In vivo OVA-specific Cytotoxic CD8$^+$ T Cell Killing Assay

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[Abstract] Cytotoxic CD8$^+$ T cells are responsible for the lysis of cells expressing peptides associated with MHC class I molecules and derived from infection with a pathogen or from mutated antigens. In order to quantify *in vivo* this antigen-specific CD8$^+$ T cell killing activity, we use the *in vivo* killing assay (IVK). Here we describe the protocol for the lysis of cells expressing a CD8$^+$ T cell epitope of the OVA protein (SIINFEKL). Mice are previously immunized with the OVA protein and 7 days after immunization, they receive a mix of target cells, prepared from naive C57BL/6 spleen cells pulsed with the SIINFEKL peptide and labeled with high level of CFSE and of non-pulsed control cells labeled with low level of CFSE. One day later, the spleen cells of recipient mice are isolated and analyzed by FACS to measure the amount of CFSE^{high} cells and CFSE^{low} cells. The percentage of lysis is calculated by the difference between CFSE high versus low in immunized vs non-immunized mice.

Measuring the ability of antigen-specific CD8$^+$ T cell to lyse their antigen *in vivo* is very important to evaluate the adaptive cytotoxic response induced against a pathogen or a tumor antigen.

Materials and Reagents

1. 0.5 ml insulin syringe (Terumo Corporation, catalog number: SS05M2713M)
2. Falcon® 50 ml high clarity PP centrifuge tube (Corning, catalog number: 352070)
3. 40 µm nylon mesh cell strainer (Corning, catalog number: 352340)
4. 6-well plate (TPP Techno Plastic Products AG., catalog number: 92006)
5. Cell counting material
6. 5 ml round bottom polystyrene test tube (Corning, catalog number: 352235)
7. C57BL/6 mice
8. CpG B-1826 (5'-TCCATGACGTTCCTGACGTT-3') (Sigma-Aldrich, PrOligo, custom made)
9. N-[1-(2, 3-dioleoyloxyl)propyl]-NNNtrimethylammoniummethyl sulfate (DOTAP) (Roche Diagnostics, catalog number: 11202375001)
10. Albumin from chicken egg white (OVA protein) (Sigma-Aldrich, catalog number: A5503)

11. Sterile DPBS, 1x (Life Technologies, Gibco, catalog number: 14190-094)
    Note: Currently, it is "DPBS, no calcium, no magnesium (Thermo Fisher Scientific, Gibco™, catalog number: 14190-094)".
12. Carboxyfluorescein succinimidyl ester (CFSE) (Life Technologies, catalog number: C1157)
    Note: Currently, it is "(5-(and-6)-carboxyfluorescein diacetate, succinimidyl ester) (CFSE) (Thermo Fisher Scientific, Molecular Probes™, catalog number: C1157)".
13. ACK lysing buffer (1x) (Lonza Group Ltd., catalog number: 10548E)
14. RPMI 1640 Medium, GlutaMAX™ (Thermo Fisher Scientific, Gibco™, catalog number: 61870-010)
15. Penicillin/streptomycin (Life Technologies, Gibco™, catalog number: 15140-12)
    Note: Currently, it is "Penicillin/streptomycin (Thermo Fisher Scientific, Gibco™, catalog number: 15140122)".
16. Peptide SIINFEKL (PolyPeptide Group, custom made)
17. Fetal calf serum (Thermo Fischer Scientific, Hyclone™, catalog number: SV30160.03)
18. Bovine serum albumin (Sigma-Aldrich, catalog number: A7906)
19. FACS buffer (see Recipes)

Equipment

1. Dissection kit (containing scissors, curved forceps)
2. Centrifuge (Eppendorf, model: 5810R)
3. MyBath™ 4 mini water bath (Benchmark Scientific, model: B2000-4)
4. Flow cytometer: Cyan (Beckman Coulter) or Fortessa (Becton Dickinson)

Procedure

A. Day 0. Immunize mice with:
   1. CpG-B 1826 (30 µg/mouse)
   2. DOTAP (60 µg/mouse)
   3. Ovalbumin (100 µg/mouse)

   All reagents are diluted in sterile 1x DPBS (to a final total volume of 200 µl/mouse) and the final solution is inoculated by intravenous *(i.v.)* retro-orbital injection in the eye of each mouse. As control for immunization, some mice receive retro-orbital *i.v.* injections of sterile 1x DPBS: these naive/non-immunized mice are used to calculate the % of specific lysis, as compared to immunized mice.

   Naive non-injected mice will also be used for isolating splenocytes and pulsing them with peptides, and will also be used as control mice (see step B14).

