Video Article

Generation of Induced Regulatory T Cells from Primary Human Naïve and Memory T Cells

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Abstract

The development and maintenance of immunosuppressive CD4⁺ regulatory T cells (Tregs) contribute to the peripheral tolerance needed to remain in immunologic homeostasis with the vast amount of self and commensal antigens in and on the human body. Perturbations in the balance between Tregs and inflammatory conventional T cells can result in immunopathology or cancer. Although therapeutic injection of Tregs has been shown to be efficacious in murine models of colitis¹, type I diabetes², rheumatoid arthritis and graft versus host disease,⁴ several fundamental differences in human versus mouse Treg biology⁵ has thus far precluded clinical use. The lack of sufficient number, purity, stability and homing specificity of therapeutic Tregs necessitated a dynamic platform of human Treg development on which to optimize conditions for their *ex vivo* expansion⁶.

Here we describe a method for the differentiation of induced Tregs (iTregs) from a single human peripheral blood donor which can be broken down into four stages: isolation of peripheral blood mononuclear cells, magnetic selection of CD4⁺ T cells, *in vitro* cell culture and fluorescence activated cell sorting (FACS) of T cell subsets. Since the Treg signature transcription factor forkhead box P3 (FoxP3) is an activation-induced transcription factor in humans⁷ and no other unique marker exists, a combinatorial panel of markers must be used to identify T cells with suppressor activity. After six days in culture, cells in our system can be demarcated into naïve T cells, memory T cells or iTregs based on their relative expression of CD25 and CD45RA. As memory and naïve T cells have different reported polarization requirements and plasticities⁸, pre-sorting of the initial T cell population into CD45RA⁺ and CD45RO⁺ subsets can be used to examine these discrepancies. Consistent with others, our CD25^{hi}CD45RA⁺ iTregs express high levels of FoxP3⁹, GITR and CTLA-4¹¹ and low levels of CD127¹². Following FACS of each population, resultant cells can be used in a suppressor assay which evaluates the relative ability to retard the proliferation of carboxyfluorescein succinimidyl ester (CFSE)-labeled autologous T cells.

Video Link

The video component of this article can be found at <http://www.jove.com/video/3738/>

Protocol

1. Isolation of Human Peripheral Blood Mononuclear Cells (PBMCs) from Buffy Coat

1. Acquire one unit of buffy coat from hospital or nearby blood center location. Our blood center provides us with about 40-60 mL of buffy coat per unit obtained from normal blood donors.
2. Pour blood into an autoclaved 500 mL glass bottle containing sterile PBS to dilute buffy coat. The final volume of PBS + buffy coat should be 250 mL.
3. Fill ten 50 mL conical tubes each with 20 mL Lymphoprep solution.
4. Gently overlay the Lymphoprep solution with 25 mL of the diluted buffy coat, being careful not to disturb the Lymphoprep solution.
5. Spin tubes for 30 minutes at room temperature at 500 x g. Make sure to turn off the centrifuge's brake, so as to not disturb the lymphocyte fraction.
6. Collect the PBMCs at the interface between the Lymphoprep and the plasma-medium layers with a pipette. Aspirate the plasma-medium off until about 1 mL still covers the buffy coat layer containing the PBMCs. Use a 10 mL pipette to transfer PBMCs to a new 50 mL tube.
7. Wash cells twice with PBS and then resuspend cells in 50 mL of cold RPMI for counting on a hemocytometer. Typical recovery is 8×10^8 - 1×10^9 PBMCs.

This procedure can be scaled down for smaller volumes of blood. Dilution of whole blood samples is 1:1 in PBS.

2. Magnetic Negative Selection of Total CD4⁺ T Cells, CD4⁺ CD45RA⁺ naïve T Cells or CD4⁺ CD45RO⁺ Memory T Cells from PBMCs using EasySep Enrichment Kits (Stem Cell Technologies)

Steps to follow for 1×10^8 to 4.25×10^8 PBMCs.

