Chapter 2

Pulmonary Antigen Presenting Cells: Isolation, Purification, and Culture

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Abstract

Antigen presenting cells (APCs) such as dendritic cells (DCs) and macrophages comprise a relatively small fraction of leukocytes residing in lymphoid and non-lymphoid tissues. Accordingly, functional analyses of these cells have been hampered by low cell yields. Also, alveolar macrophages share several physical properties with DCs, and this has complicated efforts to prepare pure populations of lung APCs. To overcome these difficulties, we have developed improved flow cytometry-based methods to analyze and purify APCs from the lung and its draining lymph nodes (LNs). In this chapter, we describe these methods in detail, as well as methods for culturing APCs and characterizing their interactions with T cells.

Key words Antigen presenting cells, Dendritic cells, Macrophages, Monocytes, Lung, Lymph nodes, Gradient centrifugation, Flow cytometry, Autofluorescence, Sorting, Culture

1 Introduction

Pulmonary APCs take up inhaled antigens, process them, and present antigen-derived peptides to T and B lymphocytes to initiate adaptive immune responses [1]. In keeping with their ability to acquire antigens from the airspace or parenchymal tissue, DCs and macrophages are located within the airway epithelium, lung parenchyma, and alveolar spaces [2, 3]. To maintain their positions within the lung, many DCs and macrophages adhere tightly to tissue stromal cells. Protocols that yield large numbers of lung APCs must therefore disrupt molecular interactions that hold APCs and stromal cells together. Although collagenase D has been widely used for this purpose, the yield of DCs obtained from procedures that employ this enzyme has been suboptimal. To improve cell yields, we have modified a tissue digestion method that was originally designed for cardiovascular tissue digestion [4], and found that this new protocol dramatically improves the yield of APCs from the lung [5].

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Lung APCs are highly diverse in terms of both size and density. For example, alveolar macrophages are large and light, while monocytes are relatively small and dense, with lymphocytes and non-leukocytes having even higher densities. Therefore, gradient centrifugation provides a convenient and effective method to enrich for APCs [6]. We have developed simple methods that enrich for different APCs, depending on which type is needed for the individual experiment at hand. After this enrichment step, APCs are often analyzed by flow cytometry to determine their frequency and their display of cell surface molecules. Unlike most other macrophages in the body, alveolar macrophages display the pan-DC marker, CD11c, as well as MHC class II [7, 8]. Consequently, if other markers are not used, alveolar macrophages can be easily mistaken for pulmonary DCs. According, many investigators now use the autofluorescent properties of macrophages and their display of high levels of Siglec-F to distinguish them from DCs [7, 9]. In addition, pulmonary DCs are heterogeneous [2] and include plasmacytoid, inflammatory, and conventional DCs. The latter category includes the two major lung DC subsets, which express high levels of CD11b and CD103, respectively. $CD11b^{hi}$ DCs can be further segregated into pre-DC-derived and monocyte-derived DCs (moDCs) [10, 11]. In this chapter, we describe how to distinguish each DC subset from the others by flow cytometry. This technology is useful not only for characterizing APCs but also for purifying individual APC populations. Purified APCs can be subsequently studied ex vivo to identify their biologic functions. Here, we describe methods to culture lung APCs with naïve T cells to study APC-mediated T helper cell differentiation.

2 Materials

2.1 Tissue Digestion

1. Digestion buffer: PBS (Mg^- Ca^-) with 0.5 % BSA (pH 7.2–7.4), filter-sterilized and stored at 4 °C.
2. Preparation buffer: PBS (Mg^- Ca^-) with 0.5 % BSA and 2 mM EDTA (pH 7.2–7.4), filter-sterilized and stored at 4 °C.
3. 5 mg/ml Liberase TM (Roche) in PBS, stored at −20 °C.
4. 25 mg/ml Collagenase XI (approx. 12,500 U/ml) in PBS, stored at −20 °C.
5. 100 mg/ml Hyaluronidase type I-S (approx. 6,000 U/ml) in PBS, stored at −20 °C.
6. 20 mg/ml DNase I in water, stored at −20 °C (*see* **Note 1**).
7. 120 mM EDTA in PBS (pH 7.2), stored at 4 °C.
8. Nycodenz (Accurate Chemical).
9. Incubator, 37 °C.
10. Cell strainer 70 μm.

