Video Article

In Vivo Assay for Detection of Antigen-specific T-cell Cytolytic Function Using a Vaccination Model

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Abstract

Current methodologies for antigen-specific killing are limited to *in vitro* use or utilized in infectious disease models. However, there is not a protocol specifically intended to measure antigen-specific killing without an infection. This protocol is designed and describes methods to overcome these limitations by allowing for the detection of antigen-specific killing of a target cell by $CD8^+$ T cells *in vivo*. This is accomplished by merging a vaccination model with a traditional CFSE-labeled target killing assay. This combination allows the researcher to assess the antigen-specific CTL potential directly and quickly as the assay is not dependent upon tumor growth or infection. In addition, the readout is based on flow cytometry and so should be readily accessible to most researchers. The major limitation of the study is identifying the timeline *in vivo* that is appropriate to the hypothesis being tested. Variations in antigen strength and mutations in the T cells that may result in differential cytolytic function need to be carefully assessed to determine the optimal time for cell harvest and assessment. The appropriate concentration of peptide for vaccination has been optimized for $hgp100_{25-33}$ and $OVA_{257-264}$, but further validation would be needed for other peptides that may be more appropriate to a given study. Overall, this protocol allows a quick assessment of killing function *in vivo* and can be adapted to any given antigen.

Video Link

The video component of this article can be found at https://www.jove.com/video/56255/

Introduction

Multiple protocols exist to assess the cytolytic (CTL) potential of a $CD8^+$ or $CD4^+$ T-cell. This assessment can be readily done *in vitro* under controlled conditions[1,2,3]. In addition, infectious disease models, such as LCMV, have classically examined CTL function through the use of differentially CFSE (5-(and 6)-Carboxyfluorescein diacetate succinimidyl ester) labeled target cells where the $CFSE^{hi}$-labeled cells are pulsed with a peptide and $CFSE^{lo}$-labeled target cells are left unpulsed. The cells are then injected at a 1:1 ratio and assessed for loss of the $CFSE^{hi}$-labeled pulsed targets by flow cytometry[4]. Vaccine and rejection models have also used similar strategies for assessment of *in vivo* killing by both $CD8^+$ and $CD4^+$ T cells as well as NK cells[5,6]. This is a powerful assay, but requires the use of infectious agents that prime the immune system prior to target injection.

This protocol, on the other hand, requires no prior infection of the host and instead utilizes a vaccination strategy to prime the immune system prior to target injection. This vaccination is comprised of a water-based formulation of peptide vaccine which requires provision of an immunostimulatory cocktail called covax[7], consisting of a Toll-like receptor 7 (TLR7) agonist (imiquimod cream), an agonistic anti-CD40 antibody, and interleukin-2 (IL-2) leading to synergistic combination of immunostimulatory agents for the elicitation of peptide-specific priming and robust immune response. As such, this assay provides a quick readout of CTL function as the vaccine is administered along with the cells for assessment of function only three days prior to injection of the target cells. In addition, the covax priming is strong enough that the killing capacity of the primed antigen-specific T cell can be seen between 4 and 24 h after injection.

The major limitation of this protocol is identifying the timeline *in vivo* for the detection of target killing that is appropriate to both the antigen and the hypothesis being tested. Careful assessment must be performed, as variations in antigen strength as well as genetic alterations being tested in T-cells could result in differential CTL function that would require a different timing detection of target killing. In addition, while the appropriate concentration of peptide for vaccination has been optimized for human melanoma antigen glycopeptide 100 ($hgp100_{25-33}$) and $ovalbumin_{257-264}$ ($OVA_{257-264}$)[8,9], use of another antigen model that may be more appropriate to a given study would require further validation. Because of anticipated differences in a target antigens' capacity to stimulate CTL effector function in combination with the covax as an adjuvant, optimization of IL-2 dose concentration and dose frequency may be essential to achieve the desired goal. Overall, this protocol allows for a quick assessment of killing function *in vivo* and can be adapted to any given antigen.

Protocol

All methods described here have been approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Texas MD Anderson Cancer Center.

1. Preparation of Peptide for the Vaccine

1. Dissolve the lyophilized peptide with phosphate-buffered saline (PBS) to the appropriate concentration and vortex for 30 s.
   NOTE: For $hgp100_{25-33}$, the final concentration is 1 mg/mL and for $OVA_{257-264}$, the final concentration is 0.5 mg/mL. Reconstitute peptide prior to injection. Do not store after reconstitution.

