Video Article

Rapid *In Vivo* Assessment of Adjuvant's Cytotoxic T Lymphocytes Generation Capabilities for Vaccine Development

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Abstract

The assessment of modern sub-unit vaccines reveals that the generation of neutralizing antibodies is important but not sufficient for adjuvant selection. Therefore, adjuvants with both humoral and cellular immuno-stimulatory capabilities that are able to promote cytotoxic T lymphocytes (CTL) responses are urgently needed. Thus, faithful monitoring of adjuvant candidates that induce cross-priming and subsequently enhance CTL generation represents a crucial step in vaccine development. In here we present an application for a method that uses SIINFEKL-specific (OT-I) T cells to monitor the cross-presentation of the model antigen ovalbumin (OVA) *in vivo* in the presence of different adjuvant candidates. This method represents a rapid test to select adjuvants with the best cross-priming capabilities. The proliferation of CD8$^+$ T cells is the most valuable indication of cross-priming and it is also regarded as a correlate of adjuvant-induced cross-presentation. This feature can be evaluated in different immune organs like lymph nodes and spleen. The extent of the CTL generation can also be monitored, thereby giving insights on the nature of a local (draining lymph node mainly) or a systemic response (distant lymph nodes and/or spleen). This technique further allows multiple modifications for testing drugs that can inhibit specific cross-presentation pathways and also offers the possibility to be used in different strains of conventional and genetically modified mice. In summary, the application that we present here will be useful for vaccine laboratories in industry or academia that develop or modify chemical adjuvants for vaccine research and development.

Video Link

The video component of this article can be found at https://www.jove.com/video/57401/

Introduction

Cytotoxic T lymphocytes (CTL) inducing vaccines are key therapeutic interventions that have been developed to fight certain types of cancer[1]. CTL are also important for prophylactic vaccines against intracellular pathogens[2]. Moreover, CTL are one of the few immune defense mechanisms functionally active in risk populations such as neonates[3,4] whom also depend on CTL to combat early life infections[5]. In this regard, vaccines against Respiratory Syncytial Virus (RSV) that were developed with an adjuvant that does not elicit CTL responses (alum) resulted in a complete failure of the vaccine leading to serious complications upon infections in infants[6]. These negative effects of vaccination can be reversed by a CD8$^+$ T cell response[7]. We have previously demonstrated that the main cytokines (type I interferons) elicited by some stimulator of interferon genes (STING) agonists are essential for the CTL responses generated by these adjuvants[8], in part by measuring the proliferation of OT-I T cells after vaccination and using these results as a measure of CTL inducing capabilities observed in extended vaccination schedules[9]. The measurement of the proliferation of OT-I CD8$^+$ T cells in a wild type (WT) C57BL/6 recipient mouse by carboxyfluorescein succinimidyl ester (CFSE) dye dilution is a robust estimation of the capability of the adjuvant of a vaccine to generate cross-priming of SIINFEKL, (the immuno-dominant peptide of ovalbumin, OVA). Variations of this technique are widely used for the assessment of the proliferation of OT-I CD8$^+$ and OT-II CD4$^+$ T cells. For example, it has been used in the absence of selected cytokines (KO mice) or to measure vaccine efficacy after antigen recall in WT animals. We devised a short protocol (4 days experiment) in which after passive transfer of CFSE-stained OT-I CD8$^+$ T cells, a subcutaneous (s.c.) immunization consisting of one dose of 50 µg of endotoxin-free OVA supplemented with test adjuvants is administered (**Figure 1**). The follow up of the results 48 h after vaccination provides a reliable proof of the capacity of the adjuvant to generate CTL responses. By this strategy, it is possible to assess the potency of the local immune response in the draining lymph node after immunization as well as the extent of the response by measuring the CTL activity in the spleen (or distant lymph nodes).

Protocol

All mice used in this study were from the C57BL/6 background. All the animals were kept under pathogen-free conditions. All experiments were performed according to the normative of the German animal protection law (TierSchG BGBl. I S 1105; 25.05.1998) and were approved by the Lower Saxony Committee on the Ethics of Animal Experiments and the state office (Lower Saxony State Office of Consumer Protection and Food Safety), under permit number 33.4-42502-04-13/1281 and 162280.

