

Short-Term Culture of CD8 Cells and Intracellular Cytokine Staining

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Abstract

CD8 T cells play an important role in the regulation of allergic disease. Human and murine CD8 T cells have been shown to be capable of differentiating into distinct subsets defined by cytokine profiles analogous to the Th1 and Th2 subsets and termed T cytotoxic 1 (Tc1, IFN- γ producing) and 2 (Tc2, IL-4 producing).

Effector cell phenotype can be analyzed in vitro on a single cell basis using intracellular cytokine staining and flow cytometry or analysis of other phenotypic markers. Human PBMC usually contain only very low percentages of effector cells which produce relatively high levels of cytokines required for this kind of analysis. It is therefore necessary to activate the T cells to induce rapid accumulation of cytoplasmic cytokines before analysis. This makes it difficult to analyze the antigen specificity of responding T cells but will indicate the type 1/type 2 bias of the population, reflecting previous exposures to antigen. In this chapter, we provide protocols for the generation of polarized populations of CD8 T effector cells using polyclonal stimulation and for their subsequent analysis by intracellular cytokine staining.

Key Words: Effector cells; intracellular cytokine staining; Tc1; Tc2; CD8 T cells.

1. Introduction

In general, the techniques and conditions required for both short-term CD8 T-cell culture and generation of CD8 T-cell clones are the same as those used for CD4 T cells, because both subsets are able to undergo rapid clonal expansion on engagement of the TcR in the presence of IL-2 or IL-4. However, there are some special considerations when using CD8 T cells. First, CD8 cells generally produce very low levels of IL-2 or IL-4 upon activation and so their continued growth is more dependent on provision of exogenous cytokine. Secondly, CD8 T cells produce cytotoxic mediators and are more susceptible

to activation-induced cell death than their CD4 counterparts and may therefore undergo high levels of apoptotic death after prolonged culture and/or restimulation. CD8 T cells recognize MHC class I-associated peptide antigens, so generation of antigen-specific CD8 cells is inefficient when using crude antigen preparations added to the culture. Use of specific peptides or transfection of antigen-presenting cells with the antigen or gene encoding it may be beneficial. An alternative strategy is the use of dendritic cells instead of PBMC as antigen-presenting cells in the culture, as they efficiently cross-present soluble antigens in the MHC class I pathway. Lastly, although CD8 cells respond well to mitogens such as PHA, anti-CD3 antibodies, or superantigens, their requirements for costimulatory signals may be different. It is thought that CD8 cells are less dependent on costimulation through CD28 and more so through LFA-1 (*11*; B.V., unpublished) or cytokine receptors (*2*). This is an important consideration when culturing purified CD8 cells in the absence of antigen-presenting cells. In these circumstances, a combination of anti-CD3 and anti-LFA-1 antibodies may prove the most effective primary stimulus, whereas IL-7 and IL-15 are most effective at preventing CD8 cell apoptosis.

1.1. CD8 T-Cell Subsets

Functional heterogeneity within the CD8 subset of T cells has long been recognized, and they have often been classified as cytotoxic or suppressor (immunoregulatory) T cells. However, in contrast to CD4 T cells, these functions have not been clearly associated with distinct cytokine profiles. More recently, CD8 T cells have been shown to be capable of differentiating into distinct subsets defined by cytokine profiles analogous to the Th1 and Th2 subsets and termed T cytotoxic 1 (Tc1) and 2 (Tc2) (*3–5*). Tc1 and Tc2 cells have been demonstrated in both rodents and humans and are defined according to IFN- γ (Tc1) and IL-4 (Tc2) production. Tc1 cells secrete very high levels of IFN- γ in comparison to Th1 cells and low levels of IL-2. Tc2 cells produce IL-4, IL-5, IL-6, IL-10, and IL-13, often at similar or greater levels than Th2 cells. Such a clear-cut analogy between the cytokine profiles of CD4 and CD8 subsets has been challenged by studies which demonstrated patterns of cytokine production in CD8 populations that did not fit the Th1/Th2 paradigm (*6–8*).

