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Analyses of Programmed Cell Death in Dendritic Cells

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1. Introduction

Dendritic cells (DCs) are the most efficient antigen presenting cells that capture, process and present antigens for the activation of lymphocytes. After differentiation, DCs lose the proliferation potential and undergo high rates of cell death *in vivo*. Programmed cell death of DCs plays an important role in the maintenance of DC homeostasis. Defective cell death in DCs leads to abnormal DC accumulation, uncontrolled activation of lymphocytes and disruption of immune tolerance (1, 2). Here we describe the methods for determining the rates of cell death of DCs in mice by different *in vitro* and *in vivo*, including measuring spontaneous and T cell-induced cell death of DCs, estimating the rates of clearance of DCs *in vivo* in the absence or presence of antigen-specific T cells by adoptive transfer, as well as determining the rates of half-life of resident DCs in lymphoid organs by BrdU labeling (3-5).

2. Materials

1. Liberase TL research grade (Roche, cat# 05401020001): Dissolve 5 mg in 2 ml ddH₂O, aliquot and store at -20°C. This solution is stable for 2-3 months. Dilute to 0.32 mg/ml in serum free medium prior to use.
2. Streptavidin-conjugated Biomag beads (Qiagen, cat# 311714).
3. MACS beads conjugated to anti-PDCA-1, anti-CD11c, anti-CD8 or anti-CD4 (Miltenyi Biotec).
4. Biotinylated antibody against CD3, CD4, CD8, I-A^b, B220, Thy1.2, CD19, IgM, CD49b or TER119 (BD Biosciences).
5. Magnetic Stand (BD Biosciences).
6. LS column (Miltenyi Biotec).
7. 40 micron strainer and 5 ml syringe (Becton Dickinson)
8. ACK lysis buffer: 0.15 M NH₄Cl, 1 mM KHCO₃, 0.1 mM EDTA, pH 7.2
9. PBS-EDTA buffer: PBS containing 2% FCS and 2 mM EDTA
10. Fc blocker: 1 µg/ml anti-CD16/CD32 (BD Biosciences) plus 10 µg/ml rat IgG (Sigma).
11. 25-gauge needles (BD Biosciences)
12. Cytokines: mouse GM-CSF, mouse IL-4 (Invitrogen).

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13. DC media: RPMI 1640 medium containing 10%FBS 10 ng/ml of mouse GM-CSF and IL-4 and antibiotics.
14. Lympholyte-M cell separation medium (Ficoll, Accurate Chemical, cat# CL5035).
15. Mice: OT1 transgenic mice (Jackson Laboratory) carrying TCR transgenes specific for ovalbumin (OVA) peptide SIINFEKL presented by K^b; OT2 transgenic mice (Jackson Laboratory) carrying TCR specific for OVA₃₂₃₋₃₃₉ peptide presented by I-A^b; 6-8 weeks old C57BL6 mice for DC preparation if not specified.
16. Ovalbumin (OVA) peptides: SIINFEKL peptide and OVA₃₂₃₋₃₃₉ peptide.
17. Carboxyfluorescein succinimidyl ester (CFSE, Invitrogen, cat# C34554). Dissolve CFSE in DMSO to make 5 mM stock, aliquot and freeze at -20°C.
18. 7-Amino-Actinomycin D (7-AAD) staining solution (BD Biosciences, cat# 559925).
19. 10x Annexin V Binding Buffer: 0.1M HEPES, pH7.4; 1.4M NaCl; 25mM CaCl₂. Dilute to 1x prior to use.
20. FITC-Annexin V (BD Biosciences, cat# 556419)
21. Propidium iodide (PI) solution (200 µg/ml): dissolve 10 mg PI (Sigma P1470-25 mg) in 50 ml ddH₂O.
22. BrdU (Sigma B5002-500mg): dissolve in PBS to obtain 10 mg/ml or 0.8 mg/ml solution.
23. FITC-anti-BrdU Flow kit (BD Biosciences, cat# 559619).
24. Staining buffer: PBS with 2% FCS and 0.01% sodium azide.
25. Blocking buffer: staining buffer +10 µg/ml Rat IgG +1 µg/ml anti-CD16/32.
26. PE conjugated anti-PDCA-1 (Miltenyi Biotec).
27. PE-anti-CD11c, PE-Cy5-anti-CD8 and APC-anti-CD4, APC-anti-CD11c and PE-anti-CD11b (BD Biosciences).
28. Flow cytometer, e.g. LSRII.

