Published in final edited form as:

Methods Mol Biol. 2011 ; 707: 45–54. doi:10.1007/978-1-61737-979-6_4.

***In vitro* and *in vivo* Analyses of Regulatory T Cell Suppression of CD8⁺ T Cells**

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Abstract

The study of regulatory T cells (Treg) requires methods for both *in vivo* and *in vitro* analyses, both of which have different limitations, but which complement each other to give a more complete picture of physiological function. Our analyses have focused on Treg-mediated suppression of CD8⁺ T cells, and in particular Tregs induced by viral infection. One of the unique characteristics of virus-induced Tregs is that they can suppress *in vitro* without the requirement for additional stimulation. This ability correlates with their activated status *in vivo*. Furthermore, *in vivo* activated Tregs can suppress the function of CD8⁺ T cells both *in vitro* and *in vivo*, while leaving proliferation intact. Interestingly, further *in vitro* stimulation of these Tregs confers to them the ability to suppress both the function and proliferative ability of CD8⁺ T cell targets.

Keywords

regulatory T cells; CD8⁺ T cells

1. Introduction

The model system we use for the study of virus-induced Tregs is Friend virus (FV) infection of adult immunocompetent mice (6). FV is an oncogenic mouse retrovirus that induces acute infections leading to lethal leukemia in most strains of mice (5). However, some strains of mice recover from acute infection, but remain chronically infected for life (4). It is these chronically infected mice that have revealed a role for Tregs in suppressing CD8⁺ T cells (2).

Interestingly, depletion of CD8⁺ T cells during acute infection abolishes the ability of high recovery strains of mice to prevent leukemia (3), but depletion during the chronic phase has relatively little effect (4). This finding suggested that chronic FV had escaped CD8⁺ T cell control. Adoptive transfer studies then showed that chronically infected mice had defective mixed lymphocyte reactions *in vivo*, and also a decreased CD8⁺ T cell-mediated rejection of FV-induced tumors *in vivo* (7). These data suggested a change in T cell function rather than in the virus. Interestingly, further experiments showed that suppression of *in vivo* CD8⁺ T cell responses could be adoptively transferred to naïve mice with CD4⁺ T cells, but not

³Remember to not stain cells with FITC or other fluorochromes that are detected in the CFSE channel.

⁴For lower cell numbers use a 96 well round bottom plate to maximize cell-to-cell contact. Also centrifuge for 2 minutes at 500 rpm after the cultures are set up. Lower cell numbers in the well required a longer *in vitro* culture (72 hr) because the targets were slower to proliferate and upregulate effector molecules.

CD8⁺ T cells, from chronically infected mice. Analysis of the CD4⁺ T cells revealed that CD25⁺ T cells were significantly more activated in chronically infected mice than in naïve mice, the same subset of cells that Shimon Sakaguchi had shown to be involved in suppressing anti-self reactivity to prevent autoimmune diseases (10). These studies led to the development of in vivo and in vitro analysis techniques to further study the suppressive activity of virus-induced Tregs (2, 8, 9).

2. Materials

2.1 In vitro suppression assays

1. Complete Medium: IMDM (Lonza) with 25 mM Hepes, 10% heat inactivated (56°C for 30 min) FBS, 100 U/ml penicillin and streptomycin, 2 mM L-glutamine
2. Coating buffer: 0.05M NaCO₃ pH 9.6
3. 5 mM stock solution carboxyfluorescein succinimidyl ester (CFSE) (Molecular Probes)
4. Brefeldin A (Sigma): 10 µg/ml [final]
5. Buffer A for bead purification: 1x PBS, 0.5% BSA, 2 mM EDTA
6. Fixative for target cells: 1x PBS, 0.5% Paraformaldehyde (PFA) if fixing overnight or 1x PBS, 2% PFA if fixing for 30 minutes
7. Permeabilization for target cells: 1x PBS, 0.1% saponin, 0.1% NaN₃, 1% FBS
8. 96-well flat or round bottom tissue culture plates

Option 1: anti-CD3 coated, each well incubated with 1 µg anti-CD3 in 100 µl coating buffer overnight

Option 2: Peptide-loaded APC's (concentration is determined empirically depending on peptide and TCR, but we have used 4.5 µM peptide to load APC's). A gamma irradiator is required for this option.