As their name suggests, Tc1 and Tc2 effector cells are capable of cytotoxicity at similar levels, although it has been shown that murine Tc1 cells use both Fas and perforin pathways for killing target cells, whereas Tc2s are restricted to perforin killing (*9*). “Suppressor” functions of CD8 T cells have been associated with IFN- γ , IL-4, IL-10, and TGF- β in various models and therefore cannot be ascribed to a single CD8 subset (*6,7,10,11*). This is not surprising because these regulatory CD8 cells can control immune reactivity associated with both Th1 inflammatory reactions and Th2-associated antibody-mediated

immunopathology and are therefore likely to require different effector mechanisms. Further research is required to dissect the functional roles of Tc1 and Tc2 cells in different disease states.

The conditions under which Tc1 and Tc2 cells are generated during an immune response are also analogous to those for CD4 T cells. IL-12 and IL-4 are the major differentiation factors for Th1 and Th2 cells, although IFN- γ can replace IL-12 in human T-cell differentiation (12). Murine Tc1 and Tc2 cells were initially generated using IL-12 + anti-IL-4 and IL-4 + anti-IL-12, respectively, as for CD4 cells. It has since been shown that murine Tc1 development is not dependent on IL-12 (13); whether this is true in humans is unclear.

An important distinction between CD4 and CD8 T-cell differentiation lies in the distinct bias of CD8 cells toward the Tc1 pathway (5). In CD4 cells, most TcR-dependent stimuli induce a mixture of Th1 and Th2 effectors initially, unless stringent polarizing conditions are employed. In the case of CD8 cells, most stimuli induce rapid development of Tc1 cells with few, if any, Tc2s. Induction of human Tc2 cells usually requires appropriate TcR-dependent signals, IL-4, and the presence of IL-12-neutralizing antibody and varies greatly between donors. This difficulty in generating Tc2 cells accounts for their relatively recent discovery and for their apparent rarity *in vivo*. It is also possible that current cloning procedures favor the generation of Tc1 clones.

1.2. CD8 Subsets and Allergic Disease

Although CD4 Th2 cells directed against environmental antigens are the major inducers of the allergic phenotype, it has long been recognized that CD8 T cells play an important role in the regulation of allergic disease. During antigen processing of allergens, certain peptide epitopes can be presented via MHC class I molecules to CD8 T cells (14,15). These cells have the effect of suppressing the subsequent IgE response to the allergen, probably via skewing of the CD4 T-cell response toward a Th1 phenotype; such regulatory CD8 cells typically have a Tc1 cytokine profile (16). The role of Tc2 cells in allergy is far less clear, but there is evidence for the presence of such cells in the asthmatic lung (17), where they may be involved in disease pathogenesis (18,19). Further studies of Tc1/Tc2 development are likely to reveal divergent roles for CD8 T cells in the immunoregulatory and inflammatory pathways of allergic disease.

1.3. Use of Intracellular Cytokine Staining to Characterize CD8 T Cells

Preferential outgrowth of different CD8 subsets during long-term culture procedures can be avoided by using short-term cultures to generate effector cells. The phenotype of such effector cells can be analyzed on a single cell basis using intracellular cytokine staining and flow cytometry or analysis of other phenotypic markers. Such analysis is likely to more closely reflect the patterns

of cytokine production from effector cells generated *in vivo*. Human PBMC usually contain only very low percentages of effector cells which produce relatively high levels of cytokines required for this kind of analysis, because such cells are short-lived and rapidly recruited into tissues. It is therefore necessary to activate the T cells for several days in culture with antigen or mitogen to generate fresh effector cells *in vitro*, then restimulate them in the presence of a protein transport inhibitor to induce rapid accumulation of cytoplasmic cytokines before fixation and flow cytometric analysis. This approach makes it difficult to analyze the antigen specificity of responding T cells but will indicate the type 1/type 2 bias of the population, reflecting previous exposures to antigen. The technique cannot be conducted *in vivo* and hence requires cells to be removed from their immunological milieu. Moreover, this application is limited only to those tissues from which viable T cells can be isolated. Unlike previous methods which detected cytokine secretion into supernatants, this approach has the following advantages: (i) percentages of cytokine-secreting cells as well as levels of production per cell can be assessed simultaneously; (ii) coexpression of multiple cytokines can be detected, allowing analysis of subsets such as Th0/Tc0; (iii) reutilization of secreted cytokines from the medium is prevented; and (iv) only one monoclonal anti-cytokine antibody is required.

