Isolation and Generation of Human Dendritic Cells

Dendritic cells are highly specialized antigen-presenting cells (APC; see Commentary), which may be isolated or generated from human blood mononuclear cells. Although mature blood dendritic cells normally represent ~0.2% of human blood mononuclear cells, their frequency can be greatly increased by the use of cell enrichment methods (see Basic Protocol 1 and Alternate Protocol). More highly purified dendritic cell preparations can be obtained from these populations by sorting of fluorescence-labeled cells (*UNITS 5.3 & 5.4*). Alternatively, dendritic cells can be generated from monocytes by culture with the appropriate cytokines (see Basic Protocol 2). In addition, a negative selection approach may be employed to generate cell preparations that have been depleted of dendritic cells (see Basic Protocol 3) to be used for comparison in functional studies.

CAUTION: Appropriate caution is advised in working with large volumes of human blood, particularly if from unknown, untested donors. Biosafety practices must be followed (see Chapter 7 introduction).

NOTE: All procedures are to be carried out using sterile tissue culture techniques with sterile solutions and equipment.

NOTE: All incubations are performed in a humidified 37°C, 5% CO_2 incubator unless otherwise specified.

ISOLATION OF DENDRITIC CELLS FROM HUMAN BLOOD MONONUCLEAR CELLS

BASIC PROTOCOL 1

A peripheral cell population can be enriched for dendritic cells by depletion of T cells and adherent cells. The preparation is then subjected to density gradient centrifugation over metrizamide to isolate low buoyant density cells. The resulting population contains 20% to 80% dendritic cells and is largely free of lymphocytes.

Materials

Leukocyte-enriched leukapheresis packs (leukopaks; i.e., 20- to 50-ml) or buffy coats, ≤24 hr old (from blood bank or North American Biologicals; see Critical Parameters)
Complete RPMI-10 (*APPENDIX 2*), room temperature and 37°C
RPMI 1640
14.5% metrizamide solution (see recipe), room temperature
Dulbecco's PBS, Ca^{2+} and Mg^{2+} free, 4°C
Primary antibody for flow cytometry analysis (see Table 7.32.1; optional)
Fluorescence-labeled secondary antibody for flow cytometry analysis (optional)

15- and 50-ml conical polypropylene centrifuge tubes
9-in. (~23-cm) Pasteur pipets, with and without cotton plugs
100-mm tissue culture plates
Sorvall H1000B rotor (or equivalent)

Additional reagents and equipment for isolating mononuclear cells (*UNIT 7.1*), counting viable cells (*APPENDIX 3B*), rosetting with sheep red blood cells (*UNIT 7.2*), and flow cytometric analysis (*UNITS 5.3 & 5.4*) or assessment of functional activity by mixed leukocyte reaction (*UNIT 7.10*; both procedures optional)

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Table 7.32.1 Monoclonal Antibodies Useful in Routine Flow Cytometric Analysis of Human Blood Dendritic Cells

Antigen[a]	Cell type where primarily expressed	Reactivity with dendritic cells
MHC class II	Leukocytes	+++++
CD1c	Thymocytes	++
CD33	Monocytes	++
CD40	B lymphocytes	+
CD83	Dendritic cells	++
CD86	Antigen-presenting cells	++
CD3	T lymphocytes	None
CD14	Monocytes	None
CD19	B lymphocytes	None
CD56	NK cells	None

[a]All of the antigens listed except CD83 are identified by MAbs that are widely available commercially; CD83 can be obtained either from Genzyme or by request (Zhou and Tedder, 1995a, 1996). MAb reactivity is indicated on a relative linear scale, with +++++ indicating the highest levels of antigen expression.

Isolate blood mononuclear cells

1. Isolate blood mononuclear cells from a leukapheresis pack or buffy coat preparation by Ficoll-Paque density gradient or other suitable method as described in *UNIT 7.1*. Determine the total number of viable mononuclear cells by trypan blue exclusion (*APPENDIX 3B*).

   The usual number of cells obtained at this stage is 6–10 × 10^8 cells.

   Prompt processing reduces cellular and platelet aggregation and increases cell yield and purity. If platelet contamination is severe, wash the cells and centrifuge at a lower speed, as excessive platelet contamination will lower the purity of the dendritic cells due to the formation of cellular aggregates. The cell isolation procedures should be carefully monitored by cell counting using a hemacytometer to ensure that there is no significant loss of mononuclear cells.

2. Adjust the cell concentration to 1–2 × 10^7 cells/ml in complete RPMI-10.

3. Deplete the resuspended cells of T cells by E-rosetting with sheep red blood cells (SRBC) as described in *UNIT 7.2*.

   In applying this method, it is advantageous to use 50-ml conical centrifuge tubes because of the large volume of cell suspension. AET-treated SRBC give the best results. Incubate the pelleted mononuclear cells and SRBC for 20 min on ice.

4. Transfer the interface cells to a 50-ml conical centrifuge tube using a Pasteur pipet. Dilute the density gradient medium four-fold with unsupplemented RPMI 1640 and centrifuge 10 min at 450 × *g*, room temperature. Remove the supernatant fluid and resuspend the cells in complete RPMI-10. Repeat the wash, then adjust the cell concentration to 1 × 10^7 cells/ml.

   The usual number of E-rosette-negative cells obtained at this stage is 3–6 × 10^7 cells. The cells consist of B cells, monocytes, macrophages, and dendritic cells.

Deplete monocytes from the mononuclear cell preparation

5. Plate the T cell–depleted mononuclear cell suspension into 100-mm tissue culture plates using 10 ml of the cell suspension per plate. Incubate the plates overnight at

37°C to allow monocytes to adhere to the plastic and to allow the dendritic cells to further differentiate into mature dendritic cells.