3. Methods

3.1 Purification of spleen DC subsets from mice

1. Liberase treatment: inject 1 ml (0.32 mg/ml) liberase per mouse spleen. Then, cut the spleens into small pieces. Incubate at room temperature for 10-15 min.
2. Make single cell suspension by using a cell strainer and pressing with a 5 ml syringe plunger. Add 10 x volume of PBS containing 2% FCS. Harvest cells into a tube and pellet the cells by centrifugation.
3. Resuspend cells in ACK lysis buffer and leave the cells at room temperature for 2 min, Stop by adding 10x volume of PBS containing 2% FCS and pellet the cells by centrifugation.
4. Wash the cells with PBS containing 2% FCS. Pass the cells through a 40 µm strainer. Take an aliquot for cell counting. Pellet the cells by centrifugation.
5. Resuspend cell pellet in PBS-EDTA buffer (10⁸ cells/ml)
6. Incubate with anti-CD16/32 (1 µg/ml) and rat IgG (10 µg/ml) on ice for 10 min.

7. Add 1 $\mu\text{g/ml}$ biotinylated antibodies against CD3, Thy1.2, CD19, IgM, CD49b and TER119 and incubate the cells on ice for 30 min.
8. Wash Streptavidin-Biomag beads 3 times during antibody incubation. Use 750 μl beads/ 10^9 cells in 8 ml PBS-EDTA buffer.
9. Wash cells once by adding 20x volume of buffer, centrifuge for 5 min.
10. Resuspend cells in PBS-EDTA buffer (10^8 cells/ml). Add to Streptavidin-Biomag beads. Incubate at 4-8 $^{\circ}\text{C}$ for 30 min. Invert every 10 min to mix.
11. Remove cells bound to magnetic beads on the magnetic stand (see Note 1). Transfer cells unbound to the magnetic beads to a new tube. Repeat the same step 3x. Pellet cells by centrifugation.
12. Equilibrate a MACS LS column with 3 ml PBS-EDTA buffer. Resuspend cells in 3 ml PBS-EDTA buffer and pass the cells through the LS column. Wash column once with 3 ml PBS-EDTA buffer. Collect the flow-through fraction. Pellet cells by centrifugation.
13. Resuspend the cells (10^8) in 400 μl PBS-EDTA buffer. Incubate with Fc blocker and 50 μl anti-PDCA-1 MACS beads (for up to 20 spleens) at 6-12 $^{\circ}\text{C}$ for 15 min. Wash cells with 20x volume of buffer. Centrifuge for 5 min.
14. Resuspend the cells in 3 ml PBS-EDTA buffer. Pass cells through a pre-equilibrated LS column. Wash the column 3x with 3 ml PBS-EDTA buffer. Collect the flow-through fraction and the first 2 washes for the next step. Elute PDCA-1⁺ plasmacytoid DCs (pDCs) from the column in 5ml PBS-EDTA buffer into a separate tube. Pellet pDCs by centrifugation.
15. Spin down flow-through fraction. Resuspend cells in 400 μl PBS-EDTA buffer and incubate with the Fc blocker and 100 μl anti-CD11c MACS beads (for up to 20 spleens) at 6-12 $^{\circ}\text{C}$ for 15 min. Wash cells with 20x volume of buffer.
16. Equilibrate a MACS LS column with 3 ml PBS-EDTA buffer. Resuspend cells in 3 ml buffer. Pass the cells through the LS column. Wash column 3x with 3 ml PBS-EDTA buffer. Discard the flow-through fraction. Elute CD11c⁺ DCs from the column with 5 ml PBS-EDTA buffer. Pellet DCs cells by centrifugation.
17. Resuspend each DC subset in appropriate RPMI complete medium and count the cells.