A recent development arising from intracellular cytokine staining techniques has been the cytokine secretion assay. This allows the purification of rare antigen-specific cells from PBMC, based on their cytokine secretion in response to antigen, and is particularly suitable for CD8 T cells because of their propensity for high-level IFN- γ production. This technique is described elsewhere (20). In this chapter, we provide protocols for the generation of polarized populations of CD8 T effector cells using polyclonal stimulation and for their subsequent analysis by intracellular cytokine staining. The intracellular staining protocol is equally useful for the analysis of CD4 effector cells or T-cell clones.

2. Materials

1. Hanks balanced salt solution (HBSS), phosphate-buffered saline (PBS), RPMI-1640 medium, fetal calf serum (Invitrogen, Paisley, UK).
2. Whole blood buffy coat (N. Thames blood transfusion service, London, UK) or heparinized venous blood.
3. Lymphoprep (Robbins Scientific, W. Midlands, UK).
4. CD8 Dynabeads™, Detach-a-bead™ solution (Dynal Ltd, Wirral, UK).
5. Complete medium: RPMI-1640 + 10% human AB serum (Serotec, Oxford, UK) + 1% nonessential amino acids (Sigma, Poole, UK) + 1% sodium pyruvate (Sigma) + 50 μ g/mL gentamycin (Sigma).
6. Anti-CD3 UCHT1, anti-IFN- γ -FITC (clone 25723.11), anti-IL-4-PE (clone 3010.211), anti-IL-2-PE (clone 5344.111) (BD Biosciences, Oxford, UK).
7. Anti-CD28 (Eurogenetics, Hampton, UK).

8. Recombinant human cytokines: IL-2, IL-4 (Serotec); IL-12 (R&D Systems, Cambridge, UK).
9. Anti-IL-4 (BD Biosciences), anti-IL-12 (R&D Systems).
10. 4% formaldehyde fixative: 30% stock solution (Sigma) diluted to 4% in 1.5× strength PBS (using 10× PBS), stored room temperature <6 wk.
11. 1% (w/v) paraformaldehyde solution: dissolved in PBS at 60°C + one drop concentrated NaOH.
12. PMA, ionomycin, brefeldin A (Sigma): dilute freshly from 10,000× concentrated stock solutions in absolute ethanol, stored at −20°C.
13. Permeabilizing buffer: PBS + 1% bovine serum albumin (BSA; Sigma) + 0.5% saponin (Sigma). If stored for any length of time, 0.1% sodium azide preservative should be added.

3. Methods

3.1. Generation of Tc1/Tc2-Polarized Populations of Human CD8 T Cells from Peripheral Blood

3.1.1. Protocol for Purification of CD8 T Cells from Peripheral Blood or Buffy Coats

1. Dilute whole buffy coat 1:4 with HBSS or if using peripheral blood dilute 1:2 with HBSS.
2. Isolate PBMC by Lymphoprep density gradient centrifugation at 800g for 20 min at 18°C. Wash cells with cold HBSS at 600g for 10 min at 4°C.
3. Determine viable cells by trypan blue exclusion, then wash twice with HBSS at 200g for 10 min at 4°C and resuspend at 1×10^7 per mL in PBS containing 2% fetal calf serum.
4. Calculate the volume of Dynabeads required using a 3:1 bead to target ratio, assuming CD8 target cells make up 25% of PBMC. Prior to use, wash Dynabeads™ as follows: transfer the desired amount of Dynabeads™ to an Eppendorf tube, place on a magnetic particle concentrator (MPC; Dynal Ltd) for 1 min, and aspirate the fluid. Resuspend the beads in 1 mL of washing buffer (PBS containing 2% FCS), reapply to the MPC for 30 s, and discard the wash buffer. This washing procedure should be repeated twice, each time ensuring that the tube remains firmly on the MPC.
5. Transfer the washed beads to the PBMC suspension and mix by rotation for 1 h at 4°C.
6. Collect the attached cells using the MPC and decant off the unbound cells. Resuspend the beads in wash buffer and return to the MPC for a further 1 min. Discard the wash and repeat the whole washing procedure a further four times. It is important to ensure that all air bubbles are carefully removed following each wash using a sterile pastette as these may contain contaminating cells.
7. In order to elute the bound CD8 T cells, resuspend pelleted cells/beads in wash buffer and add Detach-a-bead™ antibody (1/30) for 60 min at room temperature with mild agitation.

