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Project Page Link: https://review.jove.com/files_upload.php?src=21736788

Title: In Vitro Functional Analysis of Regulatory T Cells: Focus on Proliferation and Differentiation

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Author Questionnaire

- 1. Microscopy:** Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or something similar? **No**
- 2. Software:** Does the part of your protocol being filmed include step-by-step descriptions of software usage? **Yes**
- 3. Filming location:** Will the filming need to take place in multiple locations? **No**
- 4. Testimonials (optional):** Would you be open to filming two short testimonial statements **live during your JoVE shoot**? These will **not appear in your JoVE video** but may be used in JoVE's promotional materials. **No**

Current Protocol Length

Number of Steps: 26

Number of Shots: 60 (8 SCs)

Interviews

Videographer: Obtain headshots for all authors available at the filming location.

INTRODUCTION:

~~REQUIRED: Summarize your research focus and the main questions it aims to answer.~~

- 1.1. **Dunfang Zhang:** Our lab studies T cell subset differentiation, metabolism, and interactions. Treg-effector T cell crosstalk maintains immune homeostasis, making Treg research crucial for treating autoimmune and inflammatory diseases
 - 1.1.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 3.3.1.*

~~Describe the research gap or limitation in existing methods and how this protocol addresses them.~~

- 1.2. **Dunfang Zhanag:** Traditional in vitro Treg suppression assays overlook Treg-mediated effects on effector T cell differentiation; this modified protocol evaluates both proliferation and polarization simultaneously.
 - 1.2.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera.

CONCLUSION:

~~What does this protocol allow researchers to study or measure?~~

- 1.3. **Dunfang Zhang:** In vitro functional analysis of Treg cells enables investigation of bidirectional effects between Treg and effector T cells on proliferation and polarization.
 - 1.3.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 4.10.4*

~~Describe the most important consideration or challenge when performing this protocol.~~

- 1.4. **Dunfang Zhang:** The greatest challenge while performing this protocol is how to maintain the cell viability while ensuring the purity of Treg cells and naive T cells.

- 1.4.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera.

~~Are there any additional methods or analyses that can be performed following this procedure?~~

- 1.5. **Dunfang Zhang:** Following this procedure, ELISA and other biochemical strategies can assess cytokine and metabolic factor production, while FACS-sorted cells enable downstream RNA-seq and proteomics analyses.

- 1.5.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera.

Videographer: Obtain headshots for all authors available at the filming location.

Ethics Title Card

This research has been approved by the Animal Ethics Committee at West China Hospital,
Sichuan University

Protocol

***Videographer:** Please always record an audio slate while filming each shot mentioned in the shotlist.*

2. Labeling of Naïve CD4⁺ T Cells with Cell Proliferation Dye

Demonstrator: Hao Cheng

- 2.1. To begin, prepare naïve T cells, regulatory T or Treg (*T-reg*)-enriched cells, and antigen-presenting cell-enriched non-CD4 positive cells [1]
 - 2.1.1. Establishing shot of the talent with the labeled vials of the prepared cell placed in front of him. ***Videographer:** Please obtain CU shot as well for this shot.*
- 2.2. Centrifuge the naïve CD4 positive T cell suspension at 350 g for 7 minutes at 4 degrees Celsius [1] and discard the supernatant to obtain the cell pellet [2].
 - 2.2.1. Talent placing the tube containing the naïve CD4 positive T cell suspension into the centrifuge.
 - 2.2.2. Talent discards the supernatant.
- 2.3. Dilute the Cell Proliferation Dye eFluor 450 in PBS to obtain a concentration of 20 micromolar at room temperature [1].
 - 2.3.1. Talent adding the Cell Proliferation Dye eFluor 450 solution in PBS.
- 2.4. Add 0.5 milliliters of PBS per 1×10^7 cells to the tube [1]. Keep the tube tilted at approximately 45 degrees and gently resuspend the cells by pipetting up and down 50 times [2].
 - 2.4.1. Talent adding PBS to the tube containing the cell pellet.
 - 2.4.2. Talent tilting the tube at approximately 45 degrees and pipetting the cells up and down repeatedly.
- 2.5. Next, add an equal volume of the cell proliferation dye solution quickly into the cell suspension to obtain a final concentration of 10 micromolar [1]. Hold the tube at a 45-degree angle and pipette the cells to thoroughly mix them with the cell proliferation dye [2].
 - 2.5.1. Talent adding the cell proliferation dye solution into the cell suspension.
 - 2.5.2. Talent tilting the tube and pipetting the cell suspension up and down repeatedly.
- 2.6. Immediately wrap the centrifuge tube with aluminum foil to avoid light exposure, incubate at room temperature for 20 minutes [1], while gently vortexing them every 5 minutes to prevent cell aggregation [2].
 - 2.6.1. Talent wrapping the centrifuge tube with aluminum foil and keeping it aside.

