

Video Article

Antigen Specific *In Vivo* Killing Assay using CFSE Labeled Target Cells

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URL: <https://www.jove.com/video/2250>

DOI: [doi:10.3791/2250](https://doi.org/10.3791/2250)

Keywords: Immunology, Issue 45, CTLs, CD8+ T cells, CFSE labeling, killing assay

Date Published: 11/9/2010

Citation: Durward, M., Harms, J., Splitter, G. Antigen Specific *In Vivo* Killing Assay using CFSE Labeled Target Cells. *J. Vis. Exp.* (45), e2250, doi:10.3791/2250 (2010).

Abstract

Carboxyfluorescein diacetate succinimidyl ester (CFSE) can be used to easily and quickly label a cell population of interest for *in vivo* investigation. This labeling has classically been used to study proliferation and migration. In the method presented here, we have shortened the timeline after adoptive transfer to look at survival and killing of epitope specific CFSE labeled target cells⁴⁻⁶. The level of specific killing of a CD8 + T cell clone can indicate the quality of the response, as their quantity may be misleading. Specific CD8+ T cells can become functionally exhausted over time with a decline in cytokine production and killing^{7,8}. Also, certain CD8 + T cell clones may not kill as well as others with differing TCR specificities⁹. For effective Cell Mediated Immunity (CMI), antigens must be identified that produce not only adequate numbers of responding T cells, but also functionally robust responding T cells. Here we assess the percent cell specific killing of two peptide specific T cell clones in BALB/c mice.

Video Link

The video component of this article can be found at <https://www.jove.com/video/2250/>

Protocol

1. Eliciting Target-Specific Effector CTLs by Immunization (Day 0)

1. Before beginning, decide upon a specific effector:target system. In this example of a classical *in vivo* CTL assay, we will use a purified peptide immunization to elicit specific CD8 + T cells. The same peptide will be used to pulse the syngeneic target cells, creating a specific effector:target system. Alternatively, you may choose to use whole pathogen or protein to obtain your specific effectors and targets.
2. Decontaminate the work surface inside of a biosafety hood to ensure sterility of the injections. Prepare the peptide immunization by first placing 50 µg of purified peptide into 100 µL 10% DMSO in PBS/mouse contained in a 2 mL tube. Next, add an equal amount of very cold Incomplete Freund's Adjuvant and up to the .2 mL mark with 1 mm beads for mixing. From this point, keep immunogen on ice until injection. Secure lid with Parafilm.
3. Use a bead beater set to maximum RPMs to mix the emulsion for 3 minutes, promptly placing the emulsion back on the ice after mixing. The resulting emulsion should be similar in consistency to mayonnaise. Allow emulsion to sit at 4° C overnight if time permits (up to a week), mixing further if any separation is noted.
4. Use a 1 mL syringe and 20 or 20.5 gauge needle to pull up emulsion being careful to not pull up any beads (beads should be larger than gauge of needle, but still be aware).
5. Secure mouse for subcutaneous injection at the base of the tail. We use a modified plastic 50 mL conical tube to safely and gently immobilize the mouse for injection. Inject the mouse with .2 mL of emulsion.

2. Harvesting Target Cells (Day 8)

1. You will need naïve syngeneic mice for your target cells. Euthanize naïve mice according to AAALAC standards.
2. Disinfect mouse by spraying 70% Ethanol to minimize the chance of contamination from mouse hair or other external contaminants.
3. Using dissection scissors make small snip through the skin on the left side of the mouse. Pull skin flap over to uncover peritoneal sac and internal organs.
4. Snip through the peritoneum, being careful not to compromise any of the underlying organs. The spleen is close to the surface and is similar in color to the liver (dark red) but smaller and more kidney bean in shape. Infected mice generally show notably larger spleens than uninfected controls.
5. Carefully pull out the spleen, taking care to be gentle so as not to break the spleen into multiple pieces in order to recover maximum cell numbers. Immediately place the spleen into a 15 mL conical containing 10 mLs of prepared media on ice. Continue with remaining mice.
6. Pour contents of the 15 mL conical tube (media and spleen) into a glass tissue grinder. Grind the spleen using plenty of upper arm strength. When all tissue has lost its color you may assume that you have recovered all cells out of the connective tissue.

