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Isolation of Mouse Spleen Dendritic Cells

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1. Introduction

It is now over 20 years since dendritic cells (DC) were first identified in and isolated from the spleens of mice ***(1,2)*** and they continue to be a much-studied population. Only a small proportion of spleen cells are DC, but the large size of the organ means that useful numbers of DC can still be purified. In recent years the ability to grow cells with the phenotypic and functional properties of DC from bone marrow progenitors has opened new avenues of research. However, the relationship of cells grown in this way to DC populations in vivo is unknown and the need remains to study DC present in tissues.

Spleen DC are heterogeneous with differences in phenotype, function, and microanatomical location ***(3,4)***. At least two major subsets are recognized, and these can be discriminated on the basis of the presence or absence of a cell-surface αα homodimer of the CD8 molecule. The freshly isolated CD8αα$^+$ population is DEC-205$^+$, CD24$^+$, CD11b$^-$, 33D1$^-$, CD4$^-$, whereas the CD8αα$^-$ subset is DEC205$^-$, CD24$^-$, CD11b$^+$, 33D1$^+$, CD4$^-$. Both subsets express CD11c, and this marker appears to be expressed selectively on DC and in the mouse can be used as a pan-DC marker. The CD8αα$^+$ population predominately localizes in the T-cell areas of the white pulp and corresponds to interdigitating cells. In the steady state, the CD8αα$^-$ population is probably localized predominately in the marginal zone, between the red and white pulp, but mobilizes into the T-cell areas in response to lipopolysaccharide (LPS) administration ***(5)***. This marginal zone DC population has a higher phagocytic activity and turnover rate than the interdigitaing cells ***(6)***. The CD8αα$^+$ and CD8αα$^-$ populations may be cells of lymphoid and myeloid lineages, respectively. They

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can both activate resting T cells, but may stimulate different types of responses. The CD8αα$^+$ population has been reported to drive preferentially Th1 responses, whereas presentation of antigen by the CD8αα$^-$ may favor Th2 responses *(7)*. CD8αα$^+$ DC can also kill activated T cells via Fas-mediated apoptosis *(8)*. The division of spleen DC into "lymphoid" and "myeloid" populations is probably an oversimplification, and recent evidence suggests further heterogeneity with the description of a third, CD4$^+$, spleen DC subset *(9)*.

There are many published protocols for isolating mouse spleen cells and in choosing among these methods two factors should be borne in mind. First, different methods may favor the recovery of particular DC subsets at the expense of others. This can be a problem if the intention is to recover a representative sample of the total spleen DC, but it can also be turned to the investigator's advantage in the purification of particular subsets. Second, DC may be altered phenotypically or functionally by the isolation process itself. This modulation occurs in methods in which DC are cultured for prolonged periods, because in vitro culture is sufficient to induce DC maturation. Changes in properties of DC may also occur during positive selection with monoclonal antibodies or digestion of tissue with proteolytic enzymes. For instance, collagenase preparations are likely to contain significant concentrations of endotoxin that may affect DC.

In this chapter we describe a basic method for the enrichment of mouse spleen DC that involves overnight culture and separation on hypertonic metrizamide gradients and provide a suggested protocol for the further purification of these cells. We also describe an alternative method for spleen DC that avoids the need for culture and discuss how the choice of method for initial preparation of a spleen cell suspension can be used to influence the recovery of particular DC subsets.

2. Materials

1. Specific pathogen free mice. The commonly used strains in our laboratory are BALB/c, CBA, and C3H. We have used mice of either sex, and they are usually aged 6–12 wk.
2. Dissecting board or paper tissues.
3. 70% ethanol.
4. Sterile surgical instruments (forceps and scissors).
5. Complete medium: Dutch modification of RPMI-1640 (Sigma; cat. no.R-7638) supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 m*M* L-glutamine,100U/mL penicillin/streptomycin, and 5×10^{-5}M 2-mercaptoethanol (2-ME) (*see* **Note 1**).
6. HEPES-buffered RPMI-1640 (Sigma, cat. no. R-5886)
7. Metal cell strainers.
8. 60 mm Petri dishes (Nunc or Sterilin).