- 2.6.2. Talent gently vortexing the tube.
- 2.7. Next, add 10 milliliters of precooled cell culture medium to the centrifuge tube and gently vortex the cells to terminate the staining [1]. Then, centrifuge the cells and discard the supernatant [2].
 - 2.7.1. Talent adding precooled cell culture medium to the centrifuge tube and vortexing it.
 - 2.7.2. Talent placing the centrifuge tube into the centrifuge.
- 2.8. To wash the cells, add 5 milliliters of precooled cell culture medium to the cell pellet [1]. Repeat the centrifugation and resuspend the pellet in 5 milliliters of culture medium [2].
 - 2.8.1. Talent adding precooled cell culture medium to the cell pellet.
 - 2.8.2. Talent shows the pellet, add 5 ml medium, and mix by pipetting.
- 2.9. Following cell counting, centrifuge the cells [1] and resuspend in an appropriate volume of culture medium to achieve a cell concentration of 2×10^6 cells per milliliter [2]. Keep the tube on ice [3].
 - 2.9.1. Talent places the cells in the centrifuge.
 - 2.9.2. Talent shows the pellet and resuspend the cells in culture medium.
 - 2.9.3. Talent placing the centrifuge tube on ice.

3. Setting up the In Vitro Functional Analysis of Treg Cells

- 3.1. Calculate the number of samples required for the cell culture [1]. Prepare an appropriate amount of cell culture medium containing 2.5 micrograms per milliliter anti-mouse CD3 antibody or the suitable induction cytokines [2]. Take prepared, naïve T cells, and Treg-enriched cells [3] and vortex them [4].
 - 3.1.1. Talent calculating the number of samples required for the cell culture.
 - 3.1.2. Talent adding CD3 antibody and cytokines to the medium.
 - 3.1.3. TEXT ON A PLAIN BACKGROUND
 - CD45.2⁺CD4⁺CD62L⁺ naïve T cells
 - CD45.1⁺CD4⁺CD25⁺ Treg-enriched cells
 - APC-enriched non-CD4⁺ cells
 - 3.1.4. Talent gently vortexing the tube.
- 3.2. Add 100 microliters of CD45.2-positive CD4-positive naïve T cells and 100 microliters of CD45.1-positive APC-enriched non-CD4-positive cells to the 48-well plate [1].

- 3.2.1. Talent adding CD45.1 positive antigen-presenting cell-enriched non-CD4 positive cells to the 48-well plate.
- 3.3. Transfer 100 microliters of CD45.1-positive CD4-positive CD25-positive Treg-enriched cells into the experimental wells [1], and 100 microliters of cell culture medium into the control wells [2].
 - 3.3.1. Talent adding CD45.1 positive CD4 positive CD25 positive regulatory T cell-enriched cells to the experimental wells.
 - 3.3.2. Talent adding cell culture medium to the control wells.
- 3.4. Then, pipette 100 microliters of culture medium containing anti-mouse CD3 antibody and 100 microliters of culture medium containing the suitable induction cytokines into each well [1].
 - 3.4.1. Talent adding the anti-mouse CD3 antibody-containing cell culture medium to the wells of the 48-well plate.
- 3.5. Add PBS to the remaining empty wells to maintain the humidity of the plate and reduce edge effects [1]. Incubate the 48-well plate in a 5% carbon dioxide incubator at 37 degrees Celsius for 3 days [2].
 - 3.5.1. Talent adding PBS to the empty wells of the 48-well plate.
 - 3.5.2. Talent placing the 48-well plate into the incubator.

