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Dr. Xiaoyan Cao
Review Associate
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Dear Dr. Cao,

RE: Tailoring *in vivo* cytotoxicity assays to study immunodominance in tumor-specific CD8$^+$ T cell responses (Manuscript ID: JoVE59531)

Thank you for reviewing our manuscript quickly. We are pleased to learn that the reviewers thought our manuscript was "well-written and clear" and that they only had minor concerns/comments. We wish to express our gratitude for their insightful comments.

As instructed in your decision letter, we have now provided a rebuttal document and explained whether/how we have revised our manuscript accordingly. We hope our revisions will be satisfactory.

On behalf of all the authors whose names appear on this paper, I thank you for considering it for publication in *JoVE*.

Please do not hesitate to contact me should you require additional information.

Sincerely,

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TITLE:

Tailoring In Vivo Cytotoxicity Assays to Study Immunodominance in Tumor-specific CD8$^+$ T Cell Responses

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CD8$^+$ T cells, cytotoxicity, cancer, immunodominance, tumor-derived peptides, large T antigen, regulatory T cells, PD-1, checkpoint inhibitors, drug efficacy testing, flow cytometry

SUMMARY:

We describe here a flow cytometry-based in vivo killing assay that enables examination of immunodominance in cytotoxic T lymphocyte (CTL) responses to a model tumor antigen. We provide examples of how this elegant assay may be employed for mechanistic studies and for drug efficacy testing.

ABSTRACT:

Carboxyfluorescein succinimidyl ester (CFSE)-based in vivo cytotoxicity assays enable sensitive and accurate quantitation of CD8$^+$ cytolytic T lymphocyte (CTL) responses elicited against tumor- and pathogen-derived peptides. They offer several advantages over traditional killing assays. First, they permit the monitoring of CTL-mediated cytotoxicity within architecturally intact secondary lymphoid organs, typically in the spleen. Second, they allow for mechanistic studies during the priming, effector and recall phases of CTL responses. Third, they provide useful platforms for vaccine/drug efficacy testing in a truly in vivo setting. Here, we provide an optimized protocol for the examination of concomitant CTL responses against more than one peptide epitope of a model tumor antigen (Ag), namely, simian virus 40 (SV40)-encoded large T Ag (T Ag). Like most other clinically relevant tumor proteins, T Ag harbors many potentially immunogenic peptides. However, only four such peptides induce detectable CTL responses in C57BL/6 mice. These responses are consistently arranged in a hierarchical order based on their magnitude, which forms the basis for T_{CD8} "immunodominance" in this powerful system.

Accordingly, the bulk of the T Ag-specific T_{CD8} response is focused against a single immunodominant epitope while the other three epitopes are recognized and responded to only weakly. Immunodominance compromises the breadth of antitumor T_{CD8} responses and is, as such, considered by many as an impediment to successful vaccination against cancer. Therefore, it is important to understand the cellular and molecular factors and mechanisms that dictate or shape T_{CD8} immunodominance. The protocol we describe here is tailored to the investigation of this phenomenon in the T Ag immunization model, but can be readily modified and extended to similar studies in other tumor models. We provide examples of how the impact of experimental immunotherapeutic interventions can be measured using in vivo cytotoxicity assays.

INTRODUCTION:

Conventional $CD8^+$ T cells (T_{CD8}) play important parts in anticancer immune surveillance. They primarily function in the capacity of cytolytic T lymphocytes (CTLs) that recognize tumor-specific or -associated peptide antigens (Ags) displayed within the closed cleft of major histocompatibility complex (MHC) class I molecules. Fully armed CTLs utilize their cytotoxic arsenal to destroy malignant cells. Anticancer T_{CD8} can be detected in the circulation or even inside primary and metastatic masses of many cancer patients and tumor-bearing animals. However, they are often anergic or exhausted and fail to eradicate cancer. Therefore, many immunotherapeutic modalities are designed to increase anticancer T_{CD8} frequencies and to restore and boost their functions.

Tumor proteins harbor many peptides, some of which can be immunogenic and potentially immunoprotective. However, quantifiable T_{CD8} responses are elicited with varying magnitudes against few peptides only. This creates an "immunodominance hierarchy" among T_{CD8} clones[1]. Accordingly, immunodominant (ID) T_{CD8} occupy prominent hierarchical ranks, which is commonly judged by their abundance. In contrast, T_{CD8} cells whose T cell receptor (TCR) is specific for subdominant (SD) epitopes occur in lower frequencies. We and others have identified some of the factors that dictate or shape immunodominance in T_{CD8} responses. These include, among others, the mode of Ag presentation to naïve T_{CD8} (i.e., direct presentation, cross-presentation, cross-dressing)[2-4], the type of Ag-presenting cells (APCs) participating in T_{CD8} activation[5], the abundance and stability of protein Ags[6,7] and the efficiency and kinetics of their degradation by proteasomes[7,8], the relative selectivity of transporter associated with Ag processing (TAP) for peptides[9], the affinity of liberated peptides for MHC I molecules[9,10], the presence, precursor frequencies and TCR diversity of cognate T_{CD8} in T cell pools[11-13], cross-competition among T cells for access to APCs[14,15], and the fratricidal capacity of T_{CD8} clones[16]. In addition, T_{CD8} immunodominance is subjected to immunoregulatory mechanisms mediated by several suppressor cell types such as naturally occurring regulatory T (nTreg) cells[17], the cell surface co-inhibitory molecule programmed death-1 (PD-1)[16], and certain intracellular enzymes such as indoleamine 2,3-dioxygenase (IDO)[18] and the mammalian target of rapamycin (mTOR)[19]. It is important to note, however, that the above factors do not always fully account for immunodominance.

Apart from the basic biology of T_{CD8} immunodominance, the examination of this intriguing phenomenon has important implications in cancer immunology and immunotherapy. First, an ID

status does not necessarily confer upon a given T_{CD8} clone the ability to prevent tumor initiation or progression[20]. Whether and how ID and SD T_{CD8} contribute to antitumor immunity may be dependent upon the type and the extent of malignancy and the experimental system employed. Second, it is thought that ID T_{CD8} clones may be 'too visible' to the immune system and consequently more prone to central and/or peripheral tolerance mechanisms[16,21]. Third, heterogeneic tumors may contain neoplastic cells that avoid detection by many, if not most, CTLs by displaying only a narrow spectrum of peptide:MHC complexes. Under these circumstances, T_{CD8} responses of insufficient breadth are likely to afford such tumor cells a survival advantage, thus potentiating their outgrowth[22]. It is for the above reasons that many view immunodominance as a hurdle to successful T_{CD8}-based vaccination and therapies against cancer.