9. 2 mL and 1 mL syringes (Terumo).
10. 10 mL conical-bottomed tubes (Sterilin; cat. no. 144AS).
11. Disposable Pasteur pipets (Alpha Labs; cat. no. LW4005) or sterilized glass equivalents.
12. Small filters for sterilization (GelmanSciences; cat. no. 6224184 [0.45mm] or cat. no. 6224192 [0.22 μm]).
13. Collagenase digestion mix: 1mg/mL collagenase D (Roche Molecular Products; cat. no. 1088 866), 20 μg/mL DNase I (Roche Molecular Products; cat. no. 1284 982), 2% FCS in HEPES-buffered RPMI-1640 (*see* **Notes 2** and **3**).
    To prepare collagenase stock:
    a. Dissolve 500 mg of collagenase D in 50 mL serum-free HEPES buffered RPMI-1640 (10 mg/mL).
    b. Filter sterilize (0.45 μm).
    c. Store in aliquots at –20°C.
    d. Avoid repeated freezing and thawing.
    e. Thaw aliquots as required and keep on ice until used.
    To prepare DNase I stock:
    a. Dissolve 100 mg in 10 mL dH_20 (10 mg/mL).
    b. Filter sterilize.
    c. Store in aliquots at –20°C.
    d. Avoid repeated freezing and thawing.
    To make 10 mL of digestion mix combine:
    a. 1 mL collagenase D stock.
    b. 20 μL DNase I stock.
    c. 0.2 mL FCS
    d. 8.8ml HEPES buffered RPMI-1640
    Keep on ice until use.
14. 26G × 1/2 in. needles.
15. Disposable scalpels or scalpel blades.
16. T25 tissue culture flasks (Falcon; cat. no.353014) (*see* **Note 4**).
17. Cell scrapers (Falcon; cat. no. 3085).
18. Analytical grade metrizamide (Nycomed; cat. no.22.20.10) (*see* **Note 5**).
19. Sterile 5 mL (75 mm × 12 mm) push cap round bottomed tubes (Sarstedt; cat. no. 55.476.013).
20. MiniMACS buffer: PBS containing 5% bovine serum albumin (BSA) and 5 m*M* EDTA. Filter sterilize. Handle carefully to avoid frothing (*see* **Note 6**).
21. Heat-inactivated normal mouse serum.
22. Monoclonal antibodies and immunomagnetic microbeads (*see* **Note 7**). These include:
    a. "Fc-Block" (PharMingen; cat. no. 01241A/D) (*see* **Note 8**).
    b. Microbeads coated with anti-CD11c (N418) (Miltenyi; cat. no.520-01).
    c. Anti-CD11c-FITC (clone HL3) (PharMingen; cat. no.09704A/D). For some applications the same antibody labeled with an alternative fluorochrome (e.g., phycoerythrin) may also be required.

d. Anti-CD45R-FITC (B220) (PharMingen; cat. no.01124A/D).
e. Microbeads coated with anti-FITC (Miltenyi; cat. no.487-01).

23. MiniMACS magnet and holder or the varioMACS system (Miltenyi) (*see* **Note 9**)
24. MiniMACS columns (Type MS^+/RS^+ for miniMACS or varioMACS and/or Type LS^+/VS^+ for varioMACS, Miltenyi) (*see* **Note 9**).

3. Methods

3.1. Preparation of Single Cell Suspensions from Spleens

This section describes removal of the mouse spleen and presents three different methods for producing a single-cell suspension from the organ. The way in which the choice of methods influences the recovery of DC is discussed.

3.1.1. Removal of the Spleen

1. Kill the mouse by cervical dislocation.
2. Lay mouse on dissecting board, "left side" uppermost.
3. Surface-sterilize the skin using 70% ethanol or a proprietary compound.
4. Using one set of sterile surgical instruments (forceps and scissors), cut through the skin just below the ribcage and visualize the spleen.
5. Using a second, smaller, set of instruments, remove the spleen, trimming away any fatty tissue.
6. Place spleen into complete medium at room temperature (*see* **Note 10**). Spleens from multiple animals can be pooled.