Option 3: Use tetramers to stimulate target cells during assay (concentration must be determined empirically for individual tetramers, but we have used 2 µl of stock tetramer solution from Beckman Coulter for 3–4 × 10⁶ CD8⁺ T cells)

2.2 Cell harvest from the spleen

1. Phosphate buffered balanced salt solution (such as Dulbecco's)
2. RBC lysis buffer: 0.16 M NH₄CL, brought to pH 7.2 with drops of 1M K₂CO₃
3. Nylon 100 µm cell strainer (Fisher Scientific)

2.3 Treg cell harvest from the liver

1. Perfusion solution: 1x PBS and 75 U/ml heparin (Fisher Scientific)
2. Necessary perfusion equipment: 10 ml syringe, 23 ga. needle, tissue scissors and tweezers, anesthesia
3. Phosphate buffered balanced salt solution (such as Dulbecco's)
4. RBC lysis buffer: 0.16 M NH₄CL brought to pH 7.2 with drops of 1M K₂CO₃
5. Percoll stock (keep sterile): Percoll (Sigma), 8% 10x PBS

6. 35% Percoll solution: balanced salts solution, 35% Percoll stock, 6.5 μ M Hepes, 100 U/ml heparin
7. Nylon 100 μ m cell strainer (Fisher Scientific)

2.4 Treg cell harvest from the lung

1. Perfusion solution: 1x PBS and 75 U/ml heparin
2. Necessary perfusion equipment: 10 ml syringe, 23 ga. needle, tissue scissors and tweezers, anesthesia
3. Phosphate buffered balanced salt solution (such as Dulbecco's)
4. 1.3 mM EDTA solution: balanced salts solution with EDTA disodium salt, pH adjusted to 7.0–7.4
5. Balanced salts solution containing 5% heat inactivated FBS
6. Collagenase solution (make fresh): MEM (Invitrogen) containing 5% heat inactivated FBS, 1 μ M CaCl_2 , 1 μ M MgCl_2 and 150 U/ml collagenase (Gibco)
7. Percoll stock (keep sterile): Percoll (Amersham), 8% 10x PBS
8. 44% Percoll solution: MEM (Invitrogen), 44% Percoll stock
9. 67% Percoll solution: MEM (Invitrogen), 67% Percoll stock
10. Nylon 100 μ m cell strainer (Fisher Scientific)

2.5 Adoptive transfers and in vivo suppression assays

1. Phosphate buffered balanced salt solution containing 15 U/ml of heparin sodium (SoloPak Laboratories)
2. 3ml syringes with 23 ga. needles for i.v. injections
3. Nylon 100 μ m cell strainer (BD Bioscience)

3. Methods

3.1 Harvesting Tregs from the spleen

1. To harvest Tregs from the spleen tissue, remove the spleen and crush through a nylon 100 μ m cell strainer into a 50 ml conical tube using 30 ml of balanced salts solution.
2. Centrifuge for 5 min at 1000 rpm and decant supernatant.
3. Add 2 ml ammonium chloride and incubate 5 minutes to lyse RBCs. Add 30 ml balanced salts solution to wash.
4. Centrifuge for 5 min at 1000 rpm and decant supernatant.
5. Wash cell pellet with 30 ml balanced salts solution.
6. Centrifuge for 5 min at 1000 rpm and decant supernatant and continue on with the purified lymphocytes.

3.2 Harvesting Tregs from the liver

1. To harvest Tregs from the liver tissue, first perfuse the anesthetized mouse with PBS/heparin perfusion solution to displace blood from the tissue. Clip the right atrium and insert the 23 ga. needle of the perfusion apparatus into the left ventricle.

Slowly push 10 ml of perfusion solution through the heart. To further displace blood from the liver, push an additional 5 ml of perfusion solution through the liver via the portal vein at the base of the liver.

2. After removing the gall bladder from the liver, crush the liver through a nylon 100 μm cell strainer into a 50 ml conical tube using 30 ml of balanced salts solution.
3. Centrifuge for 10 minutes at 2000 rpm with no brake.
4. Aspirate the supernatant and resuspend the pellet in 15 ml of 35% Percoll by vortexing. Centrifuge for 10 minutes at 2000 rpm with no brake.
5. Without disrupting the cell pellet, carefully aspirate the top layer of hepatocytes and the supernatant liquid.
6. To ensure no residual hepatocytes contaminate the lymphocyte cell pellet, transfer the pellet into a fresh 15 ml tube.
7. Add 2 ml ammonium chloride and incubate 5 min to lyse residual RBCs. Wash with 30 ml balanced salts solution and continue on with the purified lymphocytes.