2.2 Staining of Leukocytes

1. Preparation buffer: PBS (Mg- and Ca-free) with 0.5 % BSA and 2 mM EDTA (pH 7.2–7.4).
2. FACS buffer: 0.5 % BSA, 0.1 % NaN_3, and 2 mM EDTA in PBS.
3. Normal mouse serum.
4. Normal rat serum.
5. Antibody dilution buffer (5 % normal mouse serum, 5 % normal rat serum, and 5 μg/ml anti-CD16/32 in FACS buffer).
6. Antibodies [12–14]

   Fc block: anti-mouse CD16/CD32 (2.4G2).

   Pan DC markers: CD11c (N418 or HL3), MHC class II I-A^b (AF6-120.1), or I-A^d (AMS-32.1) (*see* **Note 2**).

   DC subset markers: CD11b (M1/70), CD14 (Sa2-8), CD103 (M290 or 2E7), CD317 (JF05-1C2.4.1, 120G8, or eBio927), Ly-6C (AL-21), Siglec-H (eBio440c).

   Macrophage markers: CD11b (M1/70), CD11c (N418 or HL3), F4/80 (BM8), Siglec-F (E50-2440).

   Monocyte markers: CD115 (AFS98), Ly-6C (AL-21), CD11b (M1/70).

   Activation/maturation markers: CD40 (1C10), CD80 (16-10A1), CD86 (GL1), CD197/CCR7 (4B12).

   Lymphocyte markers: CD3e (145-2C11), CD19 (6D5 or eBio1D3), CD49b (DX5).
7. Round- (U) bottom 96-well plate.
8. Plate rotor.
9. 15 ml conical tubes.
10. FACS tubes.
11. Flow cytometer (e.g., FACS LSR-II (Becton Dickenson))

2.3 Cell Sorting and Culture

1. Cell sorter (e.g., FACS-ARIA-II (Becton Dickenson)).
2. RPMI 1640.
3. Fetal bovine serum, certified (low endotoxin).
4. β-Mercaptoethanol.
5. Penicillin/Streptomycin.
6. Round- (U) bottom 96-well plate.
7. Flat-bottom 96-well plate.
8. CO_2 incubator, 5 % CO_2, 37 °C.

3 Methods

3.1 Tissue Digestion

1. Collect lungs from mice and place in tissue culture dish (60 mm) or 6-well plate containing 1 ml of digestion buffer (Reagent #1) in Section 2.1. Up to four lungs per dish can be included.
2. Mince tissue using scissors, razor blade, and/or forceps (Fig. 1). Scissors are recommended.
3. Add 1 ml of digestion buffer (*see* **Note 3**). Add: 40 μl of Liberase, 20 μl of DNase I, 20 μl of collagenase XI, and 20 μl of hyaluronidase.
4. Swirl the dish gently, then incubate dish at 37 °C for 60 min.
5. During the incubation, prepare Nycodenz solution. Weigh Nycodenz according to your target cell types (Fig. 2): 1.45 g for Dendritic cells (excluding pDCs) and macrophages; 1.6 g for Dendritic cells (including pDCs), macrophages, large monocytes, and large B cells; 1.8 g for Dendritic cells (including pDCs), macrophages, monocytes, and large T and B cells. Add Nycodenz to 9.5 ml PBS in 15 ml tube. Place the tube on a shaker or a rotator.
6. To stop tissue digestion, add 0.4 ml of cold 120 mM EDTA to dish.
7. Add 5 ml of preparation buffer (Reagent #2 in Section 2.1) to 15 ml empty conical centrifugation tube (or 25 ml in 50 ml tube if you have multiple dishes). Keep the tubes on ice.