2. Isolation of Splenocytes from Transgenic Mouse

NOTE: Cell isolation from the spleen must be performed in a sterile manner.

1. **Euthanize the OT-1 transgenic mouse using the approved CO_2 asphyxiation method and remove the spleen.**
   NOTE: Appropriate transgenic mouse is utilized in this step specific for the peptide of choice.
   1. Lay the mouse on its right side. Spray the left side of the mouse with 70% ethanol (EtOH). Pull up the skin using forceps and cut the skin using surgical scissors; the spleen will be visible within the peritoneal cavity. Gently cut open the peritoneal cavity to access the spleen. Remove the spleen using forceps.

2. **Disaggregate the spleen by placing it in a 70 µm filter in a petri dish with 2 mL medium A (PBS with 1% fetal bovine serum (FBS)) and smashing the spleen with the end of a plunger.**
   1. Collect the splenocytes from the petri dish and place in a 50 mL conical tube. Wash the petri dish with 5 mL of medium A twice to collect all cells. Add medium A up to 25 mL and centrifuge the cells for 5 min at room temperature at 475 x g.

3. Aspirate the cells and resuspend in 1 mL/per spleen red blood cell (RBC) lysis buffer. Incubate at room temperature for 5 min. Add 10 mL of medium A and centrifuge at room temperature at 475 x g. Resuspend the pellet in 10 mL of medium A and remove debris by filtering the cell suspension through a 70 µm filter into a clean 50 mL conical.
4. **Count cells using trypan blue and a hemocytometer. Resuspend cells in PBS to a final concentration of 10^6 - 100^6 cells/mL. For $OVA_{257-264}$ specific killing, resuspend at 10^6 cells/mL and for $hgp100_{25-33}$ specific killing, resuspend at 100^6 cells/mL.**
   1. Spin the remaining cells at room temperature for 5 min at 475 x g. Aspirate the supernatant and resuspend in 15 mL cold PBS to wash cells. Repeat the wash step once more. Spin the cells at room temperature for 5 min at 475 x g. Aspirate the supernatant and resuspend in cold PBS according to the final volume determined in step 2.4.
   2. Transfer single cell suspension to a 1.5 mL microcentrifuge tube and keep on ice until injection into recipient C57/BL6 mouse.

3. Injection of Splenocytes from Transgenic Mice

1. Place recipient C57/BL6 mouse in a restrainer with the dorsal side up. Spray injection tail base area with 70% isopropyl alcohol. Administer 100 µL of single cell suspension (section 2) intravenously into the tail vein using a 27 G needle with the bevel side facing up.

4. Covax Administration

NOTE: If cells are injected in the afternoon, covax should be administered the following morning within 18 h of cell injection.

1. Place the mouse in a clear anesthesia box with 1 - 3% isoflurane. After mice are fully anesthetized, transfer the mice to the nose cones attached to the manifold in the anesthesia chamber. Keep mice restrained with the dorsal side up.
   NOTE: Anesthetization is confirmed by a brief toe pinch to verify a withdrawal response is not elicited. Federal law restricts isoflurane use on the order of a licensed veterinarian.
2. Spray injection tail base area with 70% isopropyl alcohol. Inject 100 µL of peptide solution (from section 1) subcutaneously using a 27 G needle with the syringe penetrating 4 - 5 mm into the tail base region, the needle bevel side facing up.
   NOTE: Mice are vaccinated in one flank with subcutaneous injection at the base of the tail[10].
3. Inject 100 µL anti-CD40 antibody (0.5 mg/mL stock) lateral to the vaccine injection site.
4. Carefully apply imiquimod cream 50 mg/mouse on the vaccination sites. Rub imiquimod cream on the surface until the cream is no longer visible and is fully absorbed.
5. Inject 100 µL of 100,000 IU/mL rhIL-2 protein intraperitoneally (i.p.) at the lower abdominal region. Observe mice for 5 min after they fully recover from anesthesia.
   NOTE: Experiment is paused at this point for 3 days.

5. Isolation of Target Splenocytes for Labeling with CFSE

NOTE: Cell isolation from the spleen must be performed in a sterile manner.