B. Day 7. Injection of splenocytes pulsed with the peptide and labeled with a high concentration of CFSE + splenocytes not pulsed with the peptide but labeled with a low concentration of CFSE

1. Isolate the spleen of naive non-injected mice (1 naive non-injected mouse for every 3 OVA-immunized or DPBS-injected mice + control mouse) and place each spleen in a well (6-well plate).
2. Mechanically disrupt the spleen to obtain single-cell suspensions in a small volume of DPBS (1-2 ml), and then wash cells with DPBS and centrifuge (652 *x g*, 5 min, + 4 °C).
3. Resuspend the pellet and treat with ACK lysing buffer to lyse red blood cells: Add 2 ml of ACK per spleen and incubate for 2 min (at room temperature, RT).
4. Wash each spleen suspension with 2-3 ml of RPMI + Penicillin/Streptomycin and filter them on cell strainers (40 µm). Pool the suspensions all together in one Falcon 50 tube (or more if many spleens are used for the experiments), add RPMI + Penicillin/Streptomycin until you reach 50 ml and centrifuge (625 *x g*, 5 min, + 4 °C).
5. Discard the supernatant and resuspend the pellet in RPMI + Penicillin/Streptomycin; divide splenocytes into two halves. Add the peptide to one half (peptide SIINFEKL 10 µg/ml in the final volume of 2 ml per half spleen). For example: If 10 spleens are used for the experiment, 10 half-spleens will be pulsed with SIINFEKL (meaning 10 x 2 ml = 20 ml final volume) while the other half (equal volume as the final volume of spleen+ SIINFEKL; in our example: 20 ml) will remain without peptide.
6. Incubate both suspensions for 30 min at 37 °C in a water bath. Gently mix every 10 min.
7. Wash in RT DPBS 1x and centrifuge (625 *x g*, 5 min, + 4 °C) twice.
8. In the meantime, prepare two solutions of CFSE (dilutions in 1x DPBS) in the dark:
   a. $CFSE^{high}$ solution: 2.5 µM (diluted from the stock solution kept at -20 °C)
   b. $CFSE^{low}$ solution: 0.25 µM (dilution 1/10 from the $CFSE^{high}$ solution)
9. Discard the supernatants and resuspend each pellet in 1 ml of DPBS and count the cells.
10. Then, add the necessary volume of the CFSE solutions to their respective cell suspension to obtain a final concentration of 10^7 cells/ml as follows:
    a. $CFSE^{high}$ solution to the splenocytes pulsed with peptide (2.5 µM of CSFE)
    b. $CFSE^{low}$ solution to the splenocytes without peptide (0.25 µM of CSFE)

    Incubate 15 min at RT in the dark.
11. Wash each suspension with 40 ml of RPMI + 10% of FCS and centrifuge (625 *x g*, 5 min, 4 °C).
12. Discard the supernatant and wash again with RPMI + 10% of FCS and centrifuge (625 *x g*, 5 min, 4 °C).
13. Discard the supernatant, resuspend the pellet in 1x DPBS and count cells:

a. Splenocytes pulsed with peptide (and $CFSE^{high}$)
b. Splenocytes without peptide (and $CFSE^{low}$)

Resuspend each suspension in DPBS to obtain a final concentration of 5 x 10^6/100 µl.

14. Pool suspensions (both $CFSE^{high}$ and^{low}) together (ratio 1:1) and inject *i.v.* 200 µl of the mix to OVA-immunized and DPBS-injected mice. Do not forget to inject a naive (previously non-injected, non-immunized) mouse as control.
15. Wait 20-24 h.

C. Day 8

1. Kill recipient mice, remove their spleen and put it on a cell strainer (40 µm) on a Falcon 50 tube. Mechanically disrupt the spleens (individually) to obtain single-cell suspensions, treat splenocytes with ACK buffer (2 ml/spleen) as described in steps B1-3.
2. Wash with DPBS 1x and centrifuge (625 *x g*, 5 min, RT).
3. Resuspend pellets in 1 ml of FACS buffer.
4. Count cells and resuspend them to get 5 x 10^7 cell/ml.
5. Filter using a 40 µm cell strainer (to avoid FACS blocking).
6. FACS acquisition: On the Forward Scatter (FSC) vs. Side scatter (SSC) plot, gate cells on total splenocytes, then when plotting CFSE (in the FL-1, GFP or FITC channel) vs. SSC, gate on total $CFSE^+$ cells. Within this gate, using another CFSE vs. SSC plot, distinguish between the $CFSE^{high}$ and $CFSE^{low}$ populations. Acquire 10,000 total $CFSE^+$ events (*i.e.* of both populations: $CFSE^{high}$ and ^{low}). The number of total $CFSE^+$ events should be the same for all the samples analyzed in order to better compare between OVA-immunized and non-immunized mice. In case 10,000 events cannot be reached, 5,000 events can be acquired, as long as the final number is the same for all samples.