3.2. Generation of bone marrow-derived DCs

1. Collect two femurs and two tibias from each mouse (Mice of 5-8 weeks of age give better yield).
2. Clean the bones with scissors to remove as much muscle as possible. Place all bones from the same mouse in 15 ml tube with PBS and keep on ice.
3. Move to the tissue culture hood. Aspirate PBS from tube and immerse intact bones in 70% ethanol for 2 min and then wash 3x with PBS in the 15 ml tube.
4. Add 10 ml RPMI media, pour all the bones and media out in a 60 mm dish

¹Because DCs represents a small population of cell types *in vivo*, the purity of DCs isolated directly from lymphoid organs using magnetic beads depends on negative selection of other cell types. We have found that direct isolation of CD11c⁺ DCs using anti-CD11c MACS-beads without negative selection yields DC with approximately 50% purity. After negative selection, >95% pure CD11c⁺ DCs can be isolated using anti-CD11c MACS-beads.

5. Clip head of bone from shaft, saving the shaft in one dish with RPMI and the heads on the dish cover. Harvest bone marrow from shafts by flushing with 25" gauge needle and a 10 ml syringe. Break up bone marrow harvested from shafts by passing through a 19-gauge needle. Harvest bone marrow from bone heads by crushing with a syringe plunger. Transfer all the cells to a clean 15 ml tube.
6. Pellet cells and deplete red blood cells by resuspending in ACK lysis buffer (0.5 ml for cells from each mouse) for 2 min at room temperature. Stop by adding 10x volume of PBS containing 2%FCS. Pellet cells by centrifugation.
7. Resuspend the cells in PBS containing 2%FCS. Pass cells through a cell strainer into a 50 ml conical tube. Count the cells and pellet cells. Typical yield is around $10\text{--}30 \times 10^6$ cells per mouse.
8. Blocking: resuspend cells at 1×10^8 /ml in PBS-EDTA buffer. Block with anti-CD16/32 (1 μ g/ml) and rat IgG (10 μ g/ml) on ice for 10 min.
9. Antibody incubation: incubate cells with biotinylated antibodies (1 μ g/ml) against CD4, CD8, Thy1.2 (for removing T cells), biotinylated anti-I-A^b (to remove MHC Class II containing cells, e.g. B cells and macrophages), and biotinylated CD45R/B220 (to remove B cells) on ice for 30 min. Flick the cells every 15 min.
10. Add 20x volume of PBS-EDTA buffer to cell suspension and then pellet the cells. Discard the supernatant.
11. Wash Streptavidin-Biomag beads 3x at the same time. Use 75 μ l beads/ 10^8 cells.
12. Resuspend cells in PBS-EDTA buffer (10^8 cells/ml). Add to Streptavidin-Biomag beads. Incubate at 4-8 °C for 30 min. Invert every 10 min to mix.
13. Remove the cells bound to magnetic beads on the magnetic stand. Transfer unbound cells to a new tube. Repeat the same step 3 times. Then, pass the cells through the strainer the last time. Pellet cells by centrifugation.
14. Resuspend the cells at 3×10^6 /ml in DC media.
15. Culture the cells in 6-well plates (3 ml/well) or in 24 well plates (1.5 ml/well).
16. Feed the cells every two or three days by aspirating half of the media off the top of each well without disturbing the cells at the bottom, and replace with fresh DC media.
17. DCs should be used between days 6-8 (see Note 2).

3.3 T cell-mediated lysis of DCs

Activation of antigen-specific CD4⁺ T cells (see Note 3).

1. Add 5 ml PBS containing 2% FCS into one well of 6-well plate. Collect spleens and lymph nodes (LNs) from each mouse. Make single cell suspension by using a cell strainer and pressing with a 5 ml syringe plunger. Harvest cells into a 15 ml tube. Pellet the cells by centrifugation.

²Culturing monocytes from the bone marrow generally yield a 70-80% CD11c⁺ DCs. Further enrichment with anti-CD11c-MACS beads is important in certain experiments to achieve over 95% CD11c⁺ DCs.