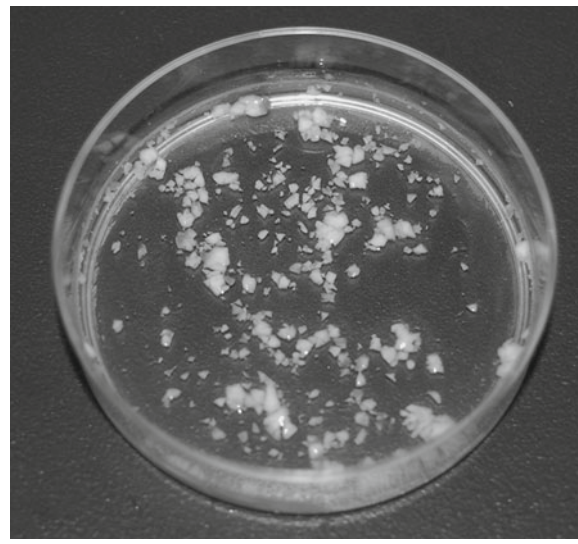

Fig. 1 Minced lung tissue. Lung tissue was minced by scissors in a 60 mm tissue culture dish. Smaller pieces (<1 mm) will result in higher cell yield

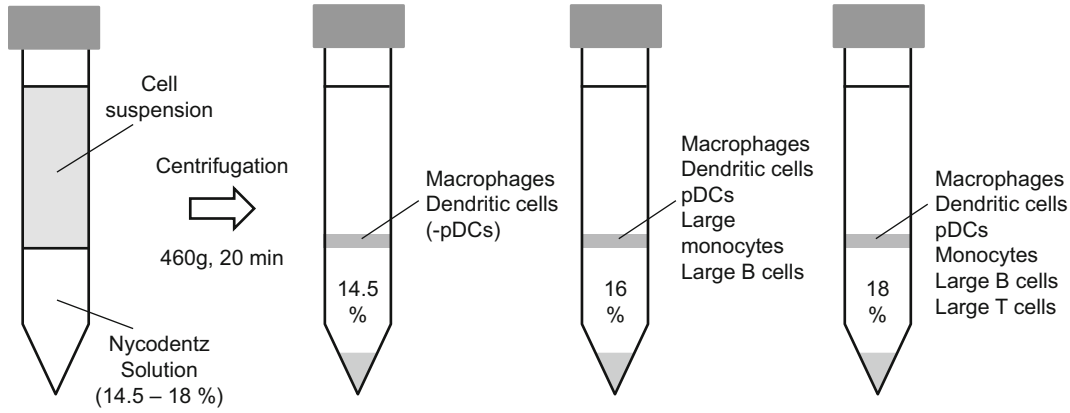

Fig. 2 Gradient centrifugation for enrichment of dendritic cells and macrophages from lung. Different concentration of Nycodenz enriches different cell types. Higher concentrations increases contamination of lymphocytes

8. Meanwhile, add 5 ml cold preparation buffer (Reagent #2 in Section 2.1) to dish. Transfer minced tissue onto a cell strainer in dish and using rubber-tipped plunger of a 3 ml syringe, push tissues through the cell strainer onto the dish.
9. Pipette the liquid in the dish back through the strainer several times to ensure a single cell suspension. Then pipette the cells several times to detach cells from dish, transfer cells (in 7 ml now) to 15 ml tube containing 5 ml of preparation buffer on ice (or 50 ml tube with 25 ml preparation buffer).
10. Centrifuge at 500 × *g* for 5 min at 4 °C. This is equivalent to 1,600 rpm in a table top Sorvall centrifuge.
11. Resuspend cells in 10 ml preparation buffer. Carefully layer 3 ml of gradient solution (e.g., 14.5–18 % Nycodenz solution in PBS) *under* the cell suspension, and spin at 450 × *g* for 20 min at room temperature with the brake OFF.
12. The enriched dendritic cells form a fuzzy white layer at the interface of the gradient solution and buffer. Remove the media until 1.5 ml of liquid is left above the interface. Collect the cell layer carefully (avoid the pellet in sample).
13. Wash cells with 5 ml preparation buffer. Spin cell suspension at 450 × *g* for 5 min at 4 °C with brake ON.
14. Resuspend cell pellets in 500–1,000 μl of preparation buffer. Count cells.

3.2 Staining of Leukocytes

1. Place 1×10^5–2×10^6 cells in each well of round-bottom 96-well plate. Afterwards, use a multichannel pipette. Spin the plate at 800 × *g* for 3 min, and then discard supernatant.
2. Add 50 μl of Ab dilution buffer, and then incubate the cells on ice for 5–10 min.

