

Isolation, Flow Cytometric Analysis, and Suppression Assay of CD4+CD25+ T-Regulatory Cells

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Abstract

Allergy and asthma are characterized by airway hyperresponsiveness and chronic mucosal inflammation mediated by CD4+ Th2 lymphocytes and their cytokines. It is unclear why allergic individuals make a Th2-type T-cell response whereas other (non-allergic) individuals do not. Recently, attention has focused on regulatory mechanisms, such as T-regulatory cells, preventing IgE responses to allergens in nonatopic individuals. Regulatory CD4+CD25+ T cells have been described in both mice and humans. The suppressive phenotype of these cells has been associated with the expression of the forkhead transcription factor, Foxp3. It has been suggested that allergic disease may arise from an inappropriate balance between allergen activation of regulatory CD4+CD25+ T cells and effector Th2 cells or from the impairment in the suppressive activity of these so-called T-regulatory cells.

The isolation of these T-regulatory cells is described in order to further our understanding of the role of these cells in allergic disease and asthma and allow us to design novel therapies.

Key Words: T-regulatory cells; tolerance; T-cell proliferation.

1. Introduction

Allergy and asthma are characterized by airway hyperresponsiveness and chronic mucosal inflammation mediated by CD4+ Th2 lymphocytes and their cytokines. It is unclear why allergic individuals make a Th2-type T-cell response whereas other (nonallergic) individuals do not. For many years, an imbalance between Th2 and Th1 cytokines has been considered to be the basis for allergic disease; however, more recently, attention has focused on regulatory mechanisms, such as T-regulatory cells, preventing IgE responses to allergens in nonatopic individuals.

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Regulatory CD4+CD25+ T cells were first characterized in mice, and the transfer of these cells has been shown to reduce the pathology of experimentally induced diseases such as thyroiditis, gastritis, and colitis (1) whereas their depletion results in the development of systemic autoimmune disease (2). A similar population of cells has since been described in human beings (3–5). The suppressive phenotype of these cells has been associated with the expression of the forkhead transcription factor, Foxp3. The CD4+CD25+ T-regulatory cell population constitutes approximately 10% of the CD4+ T-cell population.

It has been suggested that allergic disease may arise from an inappropriate balance between allergen activation of regulatory CD4+CD25+ T cells and effector Th2 cells or from the impairment in the suppressive activity of these so-called T-regulatory cells (6). In a murine model of asthma, a diminished presence of CD25+ T-regulatory cells resulted in increased airway hyper-reactivity (7). Other studies have failed to find differences in the number or functionality of these cells between atopic and nonatopic individuals. An upregulation of CD4+CD25+ T-regulatory cells has been observed in individuals following specific allergen immunotherapy (8–10).

Understanding the exact role of these cells in allergic disease and asthma will allow us to design novel therapies that are capable of targeting the direct T-regulatory cell pathway and thus leading to useful prevention and treatment of allergic disease.

2. Materials

2.1. Isolation of Peripheral Blood Mononuclear Cells

1. Dulbecco's phosphate-buffered saline (Sigma, Poole, Dorset, UK).
2. Ficoll Paque (GE Healthcare Ltd, Bucks, UK).
3. Culture medium (RPMI-1640-L-glutamine) (Sigma).
4. Sterile, individually wrapped 3-mL pastettes (VWR international, Leicestershire, UK).
5. Serological sterile, plastic pipets: 10 and 25 mL (Sarstedt, Leicester, UK).
6. 50-mL sterile centrifuge plastic tubes (Starlab Ltd, Milton Keynes, UK).
7. Trypan blue (0.4% in saline) (Sigma).
8. Neubauer hemocytometer (e.g., from Jencons, Leighton Buzzard, UK).
9. Microscope (Jencons).

