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Plasmacytoid Dendritic Cells in Tolerance

Eric Gehrie, William Van der Touw, Jonathan S. Bromberg, and Jordi C. Ochando

Abstract

Dendritic cells (DC) are professional antigen-presenting cells (APCs) that modulate the outcome of the immune response toward immunity or tolerance. There are a large variety of DC subsets according to surface phenotype, function, and tissue distribution. Murine plasmacytoid DC (pDC) represent a distinctive DC population and are characterized by the expression of CD11c, B220, Gr-1, CD45RA, Ly49Q, BST2, and siglec-H on the cell surface. PDC act as immunogenic cell sentinels by secreting large amounts of type I interferon (IFN) in the lymph nodes in response to viral stimulation. PDC also act as tolerogenic cells when expressing the inducible tolerogenic enzyme indoleamine 2,3-dioxygenase (IDO), the inducible costimulator ligand (ICOS-L), and/or the programmed death 1 ligand (PD-L1), which mediate regulatory T-cell (Treg) development and suppression of self- and alloreactive cells. The PDC ability to induce Treg development is associated with capture and presentation of antigenic peptides associated with major histocompatibility complex (MHC) class I and II. Here, we provide the tools to study PDC development from bone marrow cultures, their antigen presentation properties, and their interactions with Treg under a tolerogenic setting of sterile inflammation.

Keywords

Plasmacytoid dendritic cells; Antigen presentation; Regulatory T cells

1. Introduction

Dendritic cells (DC) are responsible for the initiation and orchestration of the adaptive immune response. To do so, DC patrol throughout the body looking for antigen that may be present within the tissues or in circulation. DC are highly efficient antigen-presenting cells (APCs) on the major histocompatibility complex (MHC), and following antigen uptake, DC migrate to secondary lymphoid organs (SLO) and present peptide/MHC complexes to antigen-specific CD4⁺ and CD8⁺ T cells. These interactions are key factors in the establishment of either immunity or tolerance, and many DC subsets are involved in these processes.

The DC family involves a large variety of subsets according to their cell surface phenotype and tissue distribution. In the mouse, all DC subsets described express the integrin CD11c. Murine plasmacytoid DC (PDC) are a DC subset that are generated from hematopoietic precursors in the bone marrow (1) and constantly circulate in blood and SLO in a quiescent immature state expressing significant levels of CD11c, B220, Gr-1, CD45RA, Ly49Q, BST2, and siglec-H (2–7). Upon Ag recognition, PDC undergo a maturation process, upregulate their expression of MHC-II on the cell surface, and migrate to T-cell areas of secondary lymphoid tissues where they can either induce activation and proliferation of immunogenic T lymphocytes, or the development of tolerogenic regulatory T cells (Treg).

Tolerogenic PDC have been shown to induce T-cell unresponsiveness under steady state conditions (8). Tolerogenic PDC cells have also been described to suppress the immune response in several inflammatory disorders such as acute graft-versus-host disease (GVHD) (9), autoimmune arthritis (10), oral tolerance (11), and a lower frequency of PDC is associated with Type 1 diabetes (12, 13) and diabetes in mice (14). As a result, protolerogenic interaction between PDC and Treg has already been suggested (8, 15) which seems to be responsible for antigen-specific suppression of CD4⁺ and CD8⁺ T-cell responses (8, 16). The mechanisms by which PDC may promote Treg development have been investigated. PDC induce in vivo differentiation of naïve CD4⁺ T cells into Treg through indoleamine 2,3-dioxygenase (IDO) (17, 18). Treg can also be induced by PD-L1 (19), and PDC expressing higher levels of PD-L1 also correlate with an elevated frequency of Treg in tolerized recipient (20); PDC poised to express ICOS-L upon maturation leads to the generation of IL-10-producing T regulatory cells (21).

IDO expression by PDC has been reported in tumor-draining lymph nodes (22), which directly activate mature Treg through PD-L1 (23). These studies suggested tumor antigen presentation by PDC in the lymph nodes, consistent with Jung and colleagues, who reported that antigen-bearing PDCs prime naïve CD4 T cell in lymph nodes, but not in the spleen (24). Although PDC are generally considered poor presenters of exogenous antigens, some in vivo studies addressing the contribution of PDC to antigen presentation suggested that PDC are able to present antigen and prime CD4 T cells during infection with *Toxoplasma gondii* (25) and during sterile inflammatory conditions of GVHD (26). Activated murine PDC are characterized by a sustained expression of MHC-II on the cell surface, which is accompanied by prolonged antigen uptake, processing, and presentation (27, 28). It is possible that prolonged antigen presentation by PDC to CD4 T cells may promote antigen-specific Treg development, since MHC-II expression by DC is required to maintain Treg cell homeostasis (29).