8. Place the tube on the MPC for 2 min and collect the released cells. Wash the attached cells with 1 mL of complete medium and reapply to the magnet for a further 30 s. Transfer the medium into a clean sterile tube and repeat this procedure twice. Pool the washes together.
9. To remove any residual Detach-a-bead™ solution, centrifuge the isolated T cells at 200g for 10 min and resuspend at 1×10^6 cells/mL in complete medium. Take 10^5 – 10^6 of these cells and stain for CD3, CD8, CD4, CD14, or CD19 using fluoro-chrome-labeled antibodies. Acquire samples by flow cytometry to determine the precise purity of the isolated populations.

3.1.2. Protocol for Generating Polarized Populations of Tc1 or Tc2 Effector Cells in Short-Term Cultures for Cytokine Analysis, Further Characterization, or Cloning

1. Coat a sterile 24-well tissue culture plate with purified anti-CD3 at a concentration of 5 μ g/mL in PBS and anti-CD28 at a concentration of 2 μ g/mL in PBS (0.25 mL/well) either overnight at 4°C or for 3 h at 37°C (see **Note 1**).
2. Wash the plate three times with PBS to remove any excess unbound antibody.
3. Plate out the cells at a density of 1×10^6 cells/mL (1 mL/well) and culture for 4 d. To generate Tc1 cells add recombinant IL-2 at a concentration of 20 U/mL, recombinant IL-12 at 200 U/mL, and anti-IL-4 (5 μ g/mL). To generate Tc2 cells, add recombinant IL-2 (20 U/mL), recombinant IL-4 (100 U/mL), and anti-IL-12 (5 μ g/mL) (see **Notes 2–4**).
4. Culture the cells in a humidified incubator at 37°C, 5% CO₂ for approximately 6 d.

3.2. Stimulation of CD8 Effector Cells and Intracellular Cytokine Analysis

The method for analyzing cytokine production by single cells was originally described by Jung et al. (21) who demonstrated the feasibility of using intracytoplasmic staining and flow cytometry to determine the frequency of cytokine-producing cells in a heterogeneous population (see **Note 5**). The basic protocol consists of in vitro stimulation of T cells in the presence of a protein transport inhibitor. This is followed by cell fixation, permeabilization, and then intracytoplasmic staining of accumulated cytokines.

3.2.1. Restimulation of Effector Cells to Induce High-Level Cytokine Production

1. Generate effector CD8 T cells under the appropriate conditions as previously described. Each sample should contain approximately 2×10^5 – 1×10^6 viable cells.
2. Gently resuspend the cells, transfer to 5-mL tubes, wash three times, and resuspend in 1 mL complete medium.
3. Restimulate cells by addition of either PMA (10 ng/mL) and ionomycin (400 ng/mL) to the medium or by coating the 24-well plates with anti-CD3 (5 μ g/mL) and

anti-CD28 (2 $\mu\text{g/mL}$) as described previously (see **Note 6**). Include an unrestimulated control sample containing effector cells cultured in only the medium.

4. Culture the cells overnight. Add brefeldin A protein transport inhibitor to a final concentration of 5 $\mu\text{g/mL}$ and culture for a further 6 h. Following incubation, transfer cells to 5-mL Falcon tubes and wash three times with PBS/0.5% BSA.