4. In Vitro Functional Analysis of Treg Cells

- 4.1. Gently pipette the cultured cells to resuspend them and transfer the cell suspension to a 1.5-milliliter centrifuge tube [1]. Centrifuge the cells [2] and remove the supernatant [3].
 - 4.1.1. Talent pipetting the cultured cells to resuspend them and transferring the suspension to a 1.5 milliliter centrifuge tube.
 - 4.1.2. Talent placing the centrifuge tube into the centrifuge.
 - 4.1.3. Talent removing supernatant.
- 4.2. Add 500 microliters of cell culture medium containing phorbol 12-myristate 13-acetate, ionomycin, and GolgiPlug Protein Transport Inhibitor to each tube [1]. Gently vortex to resuspend the cells [2] and incubate the cells in a 5% carbon dioxide incubator at 37 degrees Celsius for 3 hours [3].
 - 4.2.1. Talent adding the prepared cell culture medium to the centrifuge tube.
And
TEXT ON A PLAIN BACKGROUND
5 ng/mL PMA

1 µg/mL ionomycin

1:1,000 diluted GolgiPlug Protein Transport Inhibitor *Video editor: Please use split screen to show this*

- 4.2.2. Talent gently vortexing the centrifuge tube.
- 4.2.3. Talent placing the centrifuge tube into the incubator.
- 4.3. Following centrifugation, add 50 microliters of PBS containing one test of Zombie Yellow dye and 1 to 100 diluted anti-mouse CD16 or 32 antibodies to the pellet [1]. Vortex the samples [2] and incubate at room temperature in the dark for 15 minutes to label dead cells and block Fc (F-C) receptors [3].
 - 4.3.1. Talent adding the Zombie Yellow dye and anti-mouse CD16/32 antibody solution to the sample tube.
 - 4.3.2. Talent vortexing the sample tube.
 - 4.3.3. Talent placing the sample tube in the dark for incubation.
- 4.4. Centrifuge the cells before discarding the supernatant [1]. Add 50 microliters of surface antibody cocktail to the sample, vortex it [2], and incubate at 4 degrees Celsius in the dark for 20 minutes [3].
 - 4.4.1. Talent placing the centrifuge tube into the centrifuge.
 - 4.4.2. Talent adds the surface antibody cocktail to the sample tube and vortexes it.
 - 4.4.3. Talent placing the sample tube at 4 degrees Celsius in the dark for incubation.
- 4.5. Then, add 1 milliliter of fluorescence-activated cell sorting or FACS (Facs) buffer to each centrifuge tube [1].
 - 4.5.1. Talent adding fluorescence-activated cell sorting buffer to the centrifuge tube.
- 4.6. After centrifugation and supernatant removal, add 300 microliters of Fixation and Permeabilization Solution to each centrifuge tube and vortex the samples [1]. Incubate the samples at 4 degrees Celsius in the dark for 20 minutes [2].
 - 4.6.1. Talent adding Fixation and Permeabilization Solution to the centrifuge tube and vortexing it.
 - 4.6.2. Talent placing the centrifuge tube at 4 degrees Celsius in the dark for incubation.
- 4.7. Transfer 1 milliliter of Permeabilization and Wash Buffer to each centrifuge tube [1], followed by centrifugation and supernatant removal [2].
 - 4.7.1. Talent adding 1× Permeabilization and Wash Buffer to the centrifuge tube.
 - 4.7.2. Talent placing the centrifuge tube into the centrifuge.
- 4.8. After repeating the wash, add 50 microliters of cytokine antibody cocktail to each sample and vortex the samples [1]. Incubate the samples at 4 degrees Celsius in the

dark for 40 minutes [3].