Plating more densely than ~1 × 10^8 cells per plate reduces adherence and final yield. During the overnight incubation, initially adherent dendritic cells (and some contaminating monocytes) detach from the plastic surface and float into the overlying medium.

6. The next morning, swirl the plates gently to resuspend the nonadherent cells. Collect the nonadherent cells and transfer them to a fresh 100-mm tissue culture plate.

   Repeat until all plates have been harvested and the cells replated. It may be helpful to very gently wash each plate with 3 ml fresh medium prewarmed to 37°C to remove additional nonadherent cells.

7. Incubate the newly plated cells 30 min at 37°C to remove additional monocytes. Swirl the plates again and collect the nonadherent cells in the medium.

   Repeat this process again, if necessary, to reduce the frequency of monocytes; however, there is usually little further gain after three adherence steps. The frequency of residual adherent cells can be visualized by examining the plates by phase-contrast microscopy using an inverted microscope. The majority of residual cells in each plate should be adherent, flattened monocytes. If there are a lot of residual nonadherent cells, wash the plates very gently again and recover the wash medium containing the cells.

8. Harvest the T cell– and monocyte–depleted mononuclear cell preparation and pellet the cells 5 min at 200 × *g*, room temperature.

9. Discard the supernatant fluid and resuspend the cell preparation in complete RPMI-10. Count the number of cells and adjust the cell concentration to ≤1 × 10^7 cells/ml.

Enrich for dendritic cells by metrizamide density gradient centrifugation

10. Add 4 ml sterile 14.5% metrizamide solution to a 15-ml conical centrifuge tube at room temperature. Prepare enough tubes to accommodate the full volume of cell suspension that has to be separated.

11. Gently overlay the metrizamide cushion with 8 ml of the mononuclear cell suspension, forming a sharp interface. Centrifuge the gradient 10 min at 800 × *g* (1800 rpm in Sorvall H1000B rotor), room temperature. Accelerate the centrifuge slowly and keep the brake turned off.

    Alternatively, the metrizamide solution may be layered underneath the mononuclear cell preparation.

12. After centrifugation, aspirate off the top volume of culture medium until ~0.5 in. (~1 cm) above the interface. Collect the cells that localize at the interface with a cotton-plugged Pasteur pipet. Carefully collect the cells from the interface region, taking the culture medium and the top 1 ml of metrizamide.

    Until one becomes proficient at these procedures, one should also collect the cells that pellet through the metrizamide gradient just in case something has gone wrong during the isolation procedures. This avoids the danger of losing everything at this step. The cells can be phenotyped to determine the frequency of dendritic cells and other cell types present in each fraction.

13. Pool the cells from the metrizamide gradients into a single fresh 50-ml conical tube. Fill the 50-ml tube with cold PBS to dilute the metrizamide at least two-fold and mix the solution by gentle inversion of the tube.

14. Pellet the cells by centrifugation for 10 min at 450 × *g*. Wash the isolated cells twice with complete RPMI-10, count the viable cells by trypan blue exclusion (*APPENDIX 3B*), and resuspend at 1 × 10^7 cells/ml in fresh complete RPMI-10.

15. Further deplete monocytes from the dendritic cell preparation by plating the cells in 100-mm tissue culture plates, using 10 ml of cell suspension per plate until the entire volume has been plated. Incubate 1 hr at 37°C.

16. Gently swirl the plates and remove the supernatant fluid containing nonadherent cells.

    This is the dendritic cell–enriched fraction. Monitor the plating process by light microscopy to insure monocyte depletion is occurring without undue loss of non-adherent dendritic cells.

17. Pellet the cells by centrifugation for 5 min at 450 × *g*, room temperature.

    The usual yield of cells at this stage is 5–10 × 10^6 cells. Dendritic cells should comprise 20% to 80% of the population. While this purity may suffice for many kinds of experiments, dendritic cells may be further purified by procedures described below.