3.2.2. Staining of Restimulated Cells with Anti-cytokine Monoclonal Antibodies

1. Having stained for any necessary cell surface markers, proceed to fixing the cells. Pellet the cells by centrifugation and resuspend in 1 mL of 4% formaldehyde solution, vortex each tube immediately, and leave to stand at room temperature for 20 min in the dark.
2. Wash once in 2 mL PBS/0.5% BSA and centrifuge at 200g for 10 min at 4°C.
3. Permeabilize the cells by adding 2 mL of PBS containing 0.5% saponin and 1% BSA for 10 min at room temperature in the dark. Vortex periodically and centrifuge at 200g for 10 min at 4°C. The effects of saponin are reversible; therefore ensure its presence during each antibody incubation and washing step to maintain membrane permeability. Wash the cells once more in permeabilizing buffer, centrifuge at 200g for 10 min at 4°C, and resuspend pelleted cells (50–100 μL per tube).
4. Add 0.1–0.5 μg per tube of each fluorochrome-labeled anti-cytokine antibody: e.g., anti-IFN- γ -FITC + anti-IL-4-PE (see **Note 7**). Add the same quantity of isotype control antibodies to the control samples. It is advisable to titer each reagent for use. Leave cells to incubate for 30 min at room temperature in the dark.
5. Wash cells with 2 mL of PBS/0.5% saponin/1% BSA and centrifuge at 200g for 10 min at 4°C.
6. Resuspend cells in 1 mL PBS/0.1% BSA for direct flow cytometric analysis. If cells are not to be acquired immediately, resuspend in 1% paraformaldehyde in PBS and store at 4°C for up to 1 wk.
7. Acquire samples on a flow cytometer such as a FACScalibur (BD Biosciences) and analyze using the appropriate software.

3.3. Flow Cytometric Analysis and Expected Results

1. CD8 T-cell yields and purities: 50 mL of blood typically yields approximately 10^7 CD8 T cells. These should be >98% CD8⁺, >98% CD3⁺, <1% CD4⁺, 1% CD19⁺.
2. Cytokine staining results: typical staining profiles obtained from polarized CD8 populations are shown in **Fig. 1**. A gate should be set on the forward scatter vs side scatter plot to include only viable, nonapoptotic cells in the cytokine profile. Apoptotic cells often appear during culture and restimulation because of activation-induced cell death; they can be distinguished by their greater side scatter and reduced forward scatter compared to viable effectors (see **Note 8**). IFN- γ and IL-4 are used as the definitive Tc1 and Tc2 cytokines, respectively, but other cytokines can be analyzed simultaneously. Proportions of cytokine-positive cells are usually in the range of 10–50%, but vary greatly between donors. It should be noted that there is a large stochastic element to the acquisition of cytokine production in

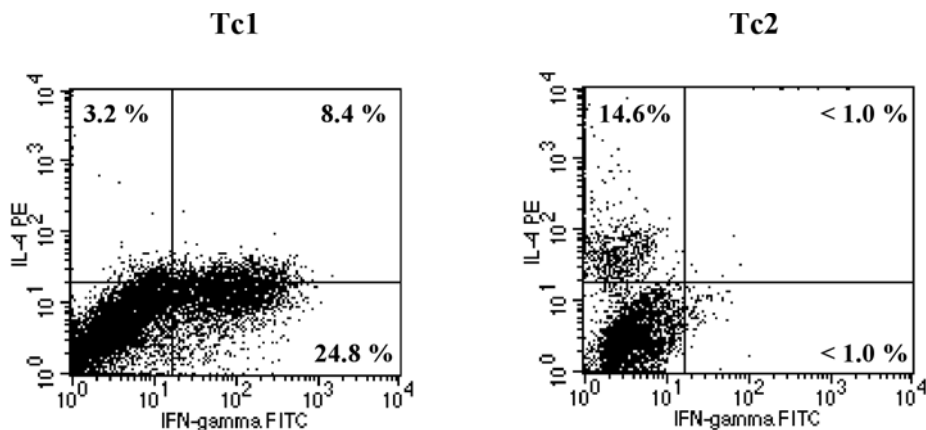


Fig. 1. Typical cytokine profiles of polarized Tc1 (left) and Tc2 (right) populations. Peripheral blood CD8 T cells from a single donor were polarized into Tc1 and Tc2 effectors as described and restimulated after 4 d with PMA + ionomycin in the presence of brefeldin A. Cells were stained with anti-IFN- γ -FITC and anti-IL-4-PE and analyzed on a FACScalibur™ flow cytometer using Cellquest software. Quadrant markers were set according to isotype control antibody-stained samples and unrestimulated controls, which had <1% positive staining.

