Isolation and purification of colon lamina propria dendritic cells from mice with colitis

Maureen L. Drakes^{1,2,*}, Steven J. Czinn¹ and Thomas G. Blanchard¹

¹*Department of Pediatrics, Case Western Reserve University School of Medicine, Cleveland, OH;* ²*Pediatrics, Rm. 737, Rainbow Babies & Children's Hospital, 11100 Euclid Avenue, Cleveland, OH 44106;* **Author for correspondence (e-mail: maureen.drakes@case.edu; phone: +216-844-7472; fax: +216-844-7642)*

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Abstract

Dendritic cells are prime antigen presenting cells for stimulation of T cell immune responses. These cells are present in trace amounts in normal tissue. At sites of disease the increased frequency of these cells interacting with T cells may provide the basis for the release of pro-inflammatory mediators and contribute to localised cell and tissue damage. Studies on dendritic cells in the colon lamina propria of inflammatory bowel disease (IBD) mice have been limited due to the difficulties encountered in the isolation and purification of sufficient numbers of these cells. This is the first detailed, reproducible method provided in the literature for the isolation of colon lamina propria dendritic cells from mice with colitis, yielding optimum purity of cells and sufficient numbers to advance the study of dendritic cell function in the colons of mice. The most frequently used identification marker of murine DC is the CD11c surface antigen. We have adapted, combined, and improved procedures developed for the isolation of other cell types, to develop an efficient procedure for the isolation of dendritic cells from colon tissue. This protocol describes a step-by-step method for optimising the purity and recovery of lamina propria CD11c+ dendritic cells from mice colons.

Abbreviations: DC – dendritic cells; PBS – Dulbecco's phosphate buffered saline; FACS – fluorescent activated cell sorting; IBD – inflammatory bowel disease; LPMC – lamina propria mononuclear cells; MACS – magnetic activated cell sorting

Introduction

Dendritic cells play a central role in innate and adaptive immune responses. These cells are equipped with surface antigens such as MHC class II molecules, co-stimulatory molecules and Toll-like recognition receptors, as well as the intracellular machinery that allows them to sense, capture, process and present antigenic peptides along with MHC class II to clonally selected lymphocytes, which mount immune responses

(Finkelman et al. 1996; Hart 1997; Banchereau and Steinman 1998; Liu 2001). DC are recruited to the lamina propria of the intestine in chronic inflammatory disease and in infection (Leithauser et al. 2001; Rescigno et al. 2001; Krajina et al. 2003; Stagg et al. 2003). It has been difficult to study the role of dendritic cells in the development and pathogenicity of intestinal disorders such as inflammatory bowel disease, primarily because protocols developed for the isolation of leukocytes from specific tissues are inadequate for the isola-

tion of significant numbers of dendritic cells from the colon. For instance, isolation of CD11c positive cells from liver non-parenchymal cells using a metrizamide density gradient separation followed by immunomagnetic beads and cell sorting (Pilarisetty et al. 2004) is not readily adaptable to the colon, given the paucity of dendritic cells and the difficulty in extracting these cells from the extracellular matrix of the gut. Additionally, a previously described procedure for isolation of unfractionated lymphoid cells from the murine intestine by collagenase digestion followed by density gradient purification (Van der Heijden and Stok 1987) has been widely accepted for preparation of semi-pure populations of lymphocytes, but does not provide for the enrichment of dendritic cells. This latter method has similarities to those reported by other authors (Cebra et al. 1977; Davies and Parrott 1981; Tseng 1982; Dillon and MacDonald 1984; Spencer et al. 2002). Here we describe a novel combination and modification of these techniques for the successful isolation of purified populations of dendritic cells from the colons of mice with colitis.

