

Video Article

Functional Characterization of Regulatory Macrophages That Inhibit Graft-reactive Immunity

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Abstract

Macrophage accumulation in transplanted organs has long been recognized as a feature of allograft rejection¹. Immunogenic monocytes infiltrate the allograft early after transplantation, mount a graft reactive response against the transplanted organ, and initiate organ rejection². Recent data suggest that suppressive macrophages facilitate successful long-term transplantation³ and are required for the induction of transplantation tolerance⁴. This suggests a multidimensional concept of macrophage ontogeny, activation, and function, which demands a new roadmap for the isolation and analysis of macrophage function⁵. Due to the plasticity of macrophages, it is necessary to provide a methodology to isolate and characterize macrophages, depending on the tissue environment, and to define their functions according to different scenarios. Here, we describe a protocol for immune characterization of graft-infiltrating macrophages and the methods we used to functionally evaluate their capacity to inhibit CD8⁺ T proliferation and to promote CD4⁺Foxp3⁺ Treg expansion *in vitro*.

Video Link

The video component of this article can be found at <https://www.jove.com/video/54242/>

Introduction

This protocol describes *in vitro* techniques to study the function of tissue-infiltrating macrophages isolated from cardiac allografts, according to their ability to modulate T-cell responses. Widely described in the literature, fluorescent cell-tracking dyes in combination with flow cytometry, are powerful tools to study the suppressive function of specific cell types *in vitro* and *in vivo*. Our protocol follows the carboxyfluorescein succinimidyl ester (CFSE) method for monitoring lymphocyte proliferation *in vitro*.

When a CFSE-labeled cell divides, its progeny acquires half the number of carboxyfluorescein-tagged molecules⁶. The corresponding decrease in cell fluorescence by flow cytometry can be used to assess cell division, monitoring the capacity of suppressive macrophages to modulate T-cell immune responses. Since CFSE is a fluorescein-based dye, it is compatible with a broad range of other fluorochromes, making it applicable to multi-color flow cytometry. The appropriate choice of fluorochromes for phenotyping is also important to avoid excessive spectral overlap and the inability to recognize antibody-positive cells, particularly with visible protein dyes such as CFSE⁷.

There are many advantages of using fluorescent dyes over alternative techniques that measure cell proliferation, such as the thymidine incorporation assay, which uses radiolabeled thymidine (TdR)⁸. This assay utilizes tritiated thymidine (³H-TdR) that is incorporated into new strands of chromosomal DNA during mitotic cell division. A safety concern associated with this assay is the use of radioisotopes, since a scintillation beta-counter is used to measure the radioactivity in DNA recovered from the cells in order to determine the extent of cell division. Methodologically, the tritiated thymidine assay is not flexible enough to fit important clinical laboratory constraints such as low number of cells and delayed analysis after staining. On the contrary, CFSE staining has been shown to prevent cell proliferation and to interfere with critical activation markers, such as CD69, HLA-DR and CD25⁹. Therefore, understanding advantages and limitations of each methodology, particularly in multicolor studies where multiple dyes are used to track different cell types, is critical for obtaining accurate and reproducible results.

Protocol

In this study, mice are housed in accordance with the United States Department of Agriculture guidelines and the recommendations of the Public Health Service Guide for the Care and Use of Laboratory Animals. All experimental techniques involving animal use were performed in accordance with Institutional Animal Care and Utilization Committee (IACUC)-approved protocols of the Mount Sinai School of Medicine.

1. Media Preparation

1. Prepare complete RPMI 1640 medium with 10% FBS and 1% penicillin-streptomycin (10,000 U/mL) by using 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM non-essential amino acids, 5 mM HEPES, and 0.05 mM 2-mercaptoethanol.

2. Allograft Isolation and single-cell Suspension

NOTE: The transplantation and anastomosis technique of the pulmonary artery and inferior cava vein was initially described by Corry and collaborators and can be visualized in Liu & Kang^{10,11} (Figure 1A).