1. CFSE Staining of OT-I T Cells and Adoptive Transfer

NOTE: OT-I mice are transgenically generated animals that express a T cell receptor (TCR) with fixed α and β chains that together recognize the immuno-dominant peptide of OVA, SIINFEKL[10,11]. As a result, these mice have a considerably high number of SIINFEKL-specific $CD8^+$ T cells (97%)[12] when compared to normal or OVA-vaccinated mice (≤ 1%)[13].

1. Isolation of traceable $CD8^+$ T cells from OT-I mice expressing T lymphocyte-specific *Thy1*^{a} (Thy1.1) allele:
    1. Euthanize 6-9 weeks old OT-I mice by CO_2 inhalation followed by cervical dislocation[14]. Dissect the spleen and major lymph nodes (inguinal, axillary and cervical pairs only) by cutting the skin with surgical scissors, detaching the skin from the body with the help of surgical pliers and forceps and place them in Petri dishes (60 x 15 mm) each containing a 100 µm pore mesh cup for tissue grinding and cell release.
    2. Maintain petri dishes (each with one mesh cup) in 3-5 mL of complete RPMI medium (RPMI 1640, 10% v/v FCS, 100 U/mL penicillin, 50 µg/mL streptomycin) kept on ice.
    3. Mash the organs with the help of a syringe plunger or a similar sterile instrument (prior cutting is not needed) and collect the resulting single cell suspension in 15 mL centrifuge tubes.
    4. Centrifuge the cell suspension at 300 x g for 10 min at 4 °C. Discard the supernatant. Wash the cells in 10 mL of cold PBS by centrifugation and lyse the erythrocytes from the spleen by re-suspending the pellet in 1 mL/spleen of ammonium chloride buffer (ACK buffer, commercially available). Incubate the cells for 1.5 min on ice and subsequently wash the cells with 10 mL of cold PBS by centrifugation (as done before).
    5. Re-suspend the pellet in the same tube in 1 mL of PBS (pH 7.2) containing 5% fetal bovine serum for magnetic isolation.
2. To perform magnetic isolation, proceed to the negative selection of $CD8^+$ T cells by using a magnetic isolation kit according to the manufacturer instructions or published protocols[15].
3. To perform CFSE staining, first count the number of $CD8^+$ T cells obtained from OT-I mice by using an automated cell counter (particle counter). Stain 1 - 5 x 10^7 cells/mL in a volume of 1-5 mL with 5 µM CFSE in PBS for 7 min at 37 °C, protected from light in a 15-ml falcon tube.
4. Quench the CFSE staining by adding the same volume (1:1) of fetal bovine serum to the cells, and incubate them for additional 7 min at 37 °C protected from light. Wash the cells with 10 mL of PBS twice.
5. Count the cells using an automated cell counter. Set the cell number to 3-5 x 10^7 cells/mL of PBS in order to inject 3-5 x 10^6 cells/mouse in 100 µL intravenously (i.v.) via the tail vein.
6. To perform tail vein injections, immobilize the mice in appropriate restrainers. Warm the back area and tail of the mice to be injected by using a red-light lamp, placed between 20 to 25 cm away from the mice for 1-3 min to allow tail vein vasodilation.
   NOTE: This helps to ease vein detection and administration of the cell suspension.
7. Ensure (with the hand placed in between the mice and the lamp) that it is not too hot for the mice and when the tail veins are clearly visible, proceed to injection. Inject the cells into the lateral or dorsal tail vein using an 1mL syringe with a 25-gauge needle[16].

2. Immunization (Endo-free OVA +/- Adjuvant)

1. Place the mice transplanted with OT-I cells (step 1.7, **Figure 1**) in an anesthesia chamber and administer isoflurane in oxygen by an anesthesia machine[14].
2. When the mouse is completely asleep under anesthesia, take it out from the chamber and shave the fur of the mouse on the area over the *gluteus superficialis* (lateral lower back) by using an electric hair trimming machine, in order to perform a clean injection with a good view of the application area.
3. Inject 50 µL of the vaccine, s.c. in the shaved area using a 25-gauge needle.