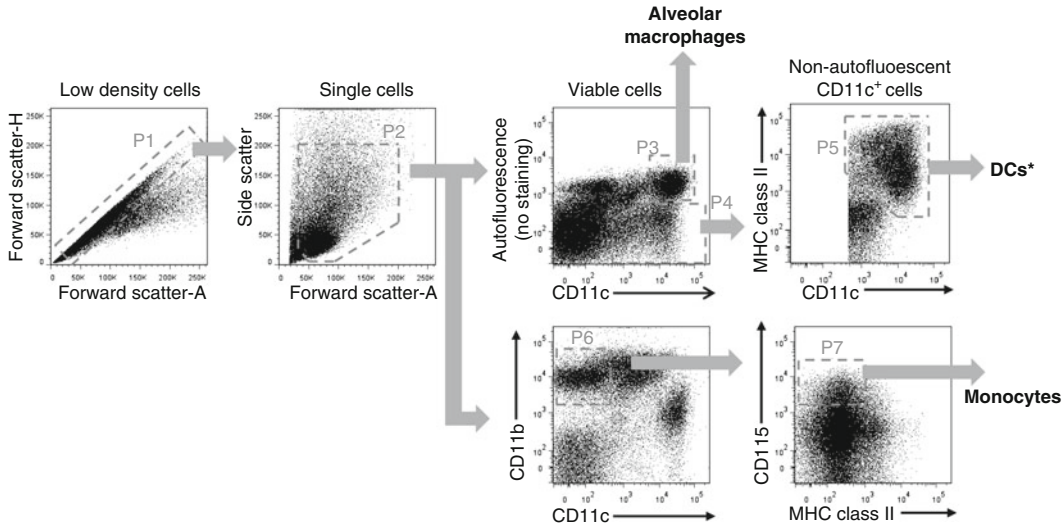

Fig. 3 An example gating strategy for lung APC analysis using flow cytometry. Gatings shown are for segregation of conventional DCs (P5) (*pDCs are not included in this gating), alveolar macrophages (P3), and monocytes (P7). Single cell gating (P1) excludes cell aggregates. P2 is the gate for viable cells. Conventional DCs are $CD11c^{+}$ MHC-II^{+} autofluorescence^{lo} (pDCs are $CD11c^{int}$ MHC-II^{lo}). Alveolar macrophages are $CD11c^{hi}$ autofluorescence^{hi}. Monocytes are $CD11b^{+}$ $CD115^{+}$ MHC-II^{lo} (P7). For lymph node APC analysis, CD3 and CD19 are used instead of autofluorescence to exclude lymphocytes. More details and additional cell markers are described in **Note 6**

3. Prepare Ab cocktail with Ab dilution buffer (2× of final concentration). The optimal final concentration is usually 0.5–2 μg/ml. Add 50 μl of 2× Ab solution to cells then mix well.

   The Ab composition of the cocktail depends on the goal of the experiment, but an example is as follows: I-A^{b}—eFluor 450; CD11b—eFluor 605NC; CD103—Phycoerythrin; CD11c—PerCP-Cy5.5; CD115—APC; and Ly-6C—APC-Cy7. FITC-labeled Ab is not used because this channel will be used for detection of autofluorescence signals (*see* **Note 4**). Protect cells from light and incubate on ice for 30 min.
4. Wash cells with FACS buffer twice. The first time, add 100 μl FACS buffer, and the second time, resuspend the pellet with 200 μl of FACS buffer. Pipette cells every time to resuspend cells.
5. Suspend cells in 200 μl FACS buffer, and transfer cells to FACS tube.

3.3 Flow Cytometric Analysis

1. Gate on single cells (P1 in FSC-A vs. FSC-H) and viable cells (P2 in FSC-A vs. SSC) (Fig. 3).
2. Set voltage of each channel (*see* **Note 5**).
3. Run compensation samples (unstained cells and cells stained with single dye).