3.1.2. Preparation of a Single-Cell Suspension using a Metal Sieve

This has been our routine method for many years. It avoids the use of proteolytic enzymes, gives good recovery of DC numbers, and, in conjunction with overnight culture and metrizamide separation, yields a mixture of $CD8\alpha\alpha^+$ and $CD8\alpha\alpha^-$ DC (*see* **Fig. 1**).

1. Strain spleens by pouring through a sterile metal cell-strainer. Discard medium.
2. Place the strainer containing spleens into a 60 mm Petri dish and add a few milliliters of fresh medium.
3. Using the barrel from a 2 mL syringe, press the spleens through the strainer. Continue until only a little fibrous tissue remains in the strainer.
4. Remove the strainer and place in upturned lid of the Petri dish.
5. Reinsert plunger into syringe barrel and use to transfer spleen cell suspension to a 10 mL conical tube. (A larger tube or replicate tubes will be required for multiple spleens.)
6. Using fresh medium and a Pasteur pipet rinse the Petri dish and the cell strainer to ensure that all cells have been recovered. Pool with the rest of the spleen cell suspension.
7. Top-up tube with complete medium to appropriate volume (*see* below).

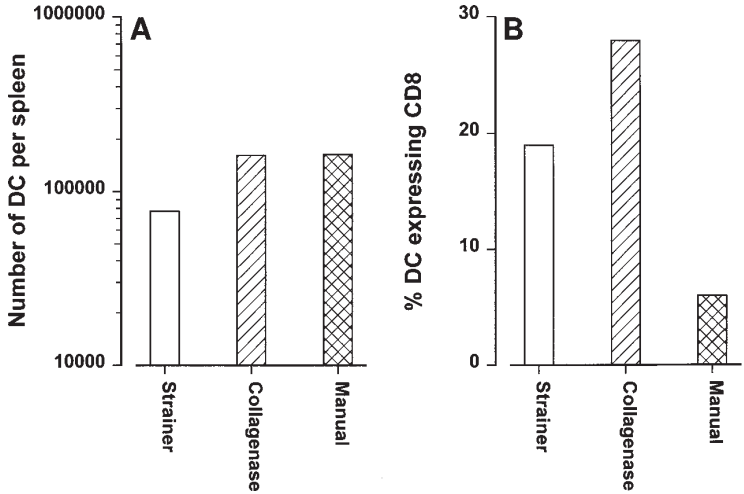

Fig. 1. Method used to prepare a singlecell suspension from spleens influences the number (**A**) and type (**B**) of DC obtained. Spleen cells were obtained by the strainer, collagenase or manual extraction techniques as described in the text. The absolute number of CD11c$^+$ DC obtained following overnight culture and separation on metrizamide was determined by flow cytometry with simultaneous acquisition of Flow Count fluorospheres (Beckman Coulter). The proportion of CD8$^+$ DC was determined by double staining with anti-CD8α.

3.1.3. Preparation of a Single-Cell Suspension using Collagenase

This method gives an overall increase in DC yield and improves the recovery of the tightly tissue-bound CD8αα$^+$ DC (**Fig. 1**). The use of proteolytic enzymes may be undesirable for some applications.

1. Place 5 mL of digestion mix into a Petri dish.
2. Put a small needle (26G × 1/2 in. is ideal) on a 1 mL syringe and fill with digestion mix.
3. Gently inject the first spleen with 0.5–1 mL of digestion mix. Initially insert the needle just inside the spleen at the narrowest part of the capsule. Inject approx 100 μL, advance the needle slightly, and then inject again. Continue in this fashion. This may take some practice. The spleen will distend and change from a dark maroon color to a reddish orange.
4. Using the needle tear open the spleen in a second (empty) Petri dish.
5. Place the spleen back in the first Petri dish containing the digestion mix.
6. Transfer the released cells in the second dish to a conical tube and place on ice. Rinse this dish with more digestion mix and pool with the other cells on ice.

