Video Article

Phenotypic and Functional Analysis of Activated Regulatory T Cells Isolated from Chronic Lymphocytic Choriomeningitis Virus-infected Mice

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Abstract

Regulatory T (T_{reg}) cells, which express Foxp3 as a transcription factor, are subsets of $CD4^+$ T cells. T_{reg} cells play crucial roles in immune tolerance and homeostasis maintenance by regulating the immune response. The primary role of T_{reg} cells is to suppress the proliferation of effector T (T_{eff}) cells and the production of cytokines such as IFN-γ, TNF-α, and IL-2. It has been demonstrated that T_{reg} cells' ability to inhibit the function of T_{eff} cells is enhanced during persistent pathogen infection and cancer development. To clarify the function of T_{reg} cells under resting or inflamed conditions, a variety of *in vitro* suppression assays using mouse or human T_{reg} cells have been devised. The main aim of this study is to develop a method to compare the differences in phenotype and suppressive function between resting and activated T_{reg} cells. To isolate activated T_{reg} cells, mice were infected with lymphocytic choriomeningitis virus (LCMV) clone 13 (CL13), a chronic strain of LCMV. T_{reg} cells isolated from the spleen of LCMV CL13-infected mice exhibited both the activated phenotype and enhanced suppressive activity compared with resting T_{reg} cells isolated from naïve mice. Here, we describe the basic protocol for *ex vivo* phenotype analysis to distinguish activated T_{reg} cells from resting T_{reg} cells. Furthermore, we describe a protocol for the measurement of the suppressive activity of fully activated T_{reg} cells.

Video Link

The video component of this article can be found at https://www.jove.com/video/54138/

Introduction

Regulatory T (T_{reg}) cells express forkhead box P3 (Foxp3) as a transcription factor for their development and function[1]. Additionally, T_{reg} cells express various other molecules such as CD25[2], lymphocyte-activation gene 3 (LAG-3)[3], glucocorticoid-induced tumor necrosis factor receptor[4], and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4)[5] on their surface or intracellular region. During chronic infection with various kinds of pathogens such as viruses[6,7], bacteria[8,9], and parasites[10-12], or in the course of cancer development[13,14], T_{reg} cells become differentiated into activated cells, displaying enhanced suppressive function targeting effector $CD4^+$ and $CD8^+$ T cells. A number of papers have suggested that expanded and activated T_{reg} cells contribute to the impaired $CD8^+$ T cell response during friend retrovirus (FV) infection[15-17]. FV-induced T_{reg} cells inhibit IFN-γ or granzyme B expression and cytotoxic reactivity of $CD8^+$ T cells[15-17]. Moreover, in a herpes simplex virus infection model, it was reported that depletion of $CD4^+CD25^+$ T_{reg} cells resulted in expansion of virus-specific $CD8^+$ T cells and severe tissue damage by infiltration of immunopathogenic $CD4^+$ T cells[18-20].

Mice infected chronically with the clone 13 strain of lymphocytic choriomeningitis virus (LCMV CL13)[21-24] have been widely used to characterize the phenotype and function of effector T cells (T_{eff}) and T_{reg} cells during chronic virus infection. During persistent LCMV infection, virus-specific T_{eff} cells progressively lose their effector function and become exhausted T (T_{exh}) cells. On the other hand, T_{reg} cells reinforce their ability to suppress virus-specific T cell response[25]. The decrease in the functioning capacity of the T_{eff} cells can be explained by several factors such as upregulation of inhibitory receptors on T_{eff} cells, altered function of antigen-presenting cells, production of immunoregulatory cytokines, and increased frequency or enhanced function of T_{reg} cells[26]. Among the factors involved in T cell suppression, programmed cell death protein-1 (PD-1)-expressing T_{exh} cells and T_{reg} cells have been widely considered as the hallmarks of antigen persistence and suppressive environment. Recently, it was reported that blockade of the PD-1 pathway and ablation of T_{reg} cells lead to enhanced T cell function and decreased viral load during LCMV chronic infection[27]. Furthermore, T_{reg} cells are activated during chronic infection of mice with LCMV[23,25] and their suppressive function is strengthened[25]. PD-1 is highly expressed on T_{reg} cells as well as T_{exh} cells, and the level of PD-1 expressed by T_{reg} cells correlates with the strength of their suppressive function to inhibit T cell proliferation[25].

Here, we describe a method to compare the characteristics of activated T_{reg} cells isolated from mice infected with LCMV CL13 and resting T_{reg} cells isolated from naïve mice. Furthermore, we explain a series of processes to separate activated T_{reg} cells and examine their *ex vivo* phenotype, as well as measure their suppressive activity *in vitro*.