1. Anesthetize a transplanted mouse with 4-5% isoflurane in an induction chamber. Sacrifice it by cervical dislocation.
2. Make a midline abdominal incision with standard scissors and remove the abdominal contents to localize the aorta.
NOTE: The transplanted heart will be on the right side of the recipient abdominal aorta (Figure 1B).
3. Pull out the graft gently using fine sharp-teeth forceps and place it immediately into ice-cold RPMI medium.
NOTE: Using a surgery scissor to separate the graft from the aorta can damage the graft and cause some tissue to remain adhered to the recipient.
4. After all grafts have been collected, move them into a sterile culture hood for tissue processing. Place the graft in a petri dish and dice the tissue into small pieces (1 mm) using sterile, blunt, microsurgery scissors.
5. With sharp-teeth forceps, transfer the pieces to a 50 mL tube with 5 mL of collagenase A (0.1 mg/mL collagenase in sterile 1x PBS). Incubate it for 1 h in a 37 °C bath.
6. Add 5 mL of RPMI medium to neutralize the collagenase and transfer the sample through a 100 µm strainer with the help of the plunger of a 1 mL syringe.
7. Spin the sample down at 400 x g for 5 min at 4 °C.
8. Resuspend the pellet in 1 mL of ACK lysis buffer. Mix well and incubate for 5 min at 4 °C.
9. Add 1 mL of RPMI medium to neutralize the lysis buffer and spin the sample down at 400 x g for 5 min at 4 °C.
10. Resuspend the cell pellet in 200 µL of RPMI medium and transfer it to a 5 mL polypropylene round-bottom tube.
NOTE: Polypropylene tubes support less adherence. Also, maintaining the cells at low temperature, such as 4 °C, reduces adhesion.

3. Isolation of Graft-infiltrating Macrophages Using Fluorescence-activated Cell Sorting

1. Block unwanted specific binding on myeloid cells by using Fc receptor blocking mAb (rat anti-mouse CD16/32). Add 1-2 µL per sample, 15 min prior to surface staining.
2. Stain with anti-mouse CD11b Percp/Cy5.5 (0.6 µg/µL final concentration in the RPMI medium), anti-mouse CD45 APC/eFluor780 (0.6 µg/µL), anti-mouse Ly6C APC (2 µg/µL), and anti-mouse Ly6G Pe/Cy7 (2 µg/µL). Cover the tubes with aluminum foil to protect the fluorescent antibodies from light and incubate the tubes in the refrigerator at 4 °C for 45 min.
NOTE: For single-stain compensations, prepare a negative control tube (no stain) and tubes with cells labeled singly with each of the fluorochromes. To help set up the gate, isotype controls can be used especially for Ly6C (IgG2a) and Ly6G (IgG2b).
3. Wash the cells twice with RPMI medium and spin them at 400 x g for 5 min at 4 °C. Count the cells using trypan blue and a hemocytometer and dilute them in 1 mL of RPMI medium per 1×10^6 cells. Before sorting, transfer the samples through a 5 mL, 70 µm cell strainer tube cap. Add DAPI (1 µg/mL) as a cell viability marker and proceed to sort.
4. Because macrophages are sensitive and fragile cells, set the sort conditions to 20 psi and use a 100 µm nozzle size to isolate macrophages using a 4-way purity mode.
5. Prepare collection tubes with 1 mL of RPMI medium in a 5 mL polypropylene tube.
6. Using the sorter software open new experiment and select blank experiment with a blank panel to define settings.
7. **Set up a dot plot that displays the forward (FSC) versus (vs.) side scatter (SSC) and gate on all leukocytes, excluding debris and clumps with the lowest forward and side scatter. From this parent gate, create a new dot plot that displays SSC vs. DAPI and gate on DAPI cells.**
 1. On this newly-gated population, create a dot plot that displays CD11b vs. CD45 and gate on double-positive (CD11b⁺ CD45⁺) macrophages and neutrophils.
 2. From here, create a final dot plot displaying Ly6C vs. Ly6G and gate the desirable populations: Ly6C^{hi} Ly6G⁻, Ly6C^{lo} Ly6G⁻, and Ly6C^{int} Ly6G⁺ (Figure 2).
8. After sorting, check for purity and cell viability (>90%) and spin the collection tubes at 400 x g for 5 min at 4 °C. Count the cells using trypan blue and a hemocytometer and resuspend them at the desired concentration (e.g., 1×10^6 macrophages/mL) in complete RPMI medium.
9. Plate 50×10^3 macrophages per well in a 96-well round-bottom plate with a final volume of 100 µL of complete RPMI medium. Leave the cells undisturbed for at least 24 h at 37 °C and 5% CO₂.