1. Euthanize the C57BL/6 mice according to approved CO_2 asphyxiation method. Remove spleen(s) as in step 2.1.1. Disaggregate the spleen and wash splenocytes as in steps 2.2.1 - 2.2.3. Lyse red blood cells as in steps 2.3 - 2.3.2.

2. Remove cells for counting using a hemocytometer and calculate for a final concentration of cells at 10^6 cells/mL in CFSE-labeling solution (described in step 7). Spin remaining cells at room temperature for 5 min at 475 x g. Aspirate supernatant.

6. Peptide Pulsing of Target Splenocytes

NOTE: Peptide pulsing must be performed in a sterile manner.

1. Resuspend cells at 10^6 cells/mL in complete media (RPMI 1640 media with 10% FBS, 1% L-glutamine (L-Gln), 1% penicillin/streptomycin (Pen/Strep)). Divide the cells into 2 tubes (15 mL conicals). Label each tube as pulsed or unpulsed.
2. **Add peptide to the tube labeled pulsed. For $OVA_{257-264}$ pulsing, add 1 µg/mL and for $hgp100_{25-33}$ pulsing, add 2 µg/mL.**
   NOTE: The unpulsed cells undergo the same incubation as the pulsed cells just without the addition of the peptide.
   1. Incubate cells + peptide at 37 °C for 1 h.
3. Add 10 mL of complete media (RPMI 1640 media with 10% FBS, 1% L-glutamine (L-Gln), 1% penicillin/streptomycin (Pen/Strep)) to each tube to wash both pulsed and unpulsed target cells. Spin remaining cells at room temperature for 5 min at 475 x g. Aspirate supernatant.

7. Preparation of CFSE for Labeling Target Splenocytes

1. **Prepare CFSE-labeling solution during the cell washing in step 6.3; the pulsed cells will be labeled as $CFSE^{hi}$ and the unpulsed as $CFSE^{lo}$. Prepare 1 mL of CFSE-labeling solution for 10^6 cells.**
   NOTE: CFSE is light sensitive and should be protected from light at all times.
   1. Prepare $CFSE^{hi}$ -labeling media by adding 1 uL/mL CFSE (5 mM stock solution) for a final concentration of 5 µM/mL in RPMI media 1640 with 2% FBS.
   2. Prepare $CFSE^{lo}$ -labeling media by adding 1 uL/mL CFSE (0.5 mM stock solution) for a final concentration of 0.5 µM/mL RPMI media 1640 with 2% FBS.

8. Labeling of Target Splenocytes with CFSE

NOTE: CFSE labeling must be performed in a sterile manner.

1. Resuspend the pulsed and unpulsed target cells (from step 6.3) at 10^6 cells/mL CFSE-labeling media. Resuspend the pulsed cells with prepared $CFSE^{hi}$-labeling media and the unpulsed cells with prepared $CFSE^{lo}$-labeling media.
2. Mix cells and CFSE-labeling media by gentle inversion or swirling. Do not mix by vortexing. Incubate cells and CFSE-labeling media at 37 °C for 15 min. Remix the cells every 5 min.
3. Add 10 mL of complete media to each cell suspension and spin cells at room temperature for 5 min at 475 x g.
4. Aspirate supernatant and resuspend cells in 10 mL cold PBS. Spin cells at 4 °C for 5 min at 475 x g.
5. Repeat step 8.4.
6. Count the cells and mix peptide-pulsed, $CFSE^{hi}$-labeled with unpulsed, $CFSE^{lo}$-labeled cells at a 1:1 ratio for injection into recipient mice. Keep an aliquot of 1x 10^6 mixed cells to use for a baseline flow cytometry assessment (section 11).
   NOTE: The final volume for injection is 100 µL per mouse. The final cell count is 10e6 cells. Loss of volume in the syringe and needle needs to be taken into account and should be estimated at 500 µL.

9. Injection of Target Cells

NOTE: Keep CFSE-labeled cells protected from light prior to and during the injection as much as possible.

1. Place recipient C57BL/6 mice in a restrainer with the dorsal side up. Spray injection tail base area with 70% isopropyl alcohol. Administer 100 µL of single cell suspension intravenously into the tail vein with the bevel side facing up.

