METHODS IN MOLECULAR MEDICINE™

Dendritic Cell Protocols

Edited by
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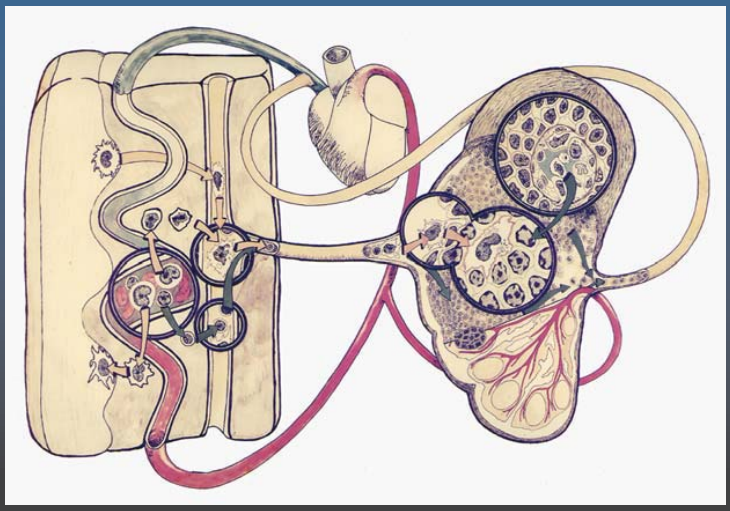
Andrew J. Stagg, PhD



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Methods in Molecular Medicine™

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Foreword

Dendritic Cell Protocols provides chapter and verse for many useful practical approaches to the art of studying dendritic cells. The book gives information on the usual techniques for derivation of human dendritic cells from precursor stem cells, such as monocytes. In addition it provides data on the difficult tasks of isolating dendritic cells directly from different tissues; whether dendritic cells from precursor cells or from tissues of mouse or human are required, this book contributes practical information. The last section of the book is devoted to functional aspects of dendritic cells ranging from information relevant to cell migration to antigen uptake and T cell stimulation.

But what is a dendritic cell? The explosion of studies on this cell type over the last few years is breathtaking. We now have so-called "myeloid" and "plasmacytoid" dendritic cells; are they of separate lineages or are they related? There are claims that dendritic cells can be derived from B cells and from granulocytes, as well as from "monocytes." Lymphocytes can be switched on or off by dendritic cells. Dendritic cells can preferentially cause Th1 or Th2 responses or some may stimulate B cells directly. When there is so much scope for exploring these new developments, it is particularly important that the data that are established and the practical basis for this work are clearly available, a task that this book fulfils. But to return to the original question of "What is a dendritic cell?" The term is generally applied to any cell that has an appropriate phenotype and can stimulate a primary allogeneic mixed leukocyte reaction. Perhaps this functional definition requires re-examination; are we applying this term "dendritic cell" too casually? Our recent studies on mixed leukocyte reactions show that dendritic cells are efficient at transferring MHC antigens to other dendritic cells and that a major route of stimulation in the mixed leukocyte reaction is the secondary presentation of alloantigens acquired by dendritic cells of responder type. We may, therefore, legitimately ask whether all the cells that we claim can stimulate primary T cell responses can actually stimulate T cells directly. They may merely be efficient at transferring MHC molecules to the cells that stimulate the primary immune responses. Re-examining this issue may eventually simplify the definition of dendritic cells. However, on current evidence, confusion may increase in the short term before these issues are finally resolved.

The two editors of this book, Stephen Robinson and Andrew Stagg, were both introduced to dendritic cells within my laboratory. In turn, the interest in dendritic cells was initiated in my laboratory in the late 1970s and early 1980s because of the influence and enthusiasm of Dr. Brigid Balfour who was studying

"veiled cells" from afferent lymphatics of pigs, rabbits, and, indeed, from herself! Since not only the editors of this chapter but also the authors of seven of the chapters belong to this particular "Dendritic Cell School," it is appropriate to pay tribute to the contribution Dr. Brigid Balfour made to the field. It was unfortunate that Dr. Balfour buried much of her work in obscure publications in the proceedings of meetings. However, the extent of her understanding of this field can be gleaned from the cover illustration, which is a picture produced by Dr. Balfour in the early 1980s. In this picture, you will see in the skin (the lower tissue) some dendritic cells arriving in the dermis, and these are shown as a separate population of dermal dendritic cells with a migration pathway into the afferent lymphatics. A second population moves to the epidermis to become Langerhans' cells that can also pass into the afferent lymph. Within the lymph nodes, the picture also shows the interchange of dendritic cells between the different areas of the lymph node. It has taken most of us a couple of decades to catch up with Brigid Balfour's understanding of this system. Brigid Balfour showed, formally, the movement of skin Langerhans' cells via the afferent lymph toward the lymph node. This work involved studies that included those of her own afferent lymphatic; she cannulated the afferent lymphatic in her leg, skin-painted herself with dinitrochlorobenzene and identified T6 (later designated CD1a) labeled cells migrating in larger numbers into her afferent lymphatic in response to the antigen challenge. She belonged to that rare early breed of scientist who believed in self-experimentation!

Brigid Balfour's enthusiasm spilled over into the work of those who were introduced by her to "veiled cells," the dendritic cells of the afferent lymph. Brigid died in 1994 but would have derived great satisfaction, not only in the fact that this subject has become so widely studied, but also that a book designed for the dissemination of techniques related to dendritic cell studies is available. She was herself an excellent experimentalist who influenced, widely, the development of dendritic studies in the UK and elsewhere. She would have been delighted to know that the general scientific community was finally a party to the knowledge of the importance of "veiled" or dendritic cells. This timely book disseminates practical information, some of which derives directly from her early studies, and provides a platform on which studies of the next generation of researchers working on dendritic cells may be soundly based.

Stella C. Knight, PhD

Preface

The last decade has witnessed a massive expansion in our understanding of the previously rather enigmatic population of antigen-presenting cells known as dendritic cells (DC). It is now apparent that DC do much more than initiate primary T cell responses. They are involved in complex interactions with T cells, B cells, and other cell types. Through the elaboration of a variety of chemokines and cytokines, DC are able to shape the evolution of an acquired immune response and play important roles in innate immunity. It has also become clear that DC are not a homogeneous population. Different developmental pathways can give rise to DC, and subpopulations of DC have distinct functional attributes allowing them to perform different roles in the immune system.

Gaining an insight into the complexity of DC biology has been made difficult by the practical challenges involved in studying these cells. In particular, their existence as a trace population in most tissues has made it difficult to isolate sufficient DC for analysis. However, in recent years, improvement in isolation techniques and the discovery of cytokine combinations that allow DC to be derived in vitro have permitted intensive investigation of these cells and driven the rapid expansion in our knowledge.

In *Dendritic Cell Protocols* we have brought together a wide range of methods currently used in the study of DC. Inevitably, as we have tried to concentrate on techniques specific to DC, particular emphasis has been placed upon how to get these cells, whether it be by isolation from a wide variety of tissues or by isolation from various progenitor cell populations. Once obtained, these DC populations are amenable to analysis by many established cellular or molecular techniques, but it should always be borne in mind that DC will vary in their phenotype and function depending on their source and the method used to obtain them. We have also included chapters that describe some of the commonly used techniques for the analysis of aspects of DC function. Often there is no "gold standard" for methods in studying DC and therefore, in some circumstances, we have included variants of similar methods by different authors. We hope this will enable the reader to gain the benefit of the experience and approaches in more than one laboratory. One of the most exciting areas of DC

research is the use of DC for immunotherapy, for instance, of malignant disease. Although this is a new field we have included one or two chapters that we hope will give some feel for the practical challenges involved in the use of DC in a clinical setting.

We hope that both novice and experienced DC researchers will find *Dendritic Cell Protocols* useful and wish everyone success in this exciting area of research.