Protocol

In this study, mice were maintained in a specific pathogen-free facility of the Yonsei Laboratory Animal Research Center of Yonsei University. All animal experiments were conducted in accordance with the Korean Food and Drug Administration guidelines using protocols approved by the International Animal Care and Use Committee of the Yonsei Laboratory Animal Research Center at Yonsei University.

1. Preparation of Solutions

1. Prepare 2% RPMI media by diluting fetal bovine serum (FBS) to 2% and penicillin-streptomycin to 1% in RPMI
2. Prepare complete RPMI media. To RPMI media add 10% of FBS, 1% of penicillin-streptomycin, 1% of L-glutamine, and 50 µM 2-mercaptoethanol.
3. Prepare fluorescence activated cell sorting (FACS) buffer. To do so, supplement phosphate-buffered saline (PBS) with 2% of FBS.
4. Prepare T cell isolation buffer. To do so, supplement PBS with 2% of FBS and 2 mM ethylenediaminetetraacetic acid.

2. Isolation of Splenic Lymphocytes

1. Remove the spleens from naïve or LCMV CL13-infected mice as described previously[19] and place them in 60 mm x 15 mm Petri dishes containing 10 ml of 2% RPMI media.
   NOTE: In this study experiments, 5 to 6 week-old C57B1L/6J female mice received 2 x 10^6 plaque-forming units (p.f.u.) of LCMV CL13 through intravenous injection via the tail vein. Sacrifice the mice at day 16 post-infection (16 d p.i.)[25]. Analyze ge-matched naive mice were on the same day.
2. Place a cell strainer into a 50 ml tube and rinse it with 2 ml of 2% RPMI media. Place the spleen on the 70 µm cell strainer and grind using the plunger of a syringe. Rinse the cell strainer with 2 ml of 2% RPMI media and remove it from the 50 ml tube.
3. Fill up the tube with 2% RPMI media and centrifuge at 300 x g for 5 min at 4 °C. Discard the supernatant and resuspend the cell pellet in 1 ml of ACK lysing buffer. Incubate the samples at room temperature (RT) for 5 min.
   NOTE: The volume of ACK lysing buffer indicated above is for one spleen. Scale up the volume accordingly for pooled spleen samples.
4. Fill up the tube with 2% RPMI media and centrifuge at 300 x g for 10 min at 4 °C. Discard the supernatant and resuspend the cells at a density of 1 x 10^7 cells/ml in complete RPMI media.
   NOTE: For live cell counting, stain cells with trypan blue and count only live cells that are not stained using hemocytometer.

3. Phenotyping of Splenic Conventional T (T_{conv}) Cells and T_{reg} Cells

NOTE: Before T_{reg} cell isolation, examine the phenotype of splenic lymphocytes isolated from naïve or infected mice by staining the cells with various antibodies and analyzing them by flow cytometry.

1. Transfer 50 µl of the cells (5 x 10^5 cells) among total splenic lymphocytes into each well of a new u-bottom 96-well plate and add 150 µl of FACS buffer per well. Centrifuge at 300 x g for 2 min at 4 °C.
2. Discard the supernatant. Agitate the cell pellet and add 200 µl of FACS buffer per well. Centrifuge at 300 x g for 2 min at 4 °C (2 times).
3. Prepare the antibody cocktail for staining cell surface markers in 50 µl of FACS buffer per well with the following antibodies and reagents. Anti-CD4 violet dye, Anti-CD25 green dye, Anti-PD-1 violet dye, Anti-CD8 PerCP-Cy5.5 dye and cell viability detection reagent near-infrared (IR) fluorescent reactive dye.
   NOTE: To further analyze the phenotype of activated T_{reg} cells, anti-CD103[23,25] can be added for cell surface-marker staining.
4. Resuspend the cell pellet with 50 µl of antibody cocktail per well. Incubate for 20 min in the dark at 4 °C. Wash the cells twice with FACS buffer by centrifugation at 300 x g for 2 min at 4 °C.
5. After the final washing step, decant the supernatant and fix the cells for 20 min in the dark at 4 °C with 100 µl of fixation buffer prepared according to the manufacturer's protocol.
6. Wash the cells twice with the permeabilization wash buffer (prepared according to manufacturer's protocol) by centrifugation at 300 x g for 2 min at 4 °C.
7. Prepare the antibody solution for intracellular Foxp3 staining, and resuspend the cell pellet with 50 µl of Anti-Foxp3 antibody solution.
   NOTE: To further analyze the phenotypes of T_{conv} and T_{reg} cells, anti-CTLA can be added at this step.
8. Incubate for 20 min in the dark at 4 °C and repeat Step 3.6. After the final washing step, resuspend the cell pellet in 200 µl of FACS buffer and examine the phenotype of the cells by flow cytometer[25].
9. Gate the live cell population. To analyze the phenotypes of T_{conv} cells during LCMV CL13 infection, examine the frequency of $Foxp3^-PD\text{-}1^+$ cells among $CD4^+$ or $CD8^+$ T cells.
   NOTE: Based on experimental results, the percentage of $Foxp3^-PD\text{-}1^+$ is more than 50% in both $CD4^+$ and $CD8^+$ T cell populations at 16 d p.i. with LCMV CL13.
   1. To analyze the frequency and phenotypes of T_{reg} cells, examine the frequencies of $Foxp3^+$ or $Foxp3^+PD\text{-}1^+$ cells among $CD4^+$ T cells.
      NOTE: Based on experimental results, the percentage of $Foxp3^+$ or $Foxp3^+PD\text{-}1^+$ cells is more than 20% in $CD4^+$ T cell population, respectively.

