of
773   mutations in HLA-A2 alpha 2 helix abolishes peptide recognition by T cells.
774   *Immunogenetics.* **34** (3), 141-148 (1991).
775   35   Crites, T. J., Chen, L. & Varma, R. A TIRF microscopy technique for real-time,
776   simultaneous imaging of the TCR and its associated signaling proteins. *Journal of*
777   *Visualized Experiments.* (61), doi:10.3791/3892 (2012).
778   36   Axmann, M., Schutz, G. J. & Huppa, J. B. Single Molecule Fluorescence Microscopy on
779   Planar Supported Bilayers. *Journal of Visualized Experiments.* (105), e53158,
780   doi:10.3791/53158 (2015).
781   37   Mariati, Koh, E. Y., Yeo, J. H., Ho, S. C. & Yang, Y. Toward stable gene expression in CHO
782   cells. *Bioengineered.* **5** (5), 340-345, doi:10.4161/bioe.32111 (2014).
783   38   Matsushita, S., Tanaka, Y., Matsuoka, T. & Nakashima, T. Clonal expansion of freshly
784   isolated CD4T cells by randomized peptides and identification of peptide ligands using
785   combinatorial peptide libraries. *European Journal of Immunology.* **31** (8), 2395-2402,
786   doi:10.1002/1521-4141(200108)31:8<2395::aid-immu2395>3.0.co;2-0 (2001).