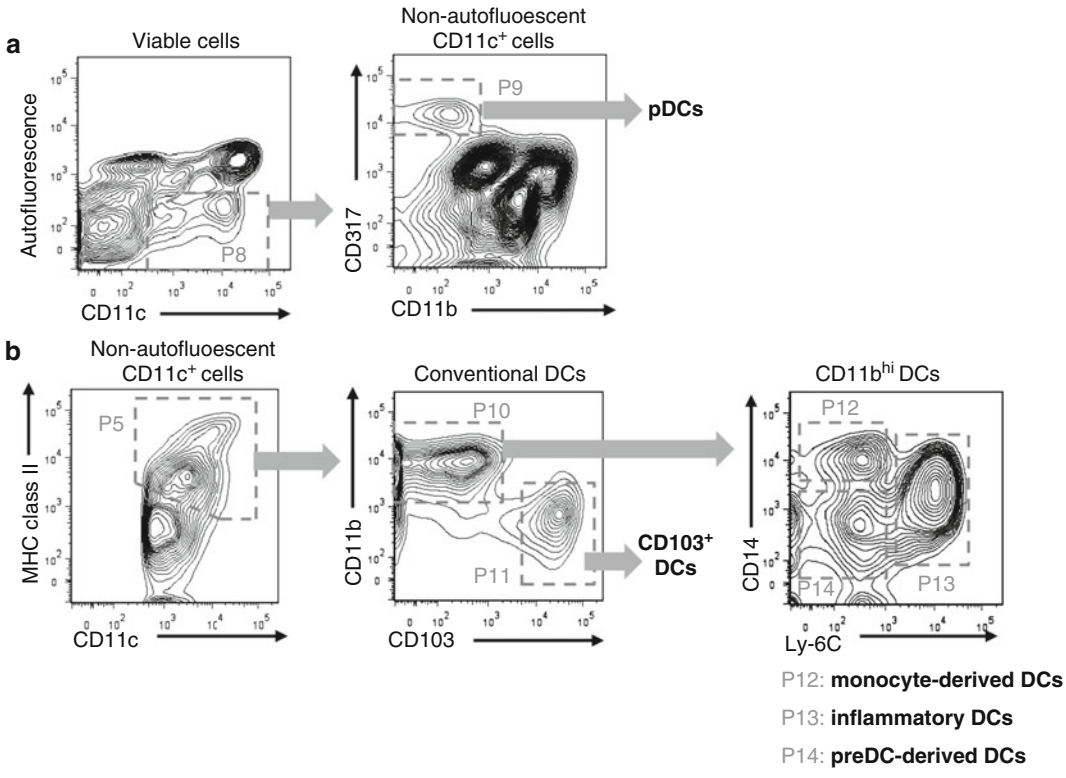

Fig. 4 Analysis of lung DC subsets. (**a**) Gating for pDC analysis; $CD11b^{lo}CD11c^{int}CD317^{+}$ (P9). (**b**) Gating for conventional DC subset analysis. Total $CD11b^{hi}$ DCs: $CD11b^{hi}CD11c^{+}MHC\text{-}Ii^{+}$ (P10); $CD103^{+}$ DCs: $CD11b^{lo}CD11c^{hi}CD103^{+}MHC\text{-}II^{hi}$ (P11); Monocyte-derived DCs: $CD11b^{hi}CD11c^{int}CD14^{hi}Ly\text{-}6C^{lo}MHC\text{-}II^{hi}$ (P12); Inflammatory DCs: $CD11b^{hi}CD11c^{int}Ly\text{-}6C^{hi}MHC\text{-}II^{+}$ (P13); PreDC-derived $CD11b^{hi}$ DCs: $CD11b^{hi}CD11c^{int}CD14^{int/lo}Ly\text{-}6C^{lo}MHC\text{-}II^{hi}$ (P14). More details and additional markers can be found in **Note 6**

4. Adjust compensation manually (*see* **Note 5**). We do not recommend using "Auto Comp," which cannot adjust compensation for DCs or macrophages. Set gates for positive cells (not autofluorescent cells) and negative cells, and then adjust the compensation value in each channel. Repeat same procedures for all channels.
5. Gate on DCs (P4 and P5; e.g., $CD11c^{+}MHC\text{-}II^{+}autofluorescence^{-}$ cells for LN DCs and/or alveolar macrophages (P3; $CD11c^{hi}$ autofluorescent)) (Fig. 2) (*see* **Notes 4** and **6**).
6. Gate on DC subsets (e.g., P10: $CD11b^{+}$, P11: $CD103^{+}$) (Fig. 4) (*see* **Note 6**).
7. Collect 10,000 cells (or as many as possible) in P5.