Materials

A. Major Equipment

1. FACSaria sorter.¹
2. Cytospin centrifuge. 74000222.²
3. Stainless steel water bath (heated at 37 °C).
4. Hemocytometer, Brightline Improved, 0.1 mm deep.⁵

B. Media, reagents, and chemicals

1. HBSS (Ca²⁺, Mg²⁺ free), 14175-095.⁴
2. Fetal Bovine Serum, 261040-079.⁴
3. EDTA, E7899.⁵
4. HEPES, 15630-080.⁴
5. Penicillin/Streptomycin/Fungizone, 17-745 E.³
6. Dithiothreitol (DTT), 15508-013.⁴

7. RPMI, 118745.⁴
8. Collagenase D, 1088874.⁶
9. DNase I, 104159.⁶
10. Bovine serum albumin, 045253.⁷
11. Dulbecco's Phosphate buffered saline, 14190-144.⁴
12. CD11c-specific, PE-labeled monoclonal hamster antibody (HL3), 553802.⁸
13. PE-labeled hamster IgG₁λ, 553954.⁸
14. Trypan blue, T8154.⁵
15. MHC class II, I-A^d (AMS-32.1), 553547.⁸

C. Plastic supplies

1. 25 cm cell scrapers, 08-773-3.⁷
2. 100 mm tissue culture dishes, 353003.⁹
3. 100 μm nylon mesh filters, 352340.⁹
4. 40 μm nylon mesh filters, 352360.⁹
5. 50 ml polypropylene conical centrifuge tubes, 352098.⁹
6. 15 ml polypropylene conical centrifuge tubes, 352097.⁹
7. 5 ml polypropylene tubes, 352064.⁹
8. 10 ml sterile disposable pipets, 357551.⁹

D. Instruments for dissociation of tissue

1. Surgical scissors, 13-808-2.⁷
2. Forceps, 13-812-39.⁷
3. 30 cc disposable syringe, 309650.⁸
4. 18 g needle, 30196.⁸

E. MACS Cell Separation System

1. Midi MACS magnet and stand, 003756.¹⁰
2. LS columns, 130-042-401.¹⁰
3. CD11c MicroBeads, 130-052-001.¹⁰

F. Morphologic analysis of cells

1. Precleaned glass microscope slides, 12-550-34.⁷
2. Cytospin filter cards, 5991022.²
3. Cytospin slide holder, 59920063.²
4. Cytospin cell chamber, 4729778.²
5. Giemsa stain, 10092-013.⁴

¹Becton Dickinson, San Jose, California., USA.

²Thermo Shandon Scientific, Industry Drive, Pittsburg, PA, USA.

³BioWhittaker, Walkersville, MD, USA.

⁴Invitrogen, Grand Island, NY, USA.

⁵Sigma Chemical Co., St. Louis, MO, USA.

⁶Roche Diagnostic Corporation, Indianapolis, IN, USA.

⁷Fisher Scientific, Pittsburgh, PA, USA.

⁸BD BioSciences, San Diego, CA, USA.

⁹Falcon, Franklin Lakes, NJ, USA.

¹⁰Miltenyi Biotec, Auburn, CA, USA.

G. Animals

1. BALB/c mice, female, 8–12 weeks old.¹¹
2. C.B-17 severe combined immunodeficient mice (SCID) mice, female, 8–12 weeks old.¹¹

Procedures

Preparation of reagents and media for cell purification

1. Preparation of HBSS buffer

- a. Supplement HBSS (Ca^{++} , Mg^{++} free) with 5% Fetal Bovine Serum, 0.05 mM EDTA, 0.6 mM HEPES, 1.2% Penicillin/Streptomycin/Fungizone, and 15 $\mu\text{g/ml}$ DTT).
- b. Store at 4 °C.

2. Preparation of RPMI buffer

- a. Supplement RPMI with 10% Fetal Bovine Serum, 0.6 mM HEPES, and 100 U/ml Penicillin/Streptomycin.
- b. Store at 4 °C.

3. Preparation of Collagenase/DNase solution (0.2 μm filtered)

- a. Supplement RPMI buffer with 1.75 mg/ml Collagenase D, and 0.05 mg/ml DNase I.
- b. Use immediately.

4. Preparation of MACS buffer.

- a. Add 0.5% bovine serum albumin and 2 mM EDTA to phosphate buffered saline.
- b. Store at 4 °C.

5. Preparation of 1% BSA/PBS

- a. Dissolve 1 g bovine serum albumin in 100 ml PBS.
- b. Use fresh.

