Chapter 2

Fluorescence-Activated Cell Sorting-Based Quantitation of T Cell Receptor Restimulation-Induced Cell Death in Activated, Primary Human T Cells

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Abstract

After initial stimulation with antigen and exposure to the growth cytokine interleukin-2, activated T lymphocytes become sensitized to apoptosis upon antigen restimulation through the T cell receptor. This self-regulatory, restimulation-induced cell death (RICD) program constrains the proliferative capacity of activated T cells to help prevent excessive T cell accumulation and associated immunopathology. Here we describe a simple FACS-based approach for measuring RICD sensitivity in activated human T cells following polyclonal restimulation in vitro. This procedure is a straightforward research and clinical diagnostic tool for assessing RICD sensitivity for T cells derived from normal donors and patients suffering from diseases causing dysregulated T cell homeostasis.

Key words: T cell receptor (TCR), Activated human T lymphocytes, IL-2, Anti-CD3, Fluoresence-activated cell sorting (FACS)

1. Introduction

Successful adaptive immune responses to infectious pathogens depend upon rapid clonal expansion of rare, antigen-specific T lymphocytes. Naïve T cells generally require three distinct signals to proliferate and acquire effector functions: (1) ligation of T cell receptor (TCR)/CD3 complexes via MHC-presented peptide antigen; (2) costimulation, primarily through the surface receptor CD28; and (3) exposure to the growth factor cytokine interleukin-2 (IL-2) in an autocrine/paracrine fashion (1). Generally, IL-2 induction of cell proliferation is a consequence of the first two signals, which upregulate expression of IL-2 as well as its high affinity receptor. Expansion of human peripheral blood T cells is readily induced in vitro using antibodies to nonspecifically cross-link CD3 and CD28 as well as exogenous, recombinant IL-2, as detailed

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in the Methods section below. In the context of infection, clonal T cell proliferation creates a potent arsenal of activated effector T cells that can directly eliminate pathogen-infected cells as well as provide assistance to B cells for effective humoral responses. An orderly transition from proliferation to contraction must take place as the adaptive immune response unfolds to eliminate excess T cells that may otherwise inflict unintended damage to host tissues, especially under conditions of recurring or persistent antigen (2). Programmed cell death via apoptosis both constrains and contracts the effector T cell pool throughout this period (3).

Restimulation-induced cell death (RICD) describes a propriocidal apoptosis mechanism by which reengagement of the TCR triggers death in a proportion of activated T cells (4). This phenomenon was originally described in vitro using mouse T cell hybridomas and/or primary, activated murine and human T cells cultured with exogenous IL-2, which readily undergo RICD upon restimulation with anti-CD3. Although the molecular details of this signaling pathway are not completely understood, RICD is proposed to act as a self-regulatory brake to set an upper limit for clonal expansion when antigen is abundant or recurrent (e.g., during chronic infections). Defective RICD can contribute to unchecked T cell accumulation and immunopathology in X-linked lymphoproliferative disease (XLP), underscoring the physiological relevance of this TCR-induced death program (5).

Here we provide a simple in vitro protocol for inducing and quantifying RICD sensitivity of activated human T cells using propidium iodide exclusion and flow cytometry. Complementary approaches for examining hallmarks of apoptosis during this process (e.g., Annexin V binding, caspase cleavage, mitochondrial depolarization, etc.) can easily be applied to the same anti-CD3-based restimulation procedure. This in vitro RICD assay provides a straightforward diagnostic tool for assessing death sensitivity in patients with aberrant T cell homeostasis, and allows basic and clinical researchers to investigate the genetic and biochemical mechanisms driving this death pathway.

2. Materials

2.1. Cell Isolation and Culture

1. Whole blood or buffy coat (see Note 1).
2. Ficoll-Paque PLUS (GE Healthcare).
3. 1× Phosphate-buffered saline (PBS).
4. 15 and 50 ml conical tubes; 1.5 ml microcentrifuge tubes (sterile).
5. ACK lysing buffer.