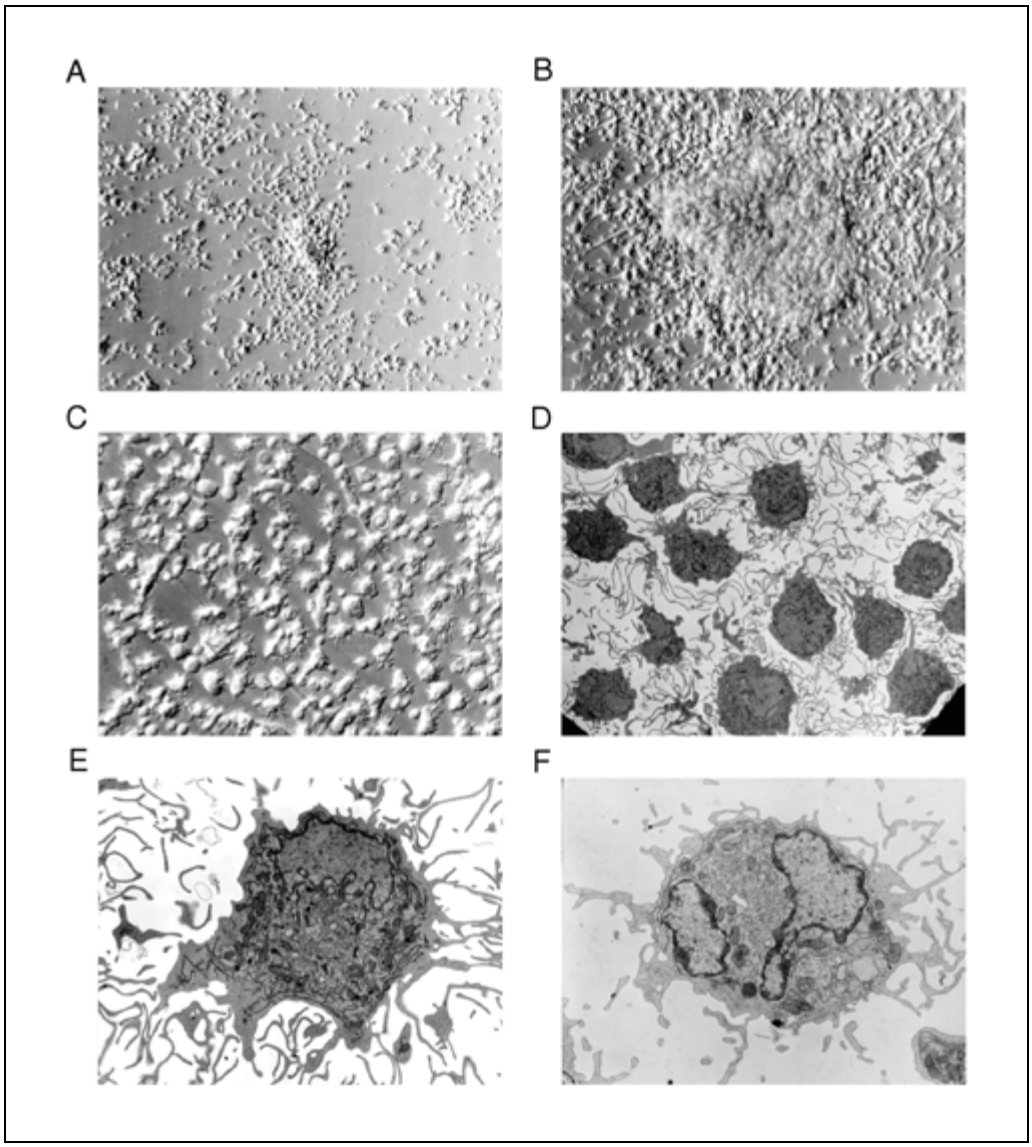

Figure 7.32.1 Generation and morphology of $CD83^+$ dendritic cells. Monocytes cultured with GM-CSF/IL-4/TNF-α develop a dendritic cell morphology (**A-C**). Monocytes ($CD14^+$) were isolated and cultured with cytokines for 2 (A) or 5 (B, C) days and examined by phase-contrast microscopy (A-B, 200× magnification; C, 400×). Representative transmission electron micrographs of (**D-E**) monocyte-derived $CD83^+$ cells isolated by cell sorting from 7-day GM-CSF/IL-4/TNF-α cultures and (**F**) blood $CD83^+$ dendritic cells (D, 1855× magnification; E-F, 5800×). This figure, originally published in Zhou and Tedder (1996), is reprinted by permission of *Proceedings of the National Academy of Sciences*.

18. Determine the purity of dendritic cells by immunofluorescence staining with flow cytometry analysis (*UNITS 5.3 & 5.4*) using appropriate primary and secondary antibodies; by assessment of cellular morphology (see Fig. 7.32.1C, D); or dry assessment of functional activity in mixed leukocyte reactions (*UNIT 7.10*).

Table 7.32.1 identifies primary antibodies specific for cell-surface antigens present on dendritic cells, and on the major contaminating cell populations obtained by routine purification. MHC class II, CD83, CD3, and CD14 constitute a recommended minimum panel. For the secondary antibody, use fluorescein-conjugated goat anti–mouse Ig antibodies.

While the purity of dendritic cells can be estimated using a variety of techniques—including hemacytometer counts and immunostaining after cytocentrifugation onto glass slides—the methods described above are generally superior. Flow cytometry, especially, offers superior quantitation and consistency (Zhou and Tedder, 1995a).

IMMUNOMAGNETIC ISOLATION OF DENDRITIC CELLS

ALTERNATE PROTOCOL

A preparation of cells enriched for $CD83^+$ dendritic cells as described in Basic Protocol 1 can be isolated using magnetic beads from a peripheral blood cell population depleted of T cells and adherent cells (see *UNIT 7.4*). Dendritic cells prepared by this method generally exhibit morphological and functional capabilities similar to those of dendritic cells prepared by negative selection (see Basic Protocol 1). It is not possible to isolate $CD83^+$ dendritic cells straight from blood mononuclear cell preparations because their frequency is too low.

Additional Materials *(also see Basic Protocol 1)*

MAb mix including anti-CD3, -CD14, -CD19, and -CD56, each at 10× saturating concentrations (Table 7.32.1)
Goat anti–mouse IgG–coated magnetic beads (Dynabeads), washed (see *UNIT 7.4*)
Anti-CD83 MAb (Genzyme) at a 10× saturating concentration

Clinical rotator
Magnetic separation apparatus

Deplete B cells, T cells, and other mononuclear cells

1. Isolate blood mononuclear cells from a leukapheresis pack or buffy coat preparation by density gradient centrifugation (see Basic Protocol 1, step 1). Adjust the cell concentration to 2×10^7 cells/ml of complete RPMI-10 in 15-ml conical polypropylene centrifuge tubes.

   The size of the container used for cell separation will depend the magnetic separation apparatus used; adequate times for cell separation must be considered to accommodate larger cell volumes or tube sizes.

2. Add $1/10$ vol of 10× MAb mix at 1× concentration to the mononuclear cell suspension and incubate 30 min with end-over-end rotation at 6 to 10 rpm, 4°C.

3. Wash the cells twice by centrifugation and resuspension with fresh medium to remove any unbound MAb, and transfer the cells to a fresh polypropylene centrifuge tube at 2×10^7 cells/ml of complete RPMI-10.

4. Deplete the resuspended cells of lymphocytes, monocytes, and NK cells by adding a 2-ml suspension of washed magnetic beads to the cell suspension and rotating 1 hr at 6 to 10 rpm, 4°C.

5. Separate the cells labeled with MAb and coated with beads using the magnetic separation apparatus. Allow ~5 min for the beads (and attached cells) to accumulate adjacent to the magnet.