D. Data analysis

1. For the analysis of the FACS data after acquisition, 2 populations (the $CFSE^{high}$ and the $CFSE^{high}$) are distinguished by using the following gating strategy: Define the splenocyte gate on FSC and SSC, then after excluding the doublets, gate on the total $CFSE^+$ cells. Within the $CFSE^+$ cells, gate on the $CFSE^{high}$ and the $CFSE^{low}$ and determine (i) the percentage of $CFSE^{high}$ within total $CFSE^+$ cells and (ii) the percentage of $CFSE^{low}$ within total $CFSE^+$ cells.
2. To calculate the percentage of specific lysis, use the following equation for each mouse (OVA or DPBS) or sample, compared to one control “naive” un-injected mouse: % specific lysis = 100 - [100 x (% $CFSE^{high}$ immunized mouse/% $CFSE^{low}$ immunized mouse)/(% $CFSE^{high}$ naive mouse/% $CFSE^{low}$ naive mouse).

Representative data

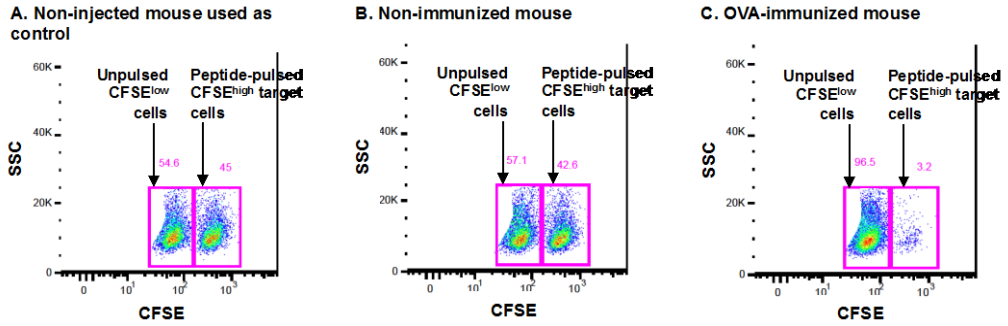

Figure 1. Representative dot plot of the FACS analysis of an *in vivo* killing assay. The dot plots in Figure 1 represent the CFSE staining (vs. side scatter, SSC) in a non-immunized control mouse (A), a non-immunized naive mouse (B) and an OVA-immunized mouse (C) after gating on total splenocytes and then on total $CFSE^+$ cells, as detailed in steps C21 and D22. The $CFSE^{high}$ and $CFSE^{low}$ gates are indicated for each mouse, and the respective percentages are shown in pink above each gate. The $CFSE^{high}$ population represents target cells pulsed with the SIINFEKL peptide, while $CFSE^{low}$ populations represent target cells not pulsed with any peptide.

C57BL/6 mice are immunized with the OVA protein (or with DPBS; non-immunized mice) and 7 days later, they receive a mix of spleen cells from C57BL/6 mice, pulsed with the SIINFEKL peptide and stained with a high concentration of CFSE and cells not pulsed with the peptide, stained with a low concentration of CFSE, at a 1:1 ration. One day later, the spleen cells from recipient mice are isolated and analyzed by FACS to determine the % of $CFSE^{high}$ and $CFSE^{low}$ cells in each mouse (OVA-immunized mouse in Figure 1C and non-immunized mice in Figure 1B).

One naive non-injected mouse is used as the “control naive mouse” (Figure 1A) in the formula detailed above (this mouse is mentioned in step B14). All OVA-immunized (ex: Figure 1C) but also the naive DPBS-injected mice (a.k.a. non-immunized mice, ex: Figure 1B) will be compared to this control mouse in order to quantify and compare the specific lysis of non-immunized mice and OVA-immunized mice.

In this example, using the formula detailed above, the % of specific lysis for the non-immunized mouse in B is calculated as follows:

% specific lysis = 100 - [100 x (42.6/57.1)/(45/54.6)]

% specific lysis = 100 - [100 x 0.746/0.824]

% specific lysis = 100 - [100 x 0.905]

% specific lysis = 100 - 90.5

% specific lysis = 9.5

The % of specific lysis for the OVA-immunized mouse in C is calculated as follows:

% specific lysis = 100 - [100 x (3.2/96.5)/(45/54.6)]

% specific lysis = 100 - [100 x 0.033/0.824]

% specific lysis = 100 - [100 x 0.040]

% specific lysis = 100 - 4.0

% specific lysis = 96

As shown in the example, the % of specific lysis of each mouse is calculated as compared to the naive non-injected mouse set at control mouse.

Notes

This protocol was optimized for the vaccination with the Ova protein and for the IVK test with the corresponding H-2^b restricted OVA peptide that activates the $CD8^+$ cytotoxic T cells. The protocol can be adapted to other vaccine candidates and their corresponding peptide with a $CD8^+$ T cell epitope. The respective concentrations should be optimized.

Recipes

1. FACS buffer
   1x DPBS
   5% fetal calf serum or bovine serum albumin

Acknowledgments

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