2.2. Isolation of CD4+CD25+ Regulatory T Cells Using Magnetic Cell Sorting – MACS®

1. CD4+CD25+ regulatory T-cell isolation kit (Miltenyi Biotec, Surrey, UK, MACS® 130-091-301) which contains:
 - 1 mL CD4+ T-cell biotin-antibody cocktail, human
 - 2 mL Anti-Biotin MicroBeads
 - 1 mL CD25 MicroBeads

2. Separation buffer (degassed): 100 mL Dulbecco's PBS (Sigma) containing 0.5% BSA and 2 mM EDTA (note: EDTA can be replaced by other supplements such as anti-coagulant citrate dextrose formula-A (ACD-A) or citrate phosphate dextrose (CPD). BSA can be replaced by other proteins such as gelatine, HSA, or fetal calf serum).
3. MACS magnets (MidiMACS™ and MiniMACS™ separation unit) and MACS columns (LD and MS).
4. Ice box.

2.3. Extracellular Flow Cytometry Staining

1. Flow cytometry tubes (BD Biosciences).
2. PBS-BSA-azide: 100 mL Dulbecco's PBS (Sigma) containing 0.1% BSA and 0.09% sodium azide.
3. Labeled antibodies and isotype controls to CD4 and CD25 (Dako UK Ltd, Cambridgeshire, UK).
4. Flow cytometer (Becton Dickinson, Cowley, Oxford, UK).

2.4. Proliferation Assay

1. Complete culture medium; RPMI supplemented with 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin (all Life Technologies, Paisley, UK) and 5% vol/vol human AB serum (Sigma).
2. Sterile repeater pipet (Jencons).
3. 96-well flat bottomed culture plates (Nunc, Fisher Scientific, Loughborough, UK).
4. Antigen solution.
5. Phytohemagglutinin (PHA) positive control (Life Technologies, Gibco, Paisley, UK).
6. Tritiated [methyl-3H]-thymidine (Amersham Pharmacia Biotech, Little Chalfont, UK), diluted 1:20 in RPMI culture medium.
7. 37°C humidified 5% CO₂ cell culture incubator (e.g., from manufacturers such as RS Biotech Laboratory Equipment Ltd, Scotland, UK).
8. Cell culture harvester (e.g., Unifilter-96, Canberra Packard, Pangbourne, Berks, UK).
9. Liquid scintillation counter (e.g., TopCount Microplate Scintillation Counter, Canberra Packard).
10. Laminar flow hood (e.g., Jencons).
11. CO₂ incubator (e.g., Jencons).

2.5. Cytokine Analysis

1. Th1/Th2 human CBA kit (BD Bioscience, Pharmingen, Oxford, UK).
2. 5-mL flow cytometry tubes (BD Biosciences, Oxford, UK).
3. Flow cytometer (e.g., BD Biosciences).

3. Methods

3.1. Isolation of PBMCs

The isolation of peripheral blood mononuclear cells (PBMCs) is an important preanalytical step for many downstream laboratory procedures such as *in vitro*

culture assays or flow cytometry. Typically, the isolation of PBMCs is carried out using density gradient centrifugation, which takes advantage of the different sizes of the cell types and their differential migration through solutions, such as Ficoll, following centrifugation. Different layers form as a result and erythrocytes and granulocytes sediment at the bottom of the tube whereas lymphocytes, monocytes, and platelets are not dense enough to penetrate into the separation solution and thus form a concentrated band at the interface between the original blood sample and the Ficoll. This enables these cells to be easily recovered in a small volume. It is important for this step to be carried out efficiently so that good yields of PBMCs are obtained.

1. Peripheral whole blood (150 mL) is collected in either heparinized vacutainers or syringes coated with heparin (0.5 mL – 500 units).
2. Blood is diluted 1:2 with Dulbecco's PBS.
3. 20 mL of Ficoll Paque (at room temperature) is poured into 50-mL tubes (Falcon).
4. Using a 25-mL pipet, the tube is tilted and blood (25 mL) is carefully trickled down the side and layered onto the Ficoll Paque.
5. Tubes are centrifuged at room temperature at 300g for 20 min (with the brake off).
6. Using a 3-mL pastette, cells at the interface, which form a thin white layer, are collected and placed into a fresh tube. Ensure that all cells are collected without taking up too much separation medium.
7. PBS is added to wash the cells, which are then centrifuged at 4°C at 300g for 10 min (with the brake off).
8. The supernatant is discarded by careful pipeting and the cells are resuspended by firmly tapping the base of the tube.
9. Add 40 mL of RPMI medium to the cells, mix well, and then remove 20 μ L, and add to 20 μ L of Trypan Blue. Store the cells on ice while counting cells.
10. Load the cells/Trypan Blue mix into a Neubauer hemocytometer, then count the cells, and calculate the number of cells per milliliter and the total cells in solution (see **Note 1**).
11. Once the cell count is completed, remove 2×10^6 cells for the proliferation assay (e.g., 2 divided by cells per milliliter to give volume for removal), 1×10^6 for staining and 9×10^6 for irradiation. Cells should be made up with complete medium and then stored on ice until needed. Autologous mononuclear cells should be made up to 2×10^6 cells/mL before being irradiated. These cells are used as antigen-presenting cells.