1. Resuspend PBMCs to a final concentration of 5×10^7 per mL in PBS containing 2% FBS and 1mM EDTA. Move cells to a fresh 14 mL polypropylene round bottom tube.
 - a. **Isolation of total human CD4⁺ T cells by negative selection:** add human CD4⁺ T Cell Enrichment Cocktail of antibodies (50 μ L per mL of PBMCs), mix and incubate for 10 minutes at room temperature.
 - b. **Isolation of human naïve T cells by negative selection:** add 50 μ L of anti-CD45RO antibody per mL of PBMC cell suspension, mix and incubate for 15 minutes at room temperature. Add enrichment cocktail provided with the kit (50 μ L of per mL of PBMCs), mix and incubate at room temperature for 10 minutes.
 - c. **Isolation of human memory T cells by negative selection:** add human Memory CD4⁺ T Cell Enrichment Cocktail of antibodies (50 μ L per mL of PBMCs), mix and incubate for 10 minutes at room temperature.
2. Mix magnetic particles well to equally distribute them throughout the solution. Do not vortex nanoparticles from naïve kit.
3. Add the magnetic particles (100 μ L per mL of PBMCs for total CD4⁺ and naïve T cell selection, 50 μ L per mL of PBMCs for memory T cell selection). Mix by gently pipetting 2-3 times and incubate at room temperature for 10 minutes for naïve T cell selection or 5 minutes for memory or total CD4⁺ T cell selection.
4. Add PBS containing 2% FBS and 1 mM EDTA to bring the volume up to 10 mL per tube. Mix by gently pipetting 2-3 times before placing uncapped tube into silver EasySep magnet for 5, 10 or 2.5 minutes for total CD4⁺ T cells, naïve and memory subsets, respectively.
5. With tube still in EasySep magnet, pour liquid into new 50 mL tube to isolate cells of interest.
6. Repeat steps 2.5 and 2.6 for better recovery.

3. Cell Culture Conditions to Induce Regulatory T Cells

1. The day before steps 1 and 2, coat tissue culture plates by first diluting anti-CD3 antibody (OKT3 clone) to a concentration of 1 μ g/mL in sterile PBS. For a 6 well plate add 2 mL of PBS + anti-CD3 per well, for a 12 well plate add 1 mL or for a 24 well plate add 500 μ L per well. Keep the coated plate at 4 °C until use.
2. Prepare polarization medium by adding 10% heat-inactivated Fetal Bovine Serum (FBS), 100 U/mL penicillin, 100 μ g/mL streptomycin and 50 μ M β -mercaptoethanol to RPMI-1640 pre-supplemented with 2.06 mM Glutamax-I and 25 mM HEPES buffer. Next, add 2 ng/mL TGF- β and 5 ng/mL IL-2. Here, one may add ligands or inhibitors of interest to the media. Resuspend cells from step 2 at a concentration of 2×10^6 cells/mL of polarization medium.
3. Pre-warm plates at 37 °C and aspirate the PBS out of anti-CD3-coated wells before adding cells. Add 4 mL per well of cell suspension for a 6 well plate, 2 mL of cells for a 12 well plate or 1 mL of cells for a 24 well plate. Fill up empty wells with PBS to reduce evaporation of media. Incubate for 3 days at 37 °C / 5% CO₂.
4. On day 3 post plating, spin down plate in a centrifuge for 5 minutes at 500 x g. Without disturbing T cells on bottom of the plate, remove half of media and replace with fresh media. Alternatively, if the well becomes overcrowded with cells (concentration above 3×10^6 /mL), split the volume equally into another anti-CD3 coated plate and add fresh media back up to original volume. Incubate for two to three more days at 37 °C / 5% CO₂.