6. Complete RPMI medium: RPMI 1640 tissue culture medium (w/ 2–4 mM L-glutamine), 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin–streptomycin. For 500 ml, add 50 ml FBS and 5 ml of 100× stock of penicillin–streptomycin. Store at 4°C.
7. Vented T75 tissue culture flasks (75 cm^2 surface area).
8. Anti-human CD3ε antibody (clone OKT3, Ortho Biotech, or clones HIT3a/UCHT1 (NA/LE), BD Biosciences).
9. Anti-human CD28 antibody (clone CD28.2 (NA/LE), BD Biosciences).
10. Recombinant human IL-2 (Peprotech or equivalent source).

2.2. RICD Assay and Flow Cytometry

1. 96-well round-bottom plate.
2. Propidium iodide (PI) (Sigma, 1 μg/ml stock diluted in 1× PBS).
3. 1.1 ml polypropylene microtubes (USA Scientific).
4. 5 ml round-bottom tubes for flow cytometry (BD Falcon, BD Biosciences).
5. Flow cytometry analyzer (FACScan, FACSCalibur, or equivalent).

3. Methods

3.1. Isolation of Peripheral Blood Mononuclear Cells Using Ficoll

1. Dilute the whole blood or buffy coat 1:2 with PBS (see Note 1). Distribute 20 ml of blood/PBS mixture per 50 ml conical tube.
2. Gently layer 12–13 ml of Ficoll under the blood/PBS mixture in each tube (see Note 2). Handle the tubes carefully to avoid disrupting the blood/Ficoll interface.
3. Spin the tubes at ~700 × *g* in a tabletop centrifuge for 20 min at room temperature (RT) with no brake (note that all subsequent centrifugations will be at RT).
4. After the spin, note the opaque layer of PBMC at the interface between the plasma (top, yellowish volume) and the Ficoll (clear). Carefully collect the PBMC from this interface using a pipette, and transfer to a new 50 ml conical tube (see Note 3).
5. Fill up to 50 ml with PBS and mix tubes by inverting several times.
6. Spin the tubes at 400 × *g* for 10 min to pellet PBMC. Carefully aspirate the PBS without disturbing pellet.
7. If excessive red blood cells (RBC) are visible in the pellet, resuspend the pellet in 5 ml ACK Lysing Buffer and mix gently by swirling the tube. Allow the cells to incubate at RT for 5 min, then fill up to 50 ml with PBS, and spin at 400 × *g* for 5 min to pellet.

8. Repeat the wash (steps 5–6) once more by resuspending PBMC pellet(s) in 5–10 ml PBS, then filling up to 50 ml PBS, and spinning at 400 × *g* for 5 min. Multiple pellets in separate tubes can be combined in one 50 ml wash.
9. Resuspend the cells in 30 ml of complete RPMI. Count PBMC using a hemocytometer.

3.2. Stimulation and Culture of Activated T Cells

1. Collect 10–20 million PBMC in a 50 ml conical tube and adjust the concentration to 2×10^6 cells/ml in complete RPMI.
2. Activate the T cells using 1 μg/ml each of anti-CD3 and anti-CD28 antibodies (1:1,000 dilution from 1 mg/ml stock). Add the antibodies directly to the tube, swirl to mix, and transfer to a T75 vented flask (see Note 4).
3. Incubate the cells in a humidified 37°C incubator (5% CO_2) for 3 days (see Note 5).
4. At ~72 h post activation, transfer cells to a 50 ml conical and pellet by spinning at 400 × *g* for 5 min. Wash the pellet twice by resuspending in ~30 ml PBS, inverting the tube several times, and spinning to pellet. Carefully aspirate PBS from the pellet after each wash.
5. After the second wash, resuspend pellet in 10 ml complete RPMI. Count cells and adjust concentration to $1–2 \times 10^6$ cells/ml.
6. Add exogenous recombinant human IL-2 to a final concentration of 100 international units (IU)/ml. Swirl to mix and transfer the volume to a fresh T75 flask.
7. Allow the cells to expand in culture for 7–10 days. Add or change media with fresh IL-2 (100 IU/ml) as needed every 2–3 days (see Note 6).

