Isolation and *in vivo* Transfer of Antigen Presenting Cells

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[Abstract] Transfer of antigen presenting cells *in vivo* is a method used by immunologists to examine the potency of antigen presentation by a selected population of cells. This method is most commonly used to analyze presentation of protein antigens to MHC class I or II restricted T cells, but it can also be used for studies of nonconventional antigens such as CD1-presented lipids. In a recent study focusing on CD1d-restricted glycolipid antigen presentation to Natural Killer T cells, we compared antigen presenting properties of splenic B cells, CD8α^{Pos} dendritc cells (DCs) and CD8α^{Neg} DCs (Arora *et al.*, 2014). This protocol describes the detailed method used for isolation of these cell populations, and their transfer into recipient mice to analyze their antigen presenting properties.

As a percentage of total mononuclear cells, an average spleen contains approximately 1-3% myeloid dendritic cells (DCs). In absolute numbers, this translates to approximately 0.6-1.8 x 10^6 DCs. To enhance the number of DCs in spleen, mice were injected subcutaneously with cells from a cultured melanoma cell line (B16.Flt3L) which has been engineered to express the fms-related tyrosine kinase 3 ligand **(**Flt3L) (Mach *et al.*, 2000). This protein is a growth factor homologous to colony stimulating factor-1 and plays a critical role in the differentiation of hematopoietic stem cells. Administration of this protein into mice as a purified protein results in the expansion of both CD8α^{Pos} and CD8α^{Neg} DC subsets in multiple organs. Similar expansion is also seen in mice that have been implanted with tumor cells overexpressing this protein (Mach *et al.*, 2000). In our experience, up to 60% of the total mononuclear cells in a spleen from a mouse with a palpable B16.Flt3L tumor can be CD11c positive dendritic cells, thereby giving a total yield of up to 5 x 10^7 DCs per mouse. A schematic illustrating the cell enrichment protocol is included in Figure 1, and representative data on purity of cell populations obtained with this protocol is shown in Figure 2.

Materials and Reagents

1. Source of splenocytes: 6-8 week old female C57BL/6 mice (Jackson ImmunoResearch Laboratories)
2. Murine B16.Flt3L melanoma cell line [as described by Mach *et al*. (2000)]

3. Ultrapure water
4. 0.05% Trypsin-EDTA (Life Technologies, Gibco®, catalog number: 25300-054)
5. Isoflurane (Sigma-Aldrich, catalog number: CDS019936-250MG)
6. Collagenase D (Roche Diagnostics, catalog number: 11088858001)
7. DNase I, dry powder (QIAGEN, catalog number: 79254)
8. 70% Ethanol (prepared from 200 proof ethanol) (Thermo Fisher Scientific, catalog number: 9-6705-004-220)
9. RBC lysis buffer (Sigma-Aldrich, catalog number: R7757)
10. RPMI-1640 medium with L-glutamine (Life Technologies, Gibco®, catalog number: 11875-119)
11. DMEM medium with L-glutamine (Life Technologies, Gibco®, catalog number: 11995-073)
12. 200 mM L-glutamine (Life Technologies, catalog number: 25030081)
13. MEM non-essential amino acids (Life Technologies, Gibco®, catalog number: 11140-050)
14. MEM essential amino acids (Life Technologies, catalog number: 11130-051)
15. β-mercaptoethanol (Life Technologies, Invitrogen™, catalog number: 21985-023)
16. Sodium pyruvate (Life Technologies, catalog number: 11360-070)
17. HEPES (Life Technologies, Invitrogen™, catalog number: 15630)
18. Phosphate buffered saline (PBS) (Ca^{2+} and Mg^{2+} free, pH 7.2) (Life Technologies, Invitrogen™, catalog number: 20012-050)
19. Dulbecco's PBS (DPBS) with Ca^{2+} and Mg^{2+} (Life Technologies, Gibco®, catalog number: 14040-182)
20. 0.5 M Ethylenediaminetetraacetate (EDTA) solution (Life Technologies, catalog number: 15575-020)
21. Bovine serum albumin (BSA) (Sigma-Aldrich, catalog number: A2153)
22. Fetal calf serum (Atlanta Biologicals, catalog number: S11050)
23. Penicillin/streptomycin (Life Technologies, Invitrogen™, catalog number: 15140-163)
24. Trypan blue (dry powder) (Sigma-Aldrich, catalog number: T6146-5G)
25. Magnetic beads conjugated with anti-mouse CD19 (Miltenyi Biotech, catalog number: 120-000-323)
26. Magnetic beads conjugated with anti-mouse CD11c (Miltenyi Biotech, catalog number: 130-152-001)
27. $CD8\alpha^{Pos}$ mouse DC isolation kit (Miltenyi Biotech, catalog number: 130-091-169)
28. Fc-gamma receptor blocking antibody (Clone 2.4G2) (BD Biosciences, catalog number: 553141).
29. Anti-mouse CD11c-FITC (BD Biosciences, catalog number: 553801)
30. Anti-mouse CD8α-PerCP (BD Biosciences, catalog number: 553036)