4. Isolation of $CD4^+CD25^+$ T_{reg} Cells

NOTE: The volumes of all reagents indicated below are for a starting cell number of 1 x 10^7 total splenocytes.

1. Prepare the cells as described in section 2 using naive and LCMV chronically infected mice. Wash the cells by adding 10 ml of T cell isolation buffer. Centrifuge at 300 x g for 10 min at 4 °C. Discard the supernatant completely and resuspend the cell pellet in 40 µl of buffer.
2. For $CD4^+$ T cell enrichment using the magnetic cell separation system, add 10 µl of biotin-antibody cocktail and mix well. Incubate for 10 min at 4 °C.
3. Add 30 µl of buffer, 20 µl of anti-biotin micro-beads for labeling of non-$CD4^+$ T cells and 10 µl of CD25-PE antibody for fluorescent labeling of $CD25^+$ cells. Mix well and incubate for 15 min in the dark.
4. Add 2 ml of buffer and pass the cells through a 40 µm cell strainer into a new 50 ml tube to remove cell debris. Wash the cells by centrifugation at 300 x g for 10 min at 4 °C. Discard the supernatant completely and resuspend the cell pellet in 500 µl buffer at a density of up to 1.25 x 10^8 cells.
5. Apply the cells onto the column and collect the unlabeled cells that pass through the column. Wash the column by adding 2 ml of buffer and centrifuge at 300 x g for 10 min at 4 °C. Discard the supernatant completely and resuspend the isolated $CD4^+$ T cells in 90 µl of buffer.
6. For magnetic labeling of $CD25^+$ cells, add 10 µl of anti-PE microbeads, mix well, and incubate for 15 min in the dark at 4 °C.
7. Add 2 ml of buffer and wash the cells by centrifugation at 300 x g for 10 min at 4 °C. Discard the supernatant completely and resuspend the cell pellet in 500 µl of buffer at a density of up to 1 x 10^8 cells.
8. To enrich the $CD4^+CD25^+$ T_{reg} cells, apply the cells onto the column for positive selection, and wash the column by adding 2 ml of buffer. Repeat the wash three times.
9. When the column is empty after the final washing step, add 1 ml of buffer onto the column, and flush out the magnetically labeled $CD4^+CD25^+$ cells using the plunger.
10. Repeat steps 4.8-4.9 to enhance the purity of the isolated $CD4^+CD25^+$ T_{reg} cells. Wash the cells with FACS buffer by centrifugation at 300 x g for 10 min at 4 °C. Discard the supernatant and resuspend the isolated $CD4^+CD25^+$ T_{reg} cells at a concentration of 2 x 10^6 cells/ml in complete RPMI media.
11. To check the purity of the isolated-$CD4^+CD25^+$ T_{reg} cells, use 2 x 10^5 cells from the total isolated-$CD4^+CD25^+$ T_{reg} cells. Stain the cells with 50 µl of FACS buffer containing anti-CD4 FITC and cell viability detecting reagent near-IR fluorescent reactive dye.
    NOTE: CD25 was already labeled with PE during isolation.
12. Incubate for 20 min in the dark at 4 °C. Wash the cells with FACS buffer by centrifugation at 300 x g for 2 min at 4 °C. After washing, resuspend the cell pellet in 100 µl of FACS buffer and check the purity by flow cytometry.
13. Gate the live-cell population. To analyze the purity of T_{reg} cells, confirm the percentage of $CD4^+CD25^+$ cells.
    NOTE: Based on experimental results, the percentage of $CD4^+CD25^+$ cells among purified cells is over about 80%.