3.3. Plating T Cells for RICD Kill Assay

1. Count the T cells and collect 2×10^6 cells in a 15 ml conical tube. Pellet the cells by spinning at 400 × *g* for 5 min.
2. Aspirate the supernatant carefully and resuspend the pellet in 4 ml complete RPMI plus 100 IU/ml IL-2 (final cell concentration = 0.5×10^6 cells/ml).
3. Aliquot 0.5×10^6 cells (1 ml each) into 4 separate 1.5 ml microcentrifuge tubes.
4. Prepare diluted stocks of anti-CD3 mAb (clone OKT3, 1 mg/ml stock concentration) in complete RPMI plus 100 IU/ml IL-2, including 1, 10, and 100 μg/ml (see Note 7).
5. Add the dilutions of OKT3 mAb into each tube as described in Table 1 (see Note 8).
6. Mix by pipetting up and down several times, and then plate cells (1×10^5 cells/200 μl/well) in triplicate in a 96-well round-bottom plate (see Fig. 1).

Table 1
Recommended dilutions for OKT3 dose–response curve in RICD assay

Dilutions	Tube 1	Tube 2	Tube 3	Tube 4
1 µg/ml OKT3 diluted stock	–	10 µl	–	–
10 µg/ml OKT3 diluted stock	–	–	10 µl	–
100 µg/ml OKT3 diluted stock	–	–	–	10 µl
Final OKT3 concentration (ng/ml)	0	10	100	1,000

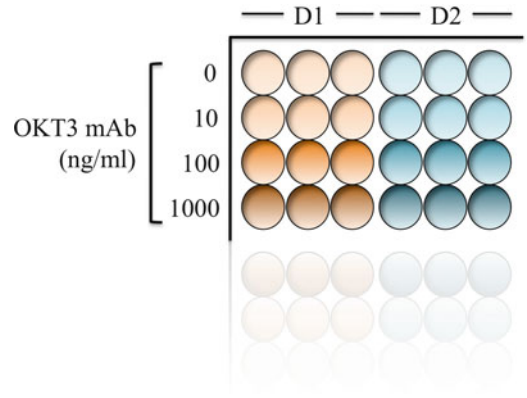

Fig. 1. Plating of activated human T cells for the RICD assay. Activated T cells (0.5×10^6 cells/ml) from different human donors (organized in columns; D1, D2, etc.) are treated with designated doses of OKT3 mAb (organized in *rows*) and subsequently plated in triplicate wells (1×10^5 cells/200 µl/well) for ~24-h incubation. PI may be added directly to the wells before transferring cells to microtubes for FACS analysis.

7. Incubate the cells in a humidified 37°C incubator (5% CO_2) for 24 h to induce apoptosis (see Note 9).

3.4. FACS-Based Quantitation of RICD

1. At 24 h post restimulation, remove the plate from the incubator and stain cells by adding 10 µl of diluted PI stock (1 µg/ml) directly to each well (see Note 10).
2. Gently pipette up and down to mix and transfer all stained cells to racked 1.1 ml microtubes using a multichannel pipette.
3. Prepare the flow cytometer for data acquisition, including a 2-parameter dot plot for forward scatter (FSC) versus FL2 (PI channel). Note that the FSC scale is linear and FL2 is logarithmic.
4. To collect the cells, place each sample (cells in microtube) into a 5 ml FACS tube and vortex gently immediately prior to placement on the flow cytometer.

