

## Murine *in vivo* CD8<sup>+</sup> T Cell Killing Assay

Myoungjoo V. Kim<sup>1\*</sup>, Weiming Ouyang<sup>2</sup>, Will Liao<sup>3</sup>, Michael Q. Zhang<sup>4</sup> and Ming O. Li<sup>5</sup>

<sup>1</sup>Department of Immunobiology, Yale University School of Medicine, New Haven, USA; <sup>2</sup>Center for Drug Evaluation and Research, U.S. Food and Drug Administration, Bethesda, USA;

<sup>3</sup>Genomics, New York Genome Center, New York, USA; <sup>4</sup>Molecular and Cellular Biology Department, University of Texas at Dallas, Richardson, USA; <sup>5</sup>Department of Immunology, Memorial Sloan Kettering Cancer Center (MSKCC), New York, USA

\*For correspondence: [myoungjoo.kim@yale.edu](mailto:myoungjoo.kim@yale.edu)

**[Abstract]** Antigen-specific killing ability of effector CD8<sup>+</sup> T cells is critical for protective immunity against infection. Here, we describe *in vivo* cytotoxic T cell assay to examine effector function of antigen-specific CD8<sup>+</sup> T cells. Mice infected with *Listeria monocytogenes* (*L. monocytogenes*) expressing chicken ovalbumin as a model antigen mount ovalbumin-specific CD8<sup>+</sup> T cell responses. Effector CD8<sup>+</sup> T cell function *in vivo* is determined by mixed transfer of OVA peptide-pulsed target cells with control target cells into the previously immunized mice. Difference in CFSE expression levels clearly marks two distinct populations: Antigen-pulsed target cells-CFSE<sup>low</sup> vs. unpulsed target cells-CFSE<sup>hi</sup>. The frequencies between antigen-pulsed target cells and control target cells are used as readouts of antigen-specific killing.

### Materials and Reagents

1. Splenocytes from a wild type mouse
2. PBS (Thermo Fisher Scientific, catalog number: BP399-20)  
*Note: 10x solution, diluted to 1x in house in distilled water and sterilized by autoclave.*
3. RBC lysis buffer (eBioscience, catalog number: 00-4333-57)
4. HBSS without Ca<sup>2+</sup> and Mg<sup>2+</sup> (Life Technologies, Gibco®, catalog number: 14175-095)
5. RPMI-1640 medium (Life Technologies, Gibco®, catalog number: 11875-119)
6. Fetal bovine Serum (Atlanta Biologicals, catalog number: S11055H)
7. Penicillin/streptomycin (Gemini Bio-Products, catalog number: F52M00E)
8. L-Glutamine (Life Technologies, Gibco®, catalog number: 25030-081)
9. Trypan blue solution (Life Technologies, Gibco®, catalog number: 15250-061)
10. OVA<sub>257-264</sub> synthetic peptide (Sigma-Aldrich, catalog number: S7951)
11. 5(6)-Carboxyfluorescein diacetate N-succinimidyl ester (CFSE) (Sigma-Aldrich, catalog number: 21888)
12. Dimethyl sulfoxide (DMSO) (Sigma-Aldrich, catalog number: D-8418)

13. Collagenase D (Sigma-Aldrich, catalog number: C-5138)
14. Percoll (Sigma-Aldrich, catalog number: P-1644)
15. Complete RPMI-1640 media (see Recipes)
16. 100% percoll solution (see Recipes)

## **Equipment**

1. Centrifuge (Thermo Fischer Scientific, Sorvall™ Legend RT )
2. 37 °C water bath
3. Hemocytometer
4. 15 ml and 50 ml Falcon tubes
5. 6 well plates (USA Scientific, CytoOne®, catalog number: CC7682-7506)
6. BD LSRII Flow Cytometer (BD)
7. 70 µm cell strainer (BD Biosciences, Falcon®, catalog number: 352350)
8. 5 ml polystyrene round-bottom tubes with cell-strainer cap (BD Biosciences, Falcon®, catalog number: 352235)
9. 3 ml syringe (BD, catalog number: 14-823-435)

## **Procedure**

### **A. Target cell preparation under sterile tissue culture conditions**

This step is for preparing peptide-pulsed target cells and stain cells with CFSE to distinguish peptide-pulsed target cells from control target cells.