Inoculation of C57BL/6 mice with simian virus 40 (SV40)-transformed cells that express large tumor Ag (T Ag) provides a powerful preclinical system to study T_{CD8} immunodominance. This model offers several benefits. First, the peptide epitopes of this clinically relevant oncoprotein are well-characterized in this mouse strain[23] (**Table 1**). Second, T Ag epitopes, which are called sites I, II/III, IV, and V, trigger T_{CD8} responses that are consistently arranged in the following hierarchical order: site IV >> site I ≥ site II/III >> site V. Accordingly, site IV-specific T_{CD8} mount the most robust response to T Ag. In contrast, sites I and II/III are subdominant, and site V-specific T_{CD8} are least abundant and usually only detectable in the absence of responsiveness to other epitopes[23,24]. Third, the T Ag^+ tumor cell line utilized in the protocol described herein, namely C57SV fibrosarcoma cells, and those used in our previous investigations[16-19,25,26], are transformed with subgenomic SV40 fragments[25]. Therefore, they are unable to assemble and release SV40 virions that could potentially infect host APCs. In addition, C57SV cells are devoid of classic costimulatory molecules such as CD80 (B7-1), CD86 (B7-2), and CD137 ligand (4-1BBL)[16]. The above attributes make these lines ideal for examination of in vivo T_{CD8} activation via cross-priming. Cross-priming is a major pathway in inducing T_{CD8} responses, especially those launched against tumor cells of non-hematopoietic origin that fail to directly prime naïve T cells[25].

Antitumor T_{CD8} frequencies and/or functions can be monitored by MHC I tetramer staining, intracellular staining for effector cytokines (e.g., interferon [IFN]-γ) or lytic molecules (e.g., perforin), enzyme-linked immunospot (ELISpot) assays and ex vivo cytotoxicity assays. Since their inception in the 1990s[27,28], carboxyfluorescein succinimidyl ester (CFSE)-based in vivo killing assays have enabled evaluation of cytotoxic responses mediated by antiviral CTLs[29-31], antitumor CTLs[16,32], natural killer (NK) cells[33], glycolipid-reactive invariant natural killer T (*i*NKT) cells[34], and preexisting and de novo donor-specific alloantibodies[26]. Therefore, their applications can be of interest to a wide readership, including but not limited to investigators working in the areas of tumor immunology and immunotherapy, anti-pathogen immunity, and preventative and therapeutic vaccine design.

To assess cell-mediated cytotoxicity in typical scenarios, two populations of naïve splenocytes that display either an irrelevant Ag or a cognate Ag(s) are labeled with two different doses of CFSE, mixed in equal numbers and injected into naïve (control) or killer cell-harboring mice. The presence/absence of each target population is then examined by flow cytometry.

We have optimized and employed in vivo killing assays in our studies on immunodominance in both antiviral and antitumor T_{CD8} responses[12,16,17]. Here, we provide a detailed protocol for the simultaneous assessment of ID and SD T_{CD8} responses to T Ag epitopes, which can be readily adopted for similar investigations in other experimental systems. We also provide representative results demonstrating that nTreg cell depletion and PD-1 blockade can selectively enhance ID T_{CD8}- and SD T_{CD8}-induced cytotoxicity, respectively. At the end, we will discuss multiple advantages of in vivo killing assays as well as some of their inherent limitations.

PROTOCOL:

The experiments described here follow animal use protocols approved by institutional entities and adhered to established national guidelines.

1. Inoculation of C57BL/6 Mice with T Ag-expressing Tumor Cells

1.1. Grow the SV40-transformed fibrosarcoma cell line C57SV (or a similar T Ag^+ adherent cell line) in Dulbecco's modified Eagle's medium (DMEM) with 4.5 g/L D-glucose and L-glutamine (1x) and supplemented with 1 mM sodium pyruvate and 10% heat-inactivated fetal bovine serum (FBS) in tissue culture-treated flasks at 37 °C in humidified atmosphere containing 10% CO_2.

1.2. Once the cells become fully confluent or slightly overconfluent, gently remove and discard the medium and rinse the monolayer with pre-warmed sterile phosphate-buffered saline (PBS).

NOTE: Maximal T Ag expression is achieved when T Ag^+ cells reach 100% confluency.

1.3. Inside a biological safety cabinet, add pre-warmed trypsin-EDTA (0.25%) to cover the monolayer at room temperature until the cells are dislodged in patches. Tap the sides of the culture flask(s) several times to release the remaining adherent cells.

NOTE: If necessary and to expedite the trypsinization process, transfer the flask(s) into a 37 °C incubator. Dislodged cells will quickly adopt a rounded shape under a light microscope. This step should last approximately 5 min.

1.4. Add 5 mL of DMEM medium and dissociate clumps to prepare a single-cell suspension by pipetting the content of each flask up and down.

1.5. Transfer the cell suspension through a cell strainer with 70-µm pores into a tube.

1.6. Spin down the tube at 400 x *g* for 5 min at 4 °C.

1.7. Discard the supernatant. Resuspend pelleted cells in 10 mL of sterile cold PBS.

1.8. Repeat steps 1.6 and 1.7 twice.

1.9. Count cells using a hemocytometer. Prepare a uniform suspension containing 4 x 10^7 cells/mL sterile PBS.

1.10. Inject 500 µL of the above suspension intraperitoneally (i.p.) into each adult (6-12-week-old) male or female C57BL/6 mouse.

2. Treatment Regimens

2.1. Treatment Regimen to Examine the Contribution of nTreg Cells to T_{CD8} Immunodominance

2.1.1. Four days before in vivo priming of C57BL/6 mice with C57SV cells (step 1.10), inject each animal once i.p. with 0.5 mg of a low-endotoxin, azide-free anti-CD25 monoclonal antibody (mAb) (clone PC-61.5.3), which depletes nTreg cells, or with a rat IgG1 isotype control (e.g., clone KLH/G1-2-2, clone HRPN, or clone TNP6A7).