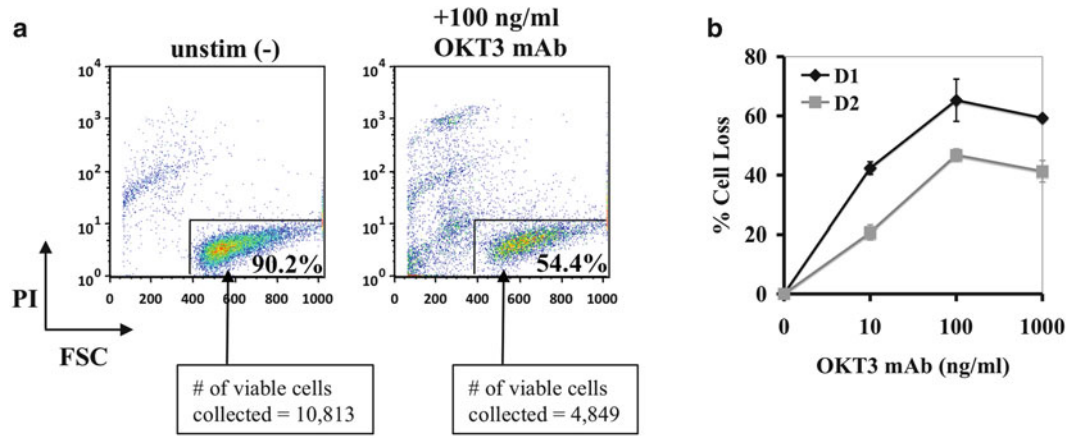

Fig. 2. Gating strategy for quantifying viable cells using PI exclusion. After restimulation (~24 h), PI-stained T cells are collected on the flow cytometer for a constant time (20–30 s/sample). (**a**) For analysis, data is visualized in a 2-color dot plot with forward scatter (FSC) on the *x*-axis and PI (FL2) on the *y*-axis. Viable cells (FSC^{hi}, PI-) are gated as shown to determine the number of viable cells collected per sample. Percent cell loss is then calculated as follows:
# of viable cells (20 sec collection): unstim (0 ng/ml) = 10813; + 100 ng/ml OKT3 = 4849; % cell loss = [1-(4849/10813)] * 100 = 55.2%
(**b**) By repeating this calculation for triplicate values, a dose–response curve for mean RICD sensitivity (–/+ SD) can be readily generated to compare different donors (*Panel B*).

5. Collect each sample using constant time (20–30 s/sample) (see Note 11).
6. For analysis, use FACS analysis software (e.g., FlowJo, FACSDiva, or equivalent) to plot FSC versus FL2 for each sample, gating on the viable cells (FSC^{hi}/PI^{-} population, see Fig. 2) to determine the number of viable cells collected.
7. Calculate the % cell loss for each dose of OKT3 as follows: % cell loss = [1 – (# of viable (PI-) restimulated cells/# of untreated viable cells)] × 100, see Note 12.
8. Plot % cell loss data on a dose–response curve (see Fig. 2 for example).

4. Notes

1. For best PBMC viability, whole blood may be collected in tubes with acid citrate dextrose (ACD) as an anticoagulant. Alternatively, the enriched PBMC fraction obtained from a whole unit blood donation after initial centrifugation (a.k.a. the "buffy coat") may be used to obtain large quantities of PBMC.
2. For best results to layer Ficoll underneath blood/PBS mixture, we recommend placing a sterile 5.75 in. glass Pasteur pipette

and adding up to 3 ml Ficoll to the top of the pipette to allow Ficoll to drip slowly from the narrow opening at the bottom of the tube. Repeat this step until 13 ml of Ficoll is distributed per tube (20 ml blood/PBS). Alternatively, blood/PBS mix can be slowly layered on top of pre-aliquoted Ficoll using a sterile pipette, being careful not to disrupt the interface.