Stephen P. Robinson, MD, PhD
Andrew J. Stagg, PhD

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I

Isolation of Dendritic Cells from Animal Tissues

1

Isolation of Dendritic Cells from Mouse Lymph Nodes

Dmitry Gabrilovich

1. Introduction

Lymph nodes are the primary sites of T-cell stimulation by dendritic cells (DC). After contact with antigens, DCs migrate to draining lymph nodes from the skin and other tissues ***(1–3)***. Investigation of the morphology and function of lymph node DCs may provide important information about the role of these cells in normal and pathological conditions. Therefore, lymph nodes are popular sites for the isolation of dendritic cells. Dendritic cells isolated from lymph nodes represent "interdigitating" DCs that are localized in T-dependent regions of lymph nodes. DCs represent about 1% of the total population of lymph node cells. Therefore, in order to perform almost any functional tests, the DC fraction should be enriched. The most practical way to enrich the DC fraction is to use a density gradient. Several gradients—metrizamide ***(4)***, Nycodenz ***(5)***, and Percoll ***(6)***—have successfully been used for enrichment of DCs obtained from different sources. When isolating DC from lymph nodes, density gradient separation produces a population of DC with a purity of 40–50%. Most contaminating cells are lymphocytes with a small fraction (usually less than 5%) of macrophages. The choice of lymph nodes is dependent on the purpose of the experiment. The most commonly used lymph nodes are axillary, inguinal, and popliteal. DCs can be further enriched using monoclonal antibodies and flow cytometric cell sorting, magnetic beads separation, panning, or cytotoxic elimination with complement. All these methods are based on the negative selection of DCs using anti-T, anti-B, and anti-macrophage antibodies. Since the first step of isolation involves gradient centrifugation, granulocyte contamination is negligible and further purification steps do not require use of anti-granulocyte antibodies.

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2. Materials

1. Sterile dissecting forceps and scissors for lymph node extraction.
2. Sterile 6-well plates, 50-mL conical tubes, and 15-mL conical tubes (Falcon, Becton Dickinson, Franklin Lakes, NJ). Sterile 70-μm cell strainers (Falcon), 5 mL syringes, and 5 mL and 10 mL pipets.
3. Fetal calf serum (FCS) (HyClone, Logan, UT). Culture media DMEM and RPMI 1640 (Gibco-BRL, Grand Island, NY) supplemented with antibiotics. We commonly use a combination of penicillin, streptomycin, and Fungizone (antibiotic–antimycotic, Gibco-BRL). DMEM can be used without serum. RPMI 1640 should be supplemented with 10% FCS (RPMI-FCS).
4. Metrizamide gradient. Dissolve 7.25 g metrizamide with 45 mL RPMI in a 50-mL tube. It usually takes 15–20 min. Sterilize the gradient by passing through a 0.45 μm filter. Add 5 mL of FCS, mix and prepare 2.5 mL aliquots. Store the gradient at –30°C. We use metrizamide produced by Nygaard, Norway. Metrizamide is also produced by Sigma, and we have had satisfactory results with Sigma's metrizamide.
5. Hemocytometer, and microscope with 400× magnification.
6. PE conjugated anti-B7-2 (CD86) antibody, FITC conjugated anti-CD11c (N418) antibody, and PE and FITC conjugated mouse IgG2a and IgG2b as isotype control (Pharmingen).

3. Methods

3.1. Isolation of DC by Density Gradient Centrifugation

1. Place a cell strainer into one well of a 6-well plate. Fill the well with DMEM. Prepare as many wells as necessary.
2. Sacrifice mice using one of the methods approved by the appropriate institutional review board. Extract the lymph nodes from at least three mice and put the lymph nodes together on the cell strainer submerged in DMEM. Make sure that the lymph nodes are covered with the medium.
3. Remove the plunger from a syringe and use it to press the lymph nodes through the mesh of the cell strainer. Make sure that lymph nodes are completely shattered on the mesh. Finally, wash the strainer with 3–4 mL of DMEM.
4. Discard cell strainers and collect cells from the well into a 15 mL conical tube. Wash cells once with DMEM by centrifuging for 5 min at 300*g*. Resuspend cells in 10 mL of RPMI-FCS.
5. Thaw the metrizamide gradient and transfer 2.5 mL into a sterile 15 mL tube. Overlay cells slowly onto the gradient. It is important not to disturb the gradient. Spin the gradients for 10 min at 500*g* at room temperature with no break on the centrifuge.
6. Collect cells from the interface using a 5 mL pipet and wash them once with RPMI-FCS. Resuspend cells in 1 mL of RPMI-FCS.
7. Count cells on the hemocytometer at magnification 400×. DCs may be clearly identified by their distinct morphology. This DC enriched fraction can now be used in

morphological or functional studies (*see* **Notes 1** and **2**). To confirm the purity of the samples, cells can be analyzed by flow cytometry (*see* **Subheading 3.3.**).

3.2. Purification of DC by Panning

If a higher purity of DCs is desired, DCs can be further enriched by panning (*see* **Note 3**).

1. Prepare a 6-well plate for panning by coating separate wells with either anti-mouse immunoglobulin or anti-rat immunoglobulin. We usually use goat anti-mouse immunoglobulin and goat anti-rat immunoglobulin from Sigma, St. Louis, MO. Add 3 mL of each antibody at a concentration of 1 mg/mL in phosphate-buffered saline (PBS) to separate wells.
2. After at least 60-min incubation at room temperature, remove the antibody solution and wash the wells four to five times with PBS (*see* **Note 4**).
3. Wash the DC fraction derived by density gradient centrifugation (*see* **Subheading 3.1.**) once in PBS and resuspend in 100 μL of hybridoma supernatants of anti-CD4 antibody (L3T4, TIB-207, ATCC, Rockville, MD), 100 μL of hybridoma supernatants of anti-CD8 antibody (Lyt-2.2, TIB-210, ATCC), and 20 μL of anti-F4/80 antibody (Serotec, Raleigh, NC) (*see* **Note 5**).
4. After a 25-min incubation on ice, wash cells twice and resuspend in 3 mL of PBS containing 0.1% mouse serum.
5. Transfer the cell suspension to a well coated with anti-rat immunoglobulin. Incubate cells on the plates for 60–90 min at 4°C.
6. Harvest the nonadherent DC enriched fraction using a 5 mL pipete. Gently wash the well with ice cold PBS to remove any partially adherent cells and add these to the nonadherent fraction (*see* **Note 5**).
7. Concentrate the harvested cells by centrifuging at 300*g*. Resuspend the cell pellet in 3 mL of cold PBS with 0.1% serum and transfer into a well coated with anti-mouse immunoglobulin. Incubate for another 60-90 min and then collect cells as described previously. Cells can now be resuspended in RPMI-FCS and used for further study (*see* **Note 5**).

3.3. Analysis of DC Purity by Flow Cytometry

We routinely use double labeling with anti-CD11c (N418) antibody and anti-B7-2 (CD86) antibody to identify DC as $CD11c^+$ $CD86^+$ cells on the flow cytometer.

1. Transfer 100 μL of the purified DC cell suspension into two tubes for flow cytometry labeled “test” and “control.”
2. Wash cells once with PBS and resuspend in 100 μL of PBS.
3. Add 5 μL of anti-B7-2 and anti-CD11c antibodies into the “test” tube and 5 μL of isotype control antibodies into the “control” tube.
4. Incubate on ice for 25 min, then wash twice with 2 mL of cold PBS and analyze on the flow cytometer (*see* **Note 6**).

4. Notes

1. Generally, one can expect to isolate around $5–10\times10^3$ DCs from one lymph node. For functional tests, we usually collect lymph nodes from three or four mice, which provides a sufficient number of cells (around 2×10^5) for several functional tests.
2. It is recommended that 10^{-5} *M* of β-mercaptoethanol is added to the culture medium while growing mouse cells. However, β-mercaptoethanol should be added after completion of all isolation procedures, since even in low concentration it may affect the binding of antibodies to the cells.
3. Owing to the relatively low number of cells, lymph nodes are not the best source of highly purified DCs. However, if the experimental goal makes it necessary to use highly purified lymph node DCs, we suggest using the panning technique as opposed to complement-dependent cytotoxicity. The latter method usually results in the nonspecific loss of some DC. If the investigator has access to a flow cell sorter, this method, as well as the magnetic bead separation technique, may provide good alternatives. For all sorting procedures, the author recommends using at least six mice per sample.
4. Panning plates may be stored overnight at 4°C. Collected antibody solution can be used again several times.
5. Anti-Thy-1.2 antibody produced by hybridoma supernatant (ATTC TIB 107) can be used instead of anti-CD4 and anti-CD8 antibodies. All these antibodies are rat $Ig2_b$. In the first panning step, T cells and macrophages are eliminated using anti-rat monoclonal antibodies. In the second step, B cells are removed using anti-mouse immunoglobulin antibody.
6. It is important to perform all procedures at 4°C, because DCs readily adhere to plastic and some cells may be lost if the incubation is performed at room temperature. The purity of DCs can be verified using anti-CD11c and B7-2 antibodies. The final purity of the DC fraction is usually above 95% following panning. One can expect to obtain at least 10^5 highly purified DCs from five or six mice.
7. A typical example of double labeling of lymph node DCs with CD11c and B7-2 antibodies is shown **Fig. 1.** Cells were isolated and labeled with antibodies as described in "Methods." Analysis was performed using FACSCalibur flow cytometer (Becton Dickinson, Mountain View, CA). It is important to note that there is no single marker that would allow for detection of 100% of lymph node DCs. N418 (CD11c) may bind to some macrophages, whereas B7-2 binds primarily to only mature DCs. The investigator may choose to use a combination of other DC markers depending on the goal of the study.