787   39   Heng, B. C., Cowan, C. M. & Basu, S. Comparison of enzymatic and non-enzymatic means
788   of dissociating adherent monolayers of mesenchymal stem cells. *Biological Procedures*
789   *Online.* **11** 161-169, doi:10.1007/s12575-009-9001-4 (2009).
790   40   Smith, K. et al. Sensory adaptation in naive peripheral CD4 T cells. *The Journal of*
791   *Experimental Medicine.* **194** (9), 1253-1261 (2001).
792   41   Wei, M. L. & Cresswell, P. HLA-A2 molecules in an antigen-processing mutant cell
793   contain signal sequence-derived peptides. *Nature.* **356** (6368), 443-446,
794   doi:10.1038/356443a0 (1992).
795   42   Vardhana, S. & Dustin, M. Supported Planar Bilayers for the Formation of Study of
796   Immunological Synapses and Kinapse. *Journal of Visualized Experiments.* (19),
797   doi:10.3791/947 (2008).
798   43   Bakker, A. H. et al. Conditional MHC class I ligands and peptide exchange technology for
799   the human MHC gene products HLA-A1, -A3, -A11, and -B7. *Proceedings of the National*
800   *Academy of Sciences of the United States of America.* **105** (10), 3825-3830,
801   doi:10.1073/pnas.0709717105 (2008).