3.4 Sorting of Dendritic Cells

1. Place up to 1×10^{8} cells in 15 ml conical tube. Fill the tube with preparation buffer (Reagent #2) in section 2.1.

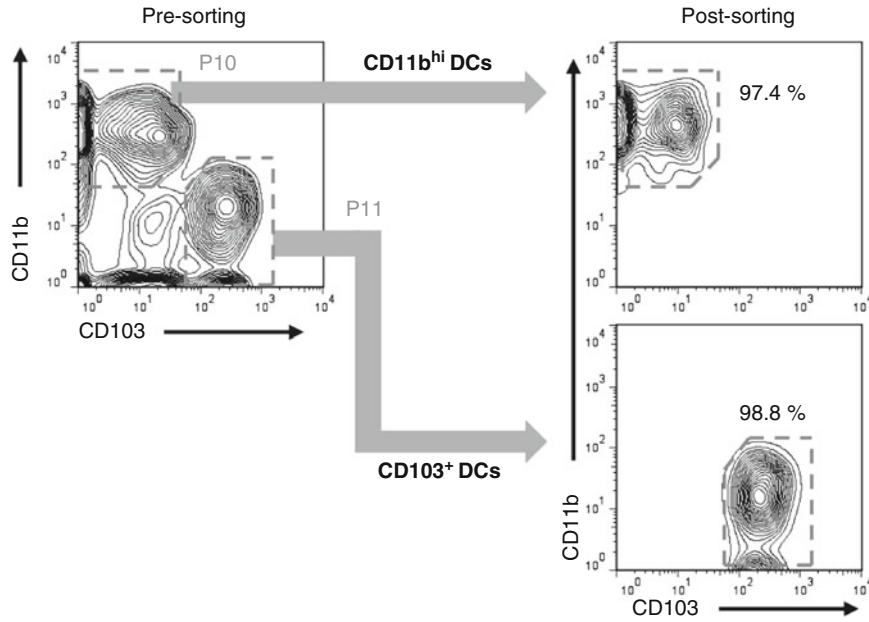

Fig. 5 DC subset sorting by flow cytometry. Total CD11b^{hi} DCs (P10) and CD103^{+} DCs (P11) were purified in a FACS ARIA-II cell sorter. Purified sorted cells are shown. Approximately 1–2 × 10^4 CD11b^{hi} DCs and 2–4 × 10^4 CD103^{+} DCs are usually obtained per mouse lung after sorting

Spin the tube at 500 × *g* for 5 min, and then remove supernatant.

2. Resuspend cells with 1 ml of Ab dilution buffer containing antibodies. Protect cells from light and incubate on ice for 30 min.
3. Meanwhile, add 4 ml of complete culture medium to each FACS collection tube.
4. Wash cells with preparation buffer (Reagent #2 in section 2.2) twice. The first time, add 14 ml of buffer, and the second time, resuspend pellet with 15 ml of buffer. Thoroughly resuspend cells every time.
5. Suspend cells in 1 ml of preparation buffer (Reagent #2 in section 2.2), and transfer cells to FACS filter cap tube.
6. Place 1 ml of preparation buffer (Reagent #2 in section 2.2) on the top of the tube three times to rinse the filter.
7. Remove 3 ml of complete culture medium from each collection tube.
8. Sort cells on FACS ARIA-II (Fig. 5) (*see* **Note 7**).

3.5 Culture of Dendritic Cells with T Cells

1. Transfer sorted dendritic cells or macrophages from the FACS tube to a 15 ml conical tube containing 10 ml of culture medium. Spin the tube at 500 × *g* for 5 min.

2. Wash cells twice with culture medium and then count the cells.
3. Resuspend dendritic cells with culture medium at 5×10^5/ml (*see* **Note 8**). Plate 5×10^4 dendritic cells (100 μl) in each well of a round-bottom, 96-well plate.
4. Add 50 μl of culture medium containing antigens, cytokines, or antibodies.
5. Adjust concentration of purified T cells to 2×10^6 cells/ml. Add 50 μl of T cell suspension to each well (final number: 1×10^5 cell/well)(*see* **Note 9**).
6. Culture cells in a CO_2 incubator (5 % CO_2, 37 °C) (*see* **Note 10**).
7. On day 3, split cells from one well into two wells. Add 100 μl of fresh culture medium to each well.
8. T cell proliferation can be assessed on day 3–5 by counting cell number, CFSE-dilution assay, or [^{3}H]-thymidine incorporation assay.
9. On day 5–6, collect cells with supernatant and centrifuge at $500 \times g$ for 5 min. Save supernatant for cytokine assay.
10. If T cell restimulation is desired, continue with **steps 11–15** (*see* **Note 11**).
11. Wash the cells with culture medium twice then count.
12. Resuspend the cells with culture medium and adjust the cell concentration to 5×10^5/ml.
13. Put 200 μl of T cell suspension (1×10^5) into a flat-bottom 96-well culture plate coated with anti-CD3e (1 μg/ml) and anti-CD28 (1 μg/ml) mAbs.
14. Culture cells in a CO_2 incubator (5 % CO_2, 37 °C).
15. 24 h later, collect supernatants for cytokine assay.