3.2. CD4+ Cell Separation Using MACS® – Negative Selection

The isolation of CD4+CD25+ cells is performed in a two-step procedure using a MACS® CD4+CD25+ Treg isolation kit. First, a depletion step takes place whereby non-CD4+ cells are labeled using a cocktail of biotin-conjugated antibodies and then subsequently with Anti-Biotin MicroBeads (see **Fig. 1**). The cells are depleted by separation over a MACS® column and the flow-through



Fig. 1. Magnetic labeling of non-CD4+ cells.

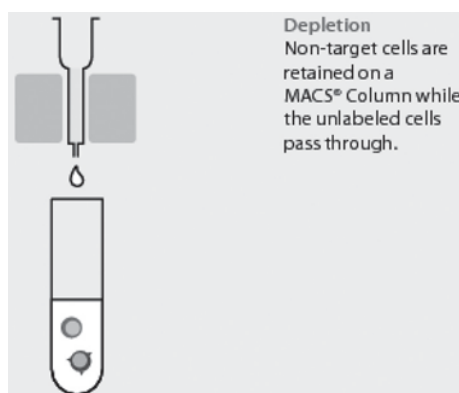


Fig. 2. Depletion of non-CD4+ cells.

fraction contains the enriched CD4+ cells (see **Fig. 2**). It is important during the next couple of steps to work quickly, keep the cells cold, and use precooled solutions. This will prevent cell loss, the capping of antibodies on the cell surface, and nonspecific labeling.

Volumes for magnetic labeling are for up to 10^7 cells; scale up accordingly for higher cell numbers, and for lower cell volumes use the same volumes as stated.

1. After the removal of PBMCs for the proliferation assay, staining, and irradiation, the cells are washed by adding RPMI and then centrifuged at 300g for 10 min at 4°C.
2. The supernatant is removed completely by careful pipeting and the cells are resuspended by firmly tapping the base of the tube.
3. 90 μ L of separation buffer is added per 10^7 cells. (It is necessary to ensure the cell suspension is well mixed to prevent the clumping of cells, which may clog the column.)
4. Add 10 μ L of CD4+ T-cell biotin-antibody cocktail per 10^7 cells and incubate for 10 min at 4–8°C.
5. 20 μ L of Anti-Biotin Microbeads are added per 10^7 cells. The cells are mixed well and incubated for an additional 15 min at 4–8°C (see **Note 2**).

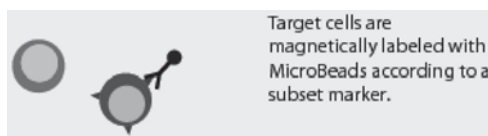


Fig. 3. Magnetic labelling of CD4+CD25+ regulatory T-cells.

6. The cells are washed by adding 1–2 mL of separation buffer and centrifuged at 300g for 10 min at 4°C. Remove the supernatant carefully using a 3-mL pastette.
7. Resuspend up to 10^8 cells in 500 μ L of buffer (for larger volumes, scale up accordingly).
8. Proceed to magnetic separation (*see* **Section 3.2.1.**).

3.2.1. Depletion of Non-CD4+ Cells with a MACS[®] LD Column

1. An LD column is placed into a MidiMACS Separator, 2 mL of separation buffer is run through the column, and the effluent is discarded.
2. The cell suspension is added to the column and the unlabeled cells which pass through the column are collected (these are the CD4-rich, negatively selected cells).
3. The column is washed with 2×1 mL of separation buffer and the buffer is added successively as soon as the column reservoir is empty.
4. The CD4+ flow-through fraction is made up to 14 mL with separation buffer and a cell count is carried out.
5. 2×10^6 cells are removed for the cell proliferation assay and 0.5×10^6 cells are removed for staining on a flow cytometer and kept on ice until needed.
6. Proceed to the positive selection of CD4+CD25+ cells.