2.2. Treatment Regimen to Test the In Vivo Significance of PD-1-PD-L1(2) Interactions in Shaping T_{CD8} Immunodominance

2.2.1. Inject C57BL/6 mice i.p. with 100 µg of the PD-1-blocking mAb (clone RMP1-14) 2 h before as well as 3 and 6 days after inoculation of mice with C57SV cells (step 1.10). Follow the same timeline to inject control animals with a rat IgG2a isotype control (e.g., clone 2A3).

NOTE: The engagement of PD-1 by PD-L1 often, but not always, mediates the co-inhibition and/or exhaustion of Ag-specific T_{CD8}. Therefore, treatment with anti-PD-1 can be performed in parallel with administration of anti-PD-L1 and anti-PD-L2 mAbs to reveal the exact intercellular interaction involved in a biological phenomenon.

3. Preparation of Target Splenocytes

3.1. Euthanize sex-matched naïve C57BL/6 mice (6-12 weeks of age) that will serve as splenocyte donors by cervical dislocation.

3.2. Position each mouse with its abdomen facing up inside a biological safety cabinet. Spray the skin with 70% (v/v) EtOH. Using sterile forceps and scissors, lift the skin and make a small ventral midline incision. Then, cut the skin in a cross-like fashion to expose the peritoneum.

3.3. Using forceps, pull up the peritoneum in a tent-like fashion without snatching any of the internal organs. Cut the peritoneum open to expose the peritoneal cavity and gently remove the spleen.

3.4. Place the spleen(s) inside a 15 mL Dounce tissue grinder containing 5 mL of sterile PBS. Apply manual pressure using the grinder's glass plunger until the splenic tissue dissipates into a red homogenous cell suspension.

NOTE: Depending on the number of recipient animals per experimental group, several donor mouse spleens may be needed for target cell preparation. Up to 3 spleens can be homogenized together inside a 15 mL grinder.

3.5. Transfer the homogenate into a 15 mL tube. Spin down the tube at 400 x *g* for 5 min at 4 °C.

3.6. Discard the supernatant. Resuspend pelleted cells in 4 mL of ammonium-chloride-potassium (ACK) lysing buffer for 4 min to eliminate erythrocytes.

NOTE: This is a time-sensitive step. Overexposing splenocytes to ACK lysing buffer will increase their fragility and render them susceptible to non-specific cell death.

3.7. To each tube, add 8 mL of RPMI 1640 medium containing 10% heat-inactivated FBS, L-alanyl-L-glutamine, 0.1 mM minimum essential media (MEM) nonessential amino acids, 1 mM sodium pyruvate, 10 mM HEPES, and 1x penicillin/streptomycin, which will hereafter be referred to as complete RPMI medium (**Table of Materials**).

3.8. Transfer the content through 70 µm pores of a cell strainer into a new 15 mL tube.

3.9. Spin down the tube at 400 x *g* for 5 min at 4 °C.

3.10. Discard the supernatant. Resuspend pelleted cells in 12 mL of complete RPMI.

3.11. Split the splenocyte suspension into 3 equal portions (4 mL each) in 3 separate tubes.

4. Coating Target Splenocytes with Irrelevant and Cognate Peptides

4.1. Label the tubes according to the peptides that will be used to pulse target splenocytes. Control splenocytes will be pulsed with an irrelevant peptide, and each population of cognate target splenocytes will be pulsed with a synthetic peptide corresponding to the T Ag-derived immunodominant epitope (site IV) or a subdominant T Ag epitope (site I or site II/III) (**Table 1**).

NOTE: The choice of irrelevant peptides depends on the experimental set-up and the mouse strain used in each investigation. The authors often use $gB_{498-505}$ (an H-$2K^b$-restricted immunodominant peptide epitope of herpes simplex virus [HSV]-1) and/or GP_{33-41} (an H-$2D^b$-restricted immunodominant peptide epitope of lymphocytic choriomeningitis virus ([LCMV]) in C57BL/6 mice (**Table 1**). These peptides are optimal choices because: (i) they are derived from pathogens not previously encountered in the mouse model described here; (ii) similar to T Ag-derived peptides, $gB_{498-505}$ and GP_{33-41} are restricted by and binds to H-2^b molecules. In 'three-peak' in vivo killing assays, each of the two peaks that correspond to cognate target cells may represent splenocytes pulsed with an immunodominant or subdominant peptide. The choice of each peptide set varies according to the objectives of each experiment. See Figure 1 and Figure 2 as examples of such variation. For the remainder of this protocol, T Ag-derived sites I and IV will represent subdominant and immunodominant peptides, respectively.

4.2. Pulse the content of each labeled tube with 1 µM of the respective peptide for 1 h at 37 °C and 5% CO_2.

4.3. Use a separate cell strainer (with 70-µm pores) for each tube to remove clumps and debris if necessary.

4.4. Spin down the tube at 400 x *g* for 5 min at 4 °C. Discard the supernatant.

4.5. Resuspend pelleted cells in 12 mL of sterile cold PBS and repeat step 4.4 once more.

NOTE: It is important to remove as much FBS as possible because FBS can bind CFSE in the next step.

5. Labeling target splenocytes with CFSE

5.1. Resuspend peptide-pulsed splenocytes in 4 mL of sterile PBS.

5.2. Add CFSE at 0.025 µM, 0.25 µM, and 2 µM into the tubes containing irrelevant peptide-, site I-, and site IV-pulsed splenocytes, respectively.

NOTE: To achieve uniform CFSE labeling, hold each tube at a 45° angle before adding CFSE to the side slightly above the cell suspension followed immediately by gentle vortexing. This will ensure the appearance of smooth histograms at the end. Batch-to-batch and age-dependent variations in CFSE intensities are not uncommon. Therefore, one may need to experiment with differential CFSE doses before deciding on optimal concentrations to be used.

CAUTION: CFSE is toxic at concentrations that are higher than 5 µM.

5.3. Place the tubes inside a 37 °C incubator for 15 min and invert them once every 5 min.

5.4. Add 3 mL of heat-inactivated FBS to each tube to stop the CFSE reaction. Top up the content with sterile PBS.

5.5. Spin down the tube at 400 x *g* for 5 min at 4 °C. Discard the supernatant.

5.6. Resuspend pelleted cells in 12 mL of sterile PBS and repeat step 5.5.

6. Examination of Adequate/Equal CFSE Labeling of Target Splenocyte Populations

6.1. Resuspend pelleted cells in 3 mL of PBS.

6.2. Vortex the tubes gently. Transfer 10 µL, each, of $CFSE^{low}$, $CFSE^{intermediate\ (int)}$, and $CFSE^{high}$ cell suspensions into a 5 mL round-bottom polystyrene fluorescence-activated cell sorting (FACS) tube containing 200 µL OF PBS.