7. Pour contents of the grinder through a 70 μ m cell filter into a 50 mL conical tube. Rinse grinder with fresh media and pour through a cell strainer.
8. Centrifuge at 4° C, 1300 RPM for 7 minutes. Pour off supernatant.
9. Resuspend in 10 mLs of prepared lysing reagent and incubate at 37° for 7 minutes.
10. Add up to 50 mLs with fresh media and centrifuge, same as above.
11. Resuspend in 50 mLs fresh media and centrifuge, same as above (wash step). *Note: At this time you can perform a magnetic cell separation for a specific cell type (i.e. macrophages) if a pure target cell population is desired for transfer into recipient mice. Also, at this time you could introduce an experimental treatment into the culture in order to study the affects of that treatment on cell specific killing.
12. Count cells and plate 1×10^7 per well in 100 μ L in a 96 well round bottom tissue culture treated plate. *This is a high concentration of cells/well, but because the assay is a quick one, we have seen no loss in viability of the cells at this concentration.

3. Treatment and Labeling of Target Cells

1. Pulse appropriate cells and replicates with pathogen specific peptide or irrelevant peptide control: Add 1 μ g/mL peptide. Incubate at 4° C for 1.5 hours. Move to 37° C incubator for last 30 minutes. During this time prepare CFSE reagent.
2. Prepare CFSE according to manufacturer specifications and dilute to a working concentration between 0.5 and 25 μ M. Make "low" concentration 10x lower than "high" concentration CFSE. For example, for [low] add 0.5 μ L CFSE to 10 mLs PBS and for [high], add 5 μ L CFSE to 10 mLs PBS. Warm prepared CFSE to 37° C before labeling.
3. Remove plate of cells from incubator and pellet in a high speed centrifuge at room temperature, 1300 RPM for 7 minutes. Flick to remove supernatant.
4. Resuspend cells in either [low] or [high] CFSE by adding 200 μ L of appropriate reagent to the corresponding wells and incubate for 15 minutes at 37° C.
5. Remove plate of cells from incubator and again pellet at room temperature, 1300 RPM for 7 minutes. Flick to remove supernatant.
6. Resuspend cells in 200 μ L/well of warmed fresh media and incubate for 30 minutes at 37°C.
7. Remove plate of cells from incubator and pellet at room temperature, 1300 RPM for 7 minutes. Flick to remove supernatant.
8. Resuspend cells in 100 μ L/well PBS.
9. For adoptive transfer, add one well [low] labeled cells to one well [high] labeled cells per mouse. Resulting injection should be 50% specific targets and 50% irrelevant cells in .2 mLs.
10. Don't forget to set aside some labeled cells to act as your non-transferred controls during data analysis and for running on the flow cytometer (See Figure 1).

4. Adoptive transfer

1. Anesthetize previously peptide immunized mice (From part #1 of this protocol) according to AAALAC standards.
2. Inject .2 mLs of labeled target cells via retro-orbital route into recipient mice.
3. Wait 6 hours or other predetermined optimal time before euthanizing the recipient mice.

5. Harvesting Labeled Cells

*Note: Steps 5.1 through 5.11 are the same as steps 2.1 through 2.11

12. Count cells and plate out at 1×10^6 per well in 100 μ L into 96 well round bottom tissue culture treated plate.
13. Fix recovered and non-transferred cells by adding 100 μ L 2% paraformaldehyde to a final concentration of 1% paraformaldehyde to all wells.

6. Flow Cytometry and Data Analysis

1. Run samples on a flow cytometer and analyze the data.

7. Representative Results

A diminishing epitope specific peak on the resulting histogram is an indicator of cell specific killing (Figure 1). Use the following equations (applied in Table 1) to determine the actual value of cell specific lysis:

Ratio = Irrelevant Percentage : Epitope Specific Percentage ([low] peak:[high] peak),

Percent Specific Lysis = $[1 - (\text{Non-transferred control ratio} / \text{Experimental ratio})] \times 100$