31. Anti-mouse B220-PE (BD Biosciences, catalog number: 553090)
32. 0.08% trypan blue (see Recipes)
33. Serum free DMEM and RPMI media (see Recipes)
34. Complete RPMI and DMEM media (see Recipes)
35. MACS buffer (see Recipes)
36. FACS staining buffer (see Recipes)
37. 10x collagenase D solution (see Recipes)

Equipment

1. 1 ml syringes (BD, catalog number: 26048)
2. 23 G1 needle (BD, catalog number: 305145)
3. 100 mm Petri dishes (Thermo Fisher Scientific, catalog number: 0875712)
4. Surgical instruments (Kent Scientific, catalog number: INSMOUSEKIT)
5. Cell strainer (70 µm) (BD, catalog number: 352350)
6. Large Petri plates (Thermo Fisher Scientific, catalog number: FB0875712)
7. Vacuum filtration system (500 ml, 0.22 um) (Corning, catalog number: 431097)
8. LS columns (Miltenyi Biotec, catalog number: 130-042-401)
9. Magnetic stand MACS separator (Miltenyi Biotec, catalog number: 130-042-302)
10. Wide-bore 200 µl pipette tips (PerkinElmer, catalog number: 111623)
11. Corning ultra-low attachment 96-well plates (Corning, catalog number: CLS3474-24EA)