4.8.1. Talent adding the cytokine antibody cocktail to the centrifuge tube and vortexes it

4.8.2. Talent placing the centrifuge tube at 4 degrees Celsius in the dark for incubation.

4.9. Wash each sample with 1 milliliter of Permeabilization and Wash Buffer [1]. Centrifuge the cells and remove the supernatant [2]. Resuspend the cell pellet in 300 microliters of FACS buffer [3].

4.9.1. Talent adding Permeabilization and Wash Buffer to the centrifuge tube.

4.9.2. Talent placing the centrifuge tube into the centrifuge.

4.9.3. Talent resuspending the cell pellet in fluorescence-activated cell sorting buffer.

4.10. Measure the proliferation and differentiation of responder T cells by flow cytometry [1]. Gate the lymphocyte population among the total cells to exclude cell debris and impurities [2]. Select the single-cell population among the lymphocytes based on scatter signals [3]. Then identify the live cell population among the single cells [4], followed by the CD4-positive T cell population among the live cells [5].

4.10.1. Talent loading the samples into the flow cytometer.

4.10.2. SCREEN: JoVE_SCREEN_71268_Step4.10.2-to-4.11.5.mp4: 00:06-00:12

4.10.3. SCREEN: JoVE_SCREEN_71268_Step4.10.2-to-4.11.5.mp4: 00:13-00:22

4.10.4. SCREEN: JoVE_SCREEN_71268_Step4.10.2-to-4.11.5.mp4: 00:23-00:28

4.11. Next, gate the CD45.1 positive regulatory T cell-enriched population and the CD45.2 positive effector T cell population among the CD4 positive T cells [1]. Then select the CD45.2 positive CD4 positive interferon gamma positive T helper 1 cells [2], CD45.2 positive CD4 positive interleukin-17A positive T helper 17 cells [3], and non-proliferated CD45.2 positive CD4 positive T cells among the CD45.2 positive T cells [4]. Perform flow cytometry data acquisition for all samples [5].

4.11.1. SCREEN: JoVE_SCREEN_71268_Step4.10.2-to-4.11.5.mp4: 00:39-00:51

4.11.2. SCREEN: JoVE_SCREEN_71268_Step4.10.2-to-4.11.5.mp4: 00:54-01:00

4.11.3. SCREEN: JoVE_SCREEN_71268_Step4.10.2-to-4.11.5.mp4: 01:02-01:09

4.11.4. SCREEN: JoVE_SCREEN_71268_Step4.10.2-to-4.11.5.mp4: 01:12-01:20

4.11.5. SCREEN: JoVE_SCREEN_71268_Step4.10.2-to-4.11.5.mp4: 01:36-01:45.

4.12. Finally, analyze the flow cytometry data with FlowJo (*Flow-Jo*) software [1], using the same gating strategy as in flow cytometry data acquisition [2].

4.12.1. Talent operating the FlowJo software to show the same gating strategy with flow cytometry data acquisition.

4.12.2. LAB MEDIA: Figure 3. *Video editor: Only display the top two rows of cytometry plots, and do not show the third row of cytometry plots in Figure 3.*