3.3 Harvesting Tregs from the lung

1. To harvest Tregs from lung tissue, first perfuse the anesthetized mouse with PBS/heparin perfusion solution. Clip the right atrium and insert the 23 ga. needle of the perfusion apparatus into the left ventricle. Slowly push 10 ml of perfusion solution through the heart.
2. Cut the lungs into small pieces with scissors and with a magnetized bar stir at 450 rpm for 30 minutes at 37°C in 40 ml of 1.3 mM EDTA in a 50 ml flask.
3. Transfer to a 50 ml conical tube, vortex, then centrifuge at 1500 rpm for 5 minutes at room temperature.
4. Carefully aspirate the supernatant from the lung pieces.
5. Wash twice with 40 ml balanced salts solution 5% FCS, using aspiration to remove the supernatant while avoiding lung pieces each time.
6. Transfer to a clean 50 ml flask with magnetized bar and stir for 1 hour at 550 rpm at 37°C in 30 ml collagenase solution.
7. Pour and crush through a nylon 100 μm cell strainer into a 50 ml conical tube. Rinse cell strainer using an additional 15 ml collagenase solution.
8. Centrifuge for 5 minutes at 1500 rpm at room temperature.
9. Wash with balanced salts solution/5% FCS and if large lung pieces remain, repeat crushing through a nylon 100 μm cell strainer into a clean 50 ml conical tube.
10. Centrifuge for 5 minutes at 1500 rpm at room temperature.
11. Suspend the cell pellet in 8 ml of 44% Percoll and then carefully pipet 5 ml of 67% Percoll under the cell suspension.
12. Centrifuge 20 minutes at 1500 rpm at room temperature with the brake off.
13. Carefully aspirate the top layer of Percoll above the visible lymphocyte layer (buffy coat). Next carefully collect the lymphocyte layer.
14. Transfer the lymphocytes into a clean 15 ml conical tube and wash once with 30 ml balanced salts solution and continue on with the purified lymphocytes.

3.4 In vitro suppression assays

Alternative materials are given in 2.1.8 that will be used depending on the type of assay to be performed. Typically both the target cells and the Tregs are stimulated with anti-CD3-coated plates (11, 12). In such co-cultures cell division of target cells is suppressed. In some situations, such as the study of virus-induced Tregs, it may be of interest to determine the suppressive capacity of the Tregs directly *ex vivo*, without further stimulation. In such cases rather than stimulating with anti-CD3, which would also activate the Tregs, the target cells may be stimulated with specific peptides, especially if TCR transgenic cells are used as targets.

1. To assay suppression of both proliferation and function, purify CD8⁺ or CD4⁺ splenocyte targets (depending on the cell type being studied) from naïve mice using MACS beads (Miltenyi MACS system) following the manufacturer's recommendations. Alternatively, TCR transgenic CD8⁺ cells may be used as targets if available.
2. Label the target cells with CFSE in culture media without FCS at a concentration of 5×10^7 cells/ml and a concentration of 5 μ M CFSE for 10 minutes at 37°C with gentle agitation every 2 minutes (*see* Note 1). Block CFSE binding by adding a saturating volume of ice-cold media containing 10% FCS and wash twice to get rid of excess CFSE.
3. Purification of the Treg population is more difficult since the most definitive marker, Foxp3, is intracellular. Tregs can be enriched using biotinylated anti-CD25 and then using streptavidin MACS beads following the manufacturers recommendations. This method yields high percentages of Foxp3⁺ Tregs, typically over 90%. Alternatively, Tregs can be obtained by FACS sorting on CD4 and CD25. First, stain lymphocytes with anti-CD4 and anti-CD25 and sort for double positive live cells on a FACS Aria by gating on the CD25^{hi} cells falling within the appropriate Forward Scatter/Side Scatter CD4⁺ population. Since most but not all CD4⁺ CD25^{hi} cells are Foxp3⁺ Tregs, stain with intracellular anti-Foxp3 (eBioscience) following the manufacturers recommendations to determine purity. If the mice are available, Tregs can also be sorted from Foxp3^{GFP} reporter mice (1) by sorting on CD4⁺ GFP⁺ double positive lymphocytes (*see* Note 2).
4. *Option 1:* Set up cultures in 200 μ l fresh complete IMDM in a 96 well flat bottom, anti-CD3-coated tissue culture plate. Use $1-4 \times 10^5$ cells of each type per well. Using fewer (10^4) cells generally gives greater variability in the assay. Set up cultures at a 1:1:1 ratio with target cells:Tregs:helper CD4⁺ T cells. CD4⁺ T helper cells are purified from a naïve mouse by anti-CD4 MACS beads following manufacturers recommendations.

Option 2: If the peptide to activate the target cell is available, target cells can be activated using peptide pulsed APC's rather than anti-CD3-coated plates. This allows the target cells to be activated without activating the Tregs. Use the negative fraction of anti-CD4 and anti-CD8 bead purified splenocytes from a naïve mouse as the APC's. Resuspend the APC's in complete IMDM media with 10% Normal Mouse Serum. Add the peptide of interest at a concentration predetermined to

¹CFSE concentrations between 2 and 10 μ M can be used to adjust the brightness of the labeled cells. Cells used for *in vivo* transfers will often lose a significant amount of label *in vivo* so concentrations at the higher end should be used.