In this chapter, we describe the protocol to follow alloantigen presentation of MHC-II-derived peptides by PDC in a model of sterile inflammation. Induction of transplantation tolerance can be achieved in mice following costimulatory blockade with anti-CD40L mAb, and interactions of alloantigen-presenting PDC with Foxp3 expressing Treg can be monitored (30).

Owing to their infrequency, PDC can be very challenging to study, making up only 0.1–0.5% of all peripheral blood mononuclear cells (PBMCs). For this reason, culturing total BM in vitro in the presence of different cytokines allows the generation of a large number of PDC that can be further used for molecular or cell biology studies. Pioneering research from Maraskovsky and colleagues described that in vivo treatment with FLT3-ligand (FLT3-L) expanded the pool of murine and human DC (31, 32), including PDC, which were characterized by their phenotype of CD4⁺CD11c[−]CD45RA⁺IL3-R⁺MHC-II⁺. Similar results were obtained in vitro by adding FLT3-L to bone marrow cultures, with the generation of two populations of CD11c⁺CD86⁺MHC-II⁺ myeloid and lymphoid DC, that differ in their expression of CD11b (33). Once PDC were identified in mice by their expression of CD11c⁺B220⁺Gr-1⁺ (2), several groups reported their specific development from FLT3-L in vitro bone marrow cultures (34, 35). Here, we describe culture conditions for the in vitro generation of PDC with FLT3-L and investigate their potential to induce Treg. In these protocols, we set out to differentiate PDC from BM precursors supplemented with FLT3-L in in vitro cultures. Treatment with FLT3-L rapidly differentiates BM cells into two distinct population of DC, which can be further identified by their expression of siglec-H.

In this chapter, we describe different systems used to differentiate BM cells into PDC, and their development into protolerogenic cells following stimulation with FLT3-L, since repetitive injections of FLT3-L results in peripheral Treg expansion (36).

2. Materials

2.1. In Vitro Generation of PDC from Murine Bone Marrow Cells

(Experiment time: 3–5 h + 10 day incubation).

2.1.1. Mouse Handling

1. BALB/c mice.
2. Ketamine HCl (100 mg/mL or 50 mg/mL).
3. Acepromazine (10 mg/mL).
4. 70% Ethanol, 100 mL.
5. Styrofoam board, ×1.
6. Surgical scissors.
7. Surgical forceps.
8. 18-Gauge 1.5" needles, ×6.

2.1.2. Cell Cultures—The following are required for the culture of BM cells:

1. Sterile PBS, 500 mL bottle.
2. RBC lysis solution.
3. 70 μ M Cell strainer, ×3.
4. 50 mL Centrifuge tubes, ×3.
5. 1.5 mL Microcentrifuge tubes, ×1.
6. Hemocytometer, ×1.
7. 0.4% Trypan blue.
8. Petri dishes, ×4.
9. 10 mL Glass pipette, ×1.
10. 10 mL Syringes, ×1 (per mouse).
11. 1 mL Syringe, ×1.
12. 3 mL Syringe, ×1.
13. 30-Gauge needles, ×1.
14. PDC Medium, see Appendix 2.
15. FLT3-L, see Appendix 3.

2.2. Sorting Murine Bone Marrow Cultures to Achieve Pure Populations of PDC

Experiment time: 2–3 h preparation + sorting time.

2.2.1. PDC Cell Sorting (see Fig. 1)

1. Mature bone marrow cultures (see Subheading 2.1).
2. 70 μ M Cell strainer, $\times 3$.
3. 5 mL Pipette, $\times 2$.
4. 10% Fetal Bovine Serum RPMI 1640 Complete Medium.
5. Staining buffer: phosphate-buffered saline (PBS) supplemented with 2% FCS (see Note 1).
6. DAPI, 1 μ L of 1 mg/mL solution.
7. Unconjugated anti-Fc receptor CD16/CD32 antibody.
8. PerCP Cy5.5-conjugated anti-mouse-CD11c antibody.
9. PerCP Cy5.5-conjugated Armenian Hamster IgG Isotype Control.
10. APC-conjugated anti-mouse-B220 antibody.
11. APC-conjugated Rat IgG2a Isotype Control.
12. FITC-conjugated anti-mouse-CD11b antibody.
13. FITC-conjugated Rat IgG2b Isotype Control.
14. PE-conjugated anti-mouse-Gr-1 antibody.
15. 4 mL Falcon tube (see Note 2).
16. 50 mL Falcon tube, $\times 6-7$.