References

1. Steinman, R. M. (1991) The dendritic cell system and its role in immunogenicity. *Annu. Rev. Immunol.* **9,** 271–296.
2. Knight, S. C., and Stagg, A. J. (1993) Antigen-presenting cell types. *Curr. Opin. Immunol.* **5,** 374–382.
3. Hart, D. N. J. (1997) Dendritic cells: Unique leukocyte populations which control the primary immune response. *Blood* **90,** 3245–3287.

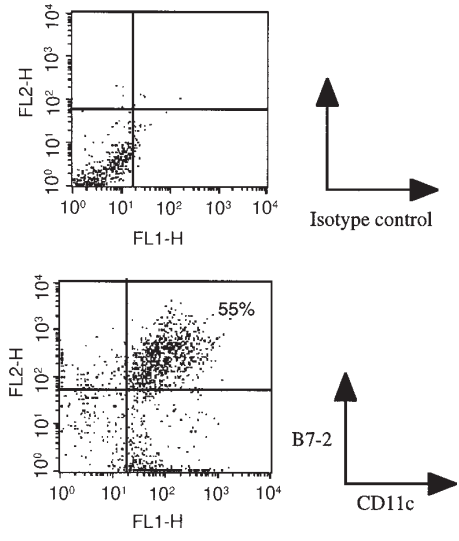

Fig. 1. Flow cytometry demonstrates the purity of DC isolated from lymph nodes following density gradient centrifugation.

4. Knight, S. C., Farrant, J., Bryant, A., et al. (1986) Non-adherent, low density cells from human peripheral bloodcontain dendritic cells and monocytes, both with veiled morphology. *Immunology* **57,** 595–598.
5. McLellan, A. D., Starling,G. C., and Hart, D. N. C. (1995) Isolation of human blood dendritic cells by Nycodenz discontinuous gradient centrifugation. *J. Immunol. Methods* **184,** 81–85.
6. Young, J. W. and Steinman, R. M. (1988) Accessory cell requirements for the MLR and polyclonal mitogens, as studied with a new technique for enriching blood dendritic cells. *Cell Immunol* **111,** 167–171.

2

Isolation of Mouse Spleen Dendritic Cells

Andrew J. Stagg, Fiona Burke, Suzanne Hill, and Stella C. Knight

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).

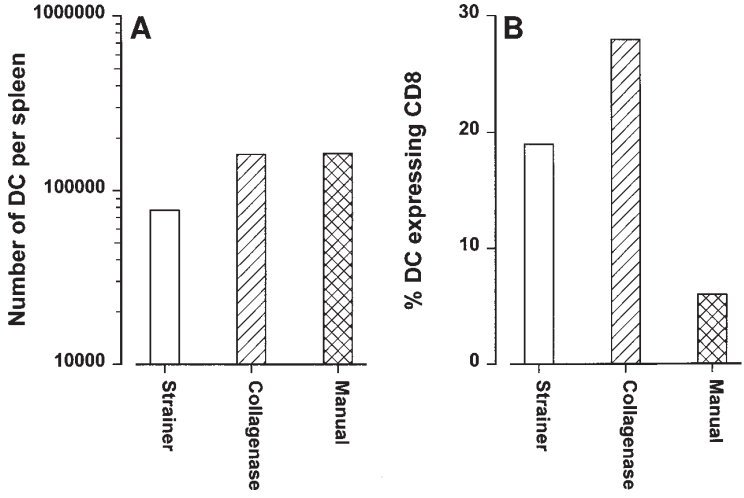

Fig. 1. Method used to prepare a singlecell suspension from spleens influences the number (**A**) and type (**B**) of DC obtained. Spleen cells were obtained by the strainer, collagenase or manual extraction techniques as described in the text. The absolute number of CD11c$^+$ DC obtained following overnight 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.

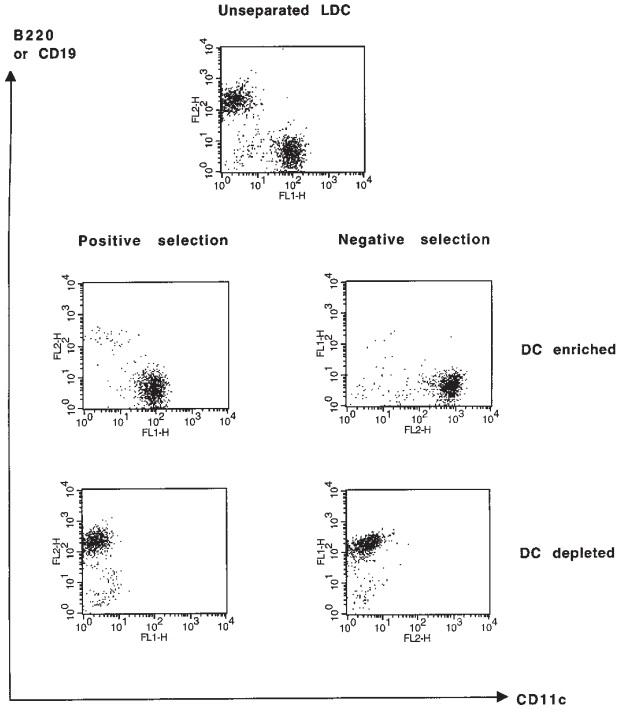

Fig. 2. DC can be further purified from the LDC preparation by either positive or negative selection using antibodies to CD11c or CD45R(B220), respectively. DC depleted populations are obtained in parallel.

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-

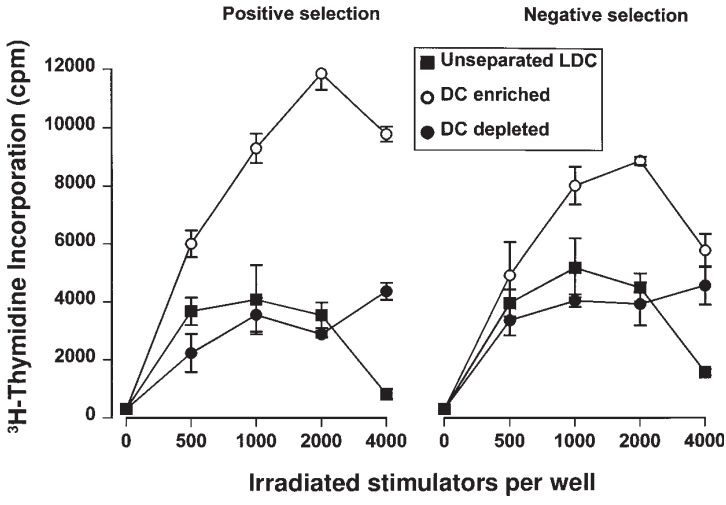

Fig. 3. DC, enriched by either positive or negative immunomagnetic selection, stimulate a Primary mixed leukocyte reaction (MLR). Unseparated LDC and DC enriched or depleted preparations were irradiated (2000r) and used to stimulate 25000 allogeneic lymph node cells in a 20 μL hanging drop culture system. 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.