3. Isolation of Lymphocytes and Staining for Flow Cytometry Analysis

1. Isolation of lymphocytes and splenocytes
    1. Euthanize the vaccinated mice by CO_2 inhalation followed by cervical dislocation.
    2. Extract the draining and distant lymph nodes and spleen and place them in separate Petri dishes containing 100 µm pore mesh cups for tissue grinding and cell release. Follow steps 1.1.2 through 1.1.3.
    3. Decant the supernatant after centrifugation and re-suspend the cell pellets in the same volume of remnant PBS (approx. 100 µL).
2. Staining for flow cytometry analysis
    1. In a 15-mL centrifuge tube prepare a master mix containing the staining antibodies in the concentrations depicted in **Table 1**, thus yielding a 2x concentrated staining mix. Prepare enough volume of master mix to have 100 µL per sample. Mix the cells and the master mix 1:1 and incubate it at 4°C for 30 min.
       NOTE: The final staining volume per sample should be 200 µL (100 µL of cell pellet +100 µL of antibody master mix).
    2. Wash the cells twice by centrifugation as described in 1.1.3, adding 10 mL of PBS, twice.
    3. Re-suspend the stained cells in 0.5-1 mL of PBS for acquisition and transfer the suspension to flow cytometer tubes. Always keep the samples on ice and protected from light.

4. Flow Cytometry

1. Always pre-filter the cell samples using 70-100 µm filters.
2. Prepare single staining compensation controls for the fluorophores detailed in Table 1 (beads or cells).
   NOTE: Compensation should be performed in cytometer or analysis software[17,18,19] and applied to all samples.

3. Follow the gating strategy depicted in **Figure 2**. Briefly, gate populations in forward scatter height vs. forward scatter area (**Figure 2A**), and again side scatter wide vs. side scatter area (SSA, **Figure 2B**) in order to exclude doublets.
4. Gate the population from **Figure 2B** by plotting BV 650 (auto-fluorescence) vs. FITC (CFSE) in order to discriminate true CFSE stained cells from high auto-fluorescent cells. Include beads or cells stained with antibody conjugated to BV 650 into compensation controls. This plot (**Figure 2C**) of BV 650 vs. FITC is used to gate the OT-I CFSE stained cells.
5. Gate the population from **Figure 2C** for Pe-Cy7 (Thy1.1 -CD90.1-) vs. APC (CD8) in order to distinguish cells derived from donor vs. recipient mice.
   NOTE: Cells derived from OT-I donor mice are Thy1.1$^+$ (CD90.1), whereas WT (C57BL/6, recipient) mice-derived cells are Thy1.2$^+$ (CD90.2) (**Figure 2D**). Some laboratories have OT-I mice in a Thy1.2 background and WT (C57BL/6) in a Thy1.1. In this case you have to use an antibody against Thy1.2 for OT-I cell gating.
6. Re-gate CD8$^+$ cells from Thy1.1$^+$ population by plotting it on a gate comprising BV 450 (CD4) vs. APC (CD8) (**Figure 2E**).
7. Display the population gated in **Figure 2E** by using a histogram display of the CD8$^+$ cells showing CFSE (FITC, 530/15 channel -blue laser-), gate the proliferated population by including intensities from 10^2 up to the level where the undivided control populations are (intensities ~10^5, depending on the efficacy of the CFSE staining and the voltage setting on the flow cytometer) (**Figure 2F**).
8. Make an additional gate (not displayed in figure 2) plotting FITC vs. L/D marker (450/20 channel, UV laser) in order to assess dead cells in parallel to the proliferation evaluation.
9. Acquire at least 5000 events for the compensation controls and 10.000 events (gate E, **Figure 2**) from the samples at a flow cytometer. Run the cytometer at low or medium flow (do not exceed flow rates of 20.000 events/s).