2.3. Harvesting and Sorting Naïve T Cells from Tea Mouse Spleens

2.3.1. Mouse Handling

1. BALB/c mice.
2. Ketamine HCl (100 mg/mL).
3. Acepromazine (10 mg/mL).
4. 70% Ethanol, 100 mL.
5. Surgical scissors.
6. Surgical forceps.
7. RPMI 1640 complete medium supplemented with 10% FCS.
8. 50 mL Centrifuge tubes, $\times 3$.

2.3.2. T-Cell Sorting

1. APC-CD4.
2. FITC-CD25.
3. PE-Foxp3.
4. 500 mL Sterile PBS.

¹FCS must be endotoxin-free to prevent DC maturation.

²In PDC processing, all tubes used during the staining or sorting purposes should be made from polypropylene to prevent PDC from adhering to the plastic.

5. RPMI 1640 complete medium supplemented with 10% FCS.
6. RBC lysis solution.
7. 70 μ M Sterile cell strainer, $\times 3$.

2.4. Culturing Bone Marrow-Derived PDC with Naïve T Cells

2.4.1. PDC and Naïve T Cell Co-culture

1. 96-well, round-bottom culture plates.
2. Murine IL-2.
3. Murine anti-CD3 mAb.
4. Human TGF β 1.
5. RPMI 1640 complete medium.

2.5. Staining for Surface Markers and Intracellular Foxp3 for FACS Analysis

2.5.1. Surface Staining for CD4 and CD25 with Intracellular Foxp3 FACS Staining (see Fig. 2)

1. PBS, 500 mL.
2. Distilled water 500 mL.
3. Fixation/permeabilization concentrate and dilution solution, 1 mL/sample (see Note 3).
4. 3 mL of prepared permeabilization solution and isotype control (see Note 4).
5. FITC-labeled anti-mouse CD25 antibody and isotype control, 1 μ L/sample.
6. APC-labeled anti-mouse CD4 antibody and isotype control, 1 μ L/sample.
7. PE-labeled anti-mouse Foxp3 antibody, 1 μ L/sample.
8. 1.5 mL Microcentrifuge tubes.
9. Tubes suitable for flow cytometry.

2.6. Identification of Alloantigen-Expressing PDC

2.6.1. PDC-YAe FACS Staining (see Fig. 3)

1. Biotin mouse IgG2b isotype control.
2. Rat serum.
3. Unconjugated anti-Fc receptor CD16/CD32 antibody.
4. Biotin YAe mAb.
5. APC anti-siglec-H mAb.
6. Streptavidin-PE.

³After addition of permeabilization buffer, cells will be slippery. Take caution when dumping supernatant. If loss of cells becomes a problem, the supernatants may be aspirated after centrifugation. Also, the cells are fragile after permeabilization. The cell pellet should be disrupted gently.

⁴Surface staining and intracellular staining may be performed consecutively; however, cells must be fixed overnight before being permeabilized.

2.6.2. PDC-YAe IHC Staining (see Fig. 4)

1. Biotin mouse IgG2b isotype control (see Note 6).
2. Rat serum.
3. Unconjugated anti-Fc receptor CD16/CD32 antibody.
4. Biotin YAe mAb.
5. Anti-siglec-H mAb.
6. Anti-Foxp3 mAb.
7. Biotinyl Tyramide Kit.
8. Amplification diluent.
9. Tyramide reagent.
10. Streptavidin–HRP.
11. Peroxidase blocking reagent.
12. Cy3-Streptavidin and Cy2 anti-rat mAb.

2.7. Visualization of PDC and Foxp3 Cells

2.7.1. PDC-Foxp3 IHC Staining (see Fig. 5)

1. Rat serum.
2. Unconjugated anti-Fc receptor CD16/CD32 antibody.
3. Anti-Foxp3 mAb.
4. Anti-siglec-H mAb.
5. Anti-Foxp3 mAb.
6. Cy3 anti-rabbit and Cy2 anti-rat mAb.

3. Methods

3.1. In Vitro Generation of PDC from Murine Bone Marrow Cells

3.1.1. Mouse Handling

1. Make a sterile solution of 2% ketamine and 0.02% acepromazine in PBS (*see* Appendix 1).
2. Fill a sterile 1 mL syringe with 200 μ L of ketamine/acepromazine solution (per mouse). Attach a sterile 30-gauge needle to the syringe.
3. Inject 200 μ L of ketamine/acepromazine solution (per mouse) intraperitoneally (IP). Wait a few minutes for the anesthesia to take effect and sacrifice the anesthetized mice by cervical dislocation.
4. Spray the sacrificed mice with a 70% ethanol solution to reduce the chance of microbial contamination.
5. Take a Styrofoam board and cover it with a clean paper towel.