4. Fluorescence-Activated Cell Sorting (FACS) of Three Populations of T Cells

1. Prepare FACS washing buffer by adding 0.5% (w/v) bovine serum albumin (BSA) and 2 mM EDTA to sterile PBS. Place buffer at 4 °C to get cold.
2. Remove cells from cell culture well and rinse each well with 2 mL of PBS to ensure complete cell recovery. Place all cells in 50 mL polypropylene tubes and centrifuge at 500 x g for 10 minutes at 4 °C.
3. Count the cells on a hemocytometer to determine cell density.
4. Place $3-5 \times 10^5$ cells per tube in four separate 5 mL round bottom polystyrene tubes for compensation controls. Place remaining cells in 50 mL polypropylene tubes, no more than 35×10^6 cells per tube.
5. Spin down cells for 5 minutes at 4 °C and aspirate media. Resuspend cells to be sorted in 90 μ L of cold washing buffer per 1×10^6 cells. Resuspend in 100 μ L of cold washing buffer the compensation control tubes.
6. To the cells to be sorted, combine 2 μ L of anti-CD25-PE, 3 μ L of anti-CD45RA-PE-Cy5 and 1 μ L of anti-CD127-APC per 1×10^6 cells. Add no antibody to the first compensation control tube, 2 μ L of anti-CD25-PE to the second tube, 3 μ L of anti-CD45RA-PE-Cy5 to the third tube and 1 μ L of anti-CD127-APC to the fourth tube. Incubate all tubes on ice for 45 minutes in the dark.
7. Add 10 mL of cold washing buffer to the cells to be sorted and 1 mL to the compensation control tubes. Centrifuge all cells at 500 x g for 5 minutes at 4 °C.
8. Aspirate buffer and resuspend cells at a concentration of 1×10^7 cells per mL of washing buffer. Add 1.5 μ L of DNase II per mL of cells before filtering through a 40 μ M nylon cell strainer. Move cells to multiple 5 mL round bottom polypropylene tubes with no more than 3.5 mL per tube. Resuspend compensation control cells in 300 μ L of washing buffer.
9. Set compensation on MoFlo flow cytometer to minimize cross detection by the PE, APC and PE-Cy5 filters. Set gates to sort iTregs (CD25^{Hi} CD127^{LOW}CD45RA⁻ cells), naïve (CD25⁻CD45RA⁺) and memory (CD25⁺CD45RA⁺) T cells into 5 mL round bottom polystyrene tubes containing 1 mL newborn calf serum.

5. Suppression Assay

1. Make suppression assay media by adding 100 U/mL of penicillin, 100 μ g/mL of streptomycin, 5 ng/mL of IL-2 and 2 ng/mL of TGF- β to AIM-V.
2. The day of the assay, purify heterologous CD4⁺ T cells from buffy coat as indicated in steps 1 and 2. These will be the *target cells* for the suppression assay and does not contain CD25⁺ Treg cells, as can be seen in **Figure 2**, Day 0.
3. Label cells with CellTrace kit as per manufacturer's instructions, except using only 1 μ L of 5 mM stock solution per mL of cells instead of 2 μ L. Keeping out from direct light, add 18 μ L of the DMSO supplied by the CellTrace kit to one vial of CFSE to make a 5 mM stock solution. Resuspend the required number of target cells (to a maximum of 1×10^7) in prewarmed PBS + 0.1% (w/v) BSA to a final concentration of 1×10^6 cells/mL. Add 1 μ L of 5 mM CFSE per mL of cells and incubate in a 37 °C water bath for 5 minutes. Add 5 volumes of complete, ice cold RPMI with 10% FBS to quench staining and incubate on ice for 5 minutes. Wash cells twice more with cold complete RPMI and resuspend 1×10^5 cells per 100 μ L of suppression assay media.
4. *Treg suppression inspector beads* are at a stock concentration of 2×10^7 beads/mL. Pellet a number of beads equal the total number of cells per experiment by quick centrifugation in an eppendorf tube. Wash beads once with RPMI and re-pellet. After aspiration of RPMI, resuspend beads so that the appropriate amount of beads per well are in 8 μ L of suppression assay media.

- To a 96 well round bottom tissue culture plate, add CFSE-stained cells (1×10^5 cells/mL), inspector beads and polarized and sorted cells (1×10^5 cells/mL) in fresh suppression assay media to a desired target (CFSE stained):effector (sorted) ratio in a final volume of 200 μ L. All conditions are set in triplicates.
- Prepare the first of two control conditions by adding 100 μ L of CFSE stained cells, 8 μ L of inspector beads and 1×10^5 of fresh, unstained cells in 92 μ L suppressor assay medium per well. Prepare the second control with the same cellular components as above but without Treg inspector beads.
- Cover plate in aluminum foil and incubate at 37 °C / 5% CO₂ for five days.
- In the dark, collect cells from each well by pipetting and place in a 5 mL round bottom polystyrene tube. Centrifuge cells at 500 x g for 5 minutes at 4 °C, aspirate media, and resuspend in 300 μ L cold FACS washing buffer from step 4. Analyze the first 3×10^4 CFSE⁺ events from the live lymphocyte gate representing target cells in a histogram with Cell Quest software.