7. Repeat steps **1–6** with the next spleen, pooling released cells and spleens.
8. Using a disposable scalpel or scalpel blade cut up spleens into small fragments.
9. Transfer to a T25 tissue culture flask and incubate with gentle shaking at 37°C for approx 60 min (*see* **Note 11**).
10. At the end of the incubation, collect contents of dish. Rinse dish with a small volume of digestion mix and pool together.
11. Press contents through a cell strainer as described previously.
12. Pool cells with those already on ice. Rinse dish in digestion mix or medium and again pool with the other cells.
13. Spin down (350*g*, 5 min), discard supernatant and gently resuspend cell pellet in 10 mL complete medium.
14. Spin down again and resuspend in complete medium. Adjust to required volume (*see* below).

3.1.4. Preparation of a Single-Cell Suspension by the "Manual Extraction" Method

This very gentle method yields DC that are almost exclusively CD8αα$^-$ (**Fig. 1**), so this approach may be useful as an early step in purifying this subset.

1. Place some complete medium in a Petri dish and place the dish at an angle by resting it partially on its lid.
2. Using forceps, make a hole in the capsule at one end of the spleen.
3. Place the spleen on the sloping Petri dish, punctured end facing "down the hill." Hold in place with forceps.
4. Using a cell scraper gently press cells out of the spleen by working the scraper from the middle to the lower end of the spleen.
5. When capsule is clear turn the spleen round, make a hole at the other end of it and work the rest of the cells out.
6. When all cells have been removed, discard the empty capsule and transfer the cells to a conical tube.
7. Repeat the procedure with additional spleens, pooling the released cells.
8. Adjust to the appropriate volume (*see* below).

3.2. Enrichment of DC Using Metrizamide Gradients

Following overnight culture, a single separation step on a metrizamide gradient enriches DC up to 100-fold. The separation is based partly on density and partly on differential shrinkage of cells following exposure to the metrizamide, which is slightly hypertonic. Following centrifugation, low density cells (LDC) that stay up on the gradient contain most of the DC, whereas the lymphocyte-rich high density cells form a pellet.

3.2.1. Preparation of Metrizamide Gradients

1. Weigh out 7.25 g of metrizamide and place in 50 mL conical tube.
2. Add a total of 45 mL HEPES-buffered RPMI-1640. To get the metrizamide into solution, it is easier to add the medium in several stages (perhaps 15 mL at a

time), mix gently, and then leave to stand for a while after each addition. Avoid inverting the tube as metrizamide is quite sticky and can be "lost" stuck to the top of the tube.
3. Add 5 mL of FCS. Adding this separately makes the metrizamide easier to dissolve. Note that the total volume of medium *added* is 50 mL so the final solution is less than 14.5% w/v (actually 13.7% w/v).
4. Filter sterilize (0.45 μm), divide into 2 mL aliquots and store at –20°C (*see* **Note 13**).
5. On the day of taking spleens calculate the required number of metrizamide aliquots (1–2 per spleen–*see* below) and place in a fridge overnight to defrost.

3.2.2. Overnight Culture of Spleen Cells

Overnight culture of spleen cells prior to centrifugation on metrizamide probably contributes to DC separation in three ways. First, "maturation" of the DC in culture increases their tendency to stay up on the gradient. In line with this, mature DC can be separated from lymph nodes without the need for prior culture of the cells (*see* Chapter 1), presumably because the lymph node DC population is more mature in vivo. Second, culture allows migration from tissue fragments of tightly bound DC (*see* **Note 14**). Third, some contaminating cells with prolonged adherence properties (e.g., macrophages) may be removed by culture.

1. Prepare single-cell suspensions from spleens by one of the methods described above.
2. Resuspend in 5-10 mL of complete medium per spleen (*see* **Note 15**).
3. Add the spleen cell suspension to T25 tissue culture flasks, putting 5ml into each flask (*see* **Note 15**).
4. Culture overnight at 37°C in a humidified incubator containing 5% CO_2 in air (*see* **Note 16**).