⁶To monitor alloantigen presentation of the Ea52-68 peptide bound to the I-A^b/MHC Class II PDC with the YAe mAb, BALB/c heart allografts were transplanted into C56BL/6 recipients.

6. Place the mouse in a supine position on the Styrofoam board. Use 18-gauge needles to pin the mouse by its four paws so that the abdomen is stretched and the mouse is secured to the Styrofoam board.
7. Using a clean pair of forceps and surgical scissors, make a small midline opening in the inferior abdomen. Make the opening as superficial as possible, being careful to avoid opening the peritoneum.
8. Insert the scissors into the inferior aspect of the opening and cut down the anterior aspect of the leg, in an inferior-lateral direction, all the way to the paw. Perform this bilaterally.
9. The incision should resemble an inverted “Y” shape. Using your gloved fingers, separate the peritoneum from the skin on both sides of the mouse and use additional 18-gauge needles to pin the abdominal skin to the Styrofoam tray. Also, using your gloved fingers and the forceps, peel the skin off of the legs.
10. Use the scissors to remove the legs from the mouse. In order to maximize yield of bone marrow and to avoid contamination, it is best for the legs to be removed whole, with the femoral head intact. Cutting through the tissue in the upper leg region carefully will eventually bring the femoral head into view. Cutting through the tissue just superior and lateral to the femoral head will dislocate the femur from the acetabulum. Place the legs, with muscle tissue still attached, into a Petri dish that is placed on ice.
11. Replace the paper towel on the Styrofoam board with a clean one. Dispose of the needles in the sharps container. Remove the muscle from each leg to the greatest extent necessary. It is recommended to start by cutting off the hind paw, inferior to the ankle joint. Then, use the scissors to cut all of the tendons around the inferior tibia and use the forceps to peel off the leg muscle in the superior direction. Remove the rest of the tissue using the scissors and forceps, working along the natural tissue planes as much as possible.
12. As the legs are cleaned of muscle, place them one by one into a new Petri dish, and keep them on ice until all of the legs are clean.
13. Place the Petri dish containing the cleaned legs inside the tissue culture hood. Fill the Petri dish with ~40 mL of 70% ethanol solution. Make sure that all of the legs are completely submerged in the ethanol solution and leave them for 90 s.
14. Once the 90 s have elapsed, transfer the bones to a new Petri dish containing about 40 mL of RPMI 1640 solution. This will rinse off the alcohol. Discard the Petri dish containing the ethanol.
15. Set up a new Petri dish, placing two sterile 70 μ M cell strainers inside it.
16. Take a few mL of RPMI 1640 and moisten the bottom of each cell strainer in the new Petri dish.
17. Take a 10 mL syringe and fill it to capacity with the RPMI. Attach a 27.5-gauge needle to each 10 mL syringe.
18. Using your forceps, pick up each leg and hold it over one of the cell strainers. Use the scissors to cut off the ends of each bone. Flush the bone marrow cavity with RPMI 1640 using the prepared syringes. When the bone marrow has been fully removed, the bones turn bright white.
19. Take a 3 mL syringe and remove the plunger. Discard the rest of the syringe. Use the rubber end of the plunger to force the harvested bone marrow through the

strainer, being forceful enough to disrupt the bone marrow plugs but gentle enough to avoid damaging the cells. Then, use the rubber plunger to mash up the bone ends contained in the other 70 μ M strainer.

20. Remove the 70 μ M cell strainers from the Petri dishes, and place them on top of sterile 50 mL centrifuge tubes. Use an automatic pipette to suck up all of the RPMI in the Petri dish and transfer it through the cell strainers, into the 50 mL centrifuge tubes. Serially rinse the Petri dish with RPMI 1640, leaving behind as few of the bone marrow cells as possible.
21. Spin the 50 mL tubes at 1,300 rpm for 6 min at 4°C.
22. When the centrifugation is complete, pour off the supernatant, leaving the pellet undisturbed, and add 5 mL of RBC lysis solution. Gently, flick the tube so that the pellet is dislodged and the solution is homogeneous. Let the tube sit undisturbed for 4 min at room temperature.
23. Pour the solution into a new 50-mL centrifuge tube, filtering the contents through a new 70 μ M cell strainer during the transfer. Then, rinse the empty 50-mL centrifuge tube with ~45 mL of RPMI 1640 and pour the rinsate through the strainer and into the new tube. Now, the RBC lysis solution has been adequately diluted. The centrifuge tube used for the RBC lysis can be discarded.
24. Centrifuge the neutralized RBC lysis solution at 1,300 rpm for 6 min at 4°C.
25. At the end of the spin, pour off the supernatant and resuspend the pellet in 1 mL of PDC growth medium (see Appendix 2).