5. Isolation of $CD8^+$ T Cells and Labeling of $CD8^+$ T Cells

NOTE: The volumes of all reagents indicated below are for a starting cell number of 1 x 10^7 total splenocytes.

1. Prepare cells as described in section 2 using naïve mice. Wash the cells by adding 10 ml of T cell isolation buffer. Centrifuge at 300 x g for 10 min at 4 °C. Discard the supernatant completely, and resuspend the cell pellet in 40 µl of buffer.
2. For $CD8^+$ T cell isolation using the magnetic cell separation system, add 10 µl of biotin-antibody cocktail for non-$CD8^+$ T cell labeling and mix well. Incubate for 5 min at 4 °C.
3. Add 30 µl of buffer and 20 µl of anti-biotin microbeads. Mix well and incubate for 10 min at 4 °C.
4. Apply the cells onto the column and collect the unlabeled cells that pass through the column. Wash the column by adding 2 ml of buffer three times.
5. Centrifuge at 300 x g for 10 min at 4 °C. Discard the supernatant completely and resuspend the isolated $CD8^+$ T cells in 2 ml of buffer.
6. To check the purity of the isolated cells, prepare 50 µl of antibody solution in FACS buffer. Resuspend the 2 x 10^5 cells among isolated $CD8^+$ T cells in 50 µl of antibody solution.
    NOTE: To prepare antibody solution, add anti-CD8 FITC, and cell viability detection reagent (near-IR fluorescent reactive dye) into FACS buffer.
7. Incubate for 20 min in the dark at 4 °C. Wash the cells with FACS buffer by centrifugation at 300 x g for 2 min at 4 °C. After washing, resuspend the cell pellet in 100 µl of FACS buffer, and check the purity of the cells by flow cytometry.
8. Gate the live-cell population. To check the purity of $CD8^+$ T cells, confirm the percentage of $CD8^+$ T cells.
    NOTE: Based on experimental results, the percentage of $CD8^+$ cells among purified cells is over about 90%.
9. Add PBS to the isolated $CD8^+$ T cells. Centrifuge at 300 x g for 10 min at 4 °C. Discard the supernatant. Resuspend the isolated $CD8^+$ T cells at a concentration of 1 x 10^7 cells/ml in PBS.
10. To label the $CD8^+$ T cells for the *in vitro* suppression assay, dilute cell proliferation tracking violet dye in PBS to obtain a concentration of 5 µM at RT.
    NOTE: The approximate excitation and emission peaks of the cell proliferation tracking violet dye used in the study are 405 and 450 nm, respectively.
11. Mix well equal volumes of cell proliferation tracking violet dye (5 µM) and cell suspension (1 x 10^7 cells/ml of $CD8^+$ T cells) in a 15 ml tube, and incubate at 20 min at 37 °C. Vortex the tube every 10 min.
12. Fill up the tube with cold complete RPMI media, and leave the tube for 10 min at RT. Centrifuge at 300 x g for 10 min at RT. Discard the supernatant completely, and resuspend the cells at a concentration of 2 x 10^6 cells/ml with pre-warmed complete RPMI media. Incubate the cells for 15 min at RT.

6. Setting Up the *In Vitro* Suppression Assay Using $CD4^+CD25^+$ T_{reg} and $CD8^+$ T Cells

1. To prepare anti-CD3/CD28-coated beads, transfer the appropriate volume of magnetic beads to a 15 ml of tube (2.5 µl/1 x 10^5 cells). Add an equal volume of PBS and mix. Wash by centrifugation at 300 x g for 2 min at 4 °C and discard the supernatant. Dilute the magnetic beads in complete media (50 µl/well).
2. Aliquot 50 µl of $CD4^+CD25^+$ T_{reg} cells per well of u-bottom 96-well plate (1 x 10^5 cells/well). Add 50 µl of $CD8^+$ T cells as responder T (T_{resp}) cells per well (1 x 10^5 cells/well). Add 50 µl of diluted anti-CD3/CD28-coated beads into per well.