6.3. Interrogate cells using a flow cytometer equipped with a 488 nm laser. Draw a lymphocyte gate based on forward scatter (FSC) and side scatter (SSC) properties of the cells before acquiring 5000 events falling within the lymphocyte gate in the FL-1 channel.

6.4. Within the 'parent' $CFSE^{+}$ population, draw additional histogram gates to identify $CFSE^{low}$, $CFSE^{int}$, and $CFSE^{high}$ subpopulations.

6.5. Confirm equal or near-equal event numbers within the three gates. If necessary, adjust cell numbers in the 'source' tubes (step 6.1) before mixing and injecting target splenocytes into naïve and primed mice in section 7.

7. Injection of CFSE-labelled Target Cells into Naïve and T-Ag-primed Recipients

7.1. Gently vortex the source tubes. Transfer the three CFSE-labeled cell suspensions in equal ratios into a new tube.

7.2. Top up the content with sterile PBS.

7.3. Spin down the tube at 400 x *g* for 5 min at 4 °C. Resuspend pelleted cells with sterile PBS.

7.5. Count cells in trypan blue by a hemocytometer to ensure cellular viability of at least 95%.

7.6. Adjust the volume in order to inject 1 x 10^7 mixed target cells/200 µL PBS intravenously (i.v.), via tail vein, into each recipient C57BL/6 mouse.

NOTE: Store the cells on ice in between injections. Gently mix target cells prior to each injection. Record the exact time of injection for each mouse, which will determine when the animal will need to be euthanized. It is important to keep the duration of in vivo cytotoxicity consistent among all animals in the same experiment.

8. Data Acquisition

8.1. Two or four hours after the injection of CFSE-labeled target cells, euthanize the recipient mice by cervical dislocation.

NOTE: The duration of in vivo cytotoxicity can vary depending on the experimental system employed, the immunogenicity of target Ags, the anticipated abundance of peptide antigen-specific T_{CD8} in the spleen, and the robustness of their lytic function among other factors.

8.2. Remove and process each spleen separately as in steps 3.2−3.9.

8.3. Discard the supernatant and resuspend the pelleted cells in 3 mL of PBS.

NOTE: Take extra care to handle the splenic tissue and cell preparations at 4 °C or on ice before cytofluorimetric analyses. This is to prevent continued cytotoxicity ex vivo.

8.4. Transfer approximately 1 x 10^7 cells from each processed spleen into a clean FACS tube.

8.5. Interrogate cells immediately using a flow cytometer equipped with a 488-nm laser. Draw a lymphocyte gate based on FSC and SSC properties of the cells.

8.6. Identify $CFSE^-$ recipient's splenocytes and $CFSE^+$ transferred target cells. Draw additional gates accommodating distinct $CFSE^{low}$, $CFSE^{int}$, and $CFSE^{high}$ target cell populations.

8.7. Acquire a total of 2000 $CFSE^{low}$ events in the FL-1 channel.

NOTE: Acquiring a constant number of $CFSE^{low}$ target cells, as opposed to a constant number of total $CFSE^+$ cells, will help avoid inconsistencies in shapes and heights of control histograms. Therefore, naïve and T Ag-primed mice shall have similar histograms corresponding to irrelevant peptide-pulsed target cells regardless of whether and to what extent cognate ($CFSE^{int/high}$) target cells may have been destroyed.

9. Data Analysis

9.1. Calculate the specific lysis of each cognate target cell population using the following formula:

$$\% \text{ Specific cytotoxicity} = \left[1 - \left\{\left((x / y) / \left({}^{a}/_{b}\right)\right)\right\}\right] \times 100$$

where $x =$ $CFSE^{int/high}$ event number in T Ag-primed mouse, $y =$ $CFSE^{low}$ event number in T Ag-primed mouse, $a =$ $CFSE^{int/high}$ event number in naïve mouse, and $b =$ $CFSE^{low}$ event number in naive mouse.

NOTE: In 'three-peak' cytotoxicity assays in which the specific lysis of more than one cognate target population is evaluated, it is not appropriate to use target cell frequencies. This is simply because the frequency of a cognate target cell population is influenced not only by the percentage of the irrelevant controls but also by that of the other cognate target splenocytes. Therefore, event numbers within each gate should be used in the above formula to accurately calculate the lysis of each cognate target cell population (either $CFSE^{int}$ or $CFSE^{high}$ cells) against $CFSE^{low}$ controls.

REPRESENTATIVE RESULTS:

The goal of the experiment whose results are depicted in **Figure 1** was to determine whether the presence and functions of nTreg cells shape or alter the immunodominance hierarchy of T Ag-

specific T_{CD8}. C57BL/6 mice were injected i.p. with PBS or with 0.5 mg of an anti-CD25 mAb (clone PC-61.5.3 [PC61]) four days before they received 2 x 10^7 C57SV tumor cells i.p. In separate experiments, a rat IgG1 isotype control was used in lieu of PBS. Successful nTreg cell depletion by PC61 was confirmed by flow cytometry[17].

Nine days after C57SV cell inoculation, a time point at which T Ag-specific T_{CD8} responses reach their maximum[23], each animal received an i.v. injection of a cell suspension containing 3 distinct populations of CFSE-labeled target cells. Control target cells were syngeneic naïve splenocytes concomitantly pulsed with two irrelevant peptides (GP_{33-41} and $gB_{498-505}$) and labeled with a low dose of CFSE (0.02 µM). To prepare cognate target cells, syngeneic naïve splenocytes were pulsed with either T Ag-derived site I peptide or site IV peptide (**Table 1**), and subsequently labeled with CFSE at 0.2 µM and 2 µM, respectively. Control and cognate target cells were washed and mixed in equal numbers (at a 1:1:1 ratio) before they were injected into naïve (control) and T Ag-primed C57BL/6 mice. Two hours after target cell injection, mice were sacrificed for their spleen in which the presence/absence of CFSE-labeled target cells was determined by flow cytometry. Target cells were distinguished based on their differential CFSE staining intensities.