3.1.2. Cell Cultures

1. Count cells using Trypan Blue and hemocytometer. Resuspend them at a final concentration of 1.5 million cells/mL in PDC growth medium.
2. Add FLT3-L to achieve a final concentration of 100 ng/mL (see Appendix 3 for dilution and storage instructions).
3. Mix the solution well, and plate 3.5 mL of FLT3-L supplemented bone marrow cell solution per well in 6-well, flat-bottom plates. Incubate at 36°C 5% CO₂ incubator for 10 days.
4. On day 5, remove 1.5 mL of medium from each well and replace with 1.5 mL of fresh PDC medium, supplemented with 100 ng/mL FLT3-L. Try to remove the medium from each well without disturbing the cultures (i.e., do not mix up and down).
5. After 10 days, the culture is complete (see Subheading 3.2).

3.2. Sorting Murine Bone Marrow Cultures to Achieve Pure Populations of PDC

3.2.1. PDC Cell Sorting—The protocol is performed at 4°C unless otherwise stated.

1. On the morning of the 11th day after starting the bone marrow cultures, remove the cultures from the incubator and examine the wells through the microscope. The medium will still retain most of its original orange color. The culture will be highly cellular with many cells floating freely in the medium. Some fibroblastic growth will be evident on the bottom of the well.
2. Using a 5 mL glass pipette, carefully mix the contents of the culture around by pipetting up and down several times. Avoid generating bubbles to the greatest extent possible, as bubbles may generate surface tension and possibly damage or

kill the cells. Pool the well-mixed cultures in a 50-mL centrifuge tube, filtering the culture medium through a sterile 70 μ M cell strainer as it is added to the centrifuge tube.

3. Rinse the culture wells with fresh RPMI 1640 complete medium, and add to a 50 mL centrifuge tube, filtering through the 70 μ M cell strainer from step 2.
4. Centrifuge for 6 min at 1,300 rpm at 4°C.
5. Discard the supernatant and resuspend the cells in 1 mL of staining buffer.
6. Count the cells.
7. Take five aliquots of 10^6 unstained cells and bring them to a final volume of 150 μ L in 1.5-mL microcentrifuge tubes. Label one of the tubes “isotype controls” and the others “unstained cells,” “FITC only,” “PerCP Cy 5.5 only,” and “APC only.”
8. Stain single cell suspensions in staining buffer with different combinations of mAbs by adding 1 μ L of FITC anti-CD11b, PerCP Cy 5.5 anti-CD11c, and APC anti-B220 per million cells to the remaining cultured cells. Also add 2 μ L of each isotype control antibody to the “isotype control” tube, and 2 μ L of each stain to the corresponding single stain tube.
9. Flick the tubes to mix the antibody, and place all five 1.5-mL tubes (unstained, isotype control, and single stains) as well as the 50 mL centrifuge tube on ice for 25 min. Be sure to cover the ice so that light does not bleach out any of the fluorescent labels.
10. After 25 min, add 1 μ L of 1 mg/mL DAPI solution to the tubes. Flick the tube with your finger to mix the DAPI solution around. Allow the sample tubes to incubate in the dark for 5 min.
11. Centrifuge the tubes for 5 min at 1,300 rpm.
12. Discard the supernatant from each tube and resuspend the pellet in 1 mL of staining buffer. Then, pipette up and down to break up the pellet.
13. Resuspend the cells to a final concentration of 20 million cells/mL, with a minimum of 1.5 mL medium (e.g., if there are six million cells, resuspend in 1.5 mL of medium) (Fig. 1).

3.3. Harvesting and Sorting Naïve T Cells from Tea Mouse Spleens

3.3.1. Mouse Handling Procedure

1. Sacrifice mice as described in Subheading 3.1, steps 1–4. Using scissors and forceps, make a horizontal incision just under the rib cage anteriorly on the left side. Identify and remove mouse spleens using a sterile technique. Place spleens in a centrifuge tube containing 15 mL RPMI 1640 complete medium supplemented with 10% FCS.

3.3.2. T Cell Sorting

1. In a tissue culture hood, pour the medium containing the spleens into a sterile Petri dish. Then, place the spleens inside a 70 μ M cell strainer and use the plunger of a 3-mL syringe to strain the spleen through the cell strainer.
2. Place the cell strainer over a sterile 50-mL centrifuge tube and pipette the cells from the Petri dish into the 50-mL tube through the strainer. Use RPMI 1640 medium to perform serial rinses of the Petri dish, making sure to leave as few cells behind as possible.