effector T cells (*1,22,23*) and therefore 100% positive staining for any cytokine is rarely observed except in long-term T-cell clones. It is extremely important to perform sufficient negative controls, such as effector cells which are not restimulated but are stained with anti-cytokine antibodies, and restimulated cells stained with isotype-matched control antibodies. Single color controls are also advisable to ensure correct compensation settings (*see Note 9*).

4. Notes

1. The choice of primary stimulus for inducing CD8 T-cell growth can obviously be altered to address specific questions. Use of certain mitogens such as PHA and superantigens requires the presence of accessory cells in the culture in addition to CD8 cells. A convenient source of accessory cells is the CD8-depleted PBMC fraction which should be irradiated at 4000 rad before addition to culture.
2. Generation of Tc2 effectors is sometimes problematic and varies greatly between donors. We have observed that addition of steroids to the cultures, or use of PMA + ionomycin as a primary stimulus, can greatly enhance development of Tc2 cells. Extending the initial culture period (7–10 d) may also favor Tc2 generation, because this subset is less prone to activation-induced cell death.
3. Generation of allergen-specific CD8 cells is highly problematic resulting from low precursor frequencies and donor-to-donor variation. In this case, the concentration of purified CD8 cells should be increased and the culture supplemented with

autologous, irradiated PBMC as a source of antigen-presenting cells. Introduction of the protein allergens into the cytoplasm of antigen-presenting cells using transfection reagents or electroporation before addition of T cells will enhance MHC class I presentation and thus amplify the proliferative response. Longer culture periods will be required to allow sufficient expansion of antigen-specific cells.

4. If accessory cells are present in the primary culture, it may be necessary to stain effector cells for CD8 and/or CD3 in addition to intracellular cytokines. Because all components of the TCR complex are rapidly downregulated after effector cell activation, the cells should be labeled with fluorochrome-labeled CD8 or CD3 antibody at the end of the *primary* culture, then washed, and restimulated. The fluorochrome will then remain detectable after receptor downregulation.
5. A disadvantage of this technique is that it is only semiquantitative in terms of the titers of cytokines produced per cell, because fluorescence intensities of individual cells are detected. Another limitation is that not all cells may produce a given cytokine synchronously and therefore the timing between stimulation and fixation is important. It would therefore be beneficial to determine the kinetics of synthesis and secretion of a given cytokine. It should also be remembered that although the presence of a cytokine may be detected within cells this does not necessarily mean that it will be secreted.
6. The secondary restimulation of effector cells prior to intracellular staining can be performed using immobilized anti-CD3 and anti-CD28 antibodies. However, the choice of secondary stimulus can affect the cytokine staining profile.
7. If suitable fluorochrome-labeled antibodies are unavailable for a cytokine of interest, biotin-labeled antibodies can be substituted. The cells should then be washed in permeabilizing buffer and stained with fluorochrome-labeled streptavidin before analysis. However, not all monoclonal antibodies are effective for intracellular staining because certain epitopes are destroyed by fixation.
8. Populations of cells grouped along the x - y axis of FL1 vs FL2 plots after compensation are indicative of nonspecific binding by dead or apoptotic cells, rather than genuine cytokine-positive cells. They should be eliminated by adjusting the gate in the forward scatter vs side scatter window.
9. The choice of fluorochromes depends on the setup of the flow cytometer to be used. For a single 488-nm laser FACScalibur™, use FITC or Alexa Fluor™ 488 (FL1 channel), PE (FL2 channel), and PerCP or PE-Cy7 (FL3). For a dual laser FACScalibur™ with an additional 635-nm laser, APC or Alexa Fluor™ 647 (FL4 channel) can be added to give simultaneous detection of four cytokines or markers.

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