As expected, near-equal peaks corresponding to control and cognate target cells were detectable in naïve mice (**Figure 1**, left panel). In contrast, site IV-displaying target cells were almost completely absent in T Ag-primed mice regardless of their prior treatment with PC61 or PBS (**Figure 1**). Interestingly, nTreg cell depletion by PC61 augmented in vivo CTL-mediated lysis of site I-pulsed target cells[17]. These results prompted us to conclude that nTreg cells selectively inhibit site I-specific cytotoxicity. Therefore, nTreg cell-depleting/inactivating agents may enhance the cytolytic effector function of CTLs recognizing certain tumor-derived epitopes.

The above set-up provides an example of how in vivo cytotoxicity assays can be employed to simultaneously test the lytic function of ID and SD CTL clones in the same animal.

[Insert Figure 1 here].

In a more recent investigation, we asked whether blocking PD-1 affects the 'breadth' of the T_{CD8} response to T Ag[16] (**Figure 2**). This was a clinically relevant question in light of the observed therapeutic benefits of PD-1-based 'checkpoint inhibitors' in several malignancies. Although such inhibitors are thought to work primarily by reversing T cell exhaustion, we were curious to know whether interfering with PD-1-PD-L1 interactions may additionally widen (or narrow) anticancer T_{CD8} responses. In our T Ag recognition model, intracellular cytokine staining (ICS) experiments revealed that treatment with either anti-PD-1 or anti-PD-L-1 selectively expands IFN-γ-producing T_{CD8} recognizing sites I and II/III[16]. We then extended our study to examine the in vivo cytolytic effector function of these SD CTLs. C57BL/6 mice were injected i.p. with 100 µg of an anti-PD-1 mAb (clone RMP1-14) or a rat IgG2a isotype control (clone 2A3) two hours before C57SV cell inoculation. Mice received two additional doses of anti-PD-1 or isotype three and six days after tumor cell injection. On day 9 post-priming, cohorts of naïve and primed mice were given, via lateral tail veins, a cell mixture containing equal numbers of $CFSE^{low}$ (CFSE labeling dose: 0.025 µM), $CFSE^{int}$ (CFSE labeling dose: 0.25 µM), and $CFSE^{hi}$ (CFSE labeling dose: 2 µM) syngeneic naïve

splenocytes pulsed with $gB_{498\text{-}505}$, site II/III, and site I, respectively. Four hours later, animals were euthanized, and CFSE-labeled target cells were tracked cytofluorimetrically in their spleen. Representative FACS plots (**Figure 2A**) and data from 3 animals per cohort (**Figure 2B**) are illustrated. While PD-1 blockade did not affect the ID T_{CD8} response against site IV[16], sites I- and II/III-specific SD responses were invigorated. We thus concluded that interfering with PD-1-PD-L1 interactions may induce 'epitope spreading' in anticancer T_{CD8} responses.

The above set-up represents in vivo killing assays that enable quantitation of cytotoxicity elicited by two SD CTL clones in the same animal.

[Insert Figure 2 here].

FIGURE AND TABLE LEGENDS:

Figure 1: Representative cytofluorimetric analysis of T_{CD8}-mediated cytotoxicity against T Ag-derived epitopes in the presence or absence of nTreg cells. Target splenocytes pulsed with control peptides, site I or site IV, which were differentially labeled with CFSE, were tracked by flow cytometry in the spleen of a naïve mouse (left panel), a PBS-injected T Ag-primed mouse (middle panel), and a PC61 (nti-CD25)-injected (nTreg-depleted) T Ag-primed mouse (right panel). Percent specific killing of target cells was calculated using the formula described in the protocol, and representative numbers are shown. This figure is adopted, with permission, from Haeryfar et al.[17]. Copyright 2005. The American Association of Immunologists, Inc.

Figure 2: In vivo cytotoxicity of T Ag-specific T_{CD8} in anti-PD-1-treated mice. (**A**) Representative histogram plots demonstrate CFSE peaks corresponding to target splenocytes pulsed with an irrelevant peptide ($CFSE^{low}$), site II/III ($CFSE^{int}$), and site I ($CFSE^{high}$) in T Ag-primed mice that received an isotype (left panel) or a PD-1-blocking mAb (right panel). (**B**) Percent specific killing of each cognate target cell population was calculated using $CFSE^{+}$ event numbers in T Ag-primed mice (n = 3 per group) and naïve recipients (not shown) and the formula described in the protocol. Error bars represent standard errors of the mean (SEM), and ** denotes a statistical difference with $p < 0.01$ by unpaired Student's *t*-tests. This figure is adopted, with permission, from Memarnejadian et al.[16]. Copyright 2017. The American Association of Immunologists, Inc.

DISCUSSION:

CFSE-based in vivo cytotoxicity assays offer several advantages over traditional killing assays such as radioactive chromium (^{51}Cr) release and colorimetric lactate dehydrogenase (LDH) release assays. First, they permit the monitoring of CTL function within an architecturally intact secondary lymphoid organ.

Second, the specific killing of target cells in in vivo cytotoxicity assays reflects the absolute number of Ag-specific T_{CD8}, which is usually, but not always, a function of T_{CD8} frequencies present in the spleen. This is in contrast with ^{51}Cr/LDH release assays in which a constant number of cells are employed as a source of effector T_{CD8}. Consequently, ^{51}Cr/LDH release assays fail to reliably estimate the total number of Ag-specific T_{CD8} that may be available to the host to eliminate tumor

cells or to combat infections. This is important since in many cases and conditions, the size and the cellularity of secondary lymphoid organs/tissues that accommodate Ag-specific T_{CD8} are altered. For instance, a hypothetical scenario can be envisaged in which a viral infection elevates the total number of T_{CD8} specific for peptide X while also expanding multiple other T_{CD8} clones harboring other specificities. As a result, the frequency of X-specific T_{CD8} among total splenic T_{CD8} may not increase, in which case a ^{51}Cr/LDH release assay will not be helpful. As another example, we recently demonstrated that certain bacterial superantigens expand memory T_{CD8} specific for $NP_{147-155}$, an immunodominant peptide epitope of influenza A viruses in BALB/c mice, which correlated well with increased in vivo lysis of $NP_{147-155}$-pulsed target cells[31]. Since exposure to superantigens provokes T cell proliferation non-specifically, it would have been highly unlikely to demonstrate substantial $NP_{147-155}$-specific cytotoxicity using ^{51}Cr/LDH release assays.