References

1. Steinman, R. M. and Cohn, Z. A. (1973) Identification of a novel cell type in peripheral lymphoid organs of mice. I. Morphology, quantitation, tissue distribution. *J. Exp. Med.* **137,** 1142–1162.
2. Steinman, R. M. and Cohn, Z. A. (1974) Identification of a novel cell type in peripheral lymphoid organs of mice. II. Functional properties in vitro. *J. Exp. Med.* **139,** 380–397.
3. Vremec, D. and Shortman, K. (1997) Dendritic cell subtypes in mouse lymphoid organs. Cross-correlation of surface markers, changes with incubation and differences among thymus, spleen, and lymph nodes. *J. Immunol.* **159,** 565–573.
4. Crowley, M., Inaba, K., Witmer-Pack, M. D., and Steinman, R. M. (1989) The cell surface of mouse dendritic cells: FACS analyses of dendritic cells from different tissues including thymus. *Cell. Immunol.* **118,** 108–125.
5. De Smedt, T., Pajak B., Muraille, E., et al. (1996) Regulation of dendritic cell numbers and maturation by lipopolysaccharide in vivo. *J. Exp. Med.* **184,** 1413–1424.
6. Leenen, P. J. M., Radosevic, K., Voerman, J. S. A., et al. (1998) Heterogeneity of mouse spleen dendritic cells: in vivo phagocytic activity, expression of macrophage markers, and subpopulation turnover. *J. Immunol.* **160,** 2166–2173.
7. Maldonado, L., De Smedt, T., and Michel, P., (1999) CD8alpha+ and CD8alpha– subclasses of dendritic cells direct the development of distinct T helper cells in vivo. *J. Exp. Med.* **189,** 587–592.
8. Suss, G. and Shortman, K. (1996) A subclass of dendritic cells kills CD4 T cells via Fas/Fas-ligand- induced apoptosis. *J. Exp. Med.* **183**, 1789–1796.
9. Vremec, D., Pooley, J., Hochrein, H., Wu, L., and Shortman, K. (2000) CD4 and CD8 expression by dendritic cell subtypes in mouse thymus and spleen. *J. Immunol.* **164,** 2978–2986.

3

Isolation of Mouse Thymic Dendritic Cells

Fabienne Anjuère and Carlos Ardavín

1. Introduction

The method described in this chapter for the isolation of mouse thymic dendritic cells (DC) is an optimization of our previously published methods ***(1,2)*** and involves the following major steps:

1. Enzymatic digestion of thymic fragments with collagenase and DNase.
2. Separation of a very-low-density cell fraction (VLDF).
3. Magnetic depletion of T-lineage cells, B cells, macrophages, and granulocytes.
4. Positive selection of DCs by magnetic cell sorting (MACS).

This isolation method has been designed on the basis of the phenotype of thymic DCs, which belong to the lymphoid DC lineage ***(3)***, and therefore are positive for CD8 and CD11c, but negative for CD3, CD4, CD25, B220, Mac-1, the macrophage antigen F4/80, and the granulocyte antigen Gr1 ***(4)***.

In contrast to previously described DC isolation methods and with the exception of the enzymatic digestion step, this isolation method is performed entirely at 4°C thereby avoiding prolonged incubation steps at 37°C, which might alter the phenotypic and functional characteristics of DC ***(5)***. The method involves the separation of a VLDF using an Optiprep (Nycomed Pharma AS) centrifugation medium with a density of 1.055 *g*/mL. This VLDF represents 0.2–0.4% of total thymocytes, i.e., 10 times less than the low-densitiy cell fractions used in previous protocols. The use of such VLDF makes the subsequent magnetic bead depletion more efficient and less expensive.

Since DC represent around 0.1% of total thymocytes ***(4)***, a minimum number of thymuses are needed in order to obtain a reasonable number of purified

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DC. The method described below is optimized for purifying DC starting from 20 4–6 wk-old mice, i.e., around 4.0×10^9 thymocytes. The protocol for scaling this method either up or down is explained in the **Subheading 3.2.**

2. Materials

1. RPMI 1640 medium: RPMI 1640 medium supplemented with 1 m*M* sodium pyruvate and 100 U/mL penicillin–streptomycin. Store at 4°C.
2. Collagenase/DNase solution: Collagenase (0.5 mg/mL collagenase A, Boehringer-Mannheim) and DNase (40 mg/mL DNase I, grade II; Boehringer-Mannheim) in RPMI 1640 medium supplemented with 5% fetal calf serum (FCS). The collagenase/DNase solution has to be freshly made. DNase can be stored at –20°C at 1 mg/mL in 2:1 glycerol:H_2O.
3. PBS-EDTA-FCS: Phosphate-buffered saline solution (PBS) supplemented with 5 m*M* EDTA and 5% FCS. Store at 4°C (*see* **Note 1**).
4. Optiprep solution: The 1.055 Optiprep solution (density: 1.055 g/mL) is prepared from a ready made Optiprep solution (60% w/v Iodixanol in water; density 1.320 g/ml; Nycomed Pharma AS) as follows: Mix V parts (chosen volume) of the Optiprep working solution with V_1 parts of the NaCl diluent following the formula: $(V \times D) + (V_1 \times D_1) = (V + V_1) \times 1.055 \longrightarrow V_1 = V \times 2.14$. Where; D = density of Optiprep working solution = 1.162 g/mL, D_1 = density of NaCl diluent = 1.005 g/mL. Example: add 21.4 mL (= 10 mL x 2.14) of NaCl diluent to 10 mL of Optiprep working solution to obtain 31.4 mL of 1.055 Optiprep solution.
5. Optiprep working solution: Mix 1 part Optiprep with 1 part of NaCl diluent.
6. NaCl diluent: NaCl 0.8% (w/v) in 10 m*M* Tricine buffer pH7.4 containing 5 m*M* EDTA. The 1.055 Optiprep solution can be stored at 4°C for up to 4 wk.
7. mAb mixture: Prepare a monoclonal antibody (mAb) mixture containing anti-CD3 (clone KT3-1.1), anti-CD4 (clone GK1.5), anti-IL-2Rα (clone PC61.5), anti-B220 (clone RA3-6B2), anti-macrophage antigen F4/80 (clone C1.A3-1), and anti-granulocyte antigen Gr1 (clone RB6-8C5). All the mAbs must be purified from culture supernatants by affinity chromatography with Protein G Sepharose (Pharmacia Biotech) and used at 10 μg/mL in PBS-EDTA-FCS. Make aliquots and store at –20°C. The aliquot in current use may be kept at 4°C for up to 3 wk. Prolonged refrigerator storage and repeated freeze–thaw cycles must be avoided.

3. Methods

1. Pool thymuses from 20 mice and cut into small fragments with blunt scissors in empty 50 mL polypropylene conical centrifuge tubes. (Do not add any liquid medium in this step).
2. Add 5 mL of collagenase/DNase solution and incubate for 10 min at 37°C with continuous agitation.
3. Filter the digested fragments through a stainless-steel sieve with a 60-mesh screen (Sigma) and make a cell suspension in a 50-mL polypropylene conical centrifuge

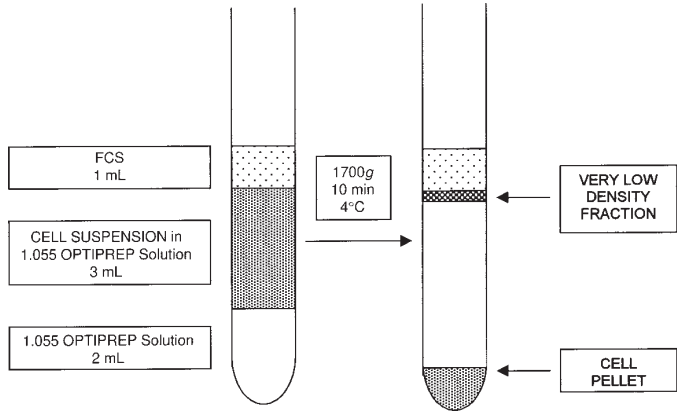

Fig. 1. Preparation of density gradients for the generation of the DC enriched VLDF.

tube by washing the digested tissue with 50 mL of RPMI 1640 medium containing 5 μg/mL DNase I (pre-warmed to 37°C) (*see* **Note 2**).