6. Preparation of 3% BSA/PBS

- a. Dissolve 3 g bovine serum albumin in 100 ml PBS.
- b. Use fresh.

Induction of colitis

1. The induction of colitis in CB.17 SCID mice is performed by intravenous transfer of $0.5\text{--}1 \times 10^6$ $\text{CD45RB}^{\text{hi}}\text{CD4}^+$ BALB/c spleen T cells (obtained by fluorescence-activated cell sorting) by a modification of the method previously described (Morrissey et al. 1993; Powrie et al. 1993). When mice exhibit diarrhea, an overt indicator of colitis, sacrifice and remove colons to obtain sections for hematoxylin and eosin staining of paraffin embedded colon sections, to verify the presence of colitis. In other experiments, remove colons for digestion to obtain CD11c^+ dendritic cells.

Preparation of single cell suspensions from mouse colons

1. Sacrifice two mice by asphyxiation with CO_2 .
2. Surgically excise the entire colons under sterile conditions and remove extraneous fat and mesenteric lymph node tissue.
3. Cut each colon in half and place in a 100 mm petri dish. Squeeze bulk luminal contents out of each colon segment using the edge of a blunt forceps, and flush out each colon half with 15 cc HBSS using a 30 cc syringe attached to an 18 gauge needle.
4. Open colons longitudinally using scissors, and clean the lumens by gentle scraping with a tissue culture cell scraper.
5. Wash colons by gentle agitation in HBSS and place in a 100 mm tissue culture dish.
6. Mince with fine scissors until a uniform slurry is obtained.
7. Transfer the contents of each petri dish into to a 50 ml conical tube containing 30 ml of 37 °C HBSS buffer and mix thoroughly by rapid pipetting. Incubate tissue at 37 °C for 15 min with intermittent pipetting every 5 min. Remove bubbles formed from repeated pipetting of the tissue from the top of the suspension prior to incubation.
8. At the end of the incubation period, pass the tissue over a 100 μm nylon gauze filter placed in a 50 ml conical centrifuge tube.
9. When the supernatant has drained away, place the tissue retained in the filter in a second 50 ml

¹¹Taconic, Germantown, NY, USA.

tube containing 25 ml HBSS buffer, and agitate by rapid pipetting during a second 15 min incubation at 37 °C.

10. Remove the supernatant by filtration over a 100 µm nylon gauze filter, transfer the tissue to a 50 ml tube containing 25 ml RPMI buffer and incubate at 37 °C for 15 min, with periodic pipetting as in the previous two incubations.
11. Filter the tissue suspension using a 100 µm strainer, and transfer the tissue retained on the filter to 25 ml collagenase/DNAse solution. Mix the suspension thoroughly and incubate for 1 h at 37 °C with periodic pipetting at 20, 40, 50 and 55 min, to assist in the dispersion of the tissue.
12. Gently force the enzyme/tissue mixture through a 100 µm filter placed on a 50 ml conical centrifuge tube, and add an additional 10 ml RPMI buffer to the filter to flush through any remaining tissue.
13. Strain the tissue suspension a second time using a 40 µm filter.
14. Wash the filtrate consisting of LPMC by centrifugation at 4 °C for 5 min at 500×g, and then resuspend in 50 ml RPMI buffer.

Enrichment of dendritic cells from LPMC by immunomagnetic sorting

1. Strain the cells a third time using a 40 µm filter, wash as before and resuspend in 50 ml MACS buffer.
2. Again strain the cells using a 40 µm filter in preparation for enrichment using MACS columns.
3. Determine the number of LPMC using a hemocytometer, taking care not to enumerate epithelial cells.
4. For the purposes of optimising cell yield when isolating CD11c positive cells using MACS technology, assume the presence of twice as many mononuclear cells as determined by counting. Additionally, in contrast to manufacturer's recommendation, add CD11c beads at a ratio of 10 µl of bead suspension per 5×10^6 mononuclear cells.
5. Add MACS buffer at a quantity of 90 µl per 5×10^6 mononuclear cells. Incubate the cells

on ice for 20 min for direct labeling with the CD11c MicroBeads.