³To assay T cell-mediated killing of DCs *in vitro*, T cells need to be activated in advance. In addition, the killing by CD4⁺ or CD8⁺ T effector cells is antigen-dependent. It is important to pulse DCs with appropriate antigens before using them as target cells in the killing assays.

2. Resuspend cells in ACK lysis buffer and leave at room temperature for 2 min, Stop by adding 10 times volume of PBS containing 2% FCS and pellet the cells by centrifugation.
3. Discard the supernatant. Wash the cells with PBS containing 2% FCS. Pass cells through a 40 μ m strainer. Take an aliquot for counting. Pellet the cells by centrifugation.
4. Resuspend cell pellet at 2×10^8 cells/ml in PBS-EDTA buffer. Incubate with anti-CD16/32 (1 μ g/ml) and rat IgG (10 μ g/ml) on ice for 10 min.
5. Incubate with 50 μ l anti-CD4 MACS beads/ 10^8 cells at 6-12°C for 15 min. Wash cells 1x with 20x volume of PBS-EDTA buffer. Centrifuge for 5 min.
6. Equilibrate a MACS LS column with 3 ml PBS-EDTA buffer. Resuspend cell pellets in 3 ml PBS-EDTA buffer and pass cells through the LS column. Wash column 3x with 3ml PBS-EDTA buffer. Discard the flow-through fraction. Elute the column-bound CD4⁺ cells using 5 ml PBS-EDTA buffer. Pellet cells by centrifugation.
7. Resuspend CD4⁺ cells in RPMI complete medium. Count the cells.
8. Stimulate CD4⁺ OT2 T cells (10^6 /ml) with DCs (10^5 /ml) pulsed with OVA₃₂₃₋₃₃₉ peptide (see the following protocol: Pulse DCs with antigenic peptide) for 4 days.

Activation of antigen-specific CD8⁺ T cells (*see* Note 3).

1. Purify CD8⁺ cells from OT1 mice using the same procedures as those used for CD4⁺ cell purification except that anti-CD8 MACS beads will be used in step 5.
 2. Activate CD8⁺ OT1 T cells (10^6 /ml) by stimulating with DCs (10^5 /ml) pulsed with OVA_{SINFEKL} for 4 days.
2. Pulse DCs with antigenic peptide (*see* Note 3).
1. Harvest day 7 culture of bone marrow-derived DCs. Pipetting up and down gently to collect loosely attached cells. Wash the plate once with 5 ml PBS. Combine the cells together and centrifuge for 5 min.
 2. Ficoll-gradient separation: Resuspend cell pellet in 5 ml PBS-EDTA buffer in a 15 ml tube. Add 2 ml Ficoll to the bottom of the tube slowly without disturbing the cell suspension. Centrifuge at room temperature at 2000rpm for 15 min. Transfer the live cells at the interphase to a new 15 ml tube. Fill the tube with PBS to 15ml and spin for 5 min.
 3. Wash cells once with 5 ml PBS-EDTA buffer and centrifuge for 5 min.
 4. Blocking: resuspend the cells (10^8) in 450 μ l Buffer, blocking with 1 μ g/ml Fc blocker and 10 μ g/ml rat IgG for 10 min on ice.
 5. MACS beads binding: Add 50 μ l anti-CD11c MACS beads, incubate at 6-12°C for 15 min. Wash cells once with 20x volume of PBS-EDTA buffer. Centrifuge 5 min and resuspend cell pellet in 3 ml Buffer.
 6. Equilibrate a MACS LS column with 3ml PBS-EDTA buffer. Pass cells through the column. Wash column 3 times with 3 ml PBS-EDTA buffer each. Discard the flow-through fraction. Elute CD11c⁺ cells from the column using 5 ml buffer and pellet the cells by centrifugation.
 7. Resuspend the cells in the DC medium. Count the cells and adjust DCs to 2×10^6 cells/ml.