3.2.3. Separation on Metrizamide

1. Remove the thawed metrizamide from the fridge and allow it to come to room temperature while preparing the spleen cells (*see* **Note 17**).
2. Take flasks containing the overnight cultures of spleen cells from the incubator.
3. Resuspend the cells and dislodge loosely adherent populations by pipeting the medium containing the cells up and down with a Pasteur pipet. This can be done fairly vigorously.
4. Transfer each 2 mL aliquot of metrizamide to a 10 mL conical centrifuge tube.
5. Carefully overlay each metrizamide gradient with 5 mL of cell suspension (0.5–1 spleen per gradient). This can be done with a Pasteur or a syringe and filling tube according to preference but does require a little practice. Monitor the gradient as the cells are added to ensure that a "clean" interface forms between the metrizamide and the cell suspension. Mixing of the two will adversely effect the separation.

6. Centrifuge at room temperature at 650*g* for 10 min. (No brake!)
7. Using a Pasteur pipet, recover the LDC from the interface between the metrizamide and medium. Avoid taking up any of the pellet cells or any fatty material that may float at the top of the medium.
8. If desired, the high density cell pellet can also be recovered (*see* **Note 18**).
9. Centrifuge the LDC suspension (650*g*, 10 min, room temperature).
10. Discard the supernatant and gently resuspend the pellet in complete medium (approx 5 mL per tube).
11. Spin down again. This step can be more gentle than previous centrifugations (350*g* for 5 min) as the majority of metrizamide has now been removed.
12. Discard the supernatant and resuspend the pellet in a small volume of medium (usually 1–2 mL depending on the number of spleens) pooling the contents of replicate tubes as appropriate.
13. Perform a cell count. Expect to recover in the order of 5×10^5 to 1×10^6 LDC per spleen depending on factors such as age and strain of mouse and the cleanliness of the animal facility (*see* **Note 19**).

3.3. Further Purification of DC by Immunomagnetic Separation

The LDC preparation obtained by centrifugation over metrizamide will generally contain 40–60% $CD11c^+$ DC (*see* **Note 20**). Some workers report up to 80% purity. Almost all of the contaminating cells are a population of B cells ($[CD19^+CD45R(B220)^+ CD11c^-]$). These are larger cells than the majority of the spleen B-cell population (which is presumably why they separate in the LDC fraction) and may correspond to marginal zone B cells. It is straightforward to purify further the DC by positive selection using CD11c or by depleting the $CD45R^+$ population (**Fig. 2**). DC prepared by both approaches stimulate an allogeneic mixed leukocyte reaction (**Fig. 3**); the $CD45R^+$ cells stimulate more weakly.

3.3.1. Positive Selection of $CD11c^+$ DC

1. In a 5 mL push-cap tube, wash LDC into cold MiniMACS buffer by diluting the cells into the buffer and pelleting by centrifugation (350*g*). In a typical experiment we might use 6×10^6 LDC.
2. Discard the supernatant and gently resuspend the cells in the residual volume (approx 100 µL) (*see* **Note 21**).
3. On ice add the following:
   15 µl heat inactivated normal mouse serum.
   1.5 µL "Fc-Block".
   20 µL immunomagnetic microbeads coated with anti-CD11c.
4. Transfer to a fridge and incubate for 15 min (*see* **Note 22**)
5. Wash cells by topping-up the tube with cold miniMACS buffer and pelleting the cells by centrifugation in the cold (400*g*).
6. Discard the supernatant and repeat washing step.