6. Transfer the unbound cell suspension to a fresh tube and perform a second magnetic separation. Count the cells and resuspend at 2×10^7 cells/ml of complete RPMI-10.

7. Repeat steps 4 and 5 and resuspend the remaining cells at 2×10^7 cells/ml of complete RPMI-10.

   This cell population will be greatly enriched for dendritic cells, but they will represent <5% of the mononuclear cell population.

8. Plate the mononuclear cell suspension into 100-mm tissue culture plates using 10 ml of the cell suspension per plate. Incubate the dishes overnight at 37°C to allow monocytes to adhere to the plastic and to allow the dendritic cells to further differentiate into mature dendritic cells.

Enrich for dendritic cells by positive selection

9. The next morning, swirl the plates gently to resuspend the nonadherent cells, harvest the lymphocyte- and monocyte-depleted mononuclear cell preparation into a polypropylene centrifuge tube, and pellet the cells 5 min at $200 \times g$, room temperature.

   It may be helpful to very gently wash each plate with 3 ml of fresh prewarmed medium to remove additional nonadherent cells.

10. Discard the supernatant fluid and resuspend the cell preparation in complete RPMI-10. Count the number of cells and adjust the cell concentration to 1×10^7 cells/ml.

11. Add $1/10$ vol of 10× anti-CD83 MAb to the mononuclear cell suspension and rotate 30 min at 6 to 10 rpm, 4°C.

12. Wash the cells twice by centrifugation and resuspension with fresh medium to remove any unbound MAb. Resuspend to a concentration of 1×10^7 cells/ml of complete RPMI-10, and transfer the cells to a fresh polypropylene centrifuge tube.

13. Isolate $CD83^+$ cells by adding a 1-ml suspension of washed magnetic beads to the cell suspension and rotating 1 hr at 6 to 10 rpm, 4°C.

14. Separate the cells coated with beads using the magnetic apparatus. After 5 min, remove the cells not bound by the magnet using an aspirator tip placed at the bottom of the flask or tube.

15. Resuspend the bound $CD83^+$ cells in fresh complete RPMI-10 to the volume used in step 10. Resuspend the cells thoroughly and perform a second magnetic separation. Resuspend the recovered cells in fresh medium and count the cells.

    Additional cycles of washing and magnetic isolation can be used to increase cell purity, but more than three cycles does not usually increase purity significantly. Dendritic cell populations isolated using these procedures are usually homogenous in morphologic appearance and display the morphologic features of dendritic cells. Procedures for removing magnetic beads from cells are outlined in UNIT 7.5. However, some of the beads attached to dendritic cells through CD83 are endocytosed with overnight culture and other methods produce only modest success at removing the beads. Flow cytometry analysis of isolated dendritic cells is not usually practical because of the attached beads.

GENERATION OF HUMAN DENDRITIC CELLS FROM MONOCYTES

A relatively homogenous population of functionally mature dendritic cells can be generated from $CD14^+$ blood monocytes by incubating them with the proper cytokines (Zhou and Tedder, 1996). Under the conditions described, monocytes differentiate into dendritic cells without cell proliferation, so that the number of monocytes used is the determining factor for dendritic cell recovery. Because monocytes are much more plentiful than dendritic cells, this method can result in higher yields than those obtained using Basic Protocol 1.

Materials

Leukocyte-enriched leukapheresis packs (leukopaks; i.e., 20- to 50-ml) or buffy coats, ≤24 hr old (from blood bank or North American Biologicals; see Critical Parameters)
Complete RPMI-10 (*APPENDIX 2*), 37°C
0.2 mM EDTA in PBS, Ca^{2+} and Mg^{2+} free (*APPENDIX 2*)
14.5% metrizamide solution (see recipe), room temperature
Recombinant human granulocyte/macrophage colony stimulating factor (GM-CSF; see recipe)
Recombinant human IL-4 (see recipe)
Recombinant human TNF-α (see recipe)

Inverted phase-contrast microscope
Sorvall H1000B rotor (or equivalent)
15-ml conical polypropylene centrifuge tube
Tissue culture flask

Additional reagents and equipment for isolation of peripheral blood monocytes (*UNIT 7.1*) followed by adherence to plastic (*UNIT 7.6*)

Isolate blood monocytes by adherence to plastic

1. Isolate peripheral blood monocytes from a leukapheresis pack or buffy coat preparation using Ficoll-Paque density gradient centrifugation and plastic adherence (*UNITS 7.1 & 7.6*).

2. Gently aspirate the medium that contains the nonadherent cells from the plate and wash each plate very gently with warm complete RPMI-10 to remove the nonadherent cells. Examine each plate using an inverted phase-contrast microscope to determine the level of contamination with small round lymphocytes.

   It will take a little practice to efficiently remove the nonadherent cells without removing dendritic cell precursors ($CD14^+$ monocytes). A balance between lymphocyte contamination and optimal yields of dendritic cells is achieved by proper washing techniques. Trial and error is unfortunately the best guide until an investigator develops a feel for how gently to wash the plates. If lymphocytes are present, rewash the plates more vigorously, yet not so vigorously as to dislodge adherent monocytes.

3. Remove adherent monocytes by gently scraping with a plastic cell scraper (*UNIT 7.6*). Alternatively, incubate the cells 10 min at 4°C in ice-cold 0.2 mM EDTA/PBS. Use a cotton-plugged Pasteur pipet with a pipet bulb to vigorously wash the adherent cells off the plate.

4. Pellet the harvested monocytes by centrifugation for 5 min at 450 × *g*, room temperature.

5. Adjust the cell density to $<1 \times 10^7$ cells/ml in PBS containing 0.2 mM EDTA.

6. Overlay 8 ml of cell suspension on 4 ml of 14.5% metrizamide solution in a 15-ml conical centrifuge tube.

7. Centrifuge the metrizamide gradients 10 min at 800 × *g*, room temperature, to pellet contaminating lymphocytes.

8. Recover the interface cells and wash the recovered cells twice with 0.2 mM EDTA in PBS.

   The harvested interface cells can be subjected to a second metrizamide gradient to remove residual lymphocytes if these represent a significant proportion of the cell population.