8. Incubate DCs with 20 $\mu\text{g/ml}$ OVA peptide at 37°C for 2-3 hrs. Mix gently every 30 min.
9. Pellet DCs by centrifugation. Wash cells once with 20x volume of PBS-EDTA buffer. Centrifuge again.
10. Resuspend the cells in 3 ml RPMI complete media. Count the cells and adjust DCs to 2×10^6 cells/ml.

Harvesting activated antigen specific T cells

1. Harvest mixtures of T cells and DCs into a 15 ml tube and centrifuge for 5 min. Discard the supernatant and resuspend the cell pellet in 5 ml PBS-EDTA buffer.
2. Add 2 ml Ficoll to the bottom of the tube slowly without disturbing the cell suspension. Centrifuge at room temperature at 2000 rpm for 15 min. Transfer live cells to a new 15 ml tube. Fill the tube with PBS to 15ml and centrifuge for 5 min.
3. Wash cells once with 5 ml PBS-EDTA buffer and centrifuge for 5 min.
4. Resuspend the cell pellet in 450 μl PBS-EDTA buffer (10^8 cells). Add 50 μl anti-CD11c MACS beads and incubate at 6-12°C for 15 min. Wash cells once with 20x volume of PBS-EDTA buffer. Centrifuge for 5 min and resuspend the cell pellet in 3 ml Buffer.
5. Equilibrate a MACS LS column with 3 ml PBS-EDTA buffer. Pass the cells through the column. Save the flow-through fraction that contains the activated T cells. Wash column 3 times with 3 ml PBS-EDTA buffer each. Combine the flow-through fraction. Centrifuge for 5 min.
6. Resuspend cell pellet in RPMI complete medium. Count cells and adjust to $2 \times 10^6/\text{ml}$.

Set up T cell killing of DC assay

1. Take an aliquot of antigen-pulsed DCs and pellet the cells by centrifugation. Wash cell pellet once with 5 ml PBS.
2. Resuspend DC pellet in PBS at $2 \times 10^6/\text{ml}$. Add CFSE to final concentration of 2 μM and incubate at 37°C for 10 min.
3. Stop CFSE labeling by adding an equal volume of pre-warmed adult serum and incubate cells at 37°C for 10 min for efflux.
4. Centrifuge for 5 min at 200-300g and discard the supernatant. Wash cells 3 times with PBS containing 2% FCS.
5. Resuspend CFSE-labeled DCs at $2 \times 10^5/\text{ml}$ in RPMI complete medium.
6. Aliquot 100 μl (2×10^4) of DCs per well of 96-well U-bottom plates.
7. Adjust activated T cells to $2 \times 10^6/\text{ml}$, $6 \times 10^5/\text{ml}$ and $2 \times 10^5/\text{ml}$.
8. Set up killing assay with activated T cells and bone marrow derived DCs at T:DC ratios of 0:1, 1:1, 3:1 or 10:1. Total volume in each well is 200 μl . Centrifuge for 5 min.
9. Incubate at 37°C for 5 h.
10. Collect cells into 5 ml Falcon tubes (Becton Dickinson).

11. Add 5 μ l (0.25 μ g) 7-AAD per tube. Incubate for 10 min at room temperature. Collect cells at a constant flow rate on a flow cytometer for 30 seconds per sample (Figure 1).
12. Percentage of cell loss will be calculated as follows: percentages of killing of DCs by T cells = $100\% \times (\text{DC}_{\text{control}} - \text{DC}_{\text{T}}) / \text{DC}_{\text{control}}$, with $\text{DC}_{\text{control}}$ and DC_{T} representing CFSE⁺7-AAD⁻ DCs in the absence or presence of T cells, respectively.