NOTE: In this step, label and set up control wells as follows: "unstimulated $CD8^+$ T cell only" with no anti-CD3/CD28-coated beads; "$CD8^+$ T cell only" with anti-CD3/CD28-coated beads; "$CD8^+$ T cell only" with anti-CD3/CD28-coated beads; "T_{reg} cell only" with anti-CD3/CD28-coated beads. T_{reg} cells can be diluted by complete media and co-cultured with T_{resp} cells in a different ratio of T_{resp} cells:T_{reg} cells (1:0.25-1:1).

3. Add 50 µl or appropriate volume of media into all wells to total volume of 200 µl. Cover the plate with foil and incubate in a CO_2 incubator at 37 °C for 72 hr.

7. Analysis of $CD8^+$ T Cell Proliferation & Cytokine Production from $CD8^+$ T Cells

1. For the cytokine production analysis, after 3 days of culture, separate the supernatant of each well into another plate and perform enzyme-linked immunosorbent assay (ELISA).
   NOTE: The supernatant may be aliquoted and stored at -70 °C. In this experiment, anti-mouse IFN-γ antibody-coated plate was used to detect IFN-γ production according to the manufacturer s protocol. To determine IFN-γ production of proliferating $CD8^+$ T cells on a single cell level, intracellular cytokine staining can be performed.
2. After separating the supernatant from each well, wash the plate containing the cells with FACS buffer and centrifuge at 300 x g for 2 min at 4 °C (3 times).
3. After washing, discard the supernatant. Resuspend the cell pellet with 50 µl of antibody cocktail for staining of proliferated $CD8^+$ T cells. Incubate for 20 min in the dark at 4 °C.
   NOTE: To prepare antibody cocktail, add anti-CD4 FITC, anti-CD8 PerCP-Cy5.5, and cell viability detection reagent (near-IR fluorescent reactive dye) into FACS buffer.
   NOTE: Antibodies against various markers such as CD44 or CD69 can be combined with other antibodies to confirm activation of $CD8^+$ T cells. Remember that $CD8^+$ T cells have already been labeled with cell proliferation tracking violet dye at Step 5.10.
4. Wash twice by centrifugation at 300 x g for 2 min at 4 °C. After the final washing step, discard the supernatant, and fix the cells for 20 min in the dark at 4 °C with 100 µl of fixation buffer.
5. Wash twice by centrifugation at 300 x g for 2 min at 4 °C. Resuspend the cells with 200 µl of FACS buffer and measure the proliferation of cell proliferation tracking violet dye-labeled $CD8^+$ T cells by flow cytometry.
   1. Gate the $CD8^+$ T cell population among the live cells. Measure the percentages of divided and undivided cells according to the dilution of the cell proliferation tracking violet dye and $CD8^+$ T cell dilution according to the following equation. % Inhibition = [(% of proliferated $CD8^+$ T cells in the absence of T_{reg} cells - % of proliferated $CD8^+$ T cells in the presence of T_{reg} cells)/(% of proliferated $CD8^+$ T cells in the absence of T_{reg} cells)] x 100. Further analyze the data by using the flow cytometry software[25].

Representative Results

We generated mice with persistent virus infection by injecting them with 2 x 10^6 p.f.u. of LCMV CL13 intravenously. To investigate the phenotypic changes in T_{reg} cells and T_{conv} cells during chronic virus infection, splenic lymphocytes obtained from naïve and infected mice were stained with various antibodies and analyzed by flow cytometry. At 16 d p.i., PD-1 was upregulated in both $Foxp3^-CD4^+$ T_{conv} (**Figure 1A**, upper panel) and $Foxp3^-CD8^+$ T_{conv} (**Figure 1B**, upper panel) cells. The frequency of $Foxp3^+CD4^+$ T_{reg} cells was two times higher in the LCMV CL13-infected mice than in the naïve mice (**Figure 1A**). In particular, most of the $Foxp3^+CD4^+$ T_{reg} cells displayed the activated phenotype, expressing high levels of PD-1 at this time point (**Figure 1A**).

For the *in vitro* suppression assay, a considerable number of $CD8^+$ T_{conv} cells and $CD4^+CD25^+$ T_{reg} cells were required. To obtain 1 x 10^7 cell proliferation tracking violet dye-labeled $CD8^+$ T cells, splenocytes from two spleens of naïve mice were pooled. To obtain 2 x 10^6 or 3 x 10^6 of $CD4^+CD25^+$ T_{reg} cells, at least three spleens from naive and LCMV CL13-infected mice, respectively, were pooled. $CD8^+$ T cells as T_{resp} cells were separated successfully without significant contamination with other immune cells (**Figure 2A**). T_{reg} cells could also be isolated, with a dominant population of $CD25^+CD4^+$ T cells with more than 80% purity (**Figure 2B**).