3. Once the Petri dish is thoroughly rinsed, cap the centrifuge tube, and centrifuge at 1,400 rpm for 5 min at 4°C. Note the red pellet that forms at the bottom of the tube after centrifugation. Discard the cell strainer and the Petri dish.
4. In the tissue culture hood, pour out and discard all the supernatant, leaving the pellet undisturbed in the bottom of the centrifuge tube.
5. Add 4 mL of RBC lysis buffer per spleen to the centrifuge tube containing the pellet. Gently, flick the side of the tube until well mixed. Place on ice for 5 min.
6. Pour the mixture from step 6 into a sterile 50-mL centrifuge tube, pouring the solution through a sterile 70- μ M cell strainer. This will eliminate some splenic debris from the solution. Then, add ~30 mL of RPMI 1640 complete medium supplemented with 10% FCS to neutralize the RBC lysis buffer.
7. Centrifuge at 1,400 rpm for 5 min at 4°C.
8. Discard the supernatant and resuspend the pellet in 1 mL of sterile staining buffer.
9. Add 10 μ L of FITC-anti-CD25, 10 μ L of APC-anti-CD4, and 10 μ L of PE-anti-CD8a. Gently, flick the tube with your finger to mix the antibodies, and place all tubes on ice and protected from light for 25 min.
10. After 25 min, add 30 mL of sterile staining buffer to the centrifuge tube.
11. Pour the contents of the centrifuge tube into a sterile 50-mL centrifuge tube, passing the contents through a sterile 70- μ M strainer.
12. Centrifuge at 1,400 rpm for 5 min at 4°C.
13. Pour off supernatant, leaving the gray-white pellet undisturbed.
14. Resuspend the cells in sterile staining buffer diluted to 20 million cells/mL. Filter the cells into a sterile 50-mL centrifuge tube through a 70- μ M cell strainer.

3.4. Culturing Bone Marrow-Derived PDC with Naïve T Cells

3.4.1. PDC and Naïve T Cell Co-culture

1. Culture freshly sorted BALB/c BM-derived PDCs (10^4 cells/well) with CD4⁺CD25⁻ T cells (5×10^4 cells/well) in 96-well round-bottom plates.
2. Add anti-CD3 mAb (150 ng/mL), 10 ng/mL IL-2, and 10 ng/mL TGF β .
3. Culture cells for 96 h, with no medium changes or additional cytokines.

3.5. Staining for Surface Markers and Intracellular Foxp3 for FACS Analysis

3.5.1. Surface Staining for CD4 and CD25 with Intracellular Foxp3 FACS Staining

1. Remove cells from culture plates. Place cells in collecting tube and add 2 mL of staining buffer.
2. Centrifuge at 2,000 rpm for 4 min at 4°C.
3. Discard the supernatant, leaving ~100 μ L in the bottom of the centrifuge tube.
4. Take five aliquots of 10^6 unstained cells from the positive control and bring them to a final volume of 100 μ L in 1.5-mL microcentrifuge tubes. Label one of the tubes “isotype control” and the others “PE-single stain,” “FITC-single stain,” “APC-single stain,” and “unstained cells.”

5. Add 1 μ L of APC-anti-CD4 and 1 μ L FITC-anti-CD25 mAb to each experimental sample tube, including the APC and FITC single stain controls. Flick the tube with your finger to mix the antibodies and disrupt the pellet, and place all tubes on ice for 25 min. Cover the ice so that light does not bleach out any of the fluorescent labels.
6. Add 1 mL of staining buffer and centrifuge at 2,000 rpm for 4 min at 4°C.
7. Discard the supernatant, preserving the pellet at the bottom of the tube and add 1 mL of fixation solution. Leave on ice overnight in a refrigerator.
8. The next morning, centrifuge at 2,000 rpm for 4 min at 4°C and remove supernatant. The pellet may no longer be visible.
9. Add 1 mL of ice-cold PBS to each tube.
10. Centrifuge at 2,000 rpm for 4 min at 4°C.
11. Discard the supernatant and add 1 mL of diluted permeabilization buffer.
12. Centrifuge at 2,000 rpm for 4 min at 4°C.
13. Discard the supernatant and add 1 mL of diluted permeabilization buffer.
14. Centrifuge at 2,000 rpm for 4 min at 4°C.
15. Discard the supernatant, leaving ~100 μ L of liquid in each tube. Then, add 1 μ L anti-Foxp3 mAb to each experimental sample tube, including the PE single stain control from step 4. Flick each tube with your finger to mix the antibody and dislodge the pellet, and place all tubes on ice for 25 min. Be sure to cover the ice so that light does not bleach out any of the fluorescent labels.
16. Add 1 mL diluted permeabilization solution.
17. Centrifuge at 1,400 rpm for 5 min at 4°C.
18. Discard the supernatant and add 1 mL of PBS.
19. Repeat steps 17 and 18.
20. Transfer samples to tubes used for flow cytometry analysis and proceed to flow cytometry (Fig. 2) (see Note 5).