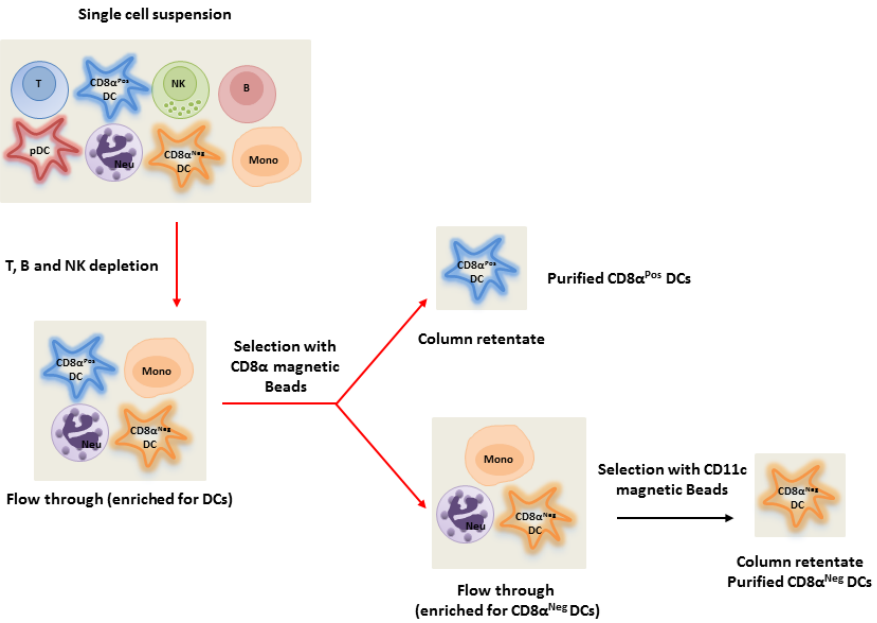

Figure1. Scheme of cell enrichment protocol

Procedure

A. Implantation of B16.Flt3L melanoma in mice

1. The B16.Flt3L melanoma cell line is grown to confluence in complete DMEM medium in standard cell culture flasks (25 cm^2), and the cells are harvested by trypsinization. For this, aspirate the medium from the culture, and wash the adherent cells with sterile PBS. After washing, add 5 ml Trypsin EDTA (0.05%) solution and incubate at 37 °C for 5 min.
2. After incubation, gently tap the flask on one side to lift the cells off of the surface of the vessel.
3. Add 20 ml of complete DMEM medium to quench the protease digestion.
4. Harvest cells from the flask by gently pipetting up and down three times, and then collecting the medium into a sterile 50 ml centrifuge tube.
5. Pellet cells by centrifuging at 300 *x g* for 5 min at 4 °C. Wash the cells three times with PBS and resuspend in PBS to a density of 10^8 cells/ml.
6. Inject mice with 100 µl cell suspension (10^7 cells) subcutaneously into a single site in the area of the shoulder at the base of the neck. This and all other procedures involving animals must be in accordance with and approved by the Institutional Animal Use and Care Committee (IACUC).
7. Allow the tumor to grow until palpable or visible (2-10 mm in diameter, usually 7-10 days) before sacrificing animals for harvesting organs.

B. Preparation of splenic single cell suspension

1. Anesthetize mice with isoflurane. A typical approach for anesthesia involves placing the animal in an induction chamber connected to an oxygen source and isofluorane vaporizer, and adjusting oxygen flow to 0.9 liters/min and the isofluorane vaporizer to 3.5%. As soon as mice become unresponsive, they are sacrificed by cervical dislocation.
2. Sterilize the left side of the abdomen by spraying the skin with 70% ethanol.
3. Working under clean, aseptic conditions, make an incision under the rib cage on the left side of the abdomen and extract spleen using scissors. Place in sterile serum free RPMI medium.
4. From this step onwards, work in the biosafety cabinet and perform all the steps under sterile conditions.
5. Wash the aseptically extracted spleen by submerging it in 5 ml of serum-free RPMI medium, and then transfer to a fresh 100 mm diameter Petri dishes. Note that because of the high yield of splenocytes from B6.Flt3L tumor bearing mice, one spleen is sufficient for most experiments, and this protocol is for the processing of a single spleen. For larger

experiments involving pooling of two or more spleens, volumes may need to be scaled up proportionately.

6. Cut spleen tissue into approximately 2 mm^3 pieces using scissors and incubate in 10 ml of collagenase D (~200 units) and DNase I (10 µg/ml) solution at 37 °C for 30 min. Make sure the tissue pieces are completely immersed in the solution.
7. Place a 70 µm cell strainer placed in a fresh Petri dish, and transfer the tissue pieces to it by pipetting with a 25 ml pipette.
8. Crush the splenic pieces by gently compressing the tissue fragments against the mesh with the syringe plunger. Wash the mesh filter with 5 ml of complete RPMI medium to wash cells through the filter and into the collection dish. Use of 70 µm mesh filter removes larger cell aggregates and helps in generation of single cell suspension.
9. Pellet cells by centrifuging at 300 *x g* for 5 min.
10. Discard supernatant and add 2 ml RBC lysis buffer to the pellet.
11. Mix gently by pipetting thrice with a 1 ml pipette and further incubate at room temperature for 5 min. Avoid longer incubations with RBC lysis buffer, since this may compromise cell viability.
12. Neutralize the RBC lysis buffer by adding complete RPMI medium (12 ml per spleen) to the resulting suspension and centrifuge at 300 *x g* for 5 min.
13. Resuspend the cell pellet in MACS buffer, using 0.5 ml per spleen. Filter through 70 µm cell strainer to remove cell clumps to prevent MACS column from clogging.

C. Purification of $CD8\alpha^{Pos}$ DCs and $CD8\alpha^{Neg}$ DCs from splenic single cell suspension

Isolation of the $CD8\alpha^{Pos}$ DCs and $CD8\alpha^{Neg}$ DCs from splenic cell suspension is a three-step protocol as illustrated in Figure 1. It relies on depletion of undesired populations like B, T and NK cells in the first step, followed by positive selection of $CD8\alpha^{Pos}$ DCs in the second step, and positive selection of $CD8\alpha^{Neg}$ dendritic cells in the third step. Keep the cells on ice and use pre-cooled buffers to maintain 2-8 °C working temperature to prevent antibody capping and non-specific binding to irrelevant cells.

