

Murine *in vivo* CD8⁺ T Cell Killing Assay

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[Abstract] Antigen-specific killing ability of effector CD8⁺ 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⁺ T cells. Mice infected with *Listeria monocytogenes* (*L. monocytogenes*) expressing chicken ovalbumin as a model antigen mount ovalbumin-specific CD8⁺ T cell responses. Effector CD8⁺ 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^{low} vs. unpulsed target cells-CFSE^{hi}. 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 Ca²⁺ and Mg²⁺ (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. OVA₂₅₇₋₂₆₄ 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. OVA₂₅₇₋₂₆₄ 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 x 10⁶/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 OVA₂₅₇₋₂₆₄ 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 5×10^7 /ml.
 - c. Thaw an aliquot of 5 mM stock CFSE solution.
 - d. Make a fresh CFSE^{low} 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^{high}): Add 1 μ l of the 5 mM stock CFSE for each milliliter of unpulsed target cells (final concentration of 5 μ M).
 - f. Incubate the pulsed target splenocytes with the lower concentration of CFSE (CFSE^{low}): Add 1 μ l of the 0.5 mM stock CFSE for each milliliter of peptide-pulsed cells (final concentration of 0.5 μ 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 6.7×10^6 /ml.
 - o. Combine an equal volume (~equal numbers) of peptide-pulsed CFSE^{low} cells with unpulsed CFSE^{hi} cells and proceed with flow cytometry analysis (Figure 1a).
- B. Intravenous injection of target splenocytes**
- To investigate OVA₂₅₇₋₂₆₄-specific CD8⁺ 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 μ l of the combined cell populations into the tail vein of each recipient. Each recipient should receive approximately 1×10^7 peptide-pulsed target cells combined with 1×10^7 unpulsed target cells.
 3. Wait for 4 h.

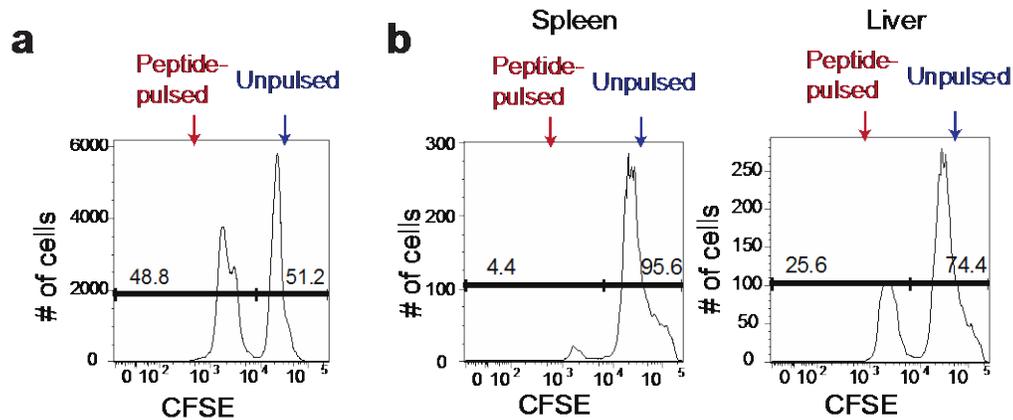


Figure 1. CFSE expression in antigen-pulsed target cells and unpulsed target cells. a. Peptide-pulsed CFSE^{low} and unpulsed CFSE^{hi} splenocytes were mixed at a 1:1 ratio before transferring to the recipients. b. The mixture of peptide-pulsed and unpulsed splenocytes was transferred into the mice 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^{low} and CFSE^{hi} cells among CFSE⁺ 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^{low} and CFSE^{hi} cell populations.

1. Prepare splenocytes for flow cytometry analysis.
 - a. Wash once with PBS, and 2-300 μ l into a 5 ml round bottom tubes through the cell strainer.
2. Isolate lymphocytes from the liver.
 - a. Harvest livers from the recipients and place them on ice.
 - b. Make a fresh collagenase D solution by diluting 20 mg/ml stock 1: 20 in PBS (a final concentration of 1 mg/ml).
 - c. Chop liver with a blade on a slide glass and transfer them into a 50 ml falcon tube.
 - d. Add 7 ml of collagenase D (1 mg/ml) and vortex well.
 - e. Incubate in water bath for 30 min at 37 $^{\circ}$ C. Vortex every 15 min.
 - f. Put tubes on ice and add supernatant on 70 μ 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.
 - g. Transfer them to a 50 ml Falcon tube and spin down at 2,000 rpm for 5 min at 4 $^{\circ}$ C.
 - h. 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

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