4. Isolation of T Cells Using Fluorescence-activated Cell Sorting

NOTE: C57BL/6-Foxp3tm1Flv/Jn is an X-linked targeted knock-in mouse strain that co-marks cells expressing the Foxp3 (forkhead box P3) gene with monomeric red fluorescent protein (mRFP).

1. Anesthetize and sacrifice C57BL/6 and C57BL/6-Foxp3tm1Flv/J (H-2b) mice as previously described in step 2.1. Isolate the spleen and lymph nodes (LN: inguinal, lumbar, axillary, brachial, and cervical) and rapidly place them in ice-cold RPMI medium.
2. In order to isolate the LN, place the mouse in a supine position, make a midline skin incision from bottom to top, carefully cutting the peritoneum and gently spreading the skin apart. Once all inguinal, lumbar, axillary, brachial, and cervical LN are collected (Figure 1C, colored in red), cut the peritoneum and isolate the spleen at the upper left side of the gut.

3. Disaggregate the spleen and LN using a 100 μ m filter placed on top of a 50 mL tube. Using the plunger of a 1-mL syringe, gently press the tissue. Rinse the filter with RPMI medium as many times as necessary until it is clean.
NOTE: LN and spleen from each mouse can be pooled together in the same tube.
4. Spin down at 400 x g for 5 min at 4 °C.
5. Resuspend the pellet in 2 mL of ACK lysis buffer. Mix well and incubate for 5 min at 4 °C.
6. Add 2 mL of RPMI medium to neutralize the lysis buffer. Spin down at 400 x g for 5 min at 4 °C.
7. Resuspend the cell pellet in 200 μ L of RPMI medium and transfer it to a 5 mL polystyrene round-bottom tube.
8. Stain for anti-mouse CD4 APC (0.6 μ g/ μ L) and/or anti-mouse CD8 PeCy7 (2 μ g/ μ L). Cover the tubes with aluminum foil to protect the fluorescent antibodies from light, and incubate the tubes in the refrigerator at 4 °C for 45 min.
NOTE: For single-stain compensations, prepare a negative control tube (no stain) and tubes with cells labeled singly with each of the fluorochromes.
9. Wash the cells twice with RPMI medium and spin them at 400 x g for 5 min at 4 °C. Count the cells using trypan blue and a hemocytometer.
NOTE: CFSE dye is provided as carboxyfluorescein diacetate succinimidyl ester powder usually at 50 μ g. Add 18 μ L DMSO to a vial of CFSE for a final stock solution of 5 mM and store at -20 °C for several months.
10. For CFSE labeling, resuspend CD8 stained cells at a concentration of up to 10⁶ cells per mL in PBS at a final working concentration of 5 μ M CFSE from the stock solution. Incubate them in a bath at 20 °C for 5 min as previously described⁶.
NOTE: (CRITICAL STEP) For cells at a low concentration (\leq 10⁶ per mL), it is essential that the cells be labeled in the presence of added protein to buffer the toxic effects of CFSE. Therefore, PBS can contain 5% FBS. Note that if medium is used, free amino acids may lower the labeling efficiency by competing for CFSE.
11. Neutralize the CFSE with 2 mL of RPMI medium. Spin at 400 x g for 5 min at 4 °C. Add 1 mL of RPMI medium per 1 x 10⁶ CD8 T cells.