1. Determine the cell number, and adjust concentration to approximately 5 x 10^8 cells/ml in MACS buffer containing 20 µg/ml Fc-gamma receptor blocking antibody (2.4G2).
2. For each 10^8 total cells, add 100 µl of biotin antibody cocktail provided in the $CD8\alpha^{Pos}$ DC isolation kit. The antibody cocktail binds to B cells, T cells and NK cells.
3. Mix thoroughly and incubate in ice cold water (4-8 °C) for 15 min.
4. For each 10^8 total cells, add 150 µl of MACS buffer and 100 µl of anti-biotin beads provided in the $CD8\alpha^{Pos}$ DC isolation kit.
5. Mix gently and incubate again in ice cold water (4-8 °C) for 15 min.
6. Add 12 ml MACS buffer and pellet cells by centrifuging at 300 *x g* for 5 min.

7. Discard supernatant and resuspend the cell pellet in 2 ml of MACS buffer.
8. Place a fresh MACS column in the magnetic field of an appropriate MACS separator.
9. Equilibrate column by washing with 5 ml of MACS buffer.
10. Pipette the cell suspension gently in the middle of the column.
11. Collect unlabeled cells that flow through the column followed by washing with 10 ml MACS buffer. The unlabeled flow through thus collected is enriched for DCs.
12. Pellet the enriched DC fraction by centrifuging at 300 *x g* for 5 min.
13. Resuspend the cells into 1 ml MACS buffer and add 200 µl of anti-CD8α conjugated Miltenyi magnetic bead suspension and incubate in ice cold water (4- 8 °C) for additional 15 min.
14. Repeat steps C6-11. The $CD8\alpha^{Pos}$ dendritic cells are bound with the anti-CD8α magnetic beads in this enrichment method, and are thus retained in the column. The flow through of the column is enriched for $CD8\alpha^{Neg}$ DCs and depleted for $CD8\alpha^{Pos}$ dendritic cells. The flow through should be retained to use for isolation of $CD8\alpha^{Neg}$ dendritic cells in steps C18-22 below.
15. To elute the $CD8\alpha^{Pos}$ DCs from the column, gently remove the column from the magnetic field, add 5 ml MACS buffer and purge the column firmly with a syringe plunger.
16. For enhanced purity, the $CD8\alpha^{Pos}$ enriched cells are passed through two consecutive columns. For this, pellet the eluted cells in step C15, resuspend with 2 ml MACS buffer and repeat steps 8-15.
17. Pellet the collected $CD8\alpha^{Pos}$ DCs by centrifugation, resuspend in 0.2 ml medium and count. Keep the cells on ice until needed to enhance viability.
18. For isolation of $CD8\alpha^{Neg}$ DCs, use the unlabeled cells collected as the column flow through in step C16. Pellet the cells by centrifugation and resuspend in 300 µl of MACS buffer.
19. Add 100 µl of anti-CD11c magnetic beads and incubate in ice cold water (4- 8 °C) for 15 min.
20. Repeat steps C6-10.
21. Wash column with 10 ml of MACS buffer. Elute the column retentate containing enriched $CD8\alpha^{Neg}$ DCs.
22. Pellet the cells and resuspend in 2 ml MACS buffer and pass them through a second MACS column to increase the purity if required.

D. Isolation of B cells from splenic cell suspension

1. Determine the cell number.
2. Add 200 µl MACS buffer containing 20 µg/ml Fc-gamma receptor blocking antibody (2.4G2), and 150 µl of anti-CD19 conjugated magnetic beads.

3. Mix thoroughly and incubate in ice cold water (4-8 °C) for 10 min.
4. Add 12 ml MACS buffer and pellet cells by centrifuging at 300 *x g* for 5 min.
5. Discard supernatant and resuspend the cell pellet 2 ml of MACS buffer.
6. Place a MACS column in the magnetic field of an appropriate MACS separator.
7. Equilibrate column by washing with 5 ml of MACS buffer.
8. Pipette the cell suspension gently in the middle of the column.
9. This is a positive selection, so the target cells will be retained in the MACS column. To elute the cells from the column, gently remove column from magnetic field, add 5 ml MACS buffer and purge the column firmly with a plunger to collect $CD8\alpha^{Pos}$ DCs.