802   44   Rhode, P. R. et al. Single-chain MHC class II molecules induce T cell activation and
803   apoptosis. *Journal of Immunology (Baltimore, Md. : 1950).* **157** (11), 4885-4891 (1996).
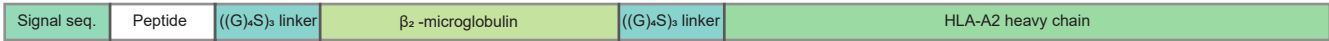804   45   Crew, M. D., Cannon, M. J., Phanavanh, B. & Garcia-Borges, C. N. An HLA-E single chain
805   trimer inhibits human NK cell reactivity towards porcine cells. *Molecular Immunology.*
806   **42** (10), 1205-1214, doi:10.1016/j.molimm.2004.11.013 (2005).
807   46   Van Kaer, L., Wu, L. & Joyce, S. Mechanisms and Consequences of Antigen Presentation
808   by CD1. *Trends in Immunology.* **37** (11), 738-754, doi:10.1016/j.it.2016.08.011 (2016).
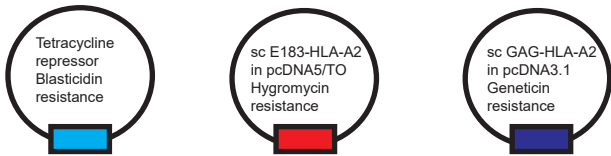809   47   Im, J. S. et al. Direct measurement of antigen binding properties of CD1 proteins using
810   fluorescent lipid probes. *The Journal of Biological Chemistry.* **279** (1), 299-310,
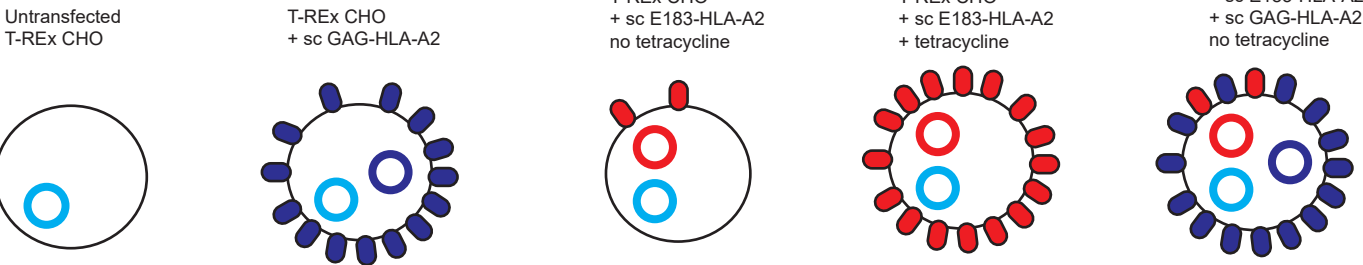811   doi:10.1074/jbc.M308803200 (2004).
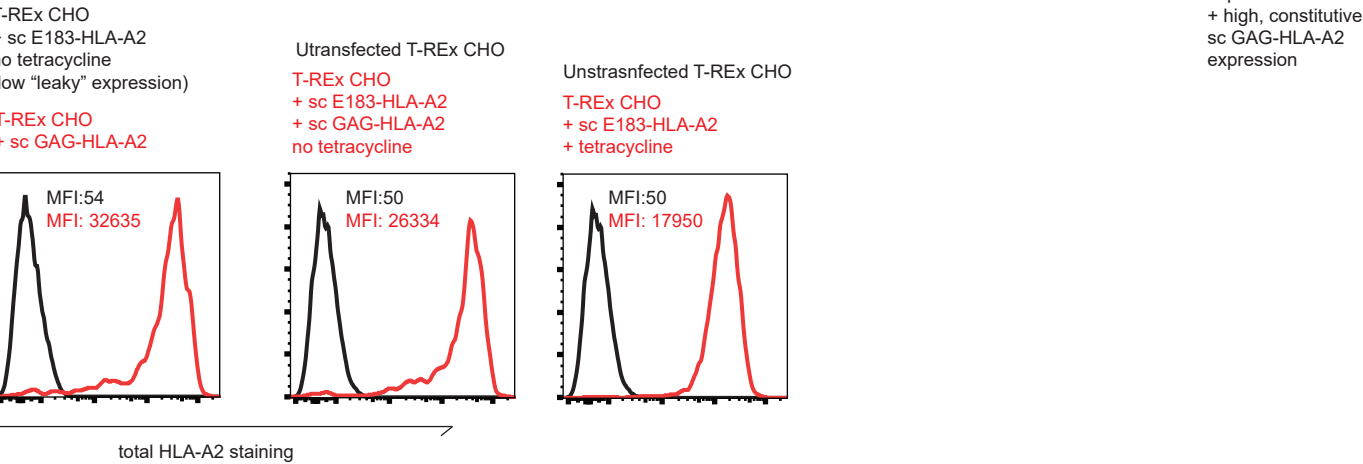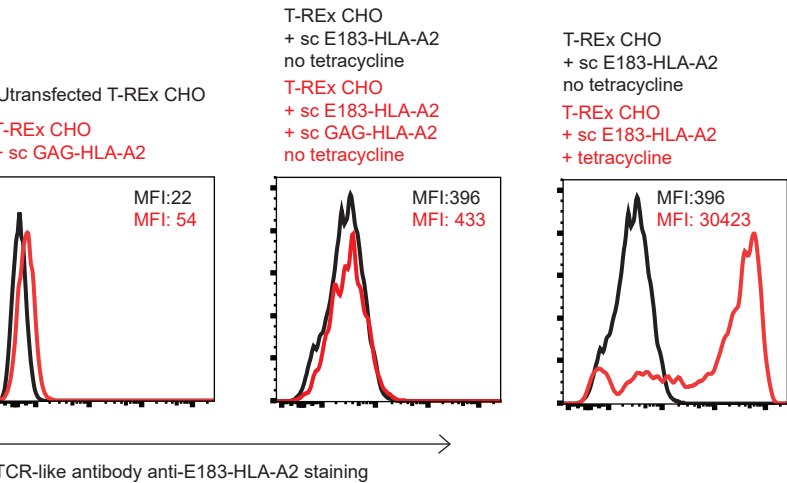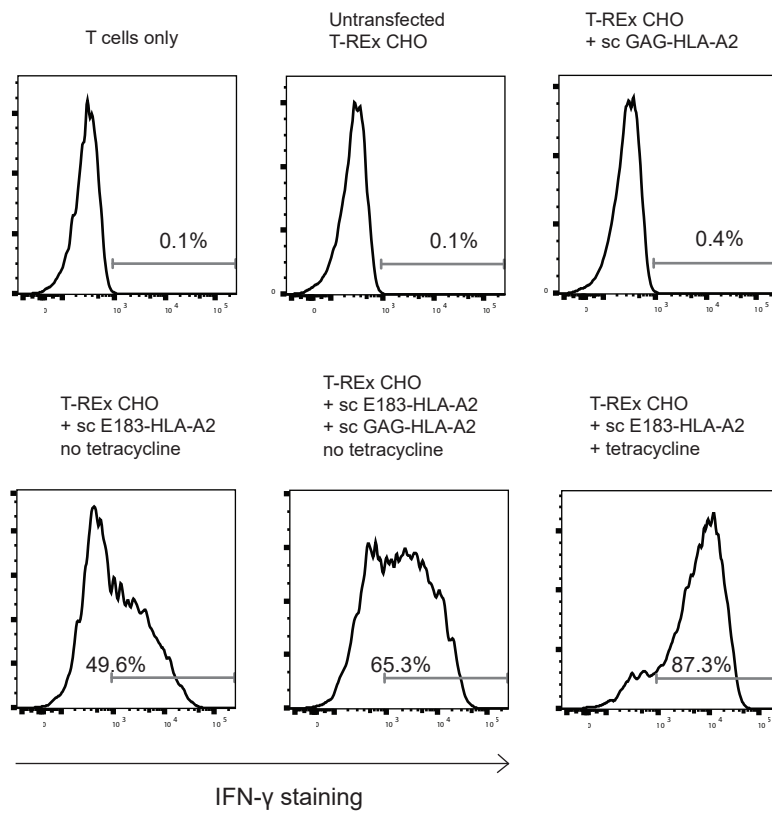812