12. Before sorting, transfer the sample through a 70 μ m cell strainer on a 5 mL tube cap. Add 50 μ L of 1x DAPI (1 μ g/mL) as a cell viability marker and proceed to sorting.
NOTE: 1x means 1:1 ratio of the reagent with respect other constituent.
13. Set the sort conditions at 20 psi and 100 μ m nozzle size to isolate double-positive CD8⁺CFSE⁺ T cells (**Figure 3A**).
14. Prepare collection tubes with 1 mL of RPMI medium in a 5 mL polypropylene tube and proceed to sort.
15. Using the sorter software open new experiment and select blank experiment with a blank panel to define settings.
16. **For CD4⁺ T cell isolation, set up a dot plot that displays the FSC vs. SSC and gate on lymphocytes, excluding debris as well as large granular cells, such as dendritic cells.**
 1. From this parent gate, create a new dot plot that displays SSC vs. DAPI and gate on DAPI cells.
 2. On this newly-gated population, create a dot plot that displays SSC vs. CD4 to isolate all CD4⁺ cells (**Figure 3B**). Alternatively, an anti-CD3 mAb can be used to ensure isolation of pure T cell populations.
NOTE: A small fraction of all CD4⁺ T cells (5-10%) should be Foxp3⁺mRFP⁺.
17. After sorting, check for purity and cell viability (>90%) and spin the collection tubes at 400 x g for 5 min at 4 °C. Count the cells using trypan blue and a hemocytometer. Resuspend using 1 mL of complete RPMI medium per 2x10⁶ T cells.
18. Set up suppression assay by culturing T cells with previously-sorted macrophages. Plate 10 x 10⁴ CD4⁺ T and 10 x 10⁴ CD8⁺CFSE⁺ T cells in the same well in a final volume of 100 μ L of complete RPMI medium. Incubate at 37 °C and 5% CO₂ for 96 h.
NOTE: Macrophages and T cells were used in a 1:4 ratio.
19. Stimulate T cell division by washing T cell-activator CD3/CD28 magnetic beads in 2 mL of PBS before using them in order to clear the azide-containing buffer. Add 5 μ L of mouse T cell-activator CD3/CD28 beads per well.
NOTE: Magnetics beads are similar in size to antigen-presenting cells and are coupled to anti-CD3 and anti-CD28 mAb, offering a simple method for the activation and expansion of T cells.

Representative Results

The representative results show the gating strategy described in the above protocol. Results also display the analysis of the T-cell proliferation activity after the co-culture with graft-infiltrating macrophages. The *in vitro* suppressive capacity of macrophage subsets was analyzed in **Figure 4**. The results indicate that the Ly6C^{lo}Ly6G⁻ macrophages obtained from tolerized recipients are suppressive. The results also indicate that Ly6C^{int}Ly6G⁺ cells display a modest suppressive capacity. Only Ly6C^{lo}Ly6G⁻ cells promoted the expansion of CD4⁺Foxp3⁺ Treg *in vitro*. Together, the data support the conclusion that graft-infiltrating CD11b⁺Ly6C^{lo}Ly6G⁻ macrophages possess many of the properties reported to be associated with monocyte-derived suppressor cells¹², including their ability to inhibit CD8 T-cell proliferation¹³ and to promote CD4⁺Foxp3⁺ Treg expansion¹⁴.