Third, target cells pulsed with peptides that bind to the same MHC class I molecule can be labeled with different doses of CFSE, mixed and used in in vivo cytotoxicity assays. The concomitant analysis of CTL functions towards such peptides is not an option in ^{51}Cr/LDH release assays.

Fourth, in vivo cytotoxicity assays allow for mechanistic studies during priming, effector and recall phases of CTL responses. For example, tumor cell inoculation or antitumor vaccination can be conducted in genetically altered mice for the assessment of CTL induction. Moreover, splenocytes from gene knock-in and knock-out mice can be used as target cells during the effector phase. Finally, various agents (e.g., pharmacological inhibitors and drug candidates) can be administered before priming, during the effector phase, or both. Therefore, in vivo cytotoxicity assays provide a powerful platform for drug/vaccine efficacy testing in a truly in vivo setting. In this body of work, we have provided examples of immunological interventions that boost in vivo CTL responses (**Figure 1 and Figure 2**).

Like other routinely used killing assays, in vivo cytotoxicity assays do not provide any direct information regarding the ability of CTLs to recycle from one target to another before they become exhausted. In addition, we have tested several tumor cell types as potential target cells in in vivo cytotoxicity assays, albeit to no avail so far. This is simply because tumor cells do not reach the spleen at least in detectable numbers after they are injected i.v. Therefore, relying on mouse splenocytes as target cells may be considered an inherent limitation of in vivo cytotoxicity assays. It is noteworthy, however, that adoptively transferred target splenocytes can be easily found in several other organs (in addition to the spleen), for instance in the liver. Therefore, Ag-specific CTL function can be assessed in multiple organs or tissues.

We have optimized in vivo cytotoxicity assays for the examination of immunodominance in T Ag-specific T_{CD8} responses[16,17]. Numerous tools and reagents are available for studying these responses in the contexts of antitumor immunity and therapy. The fibrosarcoma cell line used in the protocol described here (i.e., C57SV cells) does not give rise to tumors in immunocompetent mice. Therefore, it is a useful tool in investigating antitumor vaccination. T Ag-driven neoplastic transformation in select tissues has generated several valuable models of autochthonous cancer. For example, SV11 mice that develop choroid plexus papillomas inside their brain ventricles[35] do not harbor endogenous T Ag-specific T_{CD8} because these cells are selected against and deleted in

the thymus. However, transferring C57BL/6 splenocytes into sublethally irradiated, tumor-bearing SV11 mice leads to extended control of the tumors, which is reportedly associated with in vivo priming of site IV-specific T_{CD8}[36,37]. In the transgenic adenocarcinoma of the mouse prostate (TRAMP) model[38], the site IV-specific response dwindles away with progression of the malignancy. However, the otherwise immunorecessive site V-specific T_{CD8} cells escape negative selection in the thymus and also avoid peripheral tolerance mechanisms[21]. This provides ample opportunities for experimental therapeutic interventions revolving around site V-specific T_{CD8} functions. In vivo cytotoxicity assays should prove informative in studying and potentially reversing immunological tolerance in various model systems, including in the T Ag recognition model.

Immunodominance is a consistent feature of T_{CD8} responses generated not only against tumor Ags but also towards pathogen-derived epitopes. In fact, we have previously used in vivo cytotoxicity assays to study immunodominance in anti-influenza T_{CD8} responses[12]. Therefore, the optimized assay described in this protocol can be modified and used in a broad range of immunological applications.

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DISCLOSURES:

The authors have nothing to disclose.