Results

5. Results

- 5.1. The frequency of non-proliferated responder T cells was significantly increased under both T helper 1 [1] and T helper 17 induction conditions after co-culture with regulatory T cell-enriched cells [2].
 - 5.1.1. LAB MEDIA: Figure 4A,B. *Video editor: Highlight the plot on the right hand side and the bar Tresp plus Treg in Figure 4A.*
 - 5.1.2. LAB MEDIA: Figure 4A,B. *Video editor: Highlight the plot on the right hand side and the bar Tresp plus Treg in Figure 4B.*
- 5.2. The differentiation of CD45.2-positive CD4-positive interferon gamma-positive T helper 1 cells was significantly suppressed after co-culture with regulatory T cell-enriched cells [1].
 - 5.2.1. LAB MEDIA: Figure 4A. *Video editor: Highlight the cytometry plot on the right side and bars labelled Tresp and Tresp plus Treg*
- 5.3. The frequency of CD45.2-positive CD4-positive interleukin 17A-positive T helper 17 cells increased when responder T cells were cultured with regulatory T cell-enriched cells [1].
 - 5.3.1. LAB MEDIA: Figure 4B. *Video editor: Highlight the cytometry plot on the right side the bars labeled Tresp and Tresp plus Treg*

1. Naïve
Pronunciation link: <https://www.merriam-webster.com/dictionary/naive>
IPA: /naɪˈiːv/
Phonetic Spelling: ny·eev
2. Regulatory
Pronunciation link: <https://www.merriam-webster.com/dictionary/regulatory>
IPA: /ˈrɛɡjələˌtɔːri/
Phonetic Spelling: reg·yuh·luh·tor·ee
3. Treg
Pronunciation link: <https://www.howtopronounce.com/treg>
IPA: /ˈtiːrɛɡ/
Phonetic Spelling: tee·reg
4. Antigen-presenting
Pronunciation link: <https://www.howtopronounce.com/antigen>
IPA: /ˈæntɪdʒən priˈzɛntɪŋ/
Phonetic Spelling: an·tuh·juhn prih·zen·ting

5. Centrifuge
Pronunciation link: <https://www.merriam-webster.com/dictionary/centrifuge>
IPA: /'sentrəˌfjuːdʒ/
Phonetic Spelling: sen·truh·fyooj
6. Supernatant
Pronunciation link: <https://www.howtopronounce.com/supernatant>
IPA: /'suːpərˌneɪtənt/
Phonetic Spelling: soo·pur·nay·tuhnt
7. Pellet
Pronunciation link: <https://www.merriam-webster.com/dictionary/pellet>
IPA: /'pelət/
Phonetic Spelling: pel·uht
8. eFluor
Pronunciation link: <https://www.howtopronounce.com/efluor>
IPA: /iː'flɔr/
Phonetic Spelling: ee·floor
9. Micromolar
Pronunciation link: <https://www.howtopronounce.com/micromolar>
IPA: /ˌmaɪkroʊ'moʊləɹ/
Phonetic Spelling: my·kroh·moh·lur
10. Resuspend
Pronunciation link: <https://www.howtopronounce.com/resuspend>
IPA: /ˌriːsə'spend/
Phonetic Spelling: ree·suh·spend
11. Vortexing
Pronunciation link: <https://www.merriam-webster.com/dictionary/vortex>
IPA: /'vɔːrteks/
Phonetic Spelling: vor·teks
12. Aggregation
Pronunciation link: <https://www.merriam-webster.com/dictionary/aggregation>
IPA: /ˌægrɪ'geɪʃən/
Phonetic Spelling: ag·ruh·gay·shuhn
13. Cytokines
Pronunciation link: <https://www.merriam-webster.com/dictionary/cytokine>
IPA: /'sartəˌkaɪn/
Phonetic Spelling: sy·tuh·kyne
14. CD45.2
Pronunciation link: No confirmed link found
IPA: /ˌsiːˌdiː fɔːrti fɑːv pɔɪnt tuː/
Phonetic Spelling: see·dee forty-five point two
15. CD45.1
Pronunciation link: No confirmed link found
IPA: /ˌsiːˌdiː fɔːrti fɑːv pɔɪnt wʌn/
Phonetic Spelling: see·dee forty-five point one