9. Resuspend the cells in a small volume of complete RPMI-10 and determine the number of viable cells recovered.

   Monocytes usually comprise 5% to 10% of the mononuclear cell population isolated directly from blood and are recovered proportionally at this stage given the loss in overall cell number with each isolation step. The level of cell recovery will vary between laboratories and with the degree of vigor used to wash the plates. Few monocytes should pellet in the metrizamide gradient and most of the pelleted cells should be lymphocytes.

Culture monocytes with cytokines to induce differentiation

10. Resuspend the monocytes to a density of 1×10^6 cells/ml in complete RPMI-10 containing 800 U/ml GM-CSF and 500 U/ml IL-4.

11. Incubate the monocytes in an appropriately sized tissue culture flask or other suitable vessel. Add fresh complete RPMI-10 and cytokines to the cultures on day 3.

    Usually ~75% of spent medium is exchanged for fresh medium and cytokines. The monocytes will sediment to the bottom of the culture flasks (unless they are resuspended during movement of the tissue culture plates); thus, most of the culture medium can be aspirated without disturbing the cells. Cell differentiation within the cultures can be monitored by light microscopy.

    Monocytes cultured with GM-CSF and IL-4 generate small, distinctive clusters of cells that are primarily semiadhered to the tissue culture plates (Fig. 7.32.1A, B). The cells are tightly clustered together by day 5, with some cells located between clusters (Fig. 7.32.1C). Some cells with a dendritic cell morphology can be observed.

12. On day 5 of incubation, add fresh complete RPMI-10 containing 100 U/ml TNF-α as well as 800 U/ml GM-CSF and 500 U/ml IL-4.

13. On day 8 or 9 of incubation, resuspend the cells by vigorous pipetting to break up the cellular aggregates and to wash the semiadherent cells from the culture wells.

    At this point the cells should be predominantly $CD83^+$ dendritic cells, forming large clusters. The monocyte-derived dendritic cells should be morphologically homogenous and exhibit the characteristic cellular projections and motility of dendritic cells. While in culture, the cells will continually extend, retract, and reorient their cellular processes and veils.

    Recoveries vary, but typically range between 60% and 90% of the input monocytes. The cellular aggregates should be thoroughly disrupted before counting to obtain accurate counts. The cell preparations should remain >95% viable, as determined by trypan blue exclusion until day 14 to 15, when the cells increase in size and the cell clusters begin to deteriorate.

14. Determine the frequency of dendritic cells (see Basic Protocol 1, step 18).

DEPLETION OF DENDRITIC CELLS BY NEGATIVE SELECTION

Dendritic (i.e., $CD83^+$) cells may also be removed from mononuclear cell populations using immunomagnetic depletion. It is often useful to examine the function of cell populations in the absence of dendritic cells. Depletion of $CD83^+$ cells from mixed leukocyte populations using this protocol significantly reduces the stimulator activity of cell populations in allogeneic mixed lymphocyte reactions (Zhou and Tedder, 1995a). Because $CD83^+$ cells usually represent only a very small fraction of the cell population being examined (0.1% to 0.2% of mononuclear cells) and are such potent stimulators in mixed lymphocyte reactions, it is recommended that the cell population being depleted of $CD83^+$ cells goes through two rounds of negative selection, after which they are essentially undetectable. However, significant reductions in stimulator activity of starting cell populations are achieved with only one round. Because the $CD83^+$ cells make up such a small proportion of the initial population, there is usually no discernible loss in overall cell numbers following this procedure.

Materials

Complete RPMI-10 (*APPENDIX 2*)
CD83 MAb (Genzyme) at a 10× saturating concentration
Goat anti–mouse IgG–coated magnetic beads (Dynabeads), washed (see *UNIT 7.4)*

Tissue culture flask or petri plate
15-ml conical polypropylene centrifuge tubes
Clinical rotator
Magnetic separation apparatus

Additional reagents and equipment for isolating mononuclear cells (*UNIT 7.1*) and counting viable cells (*APPENDIX 3B*)

1. Isolate peripheral blood mononuclear cells from a leukapheresis pack or buffy coat preparation by density gradient centrifugation (see Basic Protocol 1, step 1). Culture the cells overnight at 37°C in complete RPMI-10 at 1×10^7 cells/ml in a tissue culture flask or petri plate.
2. Harvest and wash the isolated blood mononuclear cells once in complete RPMI-10 and adjust the cell concentration to 2×10^7 cells/ml of complete RPMI-10 in 15-ml conical polypropylene centrifuge tubes.
3. Add $1/10$ vol of 10× CD83 MAb to the mononuclear cell suspension and rotate 30 min at 6 to 10 rpm, 4°C.
4. Wash the cells twice with fresh medium to remove any unbound MAb and transfer the cells to a fresh polypropylene centrifuge tube at 2×10^7 cells/ml of complete RPMI-10.
5. Deplete the resuspended cells of $CD83^+$ cells by adding a 2-ml suspension of washed magnetic beads to the cell suspension and rotate 1 hr at 6 to 10 rpm, 4°C.
6. Separate the bead-coated cells using the magnetic separation apparatus for ~5 min. Transfer the unbound cell suspension to a fresh tube and repeat the magnetic separation. Harvest the unbound cells.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2****; for suppliers, see* ***APPENDIX 5****.*

Metrizamide, 14.5% (w/v)

Place 25 g metrizamide (Sigma 99% pure, grade 1) in a sterile 250-ml graduated cylinder. Rinse the bottle with 150 ml complete RPMI-10 (*APPENDIX 2*) and add this to the graduated cylinder. Dissolve the powder and then bring the total volume to 172 ml with more medium. Filter sterilize the solution into a sterile container and store at 4°C protected from light.