3. The volume of PBMC transferred to each 50 ml conical tube should not exceed 8 ml, as too much residual Ficoll may interfere with pelleting of cells by centrifugation and reduce yield. For best viability of isolated PBMC, cells must be washed thoroughly to remove all Ficoll.
4. Use of soluble anti-CD3/CD28 antibodies should activate T cells efficiently in the context of all PBMC, as the presence of Fc receptor-bearing "accessory cells" (i.e., monocytes, B cells) will aid in cross-linking. Other polyclonal stimuli may also be utilized to activate T cells in this context (e.g., concanavalin A (5 μg/ml) or phytohemagglutinin (PHA)-L (1–2 μg/ml), both available from Sigma). If T cell subsets are purified from PBMC prior to initial stimulation, we recommend using plate-bound anti-CD3/CD28 Abs or Ab-coupled beads (e.g., T cell activation/expansion kit, Miltenyi Biotec).
5. Cells should be monitored for signs of proper T cell activation during this period. T cells should blast (i.e., increase in size) and form small clumps in culture by day 2–3. Alternatively, the upregulation of certain cell surface markers (e.g., CD69 on day 1, CD25 on days 2–3) may also be tracked using flow cytometry.
6. The addition of exogenous IL-2 should result in rapid cell division and expansion of T cells in the first 7–10 days. The T cells will be largely comprising activated $CD4^+$ and $CD8^+$ cells, with the proportion of $CD8^+$ T cells increasing over time. IL-2 is always kept in excess in the culture to sensitize cells to RICD (6) and avoid inducing cytokine withdrawal-induced death (see Chapter 3). Monitor cultures every 1–2 days by watching media color (i.e., red to yellow color change of RPMI containing phenol-red accompanies the active growth and proliferation of cells since they produce metabolic acids) and counting cells. Ideally, cultures should be kept at a density of $1–2 \times 10^6$ cells/ml throughout this period. Feed or split cells accordingly using complete RPMI and fresh IL-2.
7. The anti-CD3 Ab clone OKT3 should reliably induce apoptosis in ~40–60% of activated T cells (~100 ng/ml), although sensitivity will vary depending on the donor and the relative proportion of $CD8^+$ T cells (~70–80% at the time of assay). Purification of $CD4^+$ or $CD8^+$ T cells prior to restimulation may be done to assess subset-specific RICD sensitivity and reduce donor-dependent variability. For assaying patients with

suspected RICD defects, we recommend utilizing at least 2 separate controls activated and cultured in parallel. Other anti-CD3 antibodies will also induce RICD with variable potency (e.g., clone 64.1 > OKT3; clone HIT3a ≈ OKT3). In our hands, the addition of anti-CD28 Ab does not affect the results of the RICD assay.

8. The recommended dose range of OKT3 mAb (0.01–1 μg/ml) usually encompasses maximum RICD sensitivity in normal donor T cells, typically induced at ~100–200 ng/ml. Donor-dependent differences in RICD sensitivity are often more readily observed using lower doses of OKT3, which may necessitate the addition of lower dilutions (1–5 ng/ml) to the dose–response curve. Plate-bound anti-CD3 will produce a more robust restimulation signal and increased apoptosis at all doses—while this is optional for assessing RICD sensitivity in human cells, it is required for inducing apoptosis in mouse T cells.
9. The majority of RICD-sensitive cells will die by apoptosis (i.e., become PI^+) by ~18 h post restimulation. We routinely assay cells by PI exclusion ~20–24 h post restimulation with little change in % cell loss.
10. PI rapidly intercalates into the double-stranded DNA of apoptotic cells that have lost plasma membrane integrity. Similar intercalating dyes (e.g., 7-AAD) may be substituted for PI. Moreover, earlier markers of apoptosis (e.g., Annexin V staining, cleavage of caspase-3, loss of mitochondrial membrane potential) can be measured ~4–8 h post restimulation by flow cytometry using the same basic restimulation setup.
11. Samples are collected on constant time for proper calculation of % cell loss (see Note 12). We advise waiting 2–3 s after placing each tube on the flow cytometer before starting acquisition. This allows the flow rate to stabilize and improves consistency in collecting a similar number of events (>10,000 cells) per sample.
12. By counting the *number* of viable cells collected for the same amount of time from each sample, the % cell loss calculation accounts for cells that disintegrate after apoptosis and do not register as events on the flow cytometer. This calculation therefore provides greater sensitivity than direct measurement of % PI^+ dead cells, although % dead cells and % cell loss should be similar.

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