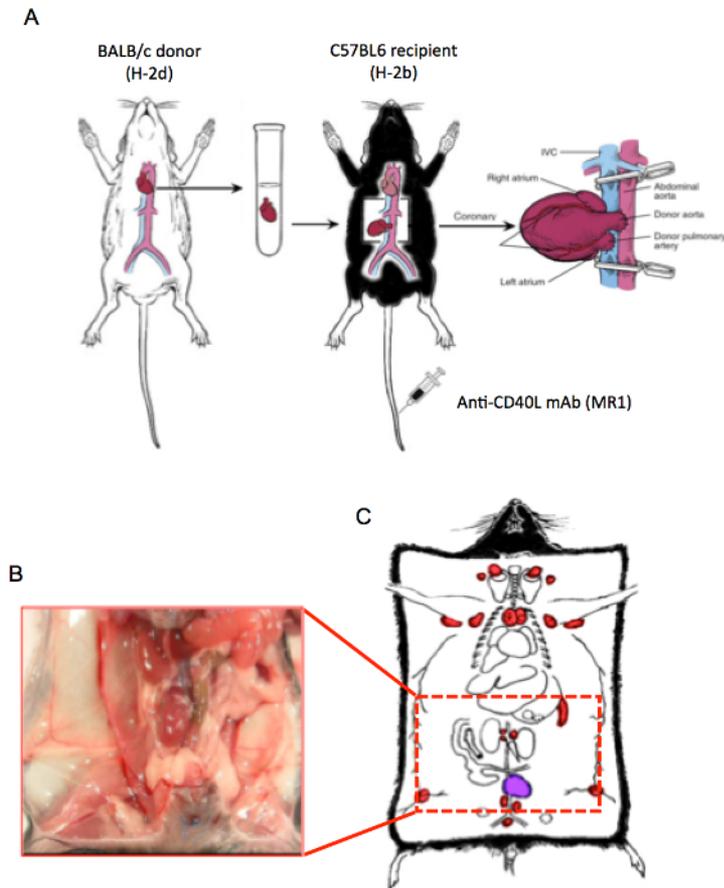


Figure 1: Animal model. (A) Balb/c hearts (H2-d) were transplanted into fully allogeneic C57BL/6 (H2-b) as as previously described¹⁰. Anastomosis, of the recipient's cava vein and abdominal aorta with the donor's pulmonary artery and ascending aorta respectively, is shown. Recipient mice were treated with 250 μ g of anti-CD40L mAb (clone MR1) for tolerance induction on days 0, 2, and 4, as we recently reported⁴. Graft function was monitored every other day by abdominal palpation. Rejection was defined as complete cessation of a palpable heartbeat and was confirmed by direct visualization at laparotomy. (B) Representative Image and (C) illustration of anatomical location of LN and spleen (colored in red) and the allograft (colored in purple) [Please click here to view a larger version of this figure.](#)

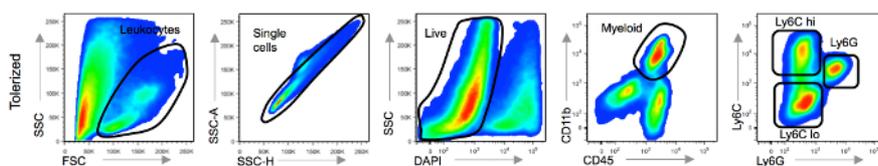


Figure 2: Macrophage sorting strategy. Starting from the upper left, leukocytes are first gated by size, and then singlet cells are discriminated from debris and clumps. From singlets, dead cells are excluded gating on the DAPI negative fraction. From live cells, $CD45^+CD11b^+$ is used to identify myeloid cells. Three myeloid cells populations are further identified based on Ly6C and Ly6G expression. [Please click here to view a larger version of this figure.](#)