6. Wash cells by centrifugation (4 °C for 5 min at 500×g).
7. Add an equal volume of MACS buffer to the cells, and resuspended in MACS buffer and apply to a prewashed LS column.
8. Remove non-column-adherent cells from the column by three separate column washes with 3 ml MACS buffer each time.
9. Elute CD11c positive cells from the MACS column by the addition of 5 ml MACS buffer and the application of minimal pressure from a column plunger (see discussion). Collect the cells in a 50 ml conical tube.
10. Wash the cells two times in RPMI buffer by centrifugation at 500×g for 5 min at 4 °C.

Enrichment of dendritic cells from colonic cell suspensions by flow sorting

1. Resuspend MACS-enriched cells at a final concentration of 1×10^8 cells/ml in RPMI buffer.
2. Set aside a 5–20 µl aliquot for staining with the hamster IgG isotype control.
3. Stain cells with 15 µg/ml CD11c PE mAb (approximately 10-fold higher concentration than typically used for cell staining, see discussion).
4. Incubate cells with either PE-labeled anti-CD11c (or isotype control) antibody for 30 min on ice.
5. Add 30 ml PBS to the cells and filter through a 40 µm nylon mesh filter.
6. Wash cells two times by centrifugation at 500×g for 5 min at 4 °C.
7. Resuspend to a final concentration of $25\text{--}40 \times 10^6$ cells/ml in 1% BSA/PBS, and place on ice.
8. Using a fluorescence-activated cell sorter to select for dendritic cells, gate out dead cells and cell aggregates in the forward vs. side scatter plots, and use the isotype control labeled cells to determine the percentage of cells positive for CD11c. Collect sorted CD11c positive cells in 5 ml polypropylene tubes containing 0.5–1.0 ml 3% BSA/PBS.
9. Pool cells in a 50 ml conical tube, add PBS to a final volume of 50 ml.

10. Wash cells two times by centrifugation.
11. Resuspend the cells in RPMI buffer and determine the total number of viable cells by trypan blue exclusion using a hemocytometer.

Morphologic analysis of isolated dendritic cells

1. Adjust cells to a concentration of 1×10^6 /ml in RPMI buffer.
2. Apply 100 μ l of cells to a cytospin cell chamber with slide and fix by centrifugation in a cytospin centrifuge.
3. Air dry slides.
4. Fix in methanol for 3 min and air dry.
5. Stain with Giemsa stain diluted 1:20 in distilled water for 40 min.

Results and Discussion

To ensure that animals had colitis, mice with diarrhea were sacrificed, colons removed and sections taken for staining with H + E. Significant colon thickening and inflammation was observed in colons of colitic mice in comparison with BALB/c mice (Figure 1a and b).

This protocol outlines the successful recovery of dendritic cells from the colons of mice by extraction of LPMC from the extracellular matrix followed by DC enrichment procedures.

Enzyme digestion and serial filtration reduced the tissue to a single cell suspension. The labeling of these cells with CD11c MicroBeads provided for the enrichment of this population by column purification. Final recovery of highly purified CD11c positive DC was obtained by mAb labeling and sorting on a high speed sorter at a flow rate of 2000 events/s. This protocol provides an adequate tool for DC isolation which will enable investigators to study the mechanism of functioning of these cells in the gut. The total estimated time for the isolation of dendritic cells from the lamina propria of colitic mice is approximately 7–10 h.

Selection of CD11c positive cells

Lamina propria mononuclear cells were obtained by collagenase/DNAse digestion of colon tissue

followed by serial filtration. Labeling of LPMC with CD11c MicroBeads and subsequent enrichment on a MACS column resulted in a cell population of 50–87% CD11c positive cells. Addition of CD11c PE mAb to this cell population and flow cytometry sorting yielded a high purity of DC (Figure 2a and b). Over 6 experiments, the mean purity obtained was $97.7 \pm 1.48\%$ CD11c positive cells.