3.2.2. CD25 Cell Separation Using MACS[®] – Positive Selection

In the second step of this T-cell separation, CD4+CD25+ T cells are directly labeled with magnetic beads specific for the CD25 low-affinity IL-2 receptor (*see* **Fig. 3**). They are isolated by positive selection from the pre-enriched CD4+ T cells (*see* **Fig. 4**).

Volumes given are for an initial cell number of 10^7 . For larger initial cell numbers, scale up accordingly.

1. The CD4+ cells are centrifuged at 300g for 10 min at 4°C (with the brake off).
2. The supernatant is removed and discarded using a 3-mL pastette.
3. The cell pellet is resuspended in 90 μ L (per 10^7 cells) of separation buffer.
4. Add 10 μ L (per 10^7 cells) of CD25 MicroBeads to the cells, mix well, and incubate for 15 min at 4–8°C (*see* **Note 2**).
5. The cells are washed with 10–20 times labeling volume of separation buffer and centrifuged at 300g for 10 min at 4°C (with the brake off). The supernatant is discarded as before.

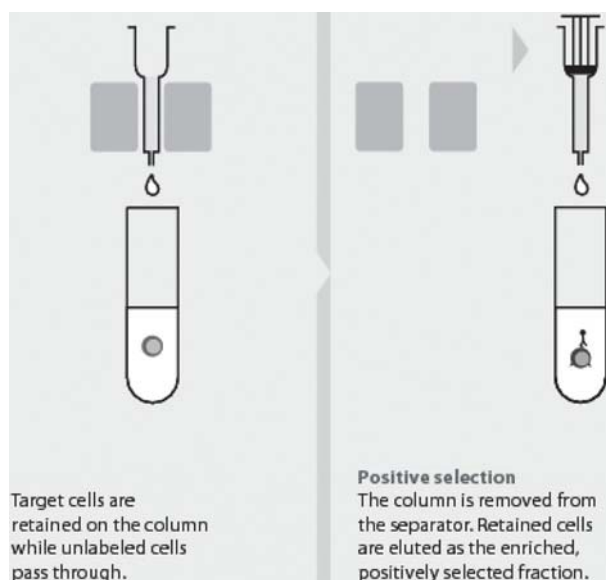


Fig. 4. Magnetic separation: Positive selection of CD4+CD25+ regulatory T cells.

6. Resuspend up to 10^8 cells in 500 μ L of separation buffer.
7. Proceed to the positive magnetic selection stage (*see Section 3.2.3.*).

3.2.3. Positive Selection of CD25+ Cells Using a MACS[®] MS Column

1. Place the MS column in a MiniMACS Separator.
2. The column is rinsed with 500 μ L of separation buffer and the effluent is discarded.
3. Just before the buffer has finished running through the column reservoir, add the cell suspension.
4. Collect the unlabeled cells, which flow through the column (these are the CD25– cells).
5. Wash the column with 3×500 μ L of separation buffer. Perform the washing step by adding the next wash just as the column reservoir is empty.
6. After completing the last washing step, remove the tube labeled CD25– and add 10 mL of separation buffer and centrifuge for 10 min at 300g at 4°C (with the brake off).
7. Meanwhile, the column is removed from the magnet and placed over a tube labeled CD25+ cells.
8. Pipet 1 mL of buffer onto the column and plunge the buffer through the column (thereby removing the CD25+ cells) by firmly applying the plunger supplied with the column.
9. A cell count of the CD25+ cells is carried out. 2×10^6 cells/mL are needed for the cell proliferation assay and 0.2×10^6 cells/mL for staining. If a large volume of

cells are recovered at the positive selection stage a second column run can be carried out where the eluted cells from the first column can be run down a second MS column. This will increase the purity of the CD4+CD25+ cells.