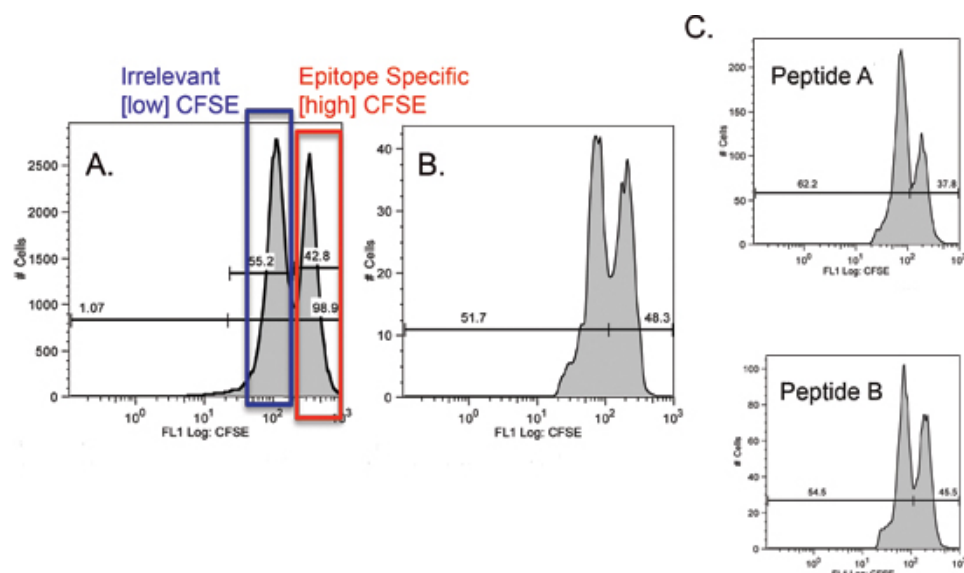


Figure 1. CFSE profile of recovered BALB/c mouse splenocytes. Numbers indicate the percentage of cells of the high or low CFSE phenotype. A) Non-transferred control. All [high] CFSE peaks represent epitope specific target cells, while [low] CFSE peaks represent targets pulsed with irrelevant peptide. B) CFSE labeled cells recovered from PBS immunized recipient mouse (Control). C) CFSE labeled cells recovered from two different peptide specific recipient mice, note the diminished epitope specific peaks.

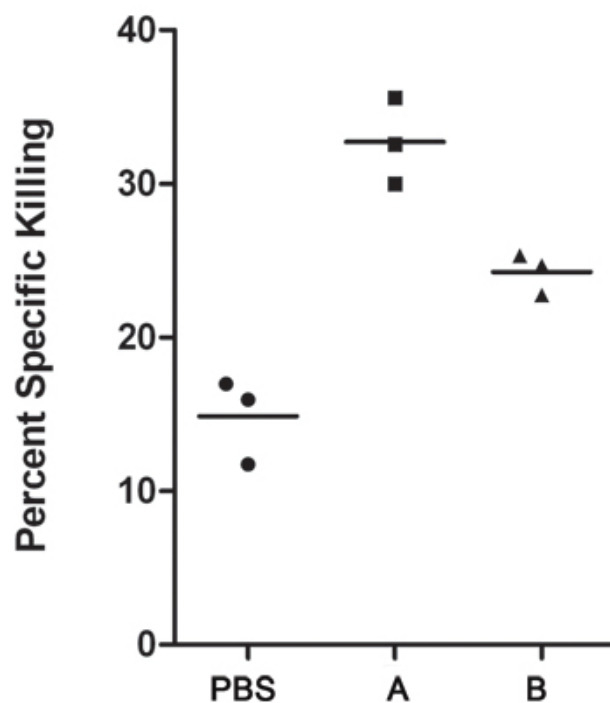


Figure 2. Graphical representation of analyzed data. Three data points shown for each group of mice, PBS, Peptide A, and Peptide B.

Sample	CFSE HIGH % Epitope Pulsed	CFSE LOW % Irrelevant	Ratio Low:High	Percent Epitope Specific Killing
PBS	42.8	57.2	1.34	16.97
PBS	43.1	56.9	1.32	15.94
PBS	44.3	55.7	1.26	11.74
Peptide A	39.7	60.3	1.52	32.56
Peptide A	38.6	61.4	1.59	35.61
Peptide A	40.6	59.4	1.46	29.99

Peptide B	38.7	61.3	1.58	24.68
Peptide B	38.5	61.5	1.60	25.32
Peptide B	39.3	60.7	1.54	22.76
PBS NT Control	47.4	52.6	1.11	
Pep A NT Control	49.4	50.6	1.02	
Pep B NT Control	45.6	54.4	1.19	

Table 1. Analysis of representative data. "NT" is non-transferred.

Discussion

This assay can be modified to investigate the proliferative capacity of cells, including clonal expansion, because CFSE divides relatively equally among progeny^{10,11}. It is also possible to use CFSE labeling to investigate cell migration^{6,12}, though there are other cell tracing methods that may be more appropriate depending on your hypothesis, for example tetramers and bioluminescence¹³, which also may be combined with CFSE labeling.

It is important to note that CFSE bleeds over into multiple channels on the flow cytometer, so if you want to co-stain for a surface marker, PeCy7 would be a good choice of fluorophore, while PE might not work as well.

Disclosures

No conflicts of interest declared. Experiments on animals were performed in accordance with the guidelines and regulations set forth by the Institutional Animal Care and Use Committee (IACUC) at The University of Wisconsin-Madison.

Acknowledgements

I would like to thank Bianca Mothé, Carla Oseroff, and Marie-France DelGuercio for introducing me to immunological assays and mouse handling. Work in the lab of G. A. Splitter is funded by NIH grant 1-RO1-AI-073558, GLRCE grant 1-U54-AI-057153, and BARD US-3829-06.

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