To compare the suppressive function of naïve and chronic T_{reg} cells, the isolated T_{reg} and T_{resp} cells were incubated together under stimulation with anti-CD3/CD28-coated beads at 37 °C for 3 days. % inhibition of $CD8^+$ T_{resp} cell proliferation was increased in a T_{reg} cell dese-dependent manner (**Figure 3A**). When $CD8^+$ T_{resp} cells were co-cultured with chronic T_{reg} cells in a ratio of 1:1, proliferation of $CD8^+$ T_{resp} cells were significantly inhibited (**Figure 3B**). IFN-γ production by $CD8^+$ T_{resp} cells were also significantly inhibited when $CD8^+$ T_{resp} cells were co-cultured with activated T_{reg} cells from chronically infected mice rather than with resting T_{reg} cells from naïve mice in a T_{reg} cell dose-dependent manner (**Figure 3C**).

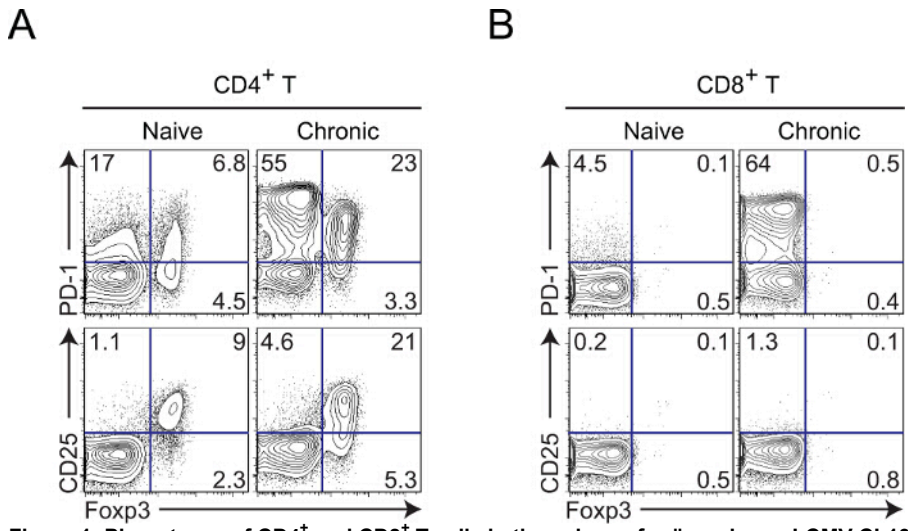

Figure 1: Phenotypes of CD4$^+$ and CD8$^+$ T cells in the spleen of naïve mice or LCMV CL13-infected mice at 16 d p.i. (**A**) Expression of PD-1 or CD25 on Foxp3$^-$CD4$^+$ T_{conv} and Foxp3$^+$CD4$^+$ T_{reg} cells. (**B**) Expression of PD-1 or CD25 on Foxp3$^-$CD8$^+$ T_{conv} cells. Splenic lymphocytes were stained with antibodies against CD4, CD8, CD25, PD-1, and Foxp3. Data are representative of three independent experiments. Panel A has been modified from[25] as a reference. Please click here to view a larger version of this figure.

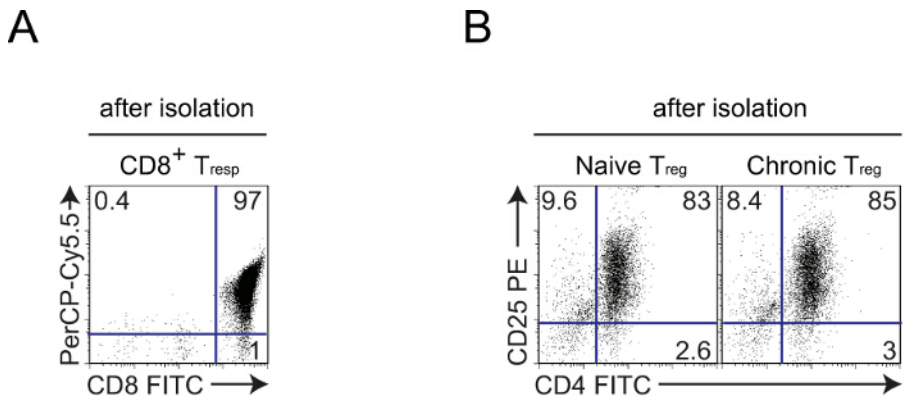

Figure 2: Purities of T_{resp} cells isolated from naïve mice and T_{reg} cells isolated from naïve or LCMV CL13-infected mice. (**A**) Percentage of CD8$^+$ T cells after isolation. (**B**) Percentage of CD25-expressing CD4$^+$ T_{reg} cells after isolation. Each quadrant in (**B**) was determined by the expression levels of CD4 and CD25. Data are representative of three independent experiments. Please click here to view a larger version of this figure.