10. Re-isolation of Target Cells

NOTE: The timing of this step is critical and dependent upon the CTL cytotoxicity and the strength of the antigen for stimulation. For assessment of killing an $OVA_{257-264}$ pulsed target, the cells need to be harvested 4 - 6 h after injection. Since CFSE-labeled cells are light sensitive, process spleens in the dark.

1. Euthanize the recipient C57BL/6 mice according to the approved CO_2 asphyxiation method.
2. Remove spleen(s) as in step 2.1.1. Disaggregate the spleen and wash splenocytes as in steps 2.2.1 - 2.2.3. Lyse red blood cells as in steps 2.3 - 2.3.2.
3. Resuspend cells in 1 mL fluorescence activated cell sorting (FACs) buffer (1% BSA+PBS) for assessment by flow cytometry.

11. Gating Logic to Determine CTL Activity by Flow Cytometry

1. **Perform assessment of CTL activity using a standard flow cytometry protocol. Acquire cells using the FITC channel on a flow cytometer with a 488 nm laser for excitation[11].**
   1. Gate on live lymphocytes using the forward scatter (FSC) vs side scatter (SSC) parameters. Subgate within the live lymphocyte gate for the total CFSE-positive population.

NOTE: The injected CFSE-labeled cells will make up a small subset of the total lymphocytes present in the spleen. The CSFE-positive cells should appear as two distinct populations on the log scale.

2. Use a histogram format to determine the percentage of the unpulsed (left peak, $CFSE^{lo}$) and pulsed (right peak, $CFSE^{hi}$) populations. Loss of the $CFSE^{hi}$ cells indicates antigen-specific CTL activity.

Representative Results

Prior to injection of CFSE-labeled target cells, the 1:1 cell mixture is run on a flow cytometer to determine the baseline frequencies of both the $CFSE^{hi}$ and $CFSE^{lo}$ target cells. **Figure 1A** shows the gating strategy to detect changes in the CFSE populations, an initial gate is made using FSC and SSC parameters. The total CFSE-positive cells are then subgated prior to assessing changes in frequency, as this population is relatively small when compared to the unlabeled endogenous splenocytes. The relative frequency of the $CFSE^{hi}$ and $CFSE^{lo}$ populations is the calculated by setting the total CFSE-positive population at 100%. This analysis can be done using a histogram or dot plot format. An example of the relative frequency of the CFSE populations prior to injection is shown in **Figure 1B**. This ratio will rarely be exactly 1:1 but should be reasonably close. The necessity of the covax priming is shown in **Figure 1C** where no killing of the antigen pulsed, $CFSE^{hi}$ target cells is observed at 24 h post injection. **Figure 1D** demonstrates the effective killing of the antigen pulsed, $CFSE^{hi}$-labeled target cells as the peak that was observed prior to injection is almost undetected and the ratio is dramatically shifted from 50% to 1% detection. The figure also shows the kinetics of antigen pulsed, $CFSE^{hi}$-labeled target cell killing by assessing loss of this population at both 6 h and 24 h post injection.