10. The second MS column is prepared in the same way by rinsing with 500 μL of buffer and the eluted CD25+ cells are run down the column and a further $3 \times 500 \mu\text{L}$ washes are performed. The flow-through fraction is discarded and the CD25+ cells are flushed through in the same way as before.
11. A cell count is carried out and 0.2×10^6 cells are removed for staining. The CD25+ cells are centrifuged at 300g for 10 min at 4°C and resuspended in the appropriate volume of complete medium to give 2×10^6 cells/mL.

3.2.4. Increasing the Purity of the CD25– Cells

1. Following centrifugation the supernatant is discarded using a pastette and the CD25– cells are resuspended in 500 μL of buffer.
2. An LD column is placed in a MidiMACS Separator and rinsed with $2 \times 1 \text{ mL}$ of separation buffer.
3. The CD25– cells are run down the column and $2 \times 1 \text{ mL}$ washes are performed.
4. The flow-through fraction is collected (this contains the pre-enriched CD25– cells).
5. Make the volume of cells up to 14 mL with separation buffer and carry out a cell count.
6. Remove 0.5×10^6 cells/mL for staining, then centrifuge the cells at 300g for 10 min at 4°C (with the brake off), and resuspend the CD25– cell pellet in the appropriate volume of complete medium to give 2×10^6 cells/mL.
7. Proceed to the cell proliferation assay (*see Section 3.3.*).

3.3. Proliferation Assay

Several factors should be considered before T-cell proliferation assays are carried out. First, it is necessary to determine the optimal antigen concentration and length of incubation by carrying out a number of experiments using a variety of antigen concentrations and time intervals. The antigen dose and incubation time that gives the highest proliferation can be considered optimal conditions for proliferation.

Positive and negative controls should be incorporated into the assay. PHA, a mitogen, is a good positive control as it induces blastogenesis *in vitro*. Cells incubated with the medium alone serve as a good negative control.

In order to compare the suppressive activity of CD4+CD25+ T cells on allergen-stimulated T-cell proliferation and cytokine production *in vitro* it is necessary to set up a coculture assay whereby both regulatory T cells and effector T cells (CD4+CD25– cells) are incubated together *in vitro* with the allergen of choice. Suppression of proliferation can be determined by comparing proliferation in the coculture system to that of the effector cells alone.

Table 1
Plate Layout for Suppression Assay

	1	2	3	4	5	6	7	8	9	10	11	12
A	PBMCs 2×10^6 cells/mL						Medium only			Rat antigen		
B	CD4+ cells only 2×10^6 cells/mL						Medium only			Rat antigen		
C	CD25- cells only 2×10^6 cells/mL						Medium only			Rat antigen		
D	CD25+ cells only 2×10^6 cells/mL						Medium only			Rat antigen		
E	CD25- cells 2×10^6 cells/mL						Medium only			Rat antigen		
	CD25+ cells 1×10^6 cells/mL											
F	CD25- cells only 3×10^6 cells/mL						Medium only			Rat antigen		
G	Irradiated cells only 2×10^6 cells/mL						Medium only			Rat antigen		
H	PBMCs PHA			CD4+ PHA			CD25- PHA					

Planning the layout of the experiment is an important stage; *see* **Table 1** for an example plate set up to examine the effect of regulatory T cells on proliferation to rat allergen.

Proliferation assay:

1. The previously identified optimal concentration of antigen is added to the appropriate wells.
2. 20 μ L of PHA (diluted 1:100 with RPMI medium) is added to the appropriate wells.
3. All cells are incubated with medium and antigen in triplicate.
4. 100 μ L of each cell type is added to the appropriate wells (*see* **Note 3**). 100 μ L of complete medium is added to the wells containing the PBMCs.
5. 100 μ L of autologous irradiated mononuclear cells are added to wells containing all other cells.
6. For the coculture, 100 μ L of CD25+ cells at 1×10^6 cells/mL and 100 μ L of CD25- cells at 2×10^6 cells/mL are added to the same wells and then 100 μ L of irradiated mononuclear cells (total volume in well = 300 μ L).
7. For the CD25- cells at 3×10^6 cells/mL, add 150 μ L of cells, 100 μ L of irradiated cells, and 50 μ L of complete medium (total volume in well = 300 μ L).
8. For the irradiated cells only, add 100 μ L of irradiated cells and 100 μ L of complete medium.
9. Incubate the plate for the number of days shown to be optimal for proliferation, e.g., 7 d at 37°C in an incubator with 5% CO₂.
10. On the last day of culture, e.g., day 7, remove 50 μ L of supernatant from each well and store at -80°C until cytokine analysis can be carried out.
11. Add 5 mCi of tritiated thymidine to each well at the end of 7 d and incubate for approximately 16 h.
12. Harvest the contents of the wells according to the manufacturer's instructions.