E. Pulsing APCs with antigens and cell transfer

1. Count live cells after MACS enrichment procedure by trypan blue exclusion.
2. Plate cells in low-binding U-bottom 96 well plates at a density of 10^6 cells per well in 250 µl complete RPMI containing antigen of interest at desired concentration. Pulse the cells with antigen (typically at concentration of 100 nM, although this may vary depending on the particular antigen) for one hour in 37 °C incubator maintaining 5% CO_2. Use of low binding plates allows for easier recovery of adherent cell populations.
3. Harvest cells by pipetting up and down 5 times with the wide-bore tips. Pool cells from multiple wells and transfer to 15 ml tube. Add complete RPMI medium to 10 ml and pellet the cells by centrifuging at 300 *x g* for 5 min. Wash twice with PBS.
4. Count the cells again and resuspend in PBS to a cell density of 10^7 cells/ml. Use these cells for injection into mice by the preferred route of administration.

Representative data

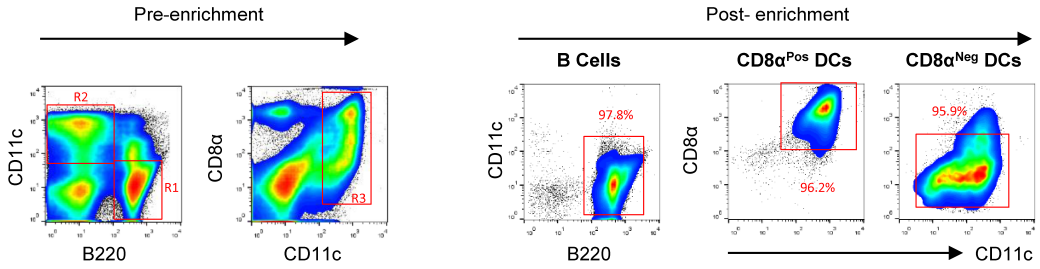

Figure 2. Pre- and post-enrichment analysis of splenic subsets. B and Dendritic cells are identified as B220 positive (R1, 38.5%) and CD11c positive (R2, 25.5%) live cells present in the spleens of B16.Flt3 melanoma bearing mice on day 8 after tumor implantation. In the second plot, gradient of CD8α expression can be observed on CD11c positive cells (R3

gate). In the post-enrichment plots, the relative purity of B cells, CD8α^{Pos} and CD8α^{Neg} DCs is ascertained. The staining for CD11c in the enriched CD8α^{Neg} DC subset competes with the anti-CD11c beads used to isolate these cells, thereby resulting in the observed gradient of CD11c-flourescence staining. In all cases, the purity of cells was found to be more than 95%.

Recipes

1. 0.08% trypan blue
   Dissolve 0.08 g trypan blue in 100 ml ultrapure water
   Filter through 70 µm strainer to remove undissolved aggregates
2. Serum free DMEM and RPMI media
   Mix all of the following ingredients in biosafety hood for either DMEM or RPMI media depending on your need
   Sterilize media by passing through 0.22 µm vacuum filtration system
   500 ml DMEM with L-glutamine, or 500 ml RPMI-1640 with L-glutamine
   5 ml MEM nonessential amino acids (100x, 10 mM)
   5 ml HEPES buffer (1 M)
   5 ml L-glutamine (200 mM)
   0.5 ml 2-mercaptoethanol (5.5 x 10^{-2} M)
3. Complete RPMI and DMEM media
   Add 50 ml of heat inactivated fetal calf serum to serum free RPMI or DMEM media to obtain complete media
4. MACS buffer
   Add 2 ml of 0.5 M EDTA and 10 ml of heat inactivated fetal calf serum to 500 ml of Phosphate-buffered saline (PBS, Ca^{2+} and Mg^{2+} free, pH 7.2)
   Filter sterile and degas the buffer by applying vacuum for at least 15 min prior to use to avoid air bubbles from blocking the column
5. FACS staining buffer
   Dissolve sodium azide to 0.05% (0.25 g per 500 ml) in MACS buffer to obtain FACS staining buffer
6. 10x collagenase D and DNase 1 stock solution
   Dissolve 1 g of collagenase D and 0.2 ml of DNase 1 stock (1 mg/ml, 100x) in 20 ml of PBS containing Ca^{2+} and Mg^{2+} to obtain a solution of approximately 1,000-2,000 Units of collagenase activity per ml
   This 10x stock solution that can be aliquoted as 1 ml vials and stored at -20 °C for several weeks. Dilute 1 ml of this with 9 ml of serum free RPMI immediately before use

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