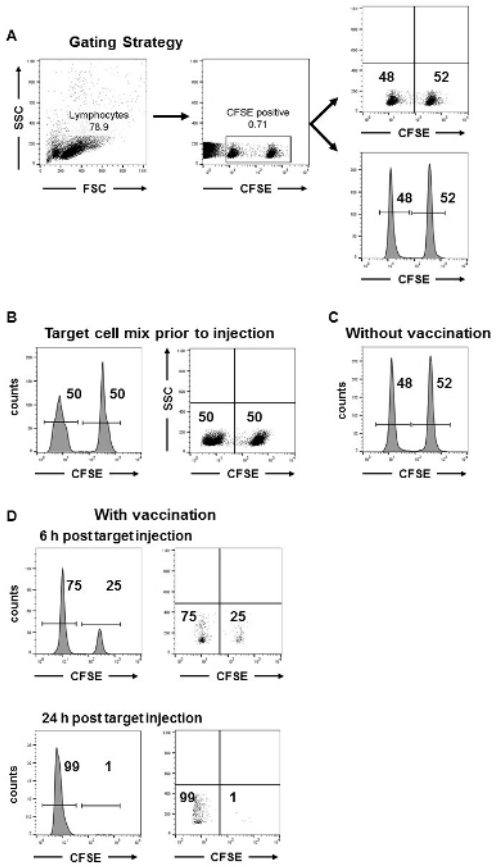

Figure 1: Comparison of labeled cells at baseline and following injection of CFSE-labeled target cells. (**A**) The gating strategy for assessment of CTL function is shown. Briefly, live lymphocytes are gated using forward scatter (FSC) vs side scatter (SSC) parameters. Total CSFE-positive cells are subgated within the live cell gate. The ratio of $CFSE^{hi}$ and $CFSE^{lo}$ is based on their respective frequency within the total CFSE-positive population. (**B**) Following CFSE labeling, target cells are mixed 1:1 and assessed for the ratio of $CFSE^{hi}$ and $CFSE^{lo}$ cells by flow cytometry. The numbers indicate frequency of the respective peaks on both a histogram (left) and CFSE vs SSC dot plot (right) formats. (**C**) This demonstrates the lack of target cells killing without prior vaccination with covax regimen. Splenocytes were harvested 24 h after injection. (**D**) This demonstrates the killing of the antigen-pulsed $CFSE^{hi}$ target cells at 6 h (top graphs) and 24 h (bottom graphs) post injection. The numbers indicate the frequency of the CFSE-labeled peaks on both a histogram (left) and CFSE vs SSC dot plot (right) format. Please click here to view a larger version of this figure.

Discussion

While this protocol is straightforward, there are a few critical steps that must be carefully performed. The covax priming following injection of the antigen-specific T-cell being tested is necessary to see any killing of the pulsed targets. While it is possible that the water-based covax

vaccination creates an acute inflammatory condition, for the chronic inflammatory phase, replacing the short-lived water-based formulation with a slow-antigen release oil-based approach may produce a better outcome[7,12].

In addition, the CFSE-labeling of the target cells following antigen pulsing needs to be uniform for an ideal flow cytometry readout. The labeling of the $CFSE^{hi}$ with 5 µM and the $CFSE^{lo}$ with 0.5 µM was found to be ideal in this system to provide a log difference in the populations that could be easily distinguished.

This protocol is simple to modify for the specific antigen system being tested. First, the concentration of peptide administered as part of the covax should be confirmed. A simple way to determine this appropriate concentration is to vaccinate and track the expansion of the injected, antigen-specific T cells in the blood. In this case, a weak antigen ($hgp100_{25-33}$) requires twice as much peptide as a strong antigen ($OVA_{257-264}$). The validated concentrations used in this protocol would be a reasonable starting point for testing other antigens based on strength of stimulation. One point of modification would be to simply immunize the wild-type mouse with an antigen of interest followed by peptide pulsed targets for assessing CTL-function. However, this modification would rely on the activation and expansion of endogenous antigen-specific responses and so the timing of the target cell transfer would likely need to occur 7 - 14 days after immunization. In addition, the level of antigen-specific killing may be greatly reduced as compared to that observed when transferring transgenic T cells. A caveat with this modification is that the level of CTL response to the target may be too low to be detected.

In addition to the amount of antigen required for T-cell priming, the timing of the re-isolation of the CFSE-labeled target cells must be optimized for a given hypothesis. Modifications to the T-cell being tested that result in differential killing capabilities may require adjustments in the timing of the re-isolation. A T-cell with an enhanced killing function will need to have targets assessed much faster than its wild-type counterpart. This kinetic profile should be carefully explored to determine the optimal timepoint to address the tested hypothesis. In general, we have found this to vary between 4 and 24 h after injection of the target cells.

One limitation of the assay is that the effector cells have not been chronically stimulated to induce an exhausted state. Therefore, CTL function is restricted to recently activated effector cells. It is possible that this method could be modified to assess recall function of a memory population or killing capability of a chronically activated antigen-specific T cell subset, which would be interesting for future applications.

This method of testing CTL function *in vivo* allows for fast detection of *in vivo* killing that overcomes some of the limitations of an *in vitro* setting. An intact immune system is in place and the priming of the transferred T cells via the covax method relies on peptide presentation and co-stimulation by endogenous antigen-presenting cells. Unlike other *in vivo* killing assays, this method does not require an infection or the presence of a tumor target. However, the addition of a tumor target would provide an additional comparator and could be added to the assay prior to the injection of antigen-specific T cells and covax administration for future applications of this method.

Disclosures

The authors have nothing to disclose.

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