6. Representative Results

Example of flow cytometric pseudocolor dot plots over a five-day time-course monitoring iTreg differentiation based on the relative co-expression of CD25 with FoxP3, CTLA-4 and CD45RA can be seen in **Figure 2**. The histogram in **Figure 3** shows a successful suppression assay in which sorted iTregs (CD25^{hi}CD45RA⁻ CD127^{-LOW} cells) are the only subset from a five-day culture that has acquired regulatory/suppressor ability. **Figure 4** shows the induction of Tregs from a naïve T cell pool (top panels) and a memory T cell pool (bottom panels), after five-day culture in standard iTreg medium. CFSE staining of initial cells demonstrate that either naïve (top right panel) or memory T cells (bottom right panel) differentiate to iTregs (highest FoxP3-expressing cells) only after several rounds of cell division.

Figure 1. Schematic of experimental procedure. PBMCs are separated out of human peripheral blood via gradient centrifugation before magnetic negative selection of CD4⁺CD25⁻ T cells. After five to six days in culture, cells undergo FACS and are co-incubated with heterologous CFSE labeled target cells to measure suppressor activity.

Figure 2. Purified human primary CD4⁺CD25⁻ T cells are cultured in iTreg medium. An aliquot of cells is collected just after isolation (day 0) and at days 1, 3 and 5 of cell culture to monitor the progress of CD45RA, FoxP3, CTLA-4 and CD25 markers. The iTreg profile corresponds to CD45RA⁻, FoxP3^{hi}, CTLA-4^{hi} and CD25^{hi} (highlighted in the in-graph window).

Tested Suppressor Cells

Figure 3. Purified CD4⁺ CFSE labeled cells (1×10^5 /well) are cultured with *Treg suppression inspector beads* in the presence of sorted naïve, memory or iTreg cells (3×10^4 / well). After five days, cells are harvested and the CFSE profile of the stained cells is analyzed by flow cytometry. The presence of iTreg cells completely abolishes the proliferation of CD4⁺ T cells. Numbers are indicative of percentage of CFSE-labeled cells that have undergone division.

Figure 4. Purified human primary naïve (CD4⁺CD25⁻CD45RA⁺) and memory (CD4⁺CD25⁺CD45RO⁺) T cells are stained with CFSE and cultured in iTreg medium. After five days, cells are phenotyped and the cell division rates estimated. As indicated in **Figure 2**, the iTreg subset differentiated from both subsets corresponds to CD45RA⁻CD25^{hi} FoxP3^{hi} and CTLA-4^{hi} (latter two not shown). Comparative CFSE staining profiles identify iTregs (here as the highest expressing FoxP3 T cells) as the most proliferative cells during the five-day culture.

Discussion

While Treg transfer holds enormous therapeutic promise in combating autoimmunity graft rejection and other immune or inflammatory-mediated disorders, methods for their efficient generation and stable maintenance have not yet been developed. As only 1-5% of circulating human T cells are Tregs, their controlled expansion and differentiation overcomes this paucity as a major deterrent of implementation of Tregs into the clinic. On the other hand, as we learned from the TGN1412 trial¹³, it is scientifically and ethically necessary to profoundly understand the molecular events that orchestrate human T cell fate decisions before implementing any therapeutic regimen. With this method of iTreg differentiation, the resulting populations of naïve, memory, effector T cells and iTregs facilitate gene expression or functional comparisons between populations of cells which derive from a single human peripheral blood donor. In our hands, sorted cells have been compared in microarray and qRT-PCR analyses to measure genetic changes, in western blots to examine key signaling events, or in patch clamping to functionally measure ion channel usage¹⁴.

Our system provides the additional benefit of the ability to manipulate key aspects of the differentiation process around a central framework and readout. Thus, one can pre-sort PBMCs to alter the input population of cells or include small molecule inhibitors and ligands to the culture medium. One can also transfect cells to overexpress or knock down a protein of interest or introduce a reporter construct. As an additional benefit, we can alter the conditions of culture to maximize the percentage of iTregs generated. Overall, this human primary T cell culture delineates a very robust experimental platform with a physiological relevance much greater than those created in murine systems. Importantly, it may also sustain a direct therapeutic value. Indeed, as most current Treg-based therapeutic approaches involve *in vitro* or *ex vivo* development or manipulation of cells, our system can be seen as an important conduit on the road towards inclusion of Treg cell therapy in the standard of care. Future expansions on this system will tailor Tregs to become activated upon encounter with specific *in vivo* inflammatory antigens rather than the polyclonal activation described.

Disclosures

The authors have nothing to disclose.

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