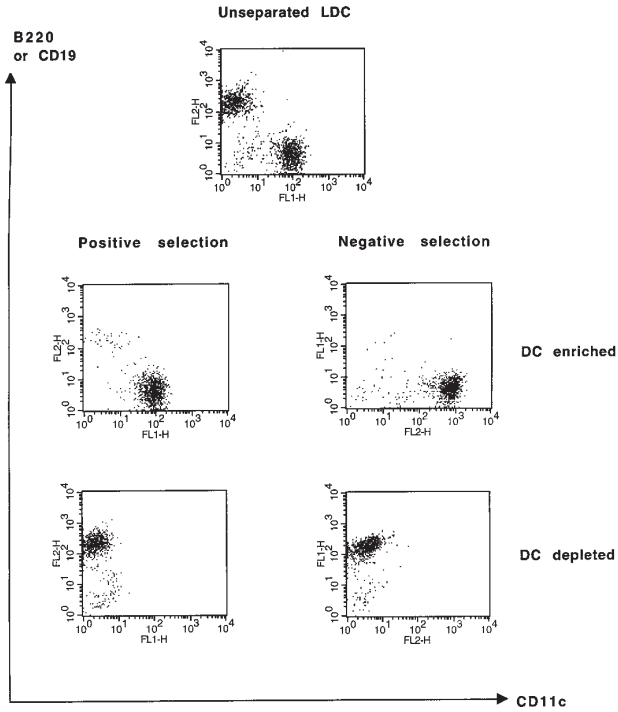

Fig. 2. DC can be further purified from the LDC preparation by either positive or negative selection using antibodies to CD11c or CD45R(B220), respectively. DC depleted populations are obtained in parallel.

7. Gently resuspend the cells in the residual volume (approx 100 µL).
8. Meanwhile prepare the miniMACS columns (Type MS):
   a. Assemble the column into the miniMACS magnet and place on holder (*see* **Note 9**). Do not use a flow restrictor (i.e., set up for positive selection).
   b. Adding 500 µL of miniMACS buffer to the top of the tube and allow to wash through the column. The washing fluid (which will be turbid) can be collected and discarded.
9. Add the cells to the top of the column and allow them to enter it.
10. Add 500 µL to of miniMACS buffer to the top of the column and allow it to wash through. If desired, these washings can be collected from the bottom of the column as a negative fraction.
11. Wash the column twice by passing 500 µL of miniMACS buffer through it on each occasion.
12. Remove column from the magnet and, at a distance well removed from the mag-

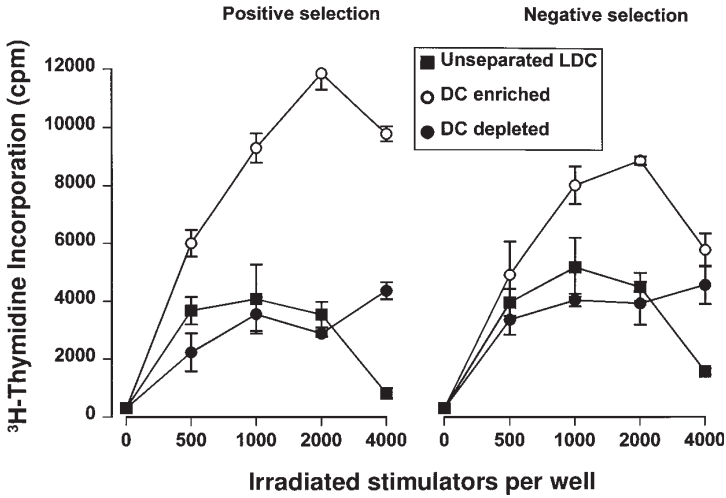

Fig. 3. DC, enriched by either positive or negative immunomagnetic selection, stimulate a Primary mixed leukocyte reaction (MLR). Unseparated LDC and DC enriched or depleted preparations were irradiated (2000r) and used to stimulate 25000 allogeneic lymph node cells in a 20 μL hanging drop culture system. Proliferation was assessed by ^{3}H-thymidine incorporation on day four of culture.

netic field, add 1 mL of miniMACS buffer to the top of the tube and press though the column using the plunger supplied.

13. Collect the positively selected cells from the bottom of the column.
14. If desired, aliquots of cells can be removed at **steps 7, 10,** and **13** and labeled with FITC conjugated anti-CD11c (clone HL3) (1.5 μL, 20 min on ice) in order to monitor the purification process.
15. The postively selected DC can be washed into complete medium (*see* **Note 23**) or pelleted and passed over a second column. A second pass can improve purity but at a cost of additional cell losses. Expect a purity of 95% (**Fig. 2**).