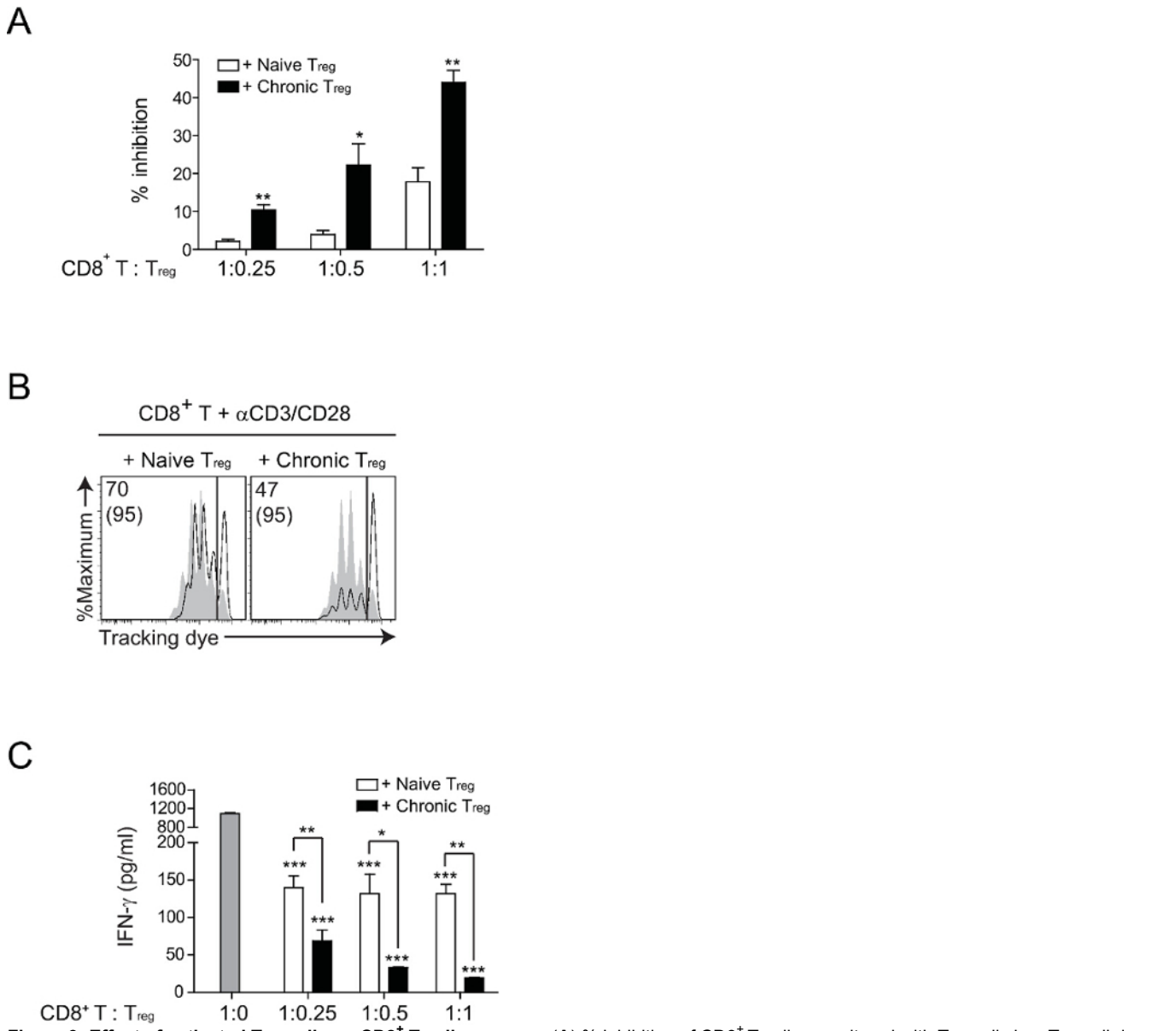

Figure 3: Effect of activated T_{reg} cells on $CD8^+$ T cell response. (**A**) % inhibition of $CD8^+$ T cells co-cultured with T_{reg} cells in a T_{reg} cell dose-dependent manner. (**B**) Proliferation of $CD8^+$ T cells co-cultured with T_{reg} cells in 1:1 ratio. (**C**) IFN-γ production of $CD8^+$ T cells co-cultured with T_{reg} cells in a T_{reg} cell dose-dependent manner. Proliferation of $CD8^+$ T cells was measured by dilution of cell proliferation tracking violet dye in proliferated $CD8^+$ T cells and IFN-γ secretion was evaluated by ELISA. Cell proliferation tracking violet dye-labeled $CD8^+$ T cells were stimulated with anti-CD3/CD28-coated beads for 3 days in the absence or presence of T_{reg} cells isolated from either naïve or chronically infected mice. In the histogram, the numbers without and with parenthesis indicate the proliferation percentage of $CD8^+$ T cells co-cultured with and without T_{reg} cells, respectively. Filled gray peaks in the histogram indicate proliferation of $CD8^+$ T cells co-cultured without T_{reg} cells. Each group was designed in triplicates. The bars represent mean + SEM. This figure has been modified from[25] as a reference. Please click here to view a larger version of this figure.

Discussion

Although only a small number of T_{reg} cells exist in mice and humans, it is important to understand their function as they play a crucial role in regulating the immune response and maintaining immune tolerance. The number and suppressive functions of T_{reg} cells increases during a chronic virus infection[15-20] as well as cancer progression[13,14]. This is probably due to continued antigen stimulation. To evaluate the T_{reg} cells function under antigen persistence and disease development, their suppressive activity needs to be measured.

Here, we describe a protocol to analyze the *ex vivo* phenotype of T_{reg} cells and measure their suppressive activity using an *in vitro* co-culture system of $CD8^+$ T cells and T_{reg} cells. Critical steps in the current protocol are analysis and isolation of T_{reg} cells. Live and freshly isolated T_{reg} cells show clear suppressive activity *in vitro*. We also examined the phenotypes of T_{conv} and T_{reg} cells during a chronic virus infection. T_{conv} cells, *i.e.*, $Foxp3^-CD4^+$ and $CD8^+$ T cells showed a decrease in cellular activity after the chronic virus infection (**Figure 1**). The PD-1 expression was upregulated in both the T_{conv} cell populations. Increase in T_{reg} cell number and up-regulation of PD-1 expression are the hallmarks of immune