The density of metrizamide is critical for optimal cell separation. Each newly prepared batch should be evaluated to determine that it optimally separates the appropriate high-density and low-density populations of cells before it is used.

Bring the solution to room temperature prior to use.

Recombinant human GM-CSF, 100×

Dilute (by ≤1:5) a 1 µg/ml vial of granulocyte/macrophage colony-stimulating factor (GM-CSF; Genzyme) in complete RPMI-10 (*APPENDIX 2*) to generate a solution containing 80,000 U/ml GM-CSF. Divide into appropriately sized aliquots and store at −70°C as recommended by manufacturer. Use or store the solution immediately after reconstitution and dilution.

Recombinant human IL-4, 100×

Resuspend a 10-µg vial of recombinant human IL-4 (Genzyme) in complete RPMI-10 (*APPENDIX 2*) at 50,000 U/ml. Divide into appropriately sized aliquots and store at −70°C as recommended by manufacturer. Use or store the solution immediately after reconstitution and dilution.

Recombinant human TNF-α, 100×

Dilute a 5-µg vial of recombinant human tumor necrosis factor α (TNF-α; Genzyme) in complete RPMI-10 (*APPENDIX 2*) to generate 10,000 U/ml. Divide into appropriately sized aliquots and store at −70°C as recommended by manufacturer. Use or store the solution immediately after reconstitution and dilution.

COMMENTARY

Background Information

Dendritic cells represent a small subpopulation of bone marrow–derived leukocytes that evolve within the monocyte lineage (Steinman, 1991; Zhou and Tedder, 1996). Dendritic cells are classified primarily based on their tissue location; thus, they are classified as interdigitating reticulum cells when present in lymphoid organs, as veiled cells when present in afferent lymph, as blood dendritic cells when in the circulation, as Langerhans cells when present in the epidermis, and as dermal dendritic cells when found in the dermis of the skin (Steinman and Cohn, 1973; Steinman, 1991). Human dendritic cells have been isolated from many of these tissues (Table 7.32.2). Although dendritic cells are widely dispersed throughout the body they exhibit many common features: an irregular shape with extensive and elongated dendritic processes (Fig. 7.32.1D, E, F), a distinct cell-surface phenotype (including very high levels of MHC class II antigens), low buoyant density, active motility, and the ability to stimulate the vigorous proliferation of unprimed T cells.

Dendritic cells are of central importance for antigen presentation during the initiation of primary immune responses such as the sensitization of MHC-restricted T cells and the formation of T cell–dependent antibodies (Steinman, 1991). They are also the principal stimulator cells of primary mixed leukocyte reactions and are actively involved in autoimmune diseases, graft rejection, and HIV infection. Therefore, the ability to isolate and further characterize dendritic cells is of considerable importance and practical utility.

Table 7.32.2 Procedures for the Isolation of Human Dendritic Cells from Tissues

Tissue	Nomenclature	References
Tonsil	Interdigitating reticulum cell	Hart and McKenzie (1988); King and Katz (1989); Prickett et al. (1992)
Thymus	Thymic dendritic cell	Landry et al. (1990); Lafontaine et al. (1992)
Skin	Dermal dendritic cell, Langerhans cell	Romani et al. (1989); Lenz et al. (1993); Nestle et al. (1993)
Synovial exudate	Synovial dendritic cell	Zvaifler (1985); Thomas et al. (1994)
Lung	Lung dendritic cell	Nicod et al. (1987)
Bone marrow	Dendritic cell precursors	Egner et al. (1993b); Egner and Hart (1995)

Isolating adequate numbers of pure dendritic cells from humans has always proven difficult because of their very low frequency in blood (~0.2%) and tissues (Zhou and Tedder, 1995a) Nonetheless, populations of human blood mononuclear cells that contain dendritic cells have been isolated by numerous laboratories and studied using a variety of methods (Van Voorhis et al., 1982; Knight et al., 1986; Young and Steinman, 1988; Freudenthal and Steinman, 1990; Egner et al., 1992; Wood and Freudenthal, 1992; Xu et al., 1992; Egner et al., 1993a; Thomas et al., 1993). Mononuclear cell preparations enriched for dendritic cells are commonly isolated by negative selection, because dendritic cells in the blood lack surface antigens found on T cells, B cells, monocytes, and NK cells (Table 7.32.1). The numbers of dendritic cells in cell preparations have been quantified primarily by determining the frequency of cells with a dendritic morphology, the frequency of cells with a limited phenotypic profile, or the ability of the cells to stimulate in mixed leukocyte reactions. However, the recent identification of CD83 as a marker preferentially expressed by human dendritic cells has greatly facilitated their isolation, identification, and characterization (Zhou et al., 1992; Engel et al., 1995; Weissman et al., 1995; Zhou and Tedder, 1995a,b, 1996).

CD83 is predominantly expressed on the surface of dendritic cells, including blood dendritic cells, skin Langerhans cells, and interdigitating reticulum cells present in the T cell zones of lymphoid organs (Zhou et al., 1992; Engel et al., 1995). $CD83^+$ cells isolated from blood are a homogenous and unique population of cells with the characteristic cellular morphology and functional activities of mature dendritic cells. $CD83^+$ cells express the highest levels of MHC class II molecules compared with other leukocyte lineages and are the most potent stimulator cells in allogeneic mixed leukocyte reactions. Other circulating leukocytes do not express detectable levels of CD83, but this antigen is weakly expressed by some germinal center lymphocytes in vivo (Zhou et al., 1992).