Representative Results

In order to test the treatments using a different combination of adjuvants (ADJ1 and ADJ2), we have assessed the CTL generation capacity by measuring the proliferation of adoptively transferred OT-I CD8$^+$ T cells by flow cytometry (**Figure 2**). For this, we previously stained isolated cells from the draining lymph nodes and spleen (**Table 1**). By measuring the proliferation of CD8$^+$ T cells in lymph nodes and spleen, we were able to corroborate a higher CTL generation capacity of ADJ2 in the draining lymph nodes (**Figure 3**) when compared to ADJ1, OVA alone or PBS control. In contrast, we have observed that ADJ2 was not able to generate a CTL response in a systemic compartment (spleen), whereas ADJ1 was showing high levels of proliferation by splenic CD8$^+$ T cells, not only compared to the controls (PBS and OVA) but also superior to ADJ1. By dissecting the action of ADJ2 using this rapid *in vivo* proliferation assay, we can confirm its strong action as a local but not a systemic CTL generator. Moreover, ADJ1 acts both at the local site of injection (draining lymph nodes) as well as systemically with an increased OT-I proliferation in the spleen. The obtained results allow us to characterize the adjuvant's activity and its extent, exemplified by the observed effects of ADJ2 (local) and ADJ1 (systemic).

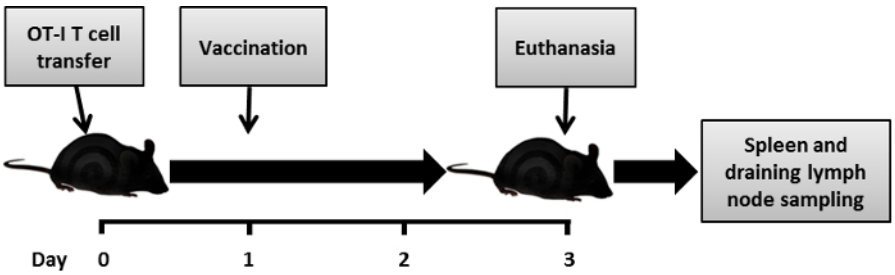

Figure 1: The assay timeline. The assay timeline represents the initial OT-I T cell transfer at day 0, the s.c. vaccination at day 1, and the sampling spleen and draining lymph nodes 2 days later. Please click here to view a larger version of this figure.