13. Count the incorporation of radioactivity using a scintillation counter according to the manufacturer's instructions.
14. Determine the mean counts per min (c.p.m.) in allergen-stimulated wells and medium controls wells.
15. Subtract the counts for the control wells from the allergen-stimulated wells.
16. Suppression of proliferation by CD4+CD25+ cells can be calculated as a difference in c.p.m. from mixed CD4+CD25+/CD4+CD25- cultures as a percentage of c.p.m. obtained with CD4+CD25- T cells alone, e.g.,

$$\frac{(\text{CD4} + \text{CD25} - \text{c.p.m.}) - (\text{mixed CD4} + 25 - / \text{CD4} + 25 + \text{c.p.m.})}{\text{CD4} + \text{CD25} - \text{c.p.m.}} \times 100$$

3.4. Extracellular Staining Using a Flow Cytometer

Cytometry refers to the measurement of the chemical and physical properties of cells. Flow cytometry is the method by which such measurements are made as the cells pass in a fluid stream through a measuring point surrounded by an array of detectors. In this way, the purity of the various cell types isolated using the MACS[®] regulatory t-cell isolation kit can be determined. It is necessary to include isotype controls in flow cytometry whereby nonreactive monoclonal antibodies of the same isotype and those conjugated with the same fluorochrome can measure the amount of conjugate binding that is unrelated to the target antigen of the human cells.

1. Label eleven 5-mL flow cytometry tubes as follows:

PBMCs 1 = unstained

2 = CD4 FITC

3 = CD25 RPE

4 = CD4 FITC/CD25 RPE

5 = IgG₁ FITC/IgG₁ RPE

CD4+ cells 1 = CD4 FITC/CD25 RPE

2 = IgG₁ FITC/IgG₁ RPE

CD4+CD25- cells 1 = CD4 FITC/CD25 RPE

2 = IgG₁ FITC/IgG₁ RPE

CD4+CD25+ cells 1 = CD4 FITC/CD25 RPE

2 = IgG₁ FITC/IgG₁ RPE

2. 0.2×10^6 of each cell type is placed into the appropriate tubes and 2 mL of buffer is added.
3. The tubes are centrifuged at 4°C for 5 min at 400g.
4. Pour off the supernatant and resuspend cells by flicking the bottom of the tube.
5. Add the relevant diluted primary antibody (e.g., FITC-conjugated CD4 antibody/ RPE-conjugated CD25 antibody) to the appropriate tubes in 20–50 µL of buffer according to the manufacturer's instructions.

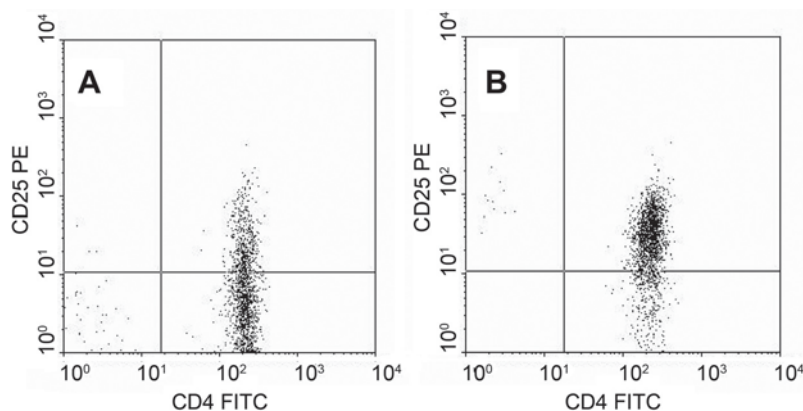


Fig. 5. Example of extracellular staining of (A) pre-enriched CD4+ T-cells after depletion of non-CD4+ cells and (B) isolated CD4+CD25+ regulatory T-cells.