Human $CD83^+$ cells have a unique phenotype, expressing several lineage-associated antigens such as the T cell markers CD2 and CD5, the B cell markers CD40 and CD78, and the myeloid cell markers CD13, CD32 (FcγR II), CD33, CD36, and CD63, as well as a large number of leukocyte-associated antigens (Zhou and Tedder, 1995a). It is therefore possible to define mature dendritic cells as being $CD83^+$ cells, rather than relying principally on the cells having a dendritic cell–like morphology or having the greatest potency in a mixed leukocyte reaction.

The use of CD83 MAbs provides a convenient and efficient way to rapidly separate dendritic cells from bulk mononuclear cells without apparent functional perturbation. Basic Protocol 1 uses CD83 MAbs as a phenotypic marker for assessing dendritic cell purity. The Alternate Protocol uses CD83 MAbs to positively select dendritic cells from populations of mononuclear cells using immunomagnetic selection. CD83 MAbs can also be used in panning procedures to isolate pure dendritic cells from dendritic-enriched populations of cells.

The CD2 and CD4 cell-surface antigens on dendritic cells are also useful. Using immunomagnetic selection protocols, CD2 and CD4 have each been used to isolate dendritic cell populations from mononuclear cell populations depleted of lymphocytes, NK cells, and monocytes. However, CD2 is expressed only at low levels by mature dendritic cells while it is expressed at high levels by T cells, and CD4 is

expressed by "immature" blood dendritic cells but not mature CD83$^+$ dendritic cells (Zhou and Tedder, 1995a, 1996). Because dendritic cells are the only cells in the blood that express CD83, CD83 MAbs can also be used to deplete dendritic cells from mononuclear cell preparations using magnetic bead isolation procedures (Zhou and Tedder, 1995a).

Three populations of cells in blood that have or can develop a dendritic cell morphology have been identified: one small subpopulation that is already CD83$^+$, one population that becomes CD83$^+$ following very brief periods of culture, and one population that derives from myelomonocytic cells but does not express CD83 even following culture or activation (Weissman et al., 1995; Zhou and Tedder, 1995a). The population of cells with a dendritic morphology that derives from myelomonocytic cells may represent a portion of precursor cells that become CD83$^+$ when cultured in the presence of GM-CSF, IL-4, and TNF-1 (Zhou and Tedder, 1996).

The above findings are consistent with other observations that many blood dendritic cells circulate as precursor cells which undergo phenotypic and morphological changes during their differentiation into functional antigen-presenting cells after a period of maturation (Knight et al., 1986; Markowicz and Engleman, 1990; O'Doherty et al., 1993; Romani et al., 1994; Thomas and Lipsky, 1994). Mixed populations of myeloid lineage cells that include cells with a dendritic cell morphology, phenotype, and function can also be generated from human bone marrow or blood progenitor cells after culture with combinations of granulocyte/monocyte colony stimulating factor (GM-CSF), and either tumor necrosis factor α (TNF-α) or interleukin 4 (IL-4; Caux et al., 1992; Reid et al., 1990, 1992; Santiago-Schwarz et al., 1992, 1993; Romani et al., 1994; Szabolcs et al., 1995). This strategy has been used to show that dendritic cells can actually be derived directly from human blood CD14$^+$ monocytes (Zhou and Tedder, 1996). Therefore, cells with a dendritic cell morphology within human blood are heterogeneous, but likely represent a common lineage of cells at different stages of differentiation.

Blood monocyte differentiation into a fairly homogenous population of functionally mature CD83$^+$ dendritic cells under the influence of a specific cascade of cytokines can be divided into several stages (Zhou and Tedder, 1996). In the first stage, CD14$^+$ CD1a$^+$ cells with a dendritic morphology are induced by GM-CSF/IL-4. In the second stage, the cells are induced by TNF-α to differentiate into CD83$^+$ CD14$^-$ CD4$^+$ cells. Finally, in a third stage involving continued culture, the monocyte-derived CD83$^+$ cells develop into cells with decreased CD1a expression that have a dermal dendritic cell phenotype. Depletion of CD14$^+$ cells from mononuclear cell preparations depletes most dendritic cell precursors (Zhou and Tedder, unpub. observ.). This finding supports and extends earlier findings in which blood monocyte subpopulations or adherent cells with dendritic cell morphology, size, cell surface phenotype, or function have been identified (Knight et al., 1986; Kabel et al., 1989; Najar et al., 1990; Peters et al., 1991; Ruppert et al., 1991; Grage-Griebenow et al., 1993). The ability to generate dendritic cells from monocytes provides a new and simple method for generating functionally mature dendritic cells from a population of cells that are relatively easy to isolate to homogeneity, as opposed to CD34$^+$ stem cells or other precursor cells.

The ability to generate dendritic cells in vitro from their precursors has considerable utility. Given the capacity of dendritic cells to elicit strong antigen-specific helper and cytotoxic T cell responses, the ability to isolate or generate large numbers of homogeneous preparations of CD83$^+$ dendritic cells will facilitate the manipulation of this cell lineage for vaccine development, organ grafting, and ex vivo therapy for a broad range of human diseases. For purposes of comparison, it is also useful to generate cell preparations depleted of dendritic cells, as detailed in Basic Protocol 3.

As outlined above, there are several ways to isolate or generate human dendritic cells. Caution should be used when dendritic cell properties or function are assessed using dendritic cell–enriched preparations of cells that are not homogeneous, as considerable variability can be obtained. Because there is tremendous variability in the way individual laboratories assess dendritic cell purity and function, there remains considerable disagreement in the literature. This heterogeneity is further magnified by the fact that dendritic cells can be at differing stages of "maturity," which may also affect their function. Although CD83$^+$ dendritic cells isolated by each of these techniques are phenotypically, functionally, and morphologically homogenous in most assays, it remains possible that there is additional heterogeneity that is not currently appreciated.