Morphology of isolated colon dendritic cells

Enzymatic digestion of colon tissue from T cell transfer SCID mice with colitis yielded LPMC cell preparations which contained approximately 15% CD11c positive cells, with other mononuclear cells and epithelial cells as contaminants. Further selection of CD11c positive cells by column sorting and flow cytometry sorting resulted in approximately 98% cells, containing large nuclei, in some cases irregular-shaped, surrounded by a large volume of cytoplasm, with or without dendrites at their cell surfaces (Figure 3a and b). The morphology of these cells was characteristic of the classic mature and immature myeloid DC (Drakes et al. 1997).

Sequential steps for the purification of colon dendritic cells

Figure 4 illustrates the sequential purification steps for the isolation of DC from the colon. The yield of LPMC from the colons varied depending upon the size of the colons and extent of disease. Cell numbers recovered over 6 experiments was $3.7 \pm 1.11 \times 10^6$ purified CD11c+ cells, from two mouse colons.

Lamina propria dendritic cells express MHC class II molecules on their cell surface

Dual staining of CD11c+ cells revealed that these DC almost exclusively expressed MHC class II, I-A^d on their cell surface (Figure 5). MHC class II on the surface of DC is a critical molecule in DC–T cell interaction and induction of immune responses.

Figure 1. SCID mice with colitis exhibit significant colon thickening and inflammation. T cell reconstituted SCID mice with colitis were sacrificed, colons removed and sections embedded in paraffin. Cross sections were stained with Hematoxylin + Eosin. Photographs are for colons of (a) BALB/c mice, (b) SCID mice with colitis. Magnification 200 $\times$. Results are representative of 3 separate experiments.

DC stimulate the release of cytokines in T cell cultures

Functional studies were performed with isolated colon DC to demonstrate their ability to induce cytokine production in autologous spleen CD4 $^{+}$ T cells. Analysis of supernatants of DC–T cell co-cultures harvested after 6 days of culture, showed significant release of pro-inflammatory cytokines IFN- γ and IL-6 above cultures with DC alone, or T cells alone (Figure 6).

Problems and limitations

Tissue digestion

Care should be taken to mince the colon tissue as fine as possible before starting the digestion process. This reduces the amount of aggregated tissue left on the filter at the end of the digestion period. Treatment of the colon tissue with EDTA and collagenase/DNAse should be carried out under a constant temperature of 37 $^{\circ}$ C. Slight fluctuations in temperature can drastically reduce the yield of

Figure 2. Histograms of sorted dendritic cells. Single cell preparations of colons from colitic SCID mice were obtained by collagenase digestion as described above. Cells were mAb stained with CD11c PE (FL2) labeled antibodies. Dead cells were gated out and CD11c cells collected. (a) Histogram shows the recovered cell population of CD11c positive cells after column enrichment, and (b) shows CD11c positive cells obtained after flow sorting. Plot shows the relative fluorescent intensity (X axis) vs. cell numbers. The percentage of CD11c positive cells is indicated. Plots are representative of 6 separate experiments.

CD11c positive cells. All steps of the digestion process should therefore be performed precisely at 37 °C and should be carefully monitored. Buffers should be prewarmed to this temperature. The efficacy of collagenase can vary greatly between vendors and therefore it should always be tested

before performing large scale LPMC preparations. In general, digestion is optimal when the quantity of collagenase is maintained between 1.5–2 mg/ml. Some batches of collagenase may vary significantly in specific activity Units. If extreme variation is observed in the stated specific activity relative to

Figure 3. Photographs of lamina propria DC. Panel a shows lamina propria cell preparations obtained from T cell reconstituted colitic SCID mice. Panel b shows photographs of CD11c+ cells isolated from LPMC of colitic SCID mice, by collagenase digestion of tissue, sorting on MACS columns, followed by flow sorting on a BD FACSaria. Cytoentrifuge preparations of cells were Giemsa stained. Magnification 400 $\times$. Results are representative of 4 separate experiments.