16. CD62L
Pronunciation link: No confirmed link found
IPA: /,si:.di: sɪksti tu: ɛl/
Phonetic Spelling: see·dee sixty-two ell
17. APC
Pronunciation link: <https://www.howtopronounce.com/apc>
IPA: /,eɪ.pi:'si:/
Phonetic Spelling: ay·pee·see
18. Anti-mouse
Pronunciation link: <https://www.howtopronounce.com/anti-mouse>
IPA: /,ænti'maʊs/
Phonetic Spelling: an·tee·mouse
19. Humidity
Pronunciation link: <https://www.merriam-webster.com/dictionary/humidity>
IPA: /hju:'mɪdəti/
Phonetic Spelling: hyoo·mih·duh·tee
20. Phorbol
Pronunciation link: <https://www.howtopronounce.com/phorbol>
IPA: /'fɔ:rbɔ:l/
Phonetic Spelling: for·ball
21. Myristate
Pronunciation link: <https://www.howtopronounce.com/myristate>
IPA: /'mɪrɪstet/
Phonetic Spelling: mir·ih·stayt
22. Ionomycin
Pronunciation link: <https://www.howtopronounce.com/ionomycin>
IPA: /,aɪənou'maɪsɪn/
Phonetic Spelling: eye·uh·noh·my·sin
23. GolgiPlug
Pronunciation link: No confirmed link found
IPA: /'goʊldʒɪ plʌɡ/
Phonetic Spelling: gohl·jee plug
24. Fc Receptors
Pronunciation link: <https://www.howtopronounce.com/fc-receptor>
IPA: /,ɛf'si: rɪ'septərz/
Phonetic Spelling: ef·see ree·sep·terz
25. Fluorescence-activated Cell Sorting
Pronunciation link: <https://www.merriam-webster.com/dictionary/fluorescence>
IPA: /flʊ'resəns 'æktə'veɪtɪd sel 'sɔ:rtɪŋ/
Phonetic Spelling: floor·ess·uhns ak·tuh·vay·tid sell sor·ting
26. FACS
Pronunciation link: <https://www.howtopronounce.com/facs>
IPA: /fæks/
Phonetic Spelling: faks

27. Fixation
Pronunciation link: <https://www.merriam-webster.com/dictionary/fixation>
IPA: /fɪk'seɪʃən/
Phonetic Spelling: fik·say·shuhn
28. Permeabilization
Pronunciation link: <https://www.howtopronounce.com/permeabilization>
IPA: /pə'rmi:ə,bɪlə'zeɪʃən/
Phonetic Spelling: pur·mee·uh·bil·uh·zay·shuhn
29. Cytokine
Pronunciation link: <https://www.merriam-webster.com/dictionary/cytokine>
IPA: /'saɪtə,kam/
Phonetic Spelling: sy·tuh·kyne
30. Flow Cytometry
Pronunciation link: <https://www.merriam-webster.com/dictionary/cytometry>
IPA: /floʊ saɪ'tɑ:mətri/
Phonetic Spelling: floh sy·tom·uh·tree
31. Lymphocyte
Pronunciation link: <https://www.merriam-webster.com/dictionary/lymphocyte>
IPA: /'lɪmfə,sait/
Phonetic Spelling: lim·fuh·syte
32. Interferon
Pronunciation link: <https://www.merriam-webster.com/dictionary/interferon>
IPA: /,ɪntər'fɪrən/
Phonetic Spelling: in·tur·feer·on
33. Interleukin-17A
Pronunciation link: <https://www.howtopronounce.com/interleukin>
IPA: /,ɪntərlu:kɪn ,sevən'ti:n eɪ/
Phonetic Spelling: in·tur·loo·kin seventeen ay
34. Effector
Pronunciation link: <https://www.merriam-webster.com/dictionary/effector>
IPA: /ɪ'fektər/
Phonetic Spelling: ih·fek·tur
35. FlowJo
Pronunciation link: <https://www.howtopronounce.com/flowjo>
IPA: /floʊdʒoʊ/
Phonetic Spelling: floh·joh