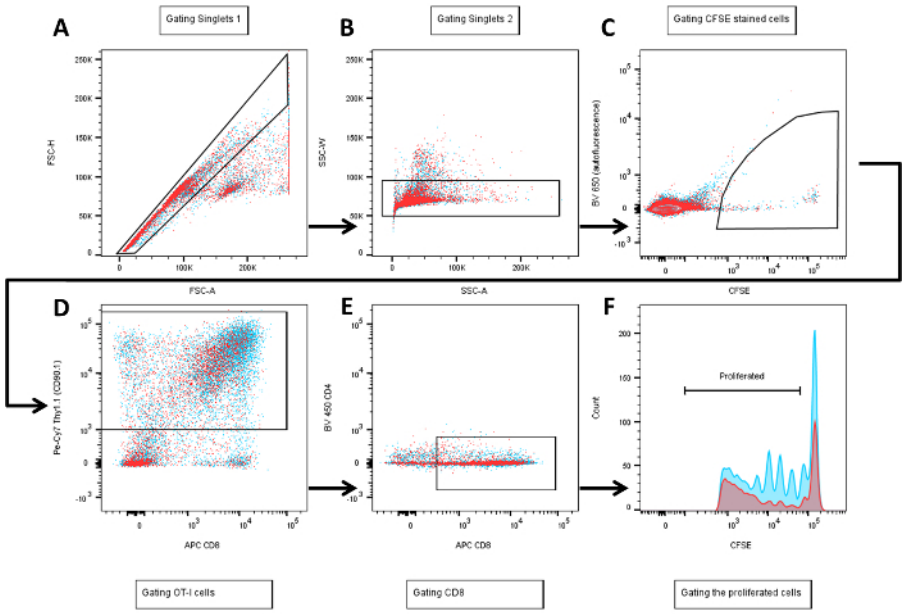

Figure 2: Flow diagram of the gating strategy followed to measure the proliferation of CD8$^+$ T cells (OT-I, Thy1.1$^+$) by flow cytometry. Two samples are represented in different colors (red and light blue) for a better visualization. **A-B.** Single cells are discriminated from doublets successively in the first two gates by plotting forward-scatter-height vs. forward-scatter-area and side-scatter-width vs. side-scatter-area. **C.** Cells gated in **B** are displayed by their fluorescence intensities of BV 650 channel (auto fluorescence) plotted against their CFSE intensity (where diming indicates cells divisions/proliferation) in the 530/30 YG channel. This gate allows the selection of true CFSE positive cells by discriminating those that have high auto fluorescence. **D.** CFSE positive cells were gated with their high fluorescence intensity of Pe-Cy7 (Thy1.1, marker for OT-I cells) vs. their APC intensity (CD8). **E.** BV 450 (CD4) plotted against APC (CD8) for previously gated Thy1.1 positive cells to accurately select CD8$^+$ T cells. **F.** Previously gated CD8$^+$ cells are plotted in a histogram against the CFSE intensity to finally gate the proliferated population. Please click here to view a larger version of this figure.

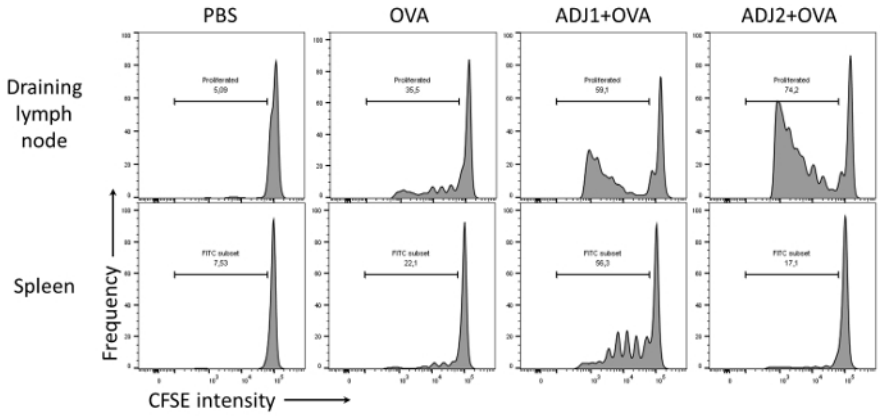

Figure 3: Adjuvant driven CTL generation. The ability to elicit local or systemic CTL responses is depicted for 2 different adjuvant treatments (ADJ1 and ADJ2) along antigen and PBS controls. The proliferation of the adoptively transferred OT-I T cells is examined in the draining lymph node (inguinal, for the exemplified subcutaneous (s.c.) administration) and in the spleen, as indicated in the figure rows. The cell proliferation is measured in all relevant treatments (columns) in order to accurately compare the extent of the immune response in its local (draining lymph node) or systemic (spleen) range of action. Please click here to view a larger version of this figure.

Dyes - Antibodies	Clone	Fluorophore/channel-filter	Concentration 2X
Thy1.1 (CD90.1)	HIS51	PE-Cy7 - 780/60 YG	1:750
CD8	53-6.7	APC - 670/14 R	1:280
CD4	RM4-5	BV 421 - 450/50 V	1:100
CFSE	-	FITC - 530/30 YG	(according to CFSE-staining protocol)
DCM (Dead Cell Marker)	-	-/ U.V. - 450/50 UV	1:500

Table 1: Antibody stainings for flow cytometry. Fluorophore-conjugated antibody clones used and recommended staining concentrations (2x).