response during a chronic virus infection. Additionally, anti-bodies for other inhibitory receptors such as TIM-3 and CTLA-4 can be used in combination with PD-1 to examine the T cell phenotype by FACS after choric virus infection.

In order to investigate the suppressive activity, T_{resp} cells and T_{reg} cells were co-cultured at a ratio of 1 to 1. Co-culture with T_{reg} cells from chronically infected mice significantly reduced $CD8^+$ T cell proliferation and IFN-γ secretion when compared to T_{reg} cells from naïve mice. This implies that the proliferation and cytokine production of $CD8^+$ T cells co-cultured with T_{reg} cells are inversely related to the suppressive function of T_{reg} cells. Although this protocol recommends the use of a magnetic cell separation system for T_{reg} cell isolation, contamination with non-T_{reg} cells cannot be ruled out. The T_{reg} cells obtained from Foxp3-GFP reporter mice[28-30] are better candidates to accurately investigate the function of T_{reg} cells than magnetic cell separation system, as CD25 is often overexpressed on activated $CD4^+$ T_{conv} as well as T_{reg} cells.

The suppressive function of T_{reg} cells has been evaluated by various *in vitro* suppression assays[16,28,31-34]. The advantage of the *in vitro* suppression assay is that it evaluates the direct effect of T_{reg} cells on the inhibition of the $CD8^+$ T cell response, because only T_{resp} cells are co-cultured with T_{reg} cells. In addition to $CD8^+$ T cells, the $CD25^-CD4^+$ T cells can be used instead of T_{resp} cells[25]. The effects of T_{reg} or T_{conv} cells on immune cells such as dendritic cells or natural killer cells can be evaluated by varying the experimental conditions.

Molecules such as the CD103[35], LAG-3[36], glycoprotein A repetitions predominant[37], CD43[38], CD11a[38], or killer cell lectin-like receptor subfamily G member 1[38] have been used as markers of activated T_{reg} cells in specific diseases. In this protocol, we used PD-1 as an indicator to identify activated T_{reg} cells during chronic virus infection. This protocol can be used for multifaceted analyses (phenotypic and functional) of T_{reg} cells under specific conditions.

Disclosures

S.-J.H. has a patent and receives patent royalties related to the PD-1 pathway. The other authors have no financial conflicts of interest.

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