Critical Parameters

The single most important factor in isolating adequate numbers of dendritic cells from human blood mononuclear cells is the availability of large numbers of starting cells. Small volumes of blood do not work well for the isolation of dendritic cells and in these cases it is advisable to generate dendritic cells from monocytes (see Basic Protocol 2). Freshly isolated mononuclear cells are best, but more than adequate results can be obtained with day-old mononuclear cell preparations that have been maintained on ice. Leukocyte-enriched "leukopaks" that have been isolated within the last 24 hr can be obtained from large blood banking facilities or from North American Biologicals. Leukopaks are usually obtained by total leukapheresis of donors, so it is possible to isolate $2–12 \times 10^8$ mononuclear cells from each leukopak. However, the number of mononuclear cells in a leukopak will vary considerably based on the source and blood donor. Alternatively, leukocyte-enriched "buffy coats" prepared from donated units of peripheral blood or from plasmapheresis procedures can be utilized. Differences in isolation procedures can affect neutrophils within leukopaks, thereby making it difficult to remove the neutrophils from the mononuclear cell preparation by Ficoll-Paque density gradient centrifugation. In these cases, the purity of the final dendritic cell preparation can be significantly diminished since neutrophils share many of the buoyant properties of dendritic cells. Units of whole blood can also be used as a starting population of cells, but the final yield of dendritic cells is closely linked with the number of starting cells, so sufficient numbers of cells are required to obtain reasonable yields. This is particularly important for experiments where dendritic cells need to be purified to homogeneity by cell sorting. Typical yields after cell sorting represent ~10% of the starting population of labeled cells that are being sorted when the frequency of $CD83^+$ cells is >50%, but vary greatly with the level of dendritic cell purity and the sort rate.

The technique-sensitive steps are those involving cell manipulation, because these can lead to large cell losses. The single most critical step is washing the tissue culture dishes free of nonadherent dendritic cells after plastic adherence (see Basic Protocol 1). Care must be taken to clear the entire surface of nonadherent cells, pipetting with sufficient force to dislodge the nonadherent cells, while at the same time avoiding excessive force which can strip off adherent cells. It is essential to use warm medium in these steps, because cold medium detaches adherent cells from plastic.

The cells that are obtained using Basic Protocol 1 are enriched for dendritic cells. The relative number of dendritic cells can be roughly assessed by phase-contrast microscopy of the cells in a hemacytometer. Dendritic cells are large and have irregular, long membrane processes (Fig. 7.32.1C), while lymphocytes are small and round. If a higher purity of dendritic cells is desired, a second metrizamide density gradient centrifugation can be performed to remove contaminating lymphocytes. Contaminating B lymphocytes and monocytes, which bind EA via their surface Fc receptors, can be further depleted from the dendritic cell preparations by EA rosetting as described in *UNIT 3.7* for mouse dendritic cells.

Alternatively, monocytes can be depleted by adherence to human Ig–coated plates and B lymphocytes depleted on plates coated with goat anti–human Ig. Depletion of contaminating cells using lineage-associated MAbs that do not bind to dendritic cells (Table 7.32.1) can also be achieved using magnetic bead–mediated depletion techniques (see Alternate Protocol) or panning. If total purity is required, cell sorting should be performed. Due to protocol variations in each laboratory, these procedures may need some optimization in order to isolate maximal numbers of dendritic cells. It is important to always start with a sufficient number of mononuclear cells, such as are present in leukapheresis packs or buffy coats from leukapheresis donors, to ensure optimal cell isolation and sufficient cell yield.

Depleting contaminating cells from the dendritic cell preparations by Fc receptor–mediated procedures is highly effective. However, the authors have found that dendritic cells express the CD32 and CD64 Fc receptors (Fanger et al., 1996), so these isolation procedures may limit the heterogeneity of the dendritic cell preparations obtained. Additionally, mature dendritic cells express the sheep erythrocyte receptor, CD2 (Zhou and Tedder, 1995a). While it is possible that some dendritic cells may be depleted from the E-rosette-negative fraction of blood mononuclear cells by rosetting with sheep red blood cells, the levels of CD2 expression are low and may not be sufficient to result in effective rosette formation.

Troubleshooting

Because of the many manipulations that are required in these protocols, there are many

things that can go wrong. Until these protocols become standard to the investigator, all cell fractions should be retained until the end of the procedure. In this way, it is possible to determine where things went wrong and, in many cases, to recover the dendritic cell–containing fraction.

Anticipated Results

The density centrifugation and immunomagnetic isolation procedures (Basic Protocol 1 and Alternate Protocol) yield cell populations consisting of 20% to 80% dendritic cells and largely free of lymphocytes. Incubation of $CD14^+$ monocytes with cytokines (Basic Protocol 2) yields a relatively homogeneous population of functionally mature dendritic cells, with typical recoveries of 60% to 90% of input monocytes.

Time Considerations

In Basic Protocol 1, 5 to 6 hr of work are required on the first day of the procedure, from the isolation of blood mononuclear cells to the overnight incubation of mononuclear cells in culture plates. On the second day, the enrichment of dendritic cells by metrizamide gradients followed by additional adherence and panning procedures requires 4 to 6 hr. It is possible to institute many shortcuts, but these usually result in decreased yields or purity of the final cell preparation, so it is best to carefully follow the protocols as outlined. The use of magnetic beads can speed up the negative and positive isolation procedures, as indicated in the Alternate Protocol.

Isolation of dendritic cells from GM-CSF/IL-4/TNF-α–induced monocytes or total mononuclear cell preparations (see Basic Protocol 2) is less time-consuming. This procedure does require an 8-day period of cell culture in addition to the time spent preparing the cells, and it is a more expensive approach, because of the cost of recombinant cytokines. Nonetheless, the generation of dendritic cells from monocytes, can yield significant numbers of cells and a fairly homogenous population of dendritic cells, which has considerable advantages for some applications.

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Details procedures for generating dendritic cells from monocytes.

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