3.6. Identification of Alloantigen-Expressing PDC

3.6.1. PDC-YAe FACS Staining

1. Resuspend 10^6 cells in 2 mL of ice-cold PBS.
2. Centrifuge at 1,400 rpm for 5 min at 4°C.
3. Discard the supernatant and block with rat serum 5% for 20 min.
4. Add 2 mL of ice-cold PBS and centrifuge at 1,400 rpm for 5 min at 4°C.
5. Discard the supernatant and block Fc receptor with anti-CD16/32 (5 μ g/mL) and biotin mouse IgG2b isotype (2.5 μ g/mL) mAb for 20 min.
6. Add YAe mAb (2.5 μ g/mL) for 30 min 4°C.

⁵Cell cultures without PDC do generate Treg in fairly high percentage numbers, but those cultures do not proliferate. The cultures only proliferate well when APCs are present in the cultures. Thus, in FACS, the percent of Treg seems to be high, but in fact the gross number is low because the cultures do not proliferate.

7. Add 2 mL of ice-cold PBS and centrifuge at 1,400 rpm for 5 min at 4°C.
8. Add Streptavidin-PE for 30 min 4°C.
9. Add 2 mL of ice-cold PBS and centrifuge at 1,400 rpm for 5 min at 4°C.
10. Add fluorescent-conjugated mAb to other cell surface molecules for 20 min 4°C.
11. Add 2 mL of ice-cold PBS and centrifuge at 1,400 rpm for 5 min at 4°C.
12. Add 1 μ L of 1 mg/mL DAPI solution to the tube for 5 min.
13. Centrifuge the tubes for 5 min at 1,300 rpm at 4°C.
14. Discard the supernatant and resuspend the cells in 0.4 mL of staining buffer (Fig. 3).

3.6.2. PDC-YAe IHC Staining

1. Cut sections 8 μ m thick in the cryostat.
2. Air-dry tissue sections for 30 min at room temperature.
3. Fix the tissue sections for 10 min in 3% paraformaldehyde at 4°C.
4. Wash three times with PBS for 5 min.
5. Block with peroxidase for 10 min.
6. Wash three times with PBS for 5 min.
7. Block slides with 5% rat serum plus anti-CD16/CD32 (5 μ g/mL) and biotin mouse IgG2b isotype control (2.5 μ g/mL) for 30 min in a humidified chamber.
8. Drain off excess liquid.
9. Add biotin-YAe (2.5 μ g/mL) or isotype control and incubate at room temperature for 1 h.
10. Wash three times with PBS on shaker for 5 min.
11. Add streptavidin-HRP (1:100 dilution) and incubate for 30 min at room temperature.
12. Wash three times with PBS on shaker for 5 min.
13. Add biotinyl tyramide working solution (1:50 dilution in Amplification diluent) to each slide and incubate 3 min on shaker at room temperature.
14. Wash three times with PBS on shaker for 5 min.
15. Add Cy3-streptavidin (1/800) and incubate for 30 min at room temperature.
16. Wash three times with PBS for 5 min.
17. Add rat anti-mouse siglec H mAb (1/100) and incubate for 30 min at room temperature.
18. Wash three times with PBS for 5 min.
19. Add Cy2 anti-rat mAb (1/600) and incubate for 30 min at room temperature.
20. Wash two times with PBS and one in water for 5 min.
21. Dry the slides at room temperature and then mount slides with DAPI mounting medium.

22. Visualize slides using fluorescence microscopy (Fig. 4).

3.7. Visualization of PDC and Foxp3 Cells

3.7.1. PDC-Foxp3 IHC Staining

1. Cut sections 8 μ m thick in the cryostat.
2. Air-dry tissue sections for 30 min at room temperature.
3. Fix the tissue sections for 10 min in acetone at -20°C .
4. Wash three times with PBS for 5 min.
5. Block slides with 5% rat serum plus anti-CD16/CD32 (5 $\mu\text{g}/\text{mL}$) for 30 min in a humidified chamber.
6. Wash three times with PBS for 5 min.
7. Add rat anti-mouse siglec H mAb (1/100), and incubate 30 min at room temperature.
8. Wash three times with PBS for 5 min.
9. Add Cy2 anti-rat mAb (1/600) and incubate for 30 min at room temperature.
10. Wash three times with PBS for 5 min.
11. Block the slides with blocking serum for 20 min (normal rat serum diluted 1/5 in 10% BSA/Tris 1 $\times$).
12. Add rabbit anti-mouse Foxp3 mAb 5 $\mu\text{g}/\text{mL}$ in blocking serum and incubate at room temperature in humid chamber for 60 min (cover the section completely 15–50 μL).
13. Wash three times with PBS on shaker for 5 min.
14. Add Cy3 anti-rabbit antibody diluted in PBS (1/800) and incubate at room temperature in humid chamber for 30 min.
15. Wash two times with PBS for 5 min.
16. Wash for 10 min in PBS at room temperature.
17. Dry the slides at room temperature and then mount slides with DAPI mounting medium.
18. Visualize slides using fluorescence microscopy (Fig. 5).