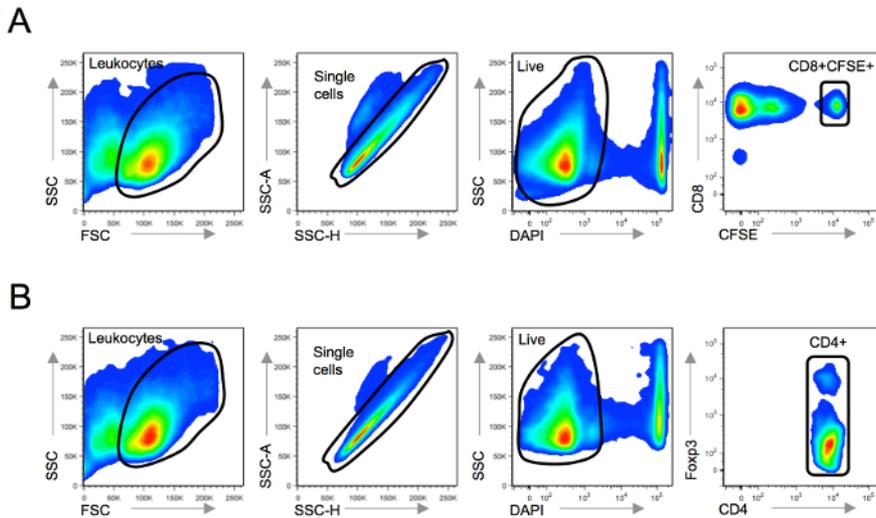


Figure 3: Lymphocyte sorting strategy. Representative flow cytometry results for the lymphocyte sorting strategy. From the upper left, gate on the lymphocyte population according to forward and side scatter. Exclude debris and clumps. From singlets, dead cells are excluded by gating on the DAPI negative fraction. From live cells, (A) CD8⁺ CFSE⁺ double-positive cells and (B) all CD4⁺ T cells, which contain a fraction of Fopx3⁺ positive cells, are gated. [Please click here to view a larger version of this figure.](#)

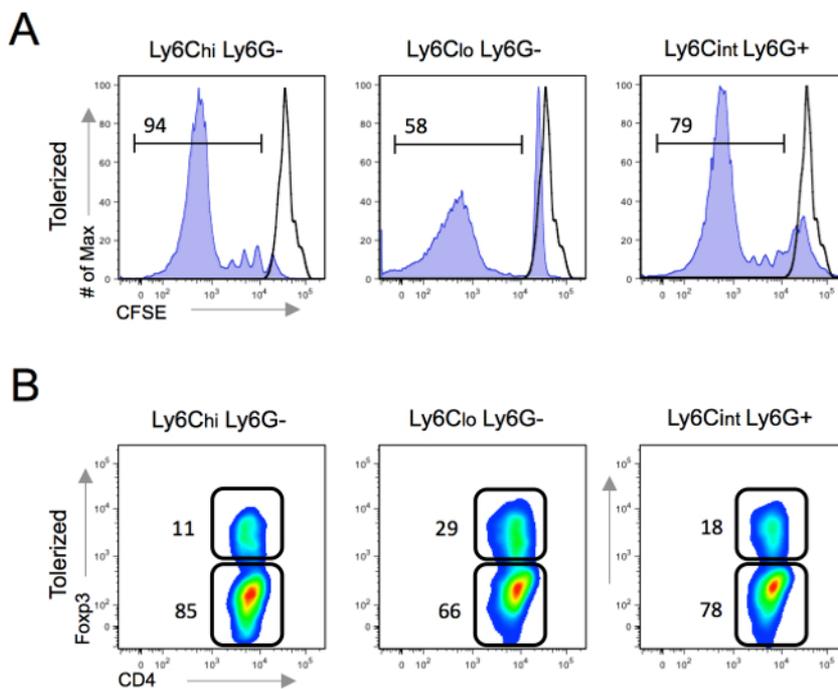


Figure 4: Suppression assay. (A) *In vitro* suppressive capacity of each myeloid subset. CD8⁺ T-cell proliferation was monitored by CFSE dilution after 96 h of co-culture with myeloid subsets. (B) *In vitro* Treg expansion of each myeloid subset. Flow cytometry analysis of Fopx3 expression on CD4⁺ T cells after 96 h of co-culture with myeloid subsets. [Please click here to view a larger version of this figure.](#)