4. Wash the cell suspension twice with 50mL cold PBS-EDTA-FCS containing 5 mg/mL DNase I, and count. For this and the following washing steps centrifuge at 540*g* for 5 min at 4°C.
5. Resuspend the cells in cold 1.055 Optiprep solution at 1.5×10^8 cells/mL.
6. Density gradient centrifugation is performed in 14 mL polypropylene round-bottom tubes (Falcon; cat. no. 2059). Using a Pasteur pipet, carefully layer 3 ml of the cell suspension over 2 ml of 1.055 Optiprep solution and then layer 1 mL of FCS above the cell suspension (*see* **Fig. 1**). The 14 mL tubes must be pre-cooled and kept at 4°C before layering the cell suspension (*see* **Note 3**); 8 to 10 tubes are generally needed when starting from 20 thymuses.
7. Centrifuge at 1700*g* for 10 min at 4°C and collect the VLDF (*see* **Fig. 1**) with a Pasteur pipet at the interface between the Optiprep and the FCS layers (select a low acceleration/deceleration rate centrifugation program).
8. Wash the VLDF three times with 15 mL PBS-EDTA-FCS in a 15 mL polystyrene conical tube and then count the cells. The VLDF constitutes a DC-enriched fraction containing 15–20% DC (*see* **Subheading 3.1. Fig. 2**).
9. Incubate the VLDF with the mAb mixture for 40 min at 4ºC. Use 25 μL of mAb mixture per 1×10^6 VLDF cells.
10. Wash the VLDF twice in 15 mL of PBS-EDTA-FCS.
11. Resuspend the cells in 25 μL of PBS-EDTA-FCS per 1×10^6 VLDF cells, and keep at 4°C while you wash the magnetic beads in order to remove the sodium azide used as preservative.
12. Wash the anti-rat Ig coated magnetic beads twice with cold PBS-EDTA (without FCS) in a 5 ml polystyrene tube (Falcon, cat. no. 2052). The beads are used at a ratio of seven beads per cell. Use a Magnetic Particle Concentrator (Dynal A.S.)

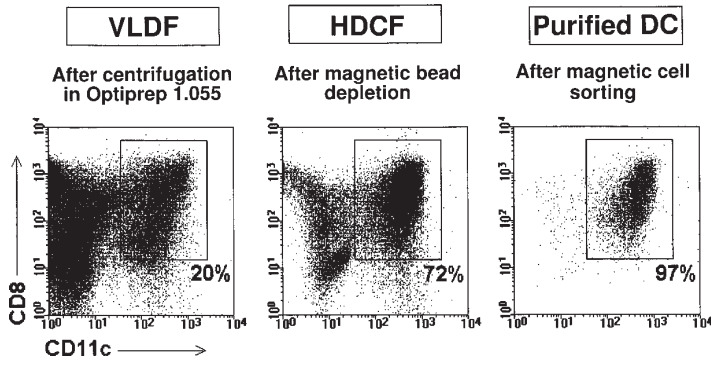

Fig. 2. Flow cytometric analyses of cell suspensions generated at different stages of DC purification. DC are identified as cells staining positively for both CD11c and CD8 following labeling with monoclonal antibodies.

to retain the magnetic beads according to the manufacturer's instructions. Remove the PBS-EDTA after the second wash.

13. Add the VLDF cell suspension to the 5 mL polystyrene tube containing the prewashed magnetic beads and mix gently.
14. Transfer the VLDF/magnetic bead suspension to a 1 mL round-bottom polypropylene CryoTube vial (Nunc; cat. no. 375353) and incubate for 30 min at 4°C with gentle rotation (e.g., in a Dynal sampler mixer).
15. Add 1 mL of PBS-EDTA-FCS to the VLDF/magnetic bead suspension, mix gently using a 1 mL micropipette, and transfer to a 5 mL polysterene tube (*see* **Note 4**).
16. Place the tube in a Magnetic Particle Concentrator and leave it for 4 min.
17. Transfer the supernatant to a clean tube with a Pasteur pipet, taking care not to disturb the magnetic bead-rosetted cells collected at the tube wall, which must be discarded. The supernatant constitutes a highly DC-enriched fraction (HDCF) containing 70–80% DC (*see* **Subheading 3.1. Fig. 2**).
18. Incubate the HDCF with a biotin-conjugated anti-CD11c mAb (clone N418) at 10 μg/mL for 20 min at 4°C. Use 25 μL of mAb per 1×10^6 HDCF cells.
19. Wash in PBS-EDTA-FCS.
20. Incubate the HDCF with strepavidin-conjugated MACS microbeads (Miltenyi Biotec) diluted at 1/10 in PBS-EDTA-FCS for 10 min at 4°C, using 100 μL of microbeads per 10^7 HDCF cells.
21. Wash in PBS-EDTA-FCS and resuspend in 500 μL of PBS-EDTA-FCS.
22. Purify the $N418^+$ DC from the HDCF by MACS, using MACS MS^+ or RS^+ positive selection columns (Miltenyi Biotec) in combination with a MACS separator (Miltenyi Biotec), following the manufacturer's instructions. After elution from the column the DC preparation has a purity > 97%.

3.1. Expected Results

Figure 2 shows the expected phenotypic profile (corresponding to a CD11c vs CD8 immunofluorescent staining analyzed by flow cytometry) of the cell fractions obtained: after enzymatic digestion and centrifugation in Optiprep 1.055 (VLDF); after depletion with magnetic beads (HDCF); after magnetic cell sorting of N418+ cells (purified DCs).

The VLDF represents 0.2-0.4% of total thymocytes and contains around 20% $N418^+$ cells. Among $N418^+$ cells of the VLDF around 80% are DCs and 20% are Mac-1^+ F4/80^+ thymic macrophages. $N418^-$ cells in the VLDF correspond mainly to immature T-lineage cells and thymic B cells.

The HDCF represents 15–20% of the VLDF and contains 70–80% of $N418^+$ DCs, the remaining $N418^-$ cells being essentially immature T-lineage cells. Excluding DCs, this cell fraction is therefore devoid of other antigen presenting cells, such as macrophages or B cells and could be used as an enriched-thymic DC fraction for certain purposes. Finally, N418+ cells represent >97% of MACS-purified DCs.

The yield of the isolation method reported here is $40–80 \times 10^3$ purified DCs per thymus of a 4-6 wk-old mouse.

3.2. Scaling the Isolation Method

Increase or decrease proportionately to the starting number of thymuses/thymocytes the quantity of the reagents and the number of tubes required, taking into consideration the following points: It is not recommended to start the isolation of thymic DCs with less than 2×10^9 thymocytes, i.e., around 10 thymuses of 4–6 wk-old mice. It is highly recommended that the 14 mL polypropylene tubes, the 5 mL polystyrene tubes, and the 1 mL Cryotubes specified in the **Subheading 3.** should be used, although other high-quality tubes with similar characteristics should also be adequate. Do not modify the conditions specified for obtaining the VLDF in terms of both the cell concentration and the volume corresponding to the different layers. In **Subheading 3., step 14** do not use more than 15×10^6 VLDF cells and the corresponding magnetic beads per 1 mL Cryotube. Finally in **Subheading 3., step 15** use one 5 mL polystyrene tube per each 1ml Cryotube required.

4. Notes

1. EDTA is added to PBS and 1.055 Optiprep solution in order to dissociate DC–thymocyte complexes and to avoid the spontaneous formation of cell clusters.
2. The RPMI 1640 medium used for obtaining a cell suspension from the colagenase/DNase-digested thymus fragments must contain DNase and be

prewarmed in order to avoid the formation of cell aggregates, which could greatly reduce the yield of the method.
3. The 14 mL polypropylene tubes used for obtaining the VLDF must be precooled in order to facilitate the setting of the discontinuous Optiprep gradient.
4. Mixing the PBS-EDTA-FCS with the VLDF magnetic bead suspension is a critical step in this isolation method. It must be done gently but efficiently in order to avoid the detachment of some contaminating cells bound loosely although specifically to the beads and on the other hand to disrupt the aggregates which are usually formed during the previous incubation.