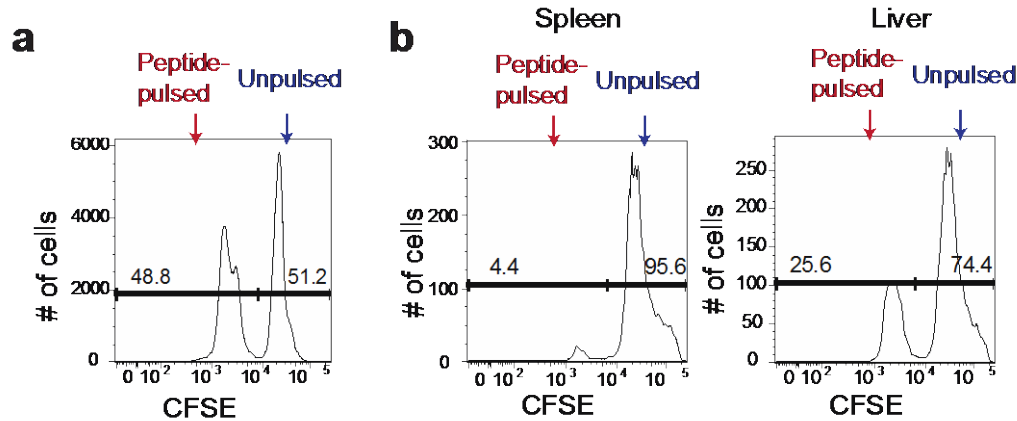
1. OVA<sub>257-264</sub> peptide-loading for the target cells.
  - a. Splenocytes are RBC lysed followed by washing with PBS twice.
  - b. Resuspend cells in RPMI-1640 complete medium.
  - c. Count the mononuclear cells by trypan blue exclusion using a hemocytometer.
  - d. Resuspend cells at  $5 \times 10^6$ /ml of RPMI-1640 complete medium.
  - e. Divide the cells equally into two separate 50 ml Falcon tubes- one for peptide-pulsed target cells, and the other for unpulsed target cells.
  - f. Add OVA<sub>257-264</sub> peptide at 1 µl/ml from a 200 µM stock to peptide-pulsed target cells.
  - g. Add an equivalent amount of PBS to the unpulsed target cells.
  - h. Incubate the cells in a 37 °C water bath for 1 h.
  - i. Wash cells twice with RPMI-1640 complete medium.
  - j. Centrifuge the cells at 1,500 rpm for 3 min at 4 °C.
  - k. Resuspend the cell pellet in HBSS.
2. CFSE cell labeling under sterile tissue culture conditions.

- a. Count all live cells by trypan blue exclusion using hemocytometer.
- b. Resuspend the cells in HBSS at  $5 \times 10^7/\text{ml}$ .
- c. Thaw an aliquot of 5 mM stock CFSE solution.
- d. Make a fresh CFSE<sup>low</sup> stock solution by diluting 5 mM stock 1:10 in DMSO (a final concentration of 0.5 mM).
- e. Incubate the unpulsed target splenocytes with the higher concentration of CFSE (CFSE<sup>high</sup>): Add 1  $\mu\text{l}$  of the 5 mM stock CFSE for each milliliter of unpulsed target cells (final concentration of 5  $\mu\text{M}$ ).
- f. Incubate the pulsed target splenocytes with the lower concentration of CFSE (CFSE<sup>low</sup>): Add 1  $\mu\text{l}$  of the 0.5 mM stock CFSE for each milliliter of peptide-pulsed cells (final concentration of 0.5  $\mu\text{M}$ ).
- g. Pipette cells up and down to mix well and incubate in water bath for 10 min at 37 °C. Gently agitate the cells periodically.
- h. Add 10x the volume of pre-warmed RPMI-1640 complete medium to the CFSE-labeled cells to stop the reaction.
- i. Pellet cells at 1,500 rpm for 3 min at 4 °C.
- j. Remove the supernatant and resuspend the pellet in cold RPMI-1640 complete medium.
- k. Wash the cells two more times with cold RPMI-1640 complete medium.
- l. Count the mononuclear cells by trypan blue exclusion using a hemacytometer.
- m. Wash the cells with cold PBS.
- n. Resuspend each cell populations in PBS at  $6.7 \times 10^6/\text{ml}$ .
- o. Combine an equal volume (~equal numbers) of peptide-pulsed CFSE<sup>low</sup> cells with unpulsed CFSE<sup>hi</sup> cells and proceed with flow cytometry analysis (Figure 1a).

## B. Intravenous injection of target splenocytes

To investigate OVA<sub>257-264</sub>-specific CD8<sup>+</sup> T cell killing ability, peptide-pulsed and unpulsed target cells were mixed at a 1:1 ratio and transferred to the previously immunized mice.