Discussion

Modern vaccines are ideally composedof purified antigen and adjuvants, with the possible addition of a delivery system like liposomes, virus-like particles, nanoparticles or live vectors. A key aspect when designing a vaccine is to choose the right adjuvant according to the clinical needs. Part of the scope could involve favoring a humoral vs. cellular immune response (or both), the election of a local vs. a systemic immune response (or both), and the kind of memory that the vaccine must generate in the target population. One crucial aspect of adjuvant evaluation is to rapidly determine its capacity to generate CTL. We presented here a method based on already known techniques, to rapidly determine the features of the CTL response *in vivo* in a mouse model by measuring OT-I CD8 T cell proliferation. This method allows for the prediction of the potency of the immune response elicited by adjuvants (proliferation of OT-I T cells) in only 4 working days. This method further facilitates the comparison of the actions of adjuvants in terms of the immune response (local vs. systemic) and its effective action. Here, we have showed an example on how immunization with different adjuvant treatments (ADJ1 vs. ADJ2) will affect the immune response. The action of ADJ2 was restricted to the local area of administration where it activates the immune response in the draining lymph nodes, whereas the action of ADJ1 with the same dose was more widespread, generating a CTL response both at the local and systemic level but with less potency than ADJ2 in the draining lymph nodes.

Critical steps for the successful evaluation of an adjuvant's CTL capacity are the choice of young OT-I animals (as a source of CD8 T cells) and the use of endotoxin-free OVA for immunization. Since OT-I mice are transgenic animals generated to produce CD8 T cells that recognize OVA peptide SIINFEKL in a MHC-I context, its artificial selection of the mouse TCR increases the appearances of hyper proliferative $CD8^+$ T cells[20] with age. An inflammation of the axillary lymph nodes is thus a more common feature of aged OT-I mice. Taking this into account, it is recommended to use young OT-I animals when possible (6-9-week-old animals) and to avoid isolating cells from any enlarged organs (either spleen of lymph nodes). The use of enlarged organs with proliferating CD8 T cells will affect the whole assay since most likely differences in proliferation between controls and treatments will not be obtained. A similar pitfall for the discrimination of adjuvant CTL generation capacity could be generated by using OVA protein with traces of endotoxin, which can elicit a more potent immune response as compared to endotoxin-free OVA[21,22] (see Materials and Reagents), since endotoxins are strong immuno-modulators *per se* [23]. Therefore, the OVA protein used for immunization must be endotoxin-free. The use of 20 or 50 µg of endotoxin-free OVA depends on the immunization route being 50 µg a suitable dose for subcutaneous testing of adjuvants. Additionally, the use of high concentrations of CFSE has been reported in the literature to kill the stained cells[24] and could also result in the failure of the assay.

Some modifications or improvements to different techniques used in the protocol were implemented in order to reduce the stress of the animals, to increase reproducibility of the experiments. For example, to minimize the heating of animals by just heating the back and the tail of the mice and not the whole animal, or to avoid subcutaneous injection related stress by anesthetizing the animals before vaccination. The anesthesia is not required by animal welfare regulations for a subcutaneous injection but in our experience, it improves reproducibility by decreasing variations introduced by the stress response.

A great limitation of this method is that since CFSE stains the membrane of cells, its high brightness usually impairs the detection of signals from the cytosol. Therefore, if the confirmation of CTL capacity is needed, the secretion of IFN-γ should be evaluated by a specific secretion assay [25], and not by intracellular cytokine staining.

The use of the measurement of proliferation to estimate the CTL generation capacity by adjuvants in just 4 days has the advantage of shorter time needed than traditional CTL assays[26] and it is a good prospective experiment in the case where further confirmation by other methods is needed.

Although restricted to OVA as an antigen, and therefore to the use of OT-I T cells, the method presented here allows the study of the properties of the activation induced by different adjuvants after sorting of the proliferated cells. One of the possible applications is the study of the metabolic signatures induced by different adjuvants in the proliferated OT-I T cells and its relationships with the development of memory[27]. Additional secondary screenings could evaluate the biological properties of the stimulated cells, such as the expression of cytokines or degranulation markers by flow cytometry or their cytotoxic capacity by performing in vivo CTL tests[8,26,28].Since this application has the limitation of its use in mice, extrapolations to humans can be performed by exploiting screenings based on humanized mice[29,30,31].

This method as we have presented here is robust enough to be used as a first screening for the selection of cellular response activators and also flexible enough to be modified or coupled to further analysis of the proliferated T cells. In summary, this rapid in vivo assessment of adjuvant CTL generation capacity is an ideal tool for vaccine laboratories in academia and industry that are interested in a fast characterization of the mode of action of their adjuvant candidate molecules.

Disclosures

The authors have nothing to disclose.

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