References

1. Vremec,D., Zorbas, M., Scollay, R. Saunders, D. J., Ardavin, C. F., and Shortman, K. (1992). The surface phenotype of dendritic cells purified from mouse thymus and spleen: Investigation of the CD8 expression by a subpopulation of dendritic cells. *J. Exp.Med.* **176,** 47–58.
2. Ardavin, C., Waanders, G., Ferrero, I., Anjuere, F., Acha-Orbea, H., and MacDonald, H. R. (1996). Expression and presentation of endogenous mouse mammary tumor virus superantigens by thymic and splenic dendritic cells and B cells. *J. Immunol.* **157,** 2789–2794.
3. Ardavin,C., Wu, L., Li, C.-L., and Shortman, K. (1993). Thymic dendritic cells and T cells develop simultaneously in the thymus from a common precursor population. *Nature* **362,** 761–763.
4. Ardavin, C. (1997). Thymic dendritic cells. *Immunol Today* **18,** 350-361.
5. Wu, L., Vremec, D., Ardavin, C., Winkel, K., Suss, G., Georgiou, H., Maraskovsky, E., Cook, W., and Shortman, K. (1995). Mouse thymus dendritic cells: kinetics of development and changes in surface markers during maturation. *Eur. J. Immunol.* **25,** 418–425.

4

Isolation of Dendritic Cells from Rat Intestinal Lymph and Spleen

Gordon MacPherson, Michelle Wykes, Fang-Ping Huang, and Chris Jenkins

1. Introduction

1.1. Lymph Dendritic Cells

Dendritic cells (DC) are rare cells in peripheral tissues, and their isolation from tissues is fraught with problems. Thus, the proportion of DC within a tissue that is extracted is unknown, isolation procedures may select for subpopulations, and the isolation procedure itself may affect their properties. As part of their life history, DC migrate from peripheral tissues, via peripheral, afferent lymph to lymph nodes, even in the absence of exogenous antigenic stimulation. They are extracted within the node and very few, if any, appear in efferent lymph. These lymph DC (L-DC) represent a population that has matured in the periphery, that may have acquired antigen (Ag), and that may be engaged in active Ag transport to lymph nodes. As such they are a physiologically relevant DC population. In large animals such as sheep and cattle, L-DC can be isolated by direct cannulation of peripheral lymphatics, but yields are relatively low. In rodents, direct cannulation of some peripheral lymphatics is possible ***(1)***, but yields of cells are minuscule. To get around this problem we and others ***(1–7)***, have utilized lymphadenectomy as a means of collecting pseudo-afferent lymph. When lymph nodes are removed, over a period of weeks, the afferent and efferent lymphatics join as part of the healing process, leaving cells in peripheral lymph free to enter central lymph. Central lymphat-

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ics are relatively easy to cannulate, and cannulation can be maintained for considerable periods of time (*see* **Note 1**).

This approach to L-DC isolation has the major advantages that DC can be collected under near-physiological conditions, that they can be collected fresh without any complicated manipulations, and that they can be metabolically "frozen" by collecting them into cooled vessels. It has the disadvantages that only DC at a particular stage of their life history can be collected, and that in the rat at least, only DC from the small intestine or liver ***(8,9)*** can be collected.

The isolation of DC by these techniques involves two stages, in the first, the lymph nodes draining the relevant tissue bed are removed. The animal is then left for a period of weeks to permit healing of afferent and efferent lymphatics, and then the thoracic duct is cannulated using Gowans' modification ***(10)*** of the original Bollman et al. method ***(11)***.

1.2. Spleen Dendritic Cells

The first DC to be isolated were derived from murine spleen ***(12)***. Isolation involved enzymatic digestion of the spleen, buoyant density separation of light cells, short-term adherence, overnight culture, and removal of FcR^+ cells. This gave a population of DC that has been used as the standard for many years. Similar methods were used to isolate DC from rat spleen ***(13)***. It has become clear, however, that there is more than one population of DC in murine spleen ***(14)***, that the original isolation procedure was selective for $33D1^+$ DC found mainly in the marginal zone and possibly red pulp, and that the classical interdigitating cells (IDC) were not being extracted ***(15)***. A modification of the original technique involving the use of EDTA was needed to extract IDC efficiently. Another problem that needs to be considered is the effect of the isolation procedure itself on the phenotype and function of the isolated DC. Thus it is clear that the properties of murine Langerhans cells ***(16)***, murine heart and kidney DC ***(17)***, and rat intestinal lymph DC ***(18)*** are markedly changed by short term *in vitro* culture. Recently, it has been shown that splenic DC isolated without overnight culture display characteristics typical of immature DC that are not found in DC isolated by standard methods ***(19)***. Thus, when isolating DC from any tissue, including the spleen, careful consideration must be given to the possible selectivity of the isolation procedure and its effects on the resulting DC (*see* **Note 2**).

In this chapter we describe the procedure that we use to isolate fresh DC without overnight culture. This has become our standard method as we consider that it provides DC that are closer to in vivo DC. Rat DC can also be prepared using the original method described for murine splenic DC ***(12)***.

2. Materials

2.1. Lymph Dendritic Cells

1. Specific pathogen-free rats around 6 wk of age are used for surgical lymphadenectomy (*see* **Note 3**).
2. Anesthetic apparatus: Any small animal apparatus suffices, but we use a halothane anaesthetic system (International Market Supply, Congleton, UK). No preoperative treatment is required.
3. Hair clippers (International Market Supply).
4. Surgical instruments including scalpel, scissors and dagger forceps (Holborn 4. Surgical Instrument Co., Margate, UK).
5. Sutures including absorbable 4/0 sutures (Ethicon) and needles (John Weiss, Milton Keynes, UK).
6. Operating light (Cold Source Fibre Optic Lamp, Schott. Scientific Laboratory Supplies, Nottingham UK).
7. Operating board (*see* **Note 4**).
8. Heparin sodium (CP Pharmaceuticals Ltd, Wrexham UK).
9. 70 μm cell strainers (Falcon 2350, Becton Dickinson).
10. Washing buffer: 2–5% fetal calf serum (FCS) (Gibco-BRL, Paisley, Scotland) and 5 m*M* EDTA in PBS.
11. Gey's solution.

Stock solution A

NH_4Cl	35.0 g
KC1	85 g
$Na_2HPO_4 \cdot 12H_2O$	1.5 g
KH_2HPO_4	0.12 g
Glucose,	5.0 g
Phenol red	50.0 mg
Make up to 1000 mL.	

Stock solution B:

$MgCl_2 \cdot 6H_2O$	0.42g
$MgSO_4 \cdot 7H_2O$	0.14 g
$CaCl_{2,}$	0.34 g
Make up to 100 mL.	

Stock solution C

$NaHCO_3$	2.25g
Make up to 100 mL.	

Mix: 20 parts stock A, 5 parts stock B, 5 parts stock C, and 70 mL distilled water.

12. Counting chambers: Fast Read 10 chambers (Immune Systems, Paington, UK).
13. NycoPrep™ 1.068 density medium (NYCOMED, Oslo, Norway).

14. Monoclonal antibodies: OX52 (Pan T cell), OX19 (CD5), OX8 (CD8), OX12 (Ig kappa-chain), OX33 (B cell CD45), OX62 (rat DC). Commercial suppliers include Serotec, Kidlington, UK and The Binding Site, Birmingham, UK.
15. Goat anti-mouse IgG labelled with immunomagnetic microbeads (Miltenyi Biotec, Order No 484-02, Bergisch Gladbach, Germany).
16. MACS buffer: 1% Bovine Serum Albumin (BSA) (Sigma), 2mM EDTA, and 0.01% sodium azide in PBS.
17. Normal rat serum (heat-inactivated, 56°C, 30 min)

2.2. Spleen Dendritic Cells

1. Specific pathogen-free rats of any strain, at 10-12 weeks of age.
2. Sterile Petri dishes.
3. Curved forceps and straight forceps with serrated ends.
4. Hanks balanced salt solution (HBSS) with Ca^{++}, Mg^{++}.
5. HBSS without Ca^{++}, Mg^{++}.
6. Enzyme cocktail: 2mg/ml Collagenase D (Boehringer Mannheim) and 0.5mg/ml DNAase (Boehringer Mannheim) in HBSS (with Ca^{++}, Mg^{++}). Keep on ice and filter sterilise just before use. Stock solutions of collagenase D (8mg/ml in HBSS with Ca^{++}, Mg^{++}) and DNAase (0.5mg/ml) can be stored frozen until required (*see* **Note 5**).
7. Collection buffer: 20mM EDTA in HBSS (without Ca^{++}, Mg^{++}). The EDTA inhibits the enzyme activity by chelation of Ca^{++}. Keep this buffer on ice at all times.
8. Gey's Solution for RBC lysis. See **Subheading 2.1.**
9. I-5 medium: Iscove's modified Dulbecco's medium supplemented with 50mg/ml penicillin, 50mg/ml streptomycin, 2 mM glutamine, 5% foetal bovine serum (FCS) and $5x10^{-5}$ M 2-β-mercaptoethanol. Medium and supplements from Gibco BRL, supplied by Life Technologies Ltd, Paisley, UK.
10. Primary and secondary antibodies: OX52 (pan T cell), OX8 (CD8, T cells and NK cells), OX 12 (anti-Ig light chain), OX33 (pan-B cells) (Serotec). Biotinylated anti-Ig μ chain and anti-Ig γ chain antibodies (The Binding Site). Rabbit anti-rat IgG, IgM. and IgA (absorbed for mouse Ig and FCS, Dako Z0494) and rabbit anti-mouse Ig (Dako Z0456).
11. Sheep red blood cells (SRBC) (TCS Microbiology, Botolph Claydon,UK).
12. Histopaque, density = 1.083 (Sigma).
13. Dynal beads coated with anti-mouse Ig and streptavidin.
14. Sterile, short cut Pasteur pipet.