3.4. Clearance of DCs after adoptive transfer

1. Inject purified CD8⁺ OT1 T cells into mice retroorbitally (2×10^6 /mouse). Plan to inject enough mice so there are at least 3 mice per group per time point.
2. One day later, prepare unpulsed and OVA_{SIINFEKL}-pulsed DCs.
3. Resuspend DCs in PBS (2×10^6 /ml). Add CFSE to final concentration of 5 μ M and incubate at 37°C for 10 min.
4. Stop CFSE labeling by adding an equal volume of pre-warmed adult serum and incubate cells at 37°C for 10 min for efflux.
5. Centrifuge for 5 min and discard the supernatant. Wash the cells 3x with PBS.
6. Resuspend CFSE-labeled DCs at 2×10^7 /ml in PBS. Inject mice with 50 μ l (1×10^6) DCs at the footpad.
7. Harvest draining popliteal lymph node on days 1, 2, 5 after DC injection. Treat lymph node with 0.32 mg/ml liberase at room temperature for 10 min.
8. Make single cell suspension by using a cell strainer and pressing with a 5 ml syringe plunger. Add 10 x volume of PBS containing 2% FCS. Pass the cells through the same strainer again. Count the cells.
9. Aliquot 2.5×10^6 cells to 5 ml tubes and pellet by centrifugation. Resuspend pellet in 50 μ l of blocking buffer and incubate on ice for 10 min.
10. Prepare antibody cocktail specific for PE-conjugated anti-CD11c (1:100) in blocking buffer. Add 50 μ l antibody cocktail per tube and mix well. Incubate cells with antibodies on ice for 20 min.
11. Wash cells 1x by adding 2 ml of staining buffer (PBS containing 2% FCS and 1 mM EDTA) per tube. Centrifuge for 5 min at 200-300g and discard the supernatant.
12. Resuspend the cells in 0.5 ml staining buffer per tube and analyze CD11c⁺CFSE⁺ cells on a flow cytometer. Quantify the total number of CD11c⁺CFSE⁺ DCs in the draining lymph node of each mouse.
13. Alternatively, inject purified CD4⁺ OT2 T cells into mice retroorbitally (2×10^6 /mouse) to analyze the effect of CD4⁺ T cells on DC survival. One day later, label DCs pulsed with OVA₃₂₃₋₃₃₉ with CFSE and inject the cells into mice at the footpad similarly as above. Quantify the total number of CD11c⁺CFSE⁺ DCs in the draining lymph node of each mouse as above.

3.5. Spontaneous cell death in DCs

1. Resuspend DCs at 5×10^5 /ml in RPMI complete medium. Add 100 μ l DCs (5×10^4 /well) to 96-well round-bottom tissue culture plates.
2. Twenty hours after culture, harvest cells to a 5 ml tube. Wash cells 1x by adding 1ml PBS per tube, centrifuge for 5 min at 200-300g, and discard supernatant.

3. Wash cells 1x with 1 ml Annexin V Binding Buffer, centrifuge for 5 min, and decant the supernatant by quickly inverting the tube and patting on the paper towel.
4. Resuspend cells in 30 μ l Annexin V Binding Buffer (staining buffer plus 1 mM CaCl_2). Add 2 μ l FITC-anti-Annexin V per sample, gently mix the cells and incubate the cells in the dark for 10 min at RT.
5. Add 1-2 μ l PI solution (200 μ g/ml) and incubate at room temperature for another 5 min. The optimal amount of PI may vary depending on the experimental systems.
6. Add 400 μ l of 1x Annexin Binding Buffer to each tube. Analyze by flow cytometry as soon as possible (within 1 h). Gate on annexin V⁻ live cells and calculate percentage of loss of live cells.