²Using CD25 expression to purify Tregs has disadvantages because even in the spleen there are CD25^{lo} Foxp3⁺ Tregs that will not be acquired using MACS beads or cell sorting using CD25 as the marker. This is especially problematic when purifying Tregs from non-lymphoid tissues, like the liver and gut, where the majority are CD25^{lo} and cannot be purified by these processes. The use of Foxp3-GFP reporter mice is necessary when obtaining CD25^{lo} Tregs from a non-lymphoid tissue or to get the total Treg population from the spleen. In this way, you can stain with anti-CD4 and by FACS cell sorting obtain >95% pure CD4⁺GFP⁺ Tregs.

activate the cells of interest and mix well by gentle agitation. Incubate at 37°C for 30–60 minutes and then irradiate with 3000 rad. Wash APC's twice using media. Use the APC's at a 1:1 ratio with the target cells. Otherwise, cultures are set up as in option 1.

Option 3: In cases where it is desirable to use target cells activated *in vivo*, such as from infected mice, harvest cells as described above. We have used activated CD8⁺ T cells harvested 4 days post-adoptive transfer into acutely infected mice, but the activation status of target cells should be determined empirically for each system. Target cells activated *in vivo* by infection can be kept stimulated *in vitro* by addition of tetramers to co-cultures with Tregs in plain plates rather than using CD3-coated plates (9). Otherwise, cultures are set up as in option 1 (*see note 7*).

5. Analyze the cultures by flow cytometry and collect the supernatants for ELISA (eg. assay for IFN γ) after 48–60 hours *in vitro*. The target cells can be surface stained for anti-CD8 or anti-CD4 and analyzed for CFSE dilution (proliferation) and intracellular IFN γ and granzyme B following a 30 min fix at 4°C in PBS 2% paraformaldehyde (PFA) or an overnight fix in PBS 0.5% PFA. Permeabilize the cells in a 0.1% saponin-PBS containing 0.1% sodium azide and 0.5% BSA. If intracellular IFN γ will be tested, add Brefeldin A at 10 μ g/ml for the last 5 h of culture.

3.5 *In vivo* suppression assays using adoptive transfers

Suppression of target cells *in vivo* may be followed using adoptive transfer of labeled cells (*see note 5*). For example, in Friend virus infections there is a burst of activated Tregs at 2 weeks post-infection (13). By adoptively transferring labeled CD8⁺ T cells into infected mice around the time of this burst, the effects of suppression on the transferred cells may be observed (2). In addition, adoptive transfer of Tregs from infected mice into naïve mice can be done to monitor their effects in, for example, a naïve mouse (7). We also use this technique to activate CD8⁺ T cells during acute infections before Treg activity begins. These physiologically activated cells can then be recovered for use in *in vitro* suppression assays (9).

- 1 Obtain the desired transfer subpopulations as described in 3.1 and suspend them at a concentration of no greater than 10⁸/ml (*see note 6*) in phosphate buffered balanced salt solution containing 15U/ml of heparin.
- 2 Label the cells with CFSE as in 3.1.2 if they are to be followed for cell division (*see note 1*).
- 2 Bring the cells to room temperature and filter through a 100 μ m cell strainer or nylon mesh to remove clumps. At this point is usually desirable to check the purity of the cells by flow cytometry. Inject the cells slowly via the intravenous route in a volume of 0.5 ml. (*see note 8*).

⁷CD8⁺ T cell function typically stops following harvest and *in vitro* culture unless the cells are kept stimulated so some type of stimulation is usually required.

⁵Donor cells can be followed by genetic markers such as Thy1, CD45, or expression of GFP. However, donor cells expressing GFP may be rejected as foreign in experiments lasting more than a week. CFSE-labeled cells can also be used but will lose signal following cells division. They can be followed for at least one month if they do not divide (*see note 1*).

⁶The cell concentration will vary depending on numerous variables such as whether the cells will divide, where they will home, how long they will be left in the animal, etc. We have had success with adoptive transfers of as few as 50 cells to as many as 5 $\times$ 10⁷. It should be noted that using high numbers of cells may give results not reflective of the true *in vivo* situation.

Acknowledgments

This research was supported by the Division of Intramural Research of the National Institutes of Health, National Institute of Allergy and Infectious Diseases.

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⁸Using a volume of 0.5 ml will help assure that the needle is in a vein and not in tissue. If the needle is in a vein the suspension should flow with very little pressure and should not distend the surrounding tissue. Better results may be obtained using the forefinger rather than the thumb on the plunger of the syringe. The retro-orbital sinus is a convenient site to do intravenous inoculations. Inclusion of heparin sodium in the injection solution is key for the prevention of clotting and pulmonary embolisms that will rapidly kill the recipient mice.