3.3.2. Depletion of CD45R$^+$ Cells.

This is an alternative approach when there are concerns about coating the selected DC population with anti-CD11c. We have generally used an indirect approach in which cells are labeled with FITC-conjugated anti-CD45R and then with anti-FITC immunomagnetic beads. However, beads coated with anti-CD45R are also available, and these could be used in a direct labeling approach.

1. Wash LDC into miniMACS buffer as in **Subheading 3.3.1**.
2. On ice add the following:
   15 μL heat inactivated normal mouse serum.

1.5 μL "Fc-Block."
5 μL anti-CD45R-FITC.

3. Incubate in fridge for 15 min.
4. Wash twice as described in **Subheading 3.3.1**.
5. Add 10 μL of anti-FITC coated immunomagnetic microbeads.
6. Incubate in fridge for 15 min.
7. Wash twice.
8. Meanwhile prepare the miniMACS column (Type MS). The procedure is as described in **Subheading 3.3.1.,** except the flow restrictor supplied with the column is fitted prior to the initial washing of the column.
9. Add the cells, in approx 100 μL of miniMACS buffer to the top of the column and let them run in.
10. Add 500 μL of buffer to the top of the column and collect the CD45R-depleted fraction as it drops from the flow restrictor (*see* **Note 24**).
11. If desired, the column can be washed and the retained $CD45R^+$ fraction can be collected (*see* **Note 25**).
12. The purification process can be monitored by retaining an aliquot of the cells prior to and after the separation. These can then be labeled with fluorochrome tagged antibodies and analyzed by flow cytometry. Be aware that the $CD45^+$ cells are already labeled with FITC, so any labeling with CD11c will have to employ an alternative fluorochrome.
13. A repeat pass over a second column can again be used to increase purity.

3.4. Isolation of Noncultured DC

In some circumstances culture of spleen cells overnight prior to separation on metrizamide may be undesirable. Here we present a method that employs the immunomagnetic microbead technology discussed above to isolate spleen DC without the need for culture.

1. Prepare a single-cell suspension from spleen tissue using one of the methods described above.
2. Resuspend spleen cells in miniMACS buffer at 400 μL of buffer per 10^8 spleen cells.
3. Add 100 μL of immunomagnetic beads coated with anti-CD11c (*see* **Note 26**)
4. Incubate for 15 min in the fridge.
5. Wash in 5–10 mL cold miniMACS buffer by centrifugation at 200*g* for 10 min. A refridgerated centrifuge is preferable.
6. Place the separation columns for separation: Use a MS^+/RS^+ column washed through with 500 μL of buffer when working with less than 2×10^8 total spleen cells; use a LS^+/VS^+ column washed through with 3 mL buffer for between 2×10^8 and 1×10^9 spleen cells. The smaller column can be used with either the miniMACS or varioMACs system; the larger column will require the varioMACS system.
7. Add the cell suspension to the top of the column(s) and allow the cells to enter the column.

8. Remove the unbound cells by washing the column with buffer: use 3×500 µL for a MS^+/RS^+ column or 3 × 3 mL for a LS^+/VS^+ column.
9. To remove bound $CD11c^+$ cells, remove the column well away from the magnet, add the appropriate volume to the top of the column (1 mL for MS^+/RS^+; 5 mL for LS^+/VS^+), and flush out using the plunger provided with the column.
10. Repeat the separation step using fresh columns (*see* **Note 27**).