Discussion

This protocol describes the methods we used to immun characterize graft-infiltrating myeloid cell subsets in an experimental murine model of heart transplantation, which is also applicable to other tissues in different murine experimental models. Low-pressure cell sorting at 20 psi was the preferred method to isolate a good yield of pure cell subsets. Maintaining the purity of each myeloid subset is critical to establish conclusive results of the suppressive capacity between the different myeloid populations. However, other methods can be used for the isolation of various leukocyte populations, such as commercial enrichment kits. Regardless of the cell population of interest, obtaining viable single-cell suspensions from the transplanted tissue is required for optimal surface staining and flow cytometry results. Incorrect tissue manipulation may cause cell loss and therefore a low yield of myeloid subsets. To increase the yield, make sure to process the sample using cold buffers and to maintain the cells in containers with low cell adherence (polypropylene tubes). In this protocol, we used Collagenase A from *C. histolyticum*, which is widely used for the disaggregation of many types of tissues (e.g., lung, heart, muscle, bone, adipose, liver, kidney, cartilage, mammary gland, placenta, blood

vessel, brain, and tumor). To prevent the loss of cells while sorting, optimizing the antibodies and generating an efficient gating strategy is highly recommended. In this protocol, we characterized murine macrophages using CD11b-Percp/Cy5.5, CD45-APC/eFluor780, Ly6C/APC, and Ly6G Pe/Cy7 fluorescent-conjugated antibodies and flow cytometry. Since the epitopes Ly6C/G do not exist in human, the use of CD14/CD15/CD16 is required for sorting human myeloid cells¹⁵.

In multicolor flow cytometry analysis, possible spectral overlaps of the chosen fluorochromes may occur. Therefore, as noted before, single-stain compensations are very helpful to avoid overlapping issues. In addition, antibody titration is highly recommended in order to reduce the background fluorescence and to get optimal results. Another important consideration to reduce non-specific binding is the use of viability dyes. In this protocol, DAPI is used as viability dye. DAPI (4', 6-diamidino-2-phenylindole) is a blue fluorescent stain that binds strongly to AT-rich regions in DNA. The excitation maximum for DAPI bound to DNA is 358 nm, and the emission maximum is 461 nm. When used according to protocol, DAPI stains nuclei specifically, with little or no cytoplasmic labeling, excluding dead cells in the flow analysis. However, depending on the panel of antibodies chosen, other viability dyes, such as 7-Aminoactinomycin D (7-AAD) and propidium iodide (PI), may be used. Accurate dead-cell exclusion and live-cell identification is a prerequisite to successfully monitor *in vitro* T cells, as it is important to have procedures that can follow lymphocyte proliferation with minimal disruption to cell viability and function.

As mentioned above, there are critical steps for cell labeling and proliferation analyses with cell tracking dyes such as CFSE. As an example described in the literature, careful attention must be paid to the exclusion of dead/dying cells in order to obtain uniform distributions and distinguishable daughter peaks when using CFSE¹⁶. There are many commercially available cell tracking dyes to choose from, depending on the staining panels. As an alternative to cell tracking dyes, thymidine titrated assay are performed to evaluate the proliferation of lymphocytes and other cells in cancer studies^{13,14}. Thymidine incorporation protocols assess the replication of DNA during cell division by the measure of radiolabeled 3H- or 14C- thymidine. Although this method is quite sensitive and well established, the major disadvantages for thymidine titrated technique is that it involves radioactivity and does not provide information at the single cell level. In the other hand, CFSE flow cytometry analysis provides clear information about responding lymphocyte subsets and has less inter and intralaboratory variability.

Disclosures

The authors have nothing to disclose.

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