3.6. Measurement of DC turnover *in vivo* (see Notes 4)

1. Inject mice intraperitoneally with 100 μ l of 10 mg/ml BrdU solution per mouse. Feed mice with 0.8 mg/ml of BrdU drinking water.
2. At the desired time point, sacrifice the labeled mice. Isolate the spleen and inject 1 ml liberase (0.32 mg/ml) per spleen. Cut the spleens into small pieces and incubate at room temperature for 10-15 min. Isolate spleen from unlabelled mice as control.
3. Make single cell suspension by using a cell strainer and pressing with a 5 ml syringe plunger. Add 10x volume of PBS containing 2% FCS. Harvest cells into a tube and pellet the cells by centrifugation.
4. Resuspend cell pellet in ACK lysis buffer and leave at room temperature for 2 min. Stop by adding 10 times volume of PBS containing 2% FCS and pellet the cells by centrifugation.
5. Wash the cells with PBS containing 2% FCS. Pass cells through a 40 μ m strainer. Take an aliquot for cell counting. Pellet the cells by centrifugation.
6. Resuspend cell pellet in PBS-EDTA buffer (10^8 cells/ml).
7. Incubate with anti-CD16/32 (1 μ g/ml) and rat IgG (10 μ g/ml) on ice for 10 min.
8. Incubate with 1 μ g/ml biotinylated antibodies against CD3, Thy1.2, CD19, and TER119 on ice for 30 min.
9. Wash Streptavidin-Biomag beads 3x during antibody incubation. Use 750 μ l beads/ 10^9 cells in 8 ml PBS-EDTA buffer.
10. Wash cells once by adding 20x volume of buffer, centrifuge for 5 min.
11. Resuspend cells in PBS-EDTA buffer (10^8 cells/ml). Add to Streptavidin-Biomag beads. Incubate at 4-8 $^{\circ}\text{C}$ for 30 min. Invert every 10 min to mix.
12. Remove magnetic beads on the magnetic stand. Transfer cells unbound to the magnetic beads to a new tube. Repeat the same step 3 times. Pellet cells by centrifugation (see Note 4).

⁴The half-life for DCs estimated by *in vivo* BrdU labeling is about 2 days for CD11c⁺CD11b⁺ myeloid DCs and 7-8 days for CD11c^{low}PDCA-1⁺ plasmacytoid DCs (Figure 2). However, DCs undergo much faster turnover *in vitro* (5). This suggests that local microenvironment plays a major role in regulating DC lifespan *in vivo*. Because different DC subsets represent small fractions of cell population, it is necessary to enrich DCs first by depleting other cell types before staining with anti-BrdU. It will be important to study cell death of DCs in different settings of immune responses, such as during an infections. The effects of various receptors on DCs, and cytokines and chemokines produced in the microenvironment may affect the lifespan of DCs at various stages of immune responses. The interplays between different cell types and soluble factors in regulating programmed cell death in DCs will be an important area for investigation.

13. Count cells. Use 1×10^6 cells to continue staining cell surface antigens followed by intracellular staining of BrdU.
14. Cell surface staining:
 1. Add 1×10^6 BrdU-labeled cells in 50 μ l of blocking buffer to flow cytometry tubes, incubate on ice for 10 min.
 2. Prepare fluorescent antibody cocktail specific for (a) anti-CD11c-PE (1:100) and anti-CD8-PE-Cy5 (1:150) and anti-CD4-APC (1:100) or (b) anti-CD11c-APC and anti-CD11b-PE or (c) anti-CD11c-APC (1:100) and anti-PDCA-PE (1:10) in blocking buffer. Add 50 μ l antibody cocktail per tube and mix well.
 3. Incubate cells with antibodies on ice for 20 min
 4. Wash the cells once by adding 2 ml of staining buffer per tube, centrifuge for 5 min at 200–300g, and discard supernatant.
15. Fix and permeabilize cells. Perform BrdU-FITC staining according to the BrdU Flow kit instructions.
16. Resuspend the cells in 0.5 ml staining buffer per tube and analyze cells on a flow cytometer. Gate on cells with specific markers and analyze the percentage of BrdU⁺ cells using a software such as Flowjo (Figure 2).

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Figure 1.

T cell-dependent killing of DCs. CFSE-labeled DCs were incubated with antigen-specific T cells at different ratios, followed by flow cytometry. CFSE⁺ DCs were gated. Forward scattering versus 7-AAD staining was plotted.

Figure 2. Labeling of DCs with BrdU *in vivo*. Two month-old C57BL/6 mice were injected with BrdU on day 0 followed by feeding with BrdU in the drinking water for different days. Percentages of BrdU⁺ cells among CD11c⁺CD11b⁺ conventional DC (cDC) or CD11c^{low}PDCA-1⁺ plasmacytoid DC (pDC) were plotted.