4. Notes

1. 2-Mercaptoethanol is an inhibitor of collagenase. Therefore it should be omitted from medium used prior to the enzymatic digestion step if collagenase is used to produce a spleen cell suspension.
2. Collagenase D is recommended for maintenance of cell-surface protein integrity.
3. There can be considerable batch-to-batch variation in collagenase. Although a concentration of 1 mg/mL usually gives satisfactory results, it may prove necessary to adjust this concentration.
4. We use Falcon flasks. Products from other manufacturers may also be suitable, but there could be variation in performance. Small-tissue culture-grade Petri dishes may also be used.
5. Do not be tempted by the cheaper centrifugation grade metrizamide—it doesn't work! We have no experience with metrizamide from other manufacturers.
6. It is important to avoid air bubbles in the buffer, as these will affect the performance of the separation column. Degassing of the buffer is recommended by the columns' manufacturers. At the very least the buffer should be prepared and handled so as to mimimize frothing. To this end we prepare our buffer well in advance of use.
7. If in doubt about the sterility of monoclonal antibodies or immunomagnetic beads, small volumes can be sterilized by centrifugation through 0.22 µm Spin-X centrifuge tube filters (Costar; cat. no. 8160) at full speed in a microfuge for 2 min. When using beads, be sure to resuspend the pellet that forms in the bottom of the tube. All antibody concentrations stated are ones that we have found to work well in general, but investigators may need to vary these for their own applications.
8. "Fc-Block" is a mixture of unconjugated monoclonal antibodies to CD16 and CD32 (FcγIII/II), and, as its name suggests, it helps reduce nonspecific binding of the labeling antibodies via Fc receptors.
9. For best performance, precool the columns, magnets, and holders in a fridge or cold room. We also use "cool packs" supplied in the packaging of many cooled products, to keep the apparatus cool during prolonged separations.
10. If proceeding directly to preparation of cell suspensions from the spleen, it is better to keep the organ at room temperature than to expose it to the "shock" of the temperature changes involved in placing on ice and then warming up again in subsequent handling.
11. The length of the incubation may need to be varied with different batches of collagenase.

12. The performance of metrizamide gradients can be affected by small variations in osmolarity of the RPMI-1640 medium used to prepare them. The osmolarity of RPMI may vary slightly between manufacturers depending, for instance, on whether the medium is intended for use primarily with human or mouse cells. The osmolarity suitable for mouse cells is required for successful enrichment of DC. If your gradients perform poorly, it may be worth switching suppliers of RPMI.
13. There is anecdotal evidence that metrizamide gradients perform better when they have been through one freeze-thaw cycle. Therefore, we do not use freshly prepared metrizamide for separations.
14. Do not be tempted to remove tissue fragments from the cell suspension before overnight culture. These fragments are probably an important source of migrating DC.
15. Ideally use 10 mL of medium per spleen. This amount will then be cultured in two flasks and separated over two metrizamide columns. If processing many spleens, this can be reduced to 5 mL per spleen to reduce handling but do not be tempted to reduce this volume further. It is also inadvisable to "scale up" the separation procedure by using bigger flasks and columns.
16. The exact length of the "overnight" culture can influence the maturity and function of the DC obtained. Therefore, it is important to be consistent in the length of this incubation.
17. Do not allow the metrizamide to become too warm. This can be a problem in non-air-conditioned labs on hot summer days.
18. The pellet can be used as a source of lymphocytes. However, these cells need to be handled gently to allow them to recover from exposure to the hypertonic metrizamide.
19. Sudden changes in DC yields can be a sensitive indicator of the presence of infection within an animal facility.
20. The purity of DC separated on metrizamide varies with mouse strain. For instance, we routinely obtain better purity of DC from the spleen of BALB/c mice than from C3H mice.
21. To avoid frothing, resuspend *gently* using a pipet tip rather than by vortexing.
22. Incubation on ice is also possible but the time of incubation will need to be extended (20–30 min).
23. Wash thoroughly to ensure all EDTA is removed.
24. Flow through the column is much slower when the flow restrictor is in place. If flow stops, restart by *gently* pushing the plunger supplied into the syringe barrel. Push no more than is absolutely necessary to restart flow.
25. With the flow restrictor in place, the purity of the retained cells may be reduced. To improve purity of the retained $CD45R^+$ population, a pass over a second column with no restrictor in place may bring benefit.
26. The volume of beads added can be reduced to 50 μL without appreciable loss on recovery or purity of DC. Further reduction is not recommended.

27. When separating DC directly from whole spleen cells, this second pass over the column is essential for good purity. Expect up to 98% of the recovered cells to be MHC class II positive. Of these cells, up to 95% express CD11c. Expect to recover approx 2.5% of the starting spleen cell suspension.

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