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Appendix 1: 10 mL Solution of 2% Ketamine, 0.02% Acepromazine Solution

2 mL Ketamine HCl (100 mg/mL) – Use 4 mL of 50 mg/mL ketamine HCl.

0.2 mL Acepromazine (10 mg/mL).

7.8 mL Sterile PBS.

Appendix 2: 500 mL of PDC Culture Medium

435 mL RPMI 1640 supplemented with L-Glutamine.

50 mL FCS (10%).

5 mL 1 M HEPES (10 mM).

10 mL (5,000 IU/ μ g) Penicillin/streptomycin (100 IU/mL penicillin and 100 μ g/mL streptomycin).

Filter and store at 4°C.

Immediately before using, add β -mercaptoethanol (2-ME) to a concentration of 50 μ M and cold filter a second time.

Appendix 3: FLT3-L Preparation

Use lyophilized FLT3-L (R&D Systems, Minneapolis, MN) to prepare 50 ng/mL aliquots:

1. Add 480 μ L of PBS to 25 μ g of lyophilized FLT3-L.
2. Add 20 μ L of 2.5% fatty-acid-free BSA in PBS.
3. Wait 10 min for the powder to dissolve. Do not vortex to avoid bubbles. It takes about 10 min to dissolve.
4. Pipet up and down to mix the solution. Avoid adding bubbles.
5. Dispense into aliquots. 35 μ L aliquot to be most efficient for 2-mouse experiments. Store at –20°C for up to 3 months.

Fig. 1.

Plasmacytoid dendritic cell phenotype. (a) BM cells were isolated from BALB/c mice and cultured for 10 days in the presence of FLT3-L. Data can be analyzed using FlowJo software (TriStar) and BM of BALB/c mice can be divided into two major subpopulations, defined as PDC and mDC. In order to gate on PDCs, 100,000 events should be acquired for each sample. The first step in the analysis is to draw a gate looking at side scatter (SSC) versus forward scatter (FSC). This gate will be gate 1. Cell duplets are excluded in gate 2 (SSC-A versus SSC-H). Dead cells are excluded in gate 3 (SSC-A versus DAPI). Making a drill down of gate 3, we can then identify PDCs within the BM culture, by making a new gate looking at CD11c intermediate versus B220 high. This cell population should be CD11b negative. The PDC population also can be gated on from BM cultures on surface staining for Siglec-H and mPDCA-1.

Fig. 2.

Induction of regulatory T cells with BM-derived plasmacytoid dendritic cells. Freshly sorted BM-derived PDC cells (2×10^4 cells/well) isolated from BALB/c mice for 10 days in the presence of FLT3-L, were cultured with CD4⁺CD25⁻ T cells (5×10^4 cells/well) for 96 h with 150 ng/mL of anti-CD3 mAb, 10 ng/mL IL-2, and 10 ng/mL TGF β . Flow cytometry data indicates percentage of Foxp3⁺ CD4⁺ T cells induced.

Fig. 3.

Alloantigen-presenting PDCs circulate systemically through blood. Percentage of alloantigen-presenting YAc⁺ cells from the blood of tolerant mice are shown. Flow cytometry results indicate that only PDC are presenting alloantigen systemically, since only YAc⁺ cells express mPDCA-1. Other cell types on freshly isolated blood do not express YAc in their cell surface. Data are representative results from three independent experiments ($n = 3$).

Fig. 4.

Alloantigen-presenting PDCs are present in secondary lymphoid organs. Images of total alloantigen-presenting YAe⁺ cells from the lymph nodes of tolerized mice at day 5 posttransplant are shown. IHC results indicate that only PDC are presenting alloantigen, since most YAe⁺ cells express mPDCA-1. Data are representative results from three independent experiments ($n = 3$). Original magnification, $\times 200$.

Fig. 5. PDCs are next to Foxp3 expressing cells in the lymph nodes of tolerized mice. IHC results indicate that Foxp3 expressing cells are in proximity of PDC in the lymph nodes of tolerized mice at day 5 posttransplant. Data are representative results from three independent experiments ($n = 3$). Original magnification, $\times 200$.