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656

Figure 1

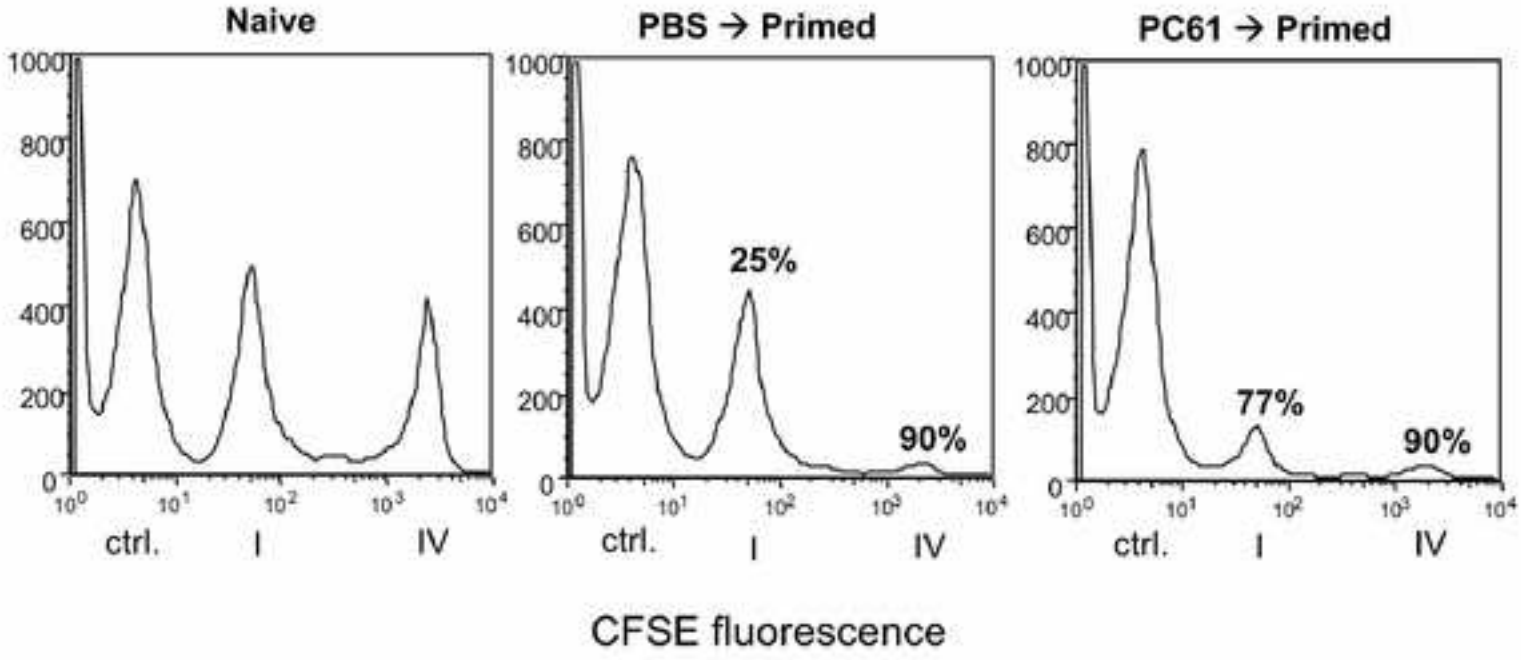

Figure 2

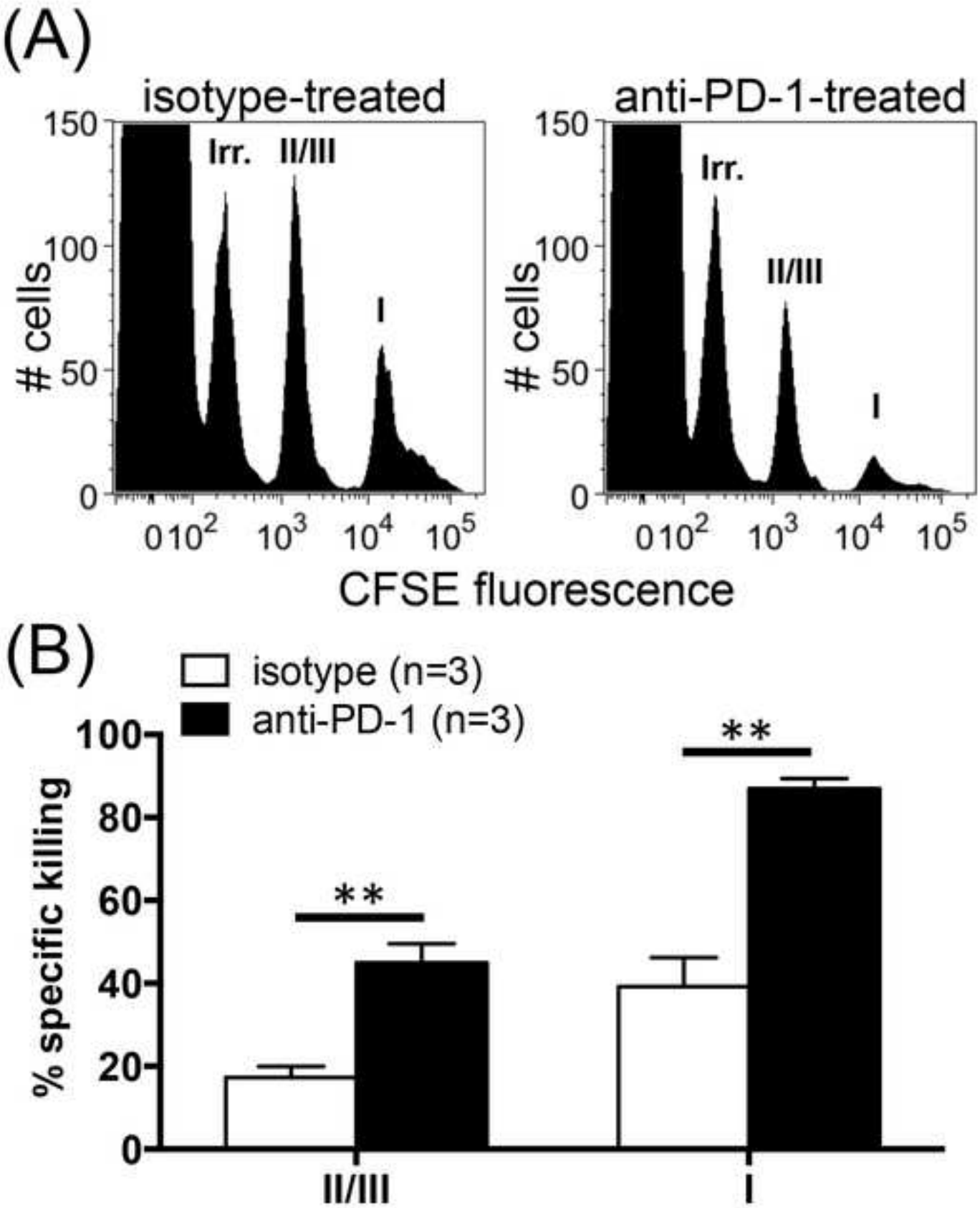

Table 1. Peptides introduced in this protocol

Protein Antigen Source	Peptide Epitope	Designation	Sequence
SV40[1] Large T Ag[2]	T $Ag_{206-215}$	Site I	SAINNYAQKL
SV40 Large T Ag	T $Ag_{223-231}$	Site II/III	CKGVNKEYL
SV40 Large T Ag	T $Ag_{404-411}$	Site IV	VVYDFLKC
SV40 Large T Ag	T $Ag_{489-497}$	Site V	QGINNLDNL
HSV-1[3] Glycoprotein B	$gB_{498-505}$*	$gB_{498-505}$	SSIEFARL
LCMV[4] Glycoprotein	GP_{33-41}*	GP_{33-41}	KAVYNFATC

[1]Simian Virus 40

[2]Large Tumor Antigen

[3]Herpes Simplex Virus type 1

[4]Lymphocytic Choriomeningitis Virus

*used as an irrelevant peptide

MHC I Restriction
H-2D^{b}
H-2D^{b}
H-2K^{b}
H-2D^{b}
H-2K^{b}
H-2D^{b}

Table of Materials

Material	Commercial Source
0.25% Trypsin-EDTA (1X)	Thermo Fisher Scientific
ACK Lysing Buffer	Thermo Fisher Scientific
Anti-mouse CD25 (clone PC-61.5.3)	Bio X Cell
Anti-mouse PD-1 (clone RMP1-14)	Bio X Cell
CFSE	Thermo Fisher Scientific
DMEM (1X)	Thermo Fisher Scientific
Fetal bovine serum (FBS)	Wisent Bioproducts
GlutaMAX (100X)	Thermo Fisher Scientific
HEPES (1M)	Thermo Fisher Scientific
MEM Non-Essential Amino Acids Solution (100X)	Thermo Fisher Scientific
Penicillin/Streptomycin	Sigma-Aldrich
Rat IgG1 (clone KLH/G1-2-2)	SouthernBiotech
Rat IgG1 (clone HRPN)	Bio X Cell
Rat IgG1 (clone TNP6A7)	Bio X Cell
Rat IgG2a (clone 2A3)	Bio X Cell
RPMI 1640 (1X)	Thermo Fisher Scientific
Sodium Pyruvate (100 mM)	Thermo Fisher Scientific