1. Recipient mice were infected with 5,000 colony-forming units (CFU) of *Listeria monocytogenes* expressing chicken ovalubmin (LM-OVA) 7 days intravenously before the CFSE-labeled cell injection.
2. Inject intravenously 300  $\mu\text{l}$  of the combined cell populations into the tail vein of each recipient. Each recipient should receive approximately  $1 \times 10^7$  peptide-pulsed target cells combined with  $1 \times 10^7$  unpulsed target cells.
3. Wait for 4 h.



**Figure 1. CFSE expression in antigen-pulsed target cells and unpulsed target cells.** a. Peptide-pulsed CFSE<sup>low</sup> and unpulsed CFSE<sup>hi</sup> splenocytes were mixed at a 1:1 ratio before transferring to the recipients. b. The mixture of peptide-pulsed and unpulsed splenocytes was transferred into the mice predisposed with LM-OVA at day 7 post infection. The spleen and the liver of the recipients were harvested after 4 h to determine percentages of CFSE<sup>low</sup> and CFSE<sup>hi</sup> cells among CFSE<sup>+</sup> cells.

#### C. Preparation of splenocytes and lymphocytes in the liver for flow cytometry analysis

This step is analyzing antigen-specific killing ability in the liver and the spleen by flow cytometric analysis of CFSE<sup>low</sup> and CFSE<sup>hi</sup> cell populations.

##### 1. Prepare splenocytes for flow cytometry analysis.

- Wash once with PBS, and 2-300  $\mu$ l into a 5 ml round bottom tubes through the cell strainer.

##### 2. Isolate lymphocytes from the liver.

- Harvest livers from the recipients and place them on ice.
- Make a fresh collagenase D solution by diluting 20 mg/ml stock 1: 20 in PBS (a final concentration of 1 mg/ml).
- Chop liver with a blade on a slide glass and transfer them into a 50 ml falcon tube.
- Add 7 ml of collagenase D (1 mg/ml) and vortex well.
- Incubate in water bath for 30 min at 37 °C. Vortex every 15 min.
- Put tubes on ice and add supernatant on 70  $\mu$ m filter on a well of a 6 well plate. Grind chunks of the chopped liver with flat portion of 3 ml syringe. Wash the tube with 5 ml of PBS and repeat grinding.
- Transfer them to a 50 ml Falcon tube and spin down at 2,000 rpm for 5 min at 4 °C.
- Make fresh 44% and 66% percoll solution: Make 44% final concentration by diluting 100% percoll in PBS, and make 66% final concentration by diluting 100% percoll in RPMI-1640 medium.

- i. Resuspend pellet in 7 ml of 44% percoll, and load them on 3 ml of 66% percoll in 15 ml tube.  
*Note: The gradient separation is sensitive to agitation. Try not to shake the tube.*
  - j. Centrifuge at 3,000 rpm for 30 min at 4 °C without brake.
  - k. Transfer the interphase lymphocytes to a new 15 ml tube.
  - l. Pellet cells at 2,000 rpm for 5 min at 4 °C.
  - m. Wash once with PBS, and transfer 2-300 µl into a 5 ml round bottom tubes through the cell strainer.
3. Proceed to flow cytometry analysis with spleen and liver samples (Figure 1b).

### **Recipes**

1. Complete RPMI-1640 medium  
10% FBS  
1% Penicillin/streptomycin with L-Glutamine
2. 100% percoll solution  
90% of percoll  
10% of 10x PBS

### **Notes**

1. The gradient separation is sensitive to agitation. Try not to shake the tube.

### **Acknowledgments**

The protocol was adapted from a previously described study (Manjunath *et al.*, 2001). This work was supported by the Starr Cancer Consortium (13-A123 to M.O.L. and M.Q.Z.), the Rita Allen Foundation (M.O.L.), the NBRPC (2012CB316503 to M.Q.Z.), and the NIH (HG001696 to M.Q.Z.).

### **References**

1. Ingulli, E. (2007). [Tracing tolerance and immunity in vivo by CFSE-labeling of administered cells](#). *Methods Mol Biol* 380: 365-376.
2. Kim, M. V., Ouyang, W., Liao, W., Zhang, M. Q. and Li, M. O. (2013). [The transcription factor Foxo1 controls central-memory CD8<sup>+</sup> T cell responses to infection](#). *Immunity* 39(2): 286-297.