3. Methods

3.1. Lymph Dendritic Cells

3.1.1. Mesenteric Lymphadenectomy

We routinely remove mesenteric nodes surgically, but it also has been shown that freezing the nodes leads to immediate loss of their ability to retain DC ***(20).***

1. Anaesthetize the rat and clip the hair from the abdomen. Tape the feet to the operating board and swab the abdomen with 70% ethanol.
2. Make a mid-line incision with a scalpel from the xiphisternum for about three-quarters of the length of the abdomen. Incise the muscle layers along the mid-line with scissors.
3. Gently reflect the intestine to the right (rat's left side) and support it on gauze soaked in saline. The chain of mesenteric nodes is clearly visible at the root of the mesentery, close to the inferior vena cava.
4. Starting at the anterior end of the chain, pick up a node with forceps. Use another pair of forceps to tear away the surrounding connective tissue and remove the node. Press with a cotton bud to stop any minor bleeding that occurs. Repeat until the whole chain has been removed.
5. Check hemostasis and replace the intestines.
6. Suture the abdominal wall in layers with interrupted sutures. Suture the muscle layers with an absorbable suture and the skin with silk (clips can be used for the skin incision).
7. Postoperative recovery is usually quick and uneventful. Infection is very rare, and the only other complication of which we are aware is postoperative ileus, again very rare.
8. Rest the rats for at least 6 wk before thoracic duct cannulation to permit healing of the lymphatics.

3.1.2. Thoracic Duct Cannulation

The technique of thoracic duct cannulation is described in detail in the following and will not be repeated here (*see* **Note 1**):

1. Ford W.L. (1978) "The preparation and labelling of lymphocytes," in Handbook of Experimental Immunology, Ed. Weir, Blackwell, Oxford, Chapter 23.
2. Waynforth, H. B. and Flecknell, P. A. (1992) *Experimental and Surgical Technique in the Rat*, Academic Press, London, pp. 264–268.

3.1.3. Lymph Collection and Cell Preparation

1. Collect lymph for suitable periods into flasks containing heparinized (20 U/mL) PBS cooled on ice. We do not normally collect for more than 20 h at a time (*see* **Note 6**).
2. Filter lymph through a sterile cell strainer to remove clumps or clots. Alternatively, two layers of lens tissue placed onto a funnel can be used.
3. Wash the remaining cells in the flasks with same volume of ice-cold PBS and filter it through the cell strainer. Combine the lymph and the PBS washing and aliquot into 50 mL centrifuge tubes.
4. Centrifuge at 400g for 8 min, resuspend the cells in 1 mL medium, and remove any contaminating RBC by lysis in Gey's solution. Add 4 mL Gey's solution and incubate for 2 min on ice.
5. Wash the cells twice with cold washing buffer, count in a hemocytometer, and

finally resuspend in the same buffer at about 2×10^7 cells per mL. Typical DC can be recognized in the counting chamber by their size and irregular outline. Usually, about $3–5 \times 10^8$ total cells per rat are obtained from a 20 h lymph collection, of which DC form about 0.3–0.5% of the total cells. The dominating cell types are T and B lymphocytes and, in some cases, contaminating RBC. Therefore, procedures that enable enrichment of DC are necessary for most studies. Depending on the purpose of the experiment and the purity required, DC can be enriched and, when necessary, further purified by using combinations of the methods described below.

3.1.4. Enrichment of L-DC by Density Centrifugation

DC have a lower average density than lymphocytes. However, the densities of the two cell types overlap, especially DC with large lymphocytes (blasts). It is difficult to separate them effectively based on density differences alone. We routinely use NycoPrep™ 1.068 (*see* **Note 7**).

1. Layer 4 mL of the DC suspension prepared above over 3 mL of the NycoPrep™ 1.068 solution in a 12-mL clear centrifuge tube (*see* **Note 8**).
2. Centrifuge the cells at 600*g* for 15–20 min at room temperature. An interface that contains DC should be visible and at the bottom of the tube is a cell pellet that consists mainly of lymphocytes (*see* **Note 9**).
3. Pour carefully and combine all cells in the suspension from three tubes into one 50-mL centrifuge tube and top up with washing buffer (at least double the original volume). Discard the cell pellets.
4. Centrifuge the cells at 600*g* for 8 min at 4°C to remove the density medium. Wash the cells twice more with washing buffer, centrifuging at 400*g* for 5 mins, and count. At this stage, DC normally represent 20–40% of the total recovered cells (*see* **Note 10**). The contaminating cells are small and in particular large lymphocytes (*see* **Note 1**).

DC can be further enriched by repeating the above steps, which normally doubles the DC purity (up to 60% can be achieved). Alternatively, DC in the partially enriched preparation may be further purified by using one of the following methods to positively select DC or by negatively selecting the remaining lymphocytes.

3.1.5. Purification of L-DC by Immunomagnetic Separation

In our lab, we routinely use positive or negative separation using the MACS system as a second step for purifying L-DC after NycoPrep enrichment (*see* **Notes 11** and **12**).

3.1.5.1. Negative Selection

DC are enriched by negative selection of T and B cells. We use a combination of monoclonal antibodies OX52 (pan T), OX19 (CD5), and OX8 (CD8) to

deplete T cells; and a combination of OX12 (kappa-chain) and OX33 (B cell CD45) to deplete B cells.

1. Incubate NycoPrep-enriched cells (**Subheading 3.1.4**) at 4°C for 20 min with a mixture of equal volumes of the above antibodies as tissue culture supernatants (TCS), supplemented with (optional) additional purified OX52 and OX12 antibodies at 10 μg/mL. Up to 10^7 total cells per ml of the antibody mixture can be efficiently labeled in this way for separation.
2. Wash the cells three times with cold washing buffer.
3. Incubate the cells with the MACS beads labeled with antibody, 1:5 diluted in the MACS buffer and used at 0.1 mL per 10^7 cells, for 15 min at 4°C. 2–5% of heat-inactivated normal rat serum can be added in the antibody diluent to reduce non-specific binding or crossreactivities.
4. Wash the cells twice with the washing buffer and then once in the MACS buffer.
5. Carefully resuspend the cell pellets in a small volume of degassed-MACS buffer, 0.5 mL per (or up to) 10^8 total cells. They are now ready for cell separation on the MACS columns (*see* **Note 12** and follow the manufacturer's instructions).
6. Details of yield and recoveries are given in **Note 13**.

3.1.5.2. Positive Selection

To positively select L-DC, we use a mouse anti-rat DC monoclonal antibody, OX62 (IgG1) (See Note 11), which is used at 10 μg/mL or as neat TCS. The staining procedure is the same as that described for the negative selection (**Subheading 3.1.5.1**). Other methods of purifying L-DC are described in **Note 14**.

3.1.6. Conclusions

Lymph DC, freshly collected, represent cells that are as close to in vivo DC as is at present possible to obtain. Their isolation is not, however, technically straightforward, requiring specialized surgical skills and equipment. They are difficult to obtain in large numbers and represent only one stage in the life history of DC from one tissue. However, by carefully following the procedure as described above, DC that are "near-physiological" can be obtained with good yields and high purity.