Catalog Number	Comments
25200-056	
A1049201	
BE0012	
BE0146	
C34554	
11965-092	
080-150	Heat-inactivate prior to use
35050-061	
15630080	10 mM final concentration
11140-050	
P0781	Stock is 100X
0116-01	Isotype control
BE0088	Isotype control
BP0290	Isotype control
BP0089	Isotype control
11875-093	
11360-070	1 mM final concentration

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CORRESPONDING AUTHOR:

Name:	Dr. S.M. Mansour Haeryfar
Department:	Microbiology and Immunology
Institution:	Western University
Article Title:	Tailoring in vivo cytotoxicity assays to study immunodominance in tumor-specific CD8+ T cell responses
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Rebuttal Letter

Dear Dr. Cao,

RE: JoVE59531

We are pleased to learn that our manuscript was reviewed quickly and also thankful for the reviewers' insightful comments. I have provided our point-by-point responses in this rebuttal document and certainly hope that you will find our revisions satisfactory.

Sincerely,

Dr. S.M. Mansour Haeryfar

Editorial comments:

Please include an ethics statement before your numbered protocol steps, indicating that the protocol follows the animal care guidelines of your institution.

We have now moved our Ethics Statement to the requested position within the manuscript.

In the JoVE Protocol format, "Notes" should be concise and used sparingly. They should only be used to provide extraneous details, optional steps, or recommendations that are not critical to a step. Any text that provides details about how to perform a particular step should either be included in the step itself or added as a sub-step. Please consider moving some of the notes about the protocol to the discussion section.

Most of our Notes conform to their intended purpose as indicated above. We have now amalgamated several Notes into one (please see our revised manuscript).

1.3: Is this done at room temperature?

Yes. We have now revised the text to make this clear.

1.7: What volume of sterile cold PBS is used?

This is typically done in 10 mL of PBS. We have revised the text.

1.9: Are the cells counted before this step? Please specify.

Yes. We have modified the text to clarify this point.

1.10, 3.1: Please specify the age and gender of the mouse.

Modified as requested!

9. 3.1, 8.1: Please specify the euthanasia method used.

Specified as requested!

4.2: Please specify the peptide used for each tube.

This may complicate the manuscript and make its flow less accessible to the readership. The readers should be able to readily refer to previous sections specifying the peptides to be used. Sorry!

4.3: What is the mesh size of the cell strainer?

Consistent with the rest of the protocol, cell strainers with 70-µm pores need to be used. We have specified this in the text now.

5.8: Is step 5.7 also repeated?

We have added a short sentence to clarify this further.

7.1: Please specify from which step the source tubes are prepared.

We have now done this actually in an earlier step where we have referred to the 'source tubes'. Accordingly, in step 6.5, we have referred to step 6.1. Please refer to our revised manuscript.

Please highlight complete sentences (not parts of sentences). Please do not highlight any steps describing anesthetization and euthanasia.

We have removed the highlights from several section titles that do not form complete sentences. We did not have any highlighted texts referring to anesthetization and euthanasia to begin with.

19. References: Please do not abbreviate journal titles.

The manuscript is now revised as instructed!

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We have previously obtained such letters, which we will upload during the re-submission.

Please upload each Table individually to your Editorial Manager account as an .xls or .xlsx file.

We have now prepared and uploaded separate tables.

Table of Materials: Please revise to include the name, company, and catalog number of all relevant supplies, reagents, equipment and software, and sort the items in alphabetical order according to the name of material/equipment.

Revised as instructed!

Reviewer 1:

"The manuscript is well written and clear."

We thank the reviewer for her/his positive feedback.

"I have few minor points that needs to be clarified.""1. In section 5, it is interesting to know if different dyes could be used instead of various CFSE titrations."

This is definitely a possibility. However, many factors need to be considered (e.g., toxicity and stability/instability of other dyes) and various conditions also need to be optimized before such dyes can be used. CFSE has worked well in various *in vivo* killing assays using different setups.

"2. In section 7.6, I suggest that the splenocyte mix is checked in FACS just before injection in the recipient mice to ensure the ratios between differently labeled cells is the same after the washing steps and the vortexing (sections 7.1-7.4)."

This is an important quality control measure. Steps 6.1-6.5 exactly address the reviewer's point.

"3. Figure 1.A and B are copied from a previous paper (Memarnejadian, A et al. J Immunol. 2017). I am not sure but some copyright issues might be concern regarding the figure. Additionally it is interesting to know why the authors did not performed cytofluorimetric analysis in order to recreate graphs represented in Figure 1."

It is our understanding, based on JoVE's instructions, that this is an acceptable practice. We did obtain formal permission from The Journal of Immunology (the flagship journal of the American Association of Immunologists) for the reproduction of figures 1 and 2, both of which are adopted from our own previously published articles. Two permission letters are now uploaded with this resubmission. We routinely perform *in vivo killing* assays and are confident about the reproducibility of our results. Our animal use protocols stipulate that we should sacrifice additional mice only if we absolutely have to.

Reviewer #2:

"Minor Concerns: Can the authors discuss adaptability of this assay to draining lymph nodes (e.g. to derive target cells)?"

This is an important question. Lymph nodes are not typically used as a source of target cells simply because they do not provide a sufficient cellular yield even if they are pooled.

Typically, CFSE-labeled target cells are not tracked in lymph nodes either since they are injected via tail vein. The intravenous route of target cell injections dictates that these cells end up in the spleen among several other organs, but not in lymph nodes, except potentially for peri-thymic nodes. It will be interesting to look for intravenously injected target cells in these nodes. We fully agree that it will also be curious to determine whether CFSE-labeled target cells that can be potentially injected subcutaneously (in other settings) show up and remain in sentinel lymph nodes draining the area of injection.

Please note that our manuscript's focus has been the application of *in vivo* cytotoxicity assays in evaluation of $CD8^+$ T cell immunodominance. Therefore, we did not discuss the above possibilities in the interest of producing a coherent manuscript.

The Journal of Immunology

AMERICAN ASSOCIATION OF IMMUNOLOGISTS FOUNDED 1913

May 4, 2018

Joshua Choi
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Canada

Email: jchoi443@uwo.ca

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Sincerely,

Todd D. Reitzel
Director of Publications
The Journal of Immunology

The Journal of Immunology

April 6, 2018

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Canada

Email: jchoi443@uwo.ca

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