Figure 4. Recovery of CD11c+ DC from colitic SCID mice lamina propria. Flow chart shows cell numbers typically recovered from freshly isolated collagenase digested colons of two pooled colitic SCID mice. LPMC were enriched on MACS columns, CD11c PE mAb labeled, and sorted on a BD FACSaria Sorter. Results are representative of 6 independent experiments.

previous lots, then equivalent units of specific activity may be a more appropriate measurement. The length of time for collagenase digestion may be increased another 10 min if visual inspection reveals large pieces of undigested tissue. On the contrary, some batches of collagenase may require only 50 min digestion time. Prolonged digestion will reduce the yield of DC. Moreover, small quantities of new brands of collagenase should be tested to ensure that it is not toxic to the cells, before the purchase of larger quantities.

Similarly, different batches of DNase should be tested to determine the optimum quantity to use for efficient digestion of tissue and recovery of cells.

Immunomagnetic sorting

Serial filtration of the LPMC preparation after isolation of these cells from colon tissue using 40 μ m pore filters is necessary to obtain a single cell suspension. Failure to adequately filter the LPMC will result in clogging of the column in the first step

Figure 5. MHC class II expression on the surface of dendritic cells. A small aliquot of LPMC obtained by collagenase digestion was removed for isotype control staining. The bulk of LPMC were stained with CD11c PE to select for these cells using a FACSARIA. Recovered CD11c+ cells were then mAb stained with MHC class II I-A^d FITC. Dot plots show dual staining for (a) isotype control staining of LPMC and (b) CD11c PE and I-A^d FITC for sorted cells. Dead cells were gated out and at least 10,000 events acquired on a FACScan (Becton Dickinson) and analysis performed using Cell Quest software. Results are representative of 2 separate experiments.

Figure 6. Lamina propria DC are potent stimulators of cytokines in spleen T cell cultures. Flow sorted colon DC from SCID mice with colitis were adjusted to a concentration of 1×10^6 /ml and 100 μ l added to 96 well flat bottomed plates. CD4+ T cells from autologous mouse spleens were purified using CD4+ MACS microbeads and column, followed by labeling with CD4 PE mAb and flow sorting. T cells were added to wells (100 μ l Vol.) at a concentration of 2×10^6 /ml. Supernatants collected after 6 days of culture in a 5% humidified CO₂ incubator, were frozen at -70°C and later assayed by ELISA for (a) IFN- γ (paired Abs, BD BioSciences) and (b) IL-6 (Duoset ELISA kit, R&D systems). Error bars are shown for standard deviations over duplicate wells, and the level of significance over cytokine release by control DC determined by Student's *t*-test.

of the DC enrichment. Additionally, when applying pressure to the MACS column for elution of CD11c positive cells, excessive force will result in elution of unlabeled cells in addition to the CD11c positive cells, thereby compromising the enrichment step and increasing the length of time required for flow sorting. Therefore only the slightest pressure should be applied when eluting the cells.

Fluorescence-activated cell sorting

Inefficient staining of cells by using too little CD11c PE mAb prevents the proper labeling of these cells and consequently limits the recovery of DC. Resuspension of cells in 1% BSA/PBS is the preferred buffer for sorting. Cells are collected in tubes containing 3% BSA/PBS buffer to maintain an adequate amount of protein during the collection process for survival of cells. The use of a similar concentration of FBS buffer in the collection tubes causes shifting of the CD11c positive cells in the gates.

Alternative and support protocols

The present method takes advantage of sterile, prepackaged tubes and filters available through several commercial vendors. Alternatively, this procedure can also be accomplished through the use of common laboratory equipment such as autoclaved glass beakers covered in foil, magnetic stir bars, and stainless steel mesh filters. The use of magnetic stir bars in a flat bottom beaker has the advantage of controlled and constant mixing during the EDTA and collagenase digestion phases. This procedure is less troublesome than intermittent removal of the 50 ml tube containing the tissue, for agitation by vigorous pipetting. However, in order to maintain solutions at a constant 37 °C, it is necessary to place the magnetic stir plate within a 37 °C incubator or controlled environment room. In the case of free-standing incubators, a model possessing an open port sufficient to run the power cable of the stir plate is required and may not be commonly available. This method details the isolation of CD11c positive dendritic cells from the intestines of mice. The use of mAbs specific for other DC subset markers can also be used in the flow cytometry sorting procedure to extend the isolation to other DC subsets as well.

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