6. Incubate on ice for 15–30 min in the dark.
7. The supernatant is discarded and the cells resuspended by flicking the bottom of the tube.
8. Add 2 mL of buffer to each tube and centrifuge at 4°C for 5 min at 400g.
9. Pour off the supernatant and resuspend cells by flicking the tube.
10. Add 200–400 μ L to each tube and analyze on a flow cytometer to determine cell purity (see **Fig. 5**).

4. Notes

1. Cell calculations: The number of cells counted in the 25 squares of the hemocytometer should be multiplied by 2 (as they are diluted 1:2 with Trypan Blue), then by 10^4 (for the volume of the graticule); this will give cells per milliliter. A further multiplication (for the total volume of sample) is required to ascertain the total cell count in solution:
e.g., $300 \text{ cells counted} \times 2 \times 10^4 = 600 \times 10^4 = 6 \times 10^6 \text{ cells/mL} \times 40 = 24,000 \times 10^4 = 2.4 \times 10^8$ in total.
2. Working on ice may require increased incubation times. Higher temperatures and/or longer incubation times lead to nonspecific cell labeling.
3. CD25[−] cells are incubated alone at a concentration of 3×10^6 cells/mL to control for amplified cell density of the CD25⁺/CD25[−] cultures.

References

1. Read, S., Malmstrom, V., and Powrie, F. (2000) Cytotoxic T lymphocyte-associated antigen 4 plays an essential role in the function of CD25(+)CD4(+) regulatory cells that control intestinal inflammation. *J. Exp. Med.* **192**, 295–302.

2. Sakaguchi, S., Fukuma, K., Kuribayashi, K., and Masuda, T. (1985) Organ-specific autoimmune diseases induced in mice by elimination of T cell subset. I. Evidence for the active participation of T cells in natural self-tolerance; deficit of a T cell subset as a possible cause of autoimmune disease. *J. Exp. Med.* **161**, 72–87.
3. Baecher-Allan, C., Brown, J. A., Freeman, G. J., and Hafler, D. A. (2001) CD4+CD25 high regulatory cells in human peripheral blood. *J. Immunol.* **167**, 1245–1253.
4. Dieckmann, D., Plottner, H., Berchtold, S., Berger, T., and Schuler, G. (2001) Ex vivo isolation and characterisation of CD(+)CD25(+) T cells with regulatory properties from human blood. *J. Exp. Med.* **193**, 1303–1310.
5. Stephens, L. A., Mottet, C., Mason, D., and Powrie, F. (2001) Human CD4(+) CD25(+) thymocytes and peripheral T cells have immune suppressive activity in vitro. *Eur. J. Immunol.* **31**, 1247–1254.
6. Ling, E. M., Smith, T., Nguyen, X. D., et al. (2004) Relation of CD4+CD25+ regulatory T-cell suppression of allergen-driven T-cell activation of atopic status and expression of allergic disease. *Lancet* **363**, 608–615.
7. Doganci, A., Eigenbrod, T., Krug, N., et al. (2005) The IL-6R alpha chain controls lung CD4+CD25+ Treg development and function during allergic airway inflammation in vivo. *J. Clin. Invest.* **115**, 313–325.
8. Akdis, C. A., Blesden, T., Akdis, M., Wuthrich, B., and Blaser, K. (2003) Role of interleukin 10 in specific immunotherapy. *J. Clin. Invest.* **102**, 98–106.
9. Francis, J. N., Till, S. J., and Durham, S. R. (2003) Induction of IL-10+CD4+ CD25+ T cells by grass pollen immunotherapy. *J. Allergy Clin. Immunol.* **111**, 1255–1264.
10. Varney V. A., Hamid, Q. A., Gaga, M., et al. (1993) Influence of grass pollen immunotherapy on cellular infiltration and cytokine mRNA expression during allergen-induced late-phase cutaneous responses. *J. Clin. Invest.* **92**, 644–651.