A

| Signal seq. | Peptide | ((G)₄S)₃ linker | β₂ -microglobulin | ((G)₄S)₃ linker | HLA-A2 heavy chain |

B

Tetracycline repressor Blasticidin resistance

sc E183-HLA-A2 in pcDNA5/TO Hygromycin resistance

sc GAG-HLA-A2 in pcDNA3.1 Geneticin resistance

C

Untransfected T-REx CHO

T-REx CHO + sc GAG-HLA-A2

T-REx CHO + sc E183-HLA-A2 no tetracycline

T-REx CHO + sc E183-HLA-A2 + tetracycline

T-REx CHO + sc E183-HLA-A2 + sc GAG-HLA-A2 no tetracycline

Very low, "leaky" sc E183-HLA-A2 expression + high, constitutive sc GAG-HLA-A2 expression

D

T-REx CHO + sc E183-HLA-A2 no tetracycline (low "leaky" expression)

T-REx CHO + sc GAG-HLA-A2

MFI:54
MFI: 32635

Utransfected T-REx CHO

T-REx CHO + sc E183-HLA-A2 + sc GAG-HLA-A2 no tetracycline

MFI:50
MFI: 26334

Unstrasnfected T-REx CHO

T-REx CHO + sc E183-HLA-A2 + tetracycline

MFI:50
MFI: 17950

total HLA-A2 staining

E

Utransfected T-REx CHO

T-REx CHO + sc GAG-HLA-A2

MFI:22
MFI: 54

T-REx CHO + sc E183-HLA-A2 no tetracycline

T-REx CHO + sc E183-HLA-A2 + sc GAG-HLA-A2 no tetracycline

MFI:396
MFI: 433

T-REx CHO + sc E183-HLA-A2 no tetracycline

T-REx CHO + sc E183-HLA-A2 + tetracycline

MFI:396
MFI: 30423

TCR-like antibody anti-E183-HLA-A2 staining

Figure 2

IFN-γ staining

CD107a staining

Figure 3

A

Untransfected T-REx CHO — 0.1%

T-REx CHO + sc E183-HLA-A2 WT + tetracycline — 75%

T-REx CHO + sc E183-HLA-A2 V152E + tetracycline — 0.2%

IFN-γ staining

B

V152E

C

T cells only — 0.01%

Untransfected T-REx CHO — 0.08%

T-REx CHO + sc GAG-HLA-A2 WT — 0.7%

T-REx CHO + sc E183-HLA-A2 no tetracycline — 49.7%

T-REx CHO + sc E183-HLA-A2 + sc GAG-HLA-A2 WT no tetracycline — 66%

T-REx CHO + sc E183-HLA-A2 + sc GAG-HLA-A2 V152E no tetracycline — 70%

T-REx CHO + sc E183-HLA-A2 + tetracycline — 86.7%

IFN-γ staining

D

T cells only — 0.4% MFI: 125

Untransfected T-REx CHO — 0.4% MFI: 124

T-REx CHO + sc GAG-HLA-A2 WT — 1.7% MFI: 211

T-REx CHO + sc E183-HLA-A2 no tetracycline — 85.2% MFI: 6646

T-REx CHO + sc E183-HLA-A2 + sc GAG-HLA-A2 WT no tetracycline — 91.7% MFI: 8063

T-REx CHO + sc E183-HLA-A2 + sc GAG-HLA-A2 V152E no tetracycline — 90.7% MFI: 8263

T-REx CHO + sc E183-HLA-A2 + tetracycline — 91.4% MFI: 9418

CD107a staining

Table of Materials

Click here to access/download;Table of Materials;JoVE_Materials co-agonism revised.xls

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Aim-V (serum free media)	Gibco	12055091	
blasticidin	Gibco	R21001	
Cytofix/Cytoperm (fixation/permealibisation kit)	BD	554714	
F12	Gibco	10565-018	
Fetal bovine serum (FBS)	HyClone	SH3007103	
geneticin	Gibco	10131035	
GolgiPlug (Brefeldin A)	BD	555029	
Human serum	Sigma-Aldrich	H4522	
hygromycin	Gibco	10687010	
Lectin from Phaseolus vulgaris (PHA)	Sigma-Aldrich	L4144	
Opti-MEM (low serum media)	Bibco	31985070	
10 X PBS	Vivantis	PB0344 – 1L	
Penicillin/Streptomycin	HyClone	SV30010	
polyethylenimine (PEI)	Sigma-Aldrich	408727	
recombinant human IL2	R&D Systems	202-IL	
recombinant human IL7	R&D Systems	207-IL	
recombinant human IL15	R&D Systems	247-ILB	
tetracycline	Sigma-Aldrich	T7660	
T-REx CHO cell line	Thermo Fisher Scientific	R71807	
10 x Trypsin/EDTA	Gibco	15400054	
Antibodies			
CD3	BD	347347	Clone SK7, RRID:AB_400287
CD8α	BD	563795	Clone RPA-T8, RRID:AB_2722501
CD107a	BD	566261	Clone H4A3, RRID:AB_2739639
HLA-A2	eBioscience	17-9876-41	Clone BB7.2, RRID:AB_11151522
IFN-γ	eBioscience	12-7319-41	Clone 4S.B3, RRID:AB_1311250

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Title of Article: Use of single chain MHC technology to investigate co-agonism in human CD8+ T cell activation

Author(s): Zhao, Hamidinia, Choo, Too, Ho, Ren, Bertoletti, MacAry, Gould, Bhostek and Gascoique

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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.

<mark>This information is provided in the revised version of the manuscript.</mark>

11. 4.1.2: Please split into several substeps.

<mark>This section of the protocol has been substantially edited for better clarity.</mark>

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.

<mark>The relevant sections are highlighted in yellow.</mark>

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.

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<mark>The revised version of the manuscript contains Representative Results and Discussion.</mark>

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

<mark>The revised version of the manuscript contains Discussion.</mark>

17. References: Please do not abbreviate journal titles.

<mark>The references have been re-formatted.</mark>

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.

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.

* 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.

* 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.

* 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.