4 Notes

1. Use distilled water to dissolve DNase I. Do not use PBS.
2. Because binding of anti-I-A/I-E mAb (M5/114) alters the phenotype and function of APCs, we recommend using anti-I-Ab (AF6-120.1), anti-I-A^d (AMS-32.1), or anti-I-E (14-4-S) mAb to detect MHC class II.
3. 1.1 ml of premixed enzymes in digestion buffer can be added.
4. Because alveolar macrophages are autofluorescent, they display positive signals in channels in which cells were not stained. Autofluorescence signal is detected in channels with violet and blue lasers (e.g., Pacific blue, AmCyan, FITC, and PE channels).

5. We recommend eliciting advice from an expert in flow cytometry to set voltage and compensation. Because different cell populations have different signal backgrounds (including autofluorescence), Auto-comp cannot adjust the compensation appropriately.
6. Surface makers of pulmonary APC populations are shown below [12–14].
   - Alveolar macrophages: $CD11b^{lo}$, $CD11c^{hi}$, $F4/80^{+}$, Siglec-F^{hi}, autofluorescence^{hi}.
   - Interstitial macrophages: $CD11b^{hi}$, $CD11c^{lo}$, $F4/80^{+}$.
   - Monocytes: $CD11b^{hi}$, $CD115^{hi}$, Ly-$6C^{hi}$, MHC-II^{lo}, autofluorescence.
   - Inflammatory DCs: $CD11b^{hi}$, $CD11c^{int}$, Ly-$6C^{hi}$, MHC-II^{+}.
   - Monocyte-derived DCs: $CD11b^{hi}$, $CD11c^{int}$, $CD14^{hi}$, Ly-$6C^{lo}$, MHC-II^{hi}.
   - PreDC-derived $CD11b^{hi}$ DCs: $CD11b^{hi}$, $CD11c^{int}$, $CD14^{hi}$, Ly-$6C^{lo}$, MHC-II^{hi}.
   - $CD103^{+}$ DCs: $CD11b^{lo}$, $CD11c^{hi}$, $CD24^{+}$, $CD103^{+}$, $CD117^{+}$, $CD207^{+}$, MHC-II^{hi}.
   - Plasmacytoid DCs: $CD11b^{lo}$, $CD11c^{int/lo}$, CD45R/$B220^{+}$, $CD317^{+}$, Ly-$6C^{+}$, MHC-II^{lo}, Siglec-H^{+}.
   - $CD8^{+}$ DCs (LNs): $CD8a^{+}$, $CD11b^{lo}$, $CD11c^{hi}$, MHC-II^{hi}.
   - B cells: $CD19^{+}$, CD45R/$B220^{+}$, $sIgM^{+}$.
7. After sorting lung DCs by flow cytometry, approximately $1–2 \times 10^4$ $CD11b^{hi}$ DCs and $2–4 \times 10^4$ $CD103^{+}$ DCs are usually obtained per mouse, although cell yields vary among different experiments depending on the treatments the mice received. Multiply mouse number based on DC number needed for experiment.
8. Complete RPMI 1640 with 10 % FBS (low endotoxin) is recommended.
9. A 1:2 ratio of DCs to T cells induces robust T cell proliferation and differentiation, although T cell responses can be detected with wide range of ratios (1:1–1:100) of DCs to T cells.
10. T cell response is affected by medium pH. Check the concentration of CO_2 in the incubator and the pH of culture medium prior to culture.
11. Restimulation allows assessment of T cell responses following their differentiation without transfer of cytokines produced by naïve or differentiating T cells during the primary culture. In addition, because an equal number of T cells are typically restimulated, this method allows measurements of T cell responses on a per-cell basis.

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