3.2. Spleen Dendritic Cells

3.2.1. Digestion of Spleens

1. Excise spleens from 10–12 wk-old rats with sterile instruments and place in a 3.5 cm sterile Petri dish with 3 mL of enzyme cocktail.
2. Fill a sterile 5 mL syringe with 2–3 mL enzyme, and using a 23G needle inject the enzyme into several sites in the spleen. When inserting the needle into the spleen, use sterile forceps to hold the tissue in place and pierce the tissue with caution so as not to exit the other side. Areas surrounding the injection site appear

as lighter colored patches. Repeat several times until a large portion of the spleen has been flushed of loose cells (*see* **Note 15**).

3. Tear the spleen into small pieces using two sterile forceps, and incubate at 37°C for 15 mins. After this, carefully remove the free cell suspension using a sterile short Pasteur pipet, put into collection buffer, and keep on ice. Add more enzyme to the remaining tissue pieces and "tease" the tissue. This involves holding the tissue with the straight serrated forceps and scraping the sides of the curved forceps against the tissue. Incubate for 15 min at 37°C.
4. Using a short pasture pipette, take up and release the tissue/cells mixture several times. This process will release cells from remaining tissue pieces. Transfer the released cells to the collection buffer on ice. If required, add remaining enzyme and repeat the incubation/ release process until no tissue pieces are visible.
5. When the digestion is complete, pellet the cells at 400*g* for 10 min, and lyse red blood cells using Gey's (**subheading 3.1.2., item 4**) or a similar solution. Wash cells in I-5, resuspend in this medium, and incubate for 2–5 min on ice. Debris should settle during this time. Transfer the cell suspension to a fresh tube, leaving behind the debris.
6. Pellet the cells at 400*g* for 5 min and incubate with the antibody cocktail (OX52, OX8, OX12, OX33) for 30–60 mins at 4°C.
7. During this incubation prepare 2 separate batches of SRBC, one coated with rabbit anti-rat IgG, IgM, and IgA (to deplete B cells) and the other with rabbit anti-mouse Ig (to deplete all antibody-coated cells) as described in Chapter 18.

3.2.2. Removal of Non-dendritic Cells Using Rosetting and Dynal Beads

Rosetting has several advantages over other methods such as the use of Dynal beads alone. The method is relatively inexpensive, it removes macrophages via FcR binding, and the gradient step removes dead cells.

1. Wash antibody-coated spleen cells twice in PBS or I-5 and resuspend with the anti-rat Ig-coated SRBC. After 2–3 min at room temperature, add the other batch of SRBC (rabbit anti-mouse Ig-coated), mix, aliquot into small sterile vials and rotate for 30 min at 4°C (*see* **Note 16**).
2. Layer onto the Histopaque (Sigma) density gradient medium and centrifuge for 20 min at 400*g*. Collect the cells at the interface and wash in I-5.
3. Resuspend the cells in 5 mL I-5 and count. Add four anti-mouse Ig and streptavidin-coated Dynal beads/cell (stock concentration is 4×10^8 beads/mL) and incubate for 30 min at 4°C, mixing occasionally. Place the tube on a Dynal magnet for 3 min, remove unbound cells to a fresh tube, and place on the magnet again. Repeat three or four times until all the magnetic beads have been removed.
4. Pellet the remaining cells and resuspend in I-5.

3.2.3. Expected results

This method consistently yields from 7×10^6 to 10^7 DC per rat spleen (*see* **Note 17**). The cells are routinely characterized by flow cytometry and immunocytochemistry, and contain at least 85% MHC class II^+ cells with less than

1% contaminating T or B cells. The level of surface MHC class II expression is very low on most cells and immunocytochemical labeling of cytospins shows that a large portion of the MHC class II is intracellular. Cytospins stained for immunoglobulin show the absence of plasma cells. The cells do not phagocytose opsonized sheep red blood cells indicating that they are not macrophages. It should also be noted that only a small proportion of the cells shows the classic dendritic morphology immediately after isolation. However, after overnight culture of these cells, 95–99% of the cells will express high levels of surface MHC class II, and the dendritic processes are more conspicuous (*see* **Note 18**).

4. Notes

1. It should be pointed out that thoracic duct cannulation requires considerable surgical skill and specialized equipment for anesthesia and restraint of the cannulated animal. The techniques involved are best learned in a laboratory where they are already in use. Lymph can be collected for periods of days; we routinely collect for up to 2 days. We have found that if lymph is collected for more than 2 d, the proportion of B blasts increases, and they tend to co-purify with the DC.
2. It is becoming apparent that in vitro manipulation of DC, even for short periods, can alter their phenotype and function. This needs to be taken into account when trying to relate in vitro and in vivo DC properties.
3. Young rats are used because the nodes are relatively free of surrounding fat at this stage and are easily visible. We use males where possible, because at the time of cannulation relatively little fat surrounds the thoracic duct. The optimal time for cannulation is when the rats are about 12-wk-old. Cannulation is possible at later stages, but the increased amounts of fat around the duct increase the technical difficulty of the procedure.
4. It may be advantageous to use a heated operating table, but this is not essential. It is essential that animals are kept warm after the operation until they are fully recovered.
5. Collagenase D is used in preference to collagenase A. We find that collagenase D gives better viability without a large decrease in yields. Batches of collagenase A vary in terms of both the viability and numbers of DC that are recovered.
6. Twenty hours represent an overnight collection, allowing a whole day for cell manipulations.
7. NycoPrep ™ 1.068 is a density medium with an adjusted osmolality and is slightly hypertonic. Because lymphocytes are more sensitive than DC to the increased osmolality, they dehydrate and become even denser resulting in much improved separation upon centrifugation. We find that cell yields and viability are better with NycoPrep than with Metrizamide. It is important not to overload the tube with cells; if large numbers are used, "streaming" will occur and DC will be dragged into the density medium. One of the major problems with DC separation is that DC form clusters with both T and B cells. The use of EDTA helps to break up clusters and keeping the cells cold at all times is essential.

8. Clear centrifuge tubes are essential to allow visualization of the interface.
9. It is possible that some DC will end up in the pellet but there are probably very few; when we use pelleted cells to stimulate an allogeneic MLR, we get little or no response.
10. DC are counted in a hemocytometer chamber. We visualize cells using Nomarski interference optics. DC are identified by their size and irregular outline. Counts done this way give similar results to those done on cytospins. At the end of long separation procedures, particularly if they are carried out in the cold, DC may not have the typical irregular outline and are thus difficult to identify. If they are left at room temperature in the hemocytometer for 10–15 min, they will regain the dendritic appearance.
11. The choice of negative or positive selection depends on the intended application. One of the advantages of using negative selection is that DC negatively selected can easily be phenotyped using cell surface markers without interference from antibodies used for the cell separation which remain bound to DC. This is also a reason for purifying DC that are to be used for functional assays when antibodies used are likely to affect DC functions. In addition, the negatively selected DCs can be subjected directly to further separation of DC into sub-populations by subsequent positive selection.
12. This note deals with the MACS separation procedure. The major problems that might be encountered are :

    Cell loss: Since the frequency of L-DC is very low and the cell separation procedure is rather complex, poor cell yields potentially are a major problem. During every step of the cell separation procedures, significant cell loss is likely to occur. This can be caused by multiple factors including incorrect cell loading and washing, cell adhesion or aggregation, cell trapping in the MACS columns, and so on.

    In our experience, it is important that: (1) all buffers used should be filtered and, for the MACS buffer, degassed; (2) cells should be kept cold throughout and, if possible, the MACS columns should be precooled and the separation procedures performed in a cold room; (3) during the NycoPrep procedure, do not over load cells; (4) during cell washing, as DC are generally less dense, sufficient *g* force should be used to ensure that cells are adequately pelleted and then resuspended without delay (do not leave DC in the cell pellet longer than necessary). When washing cells after the density separation step, add a sufficient volume for dilution of the samples which are in density medium, and use extra *g* force to bring down cells during centrifugation; (5) resuspend cells thoroughly before loading onto the MACS column but try to avoid forming bubbles.

    For general cell separation purposes, there are two types of columns—Depletion Columns (AS, BS, CS, and DS, for negative selection) and Positive Selection Columns (XS^+, VS^+ and RS^+). For isolation of DC, however, we found that DC with their long processes are likely to be trapped nonspecifically in the Depletion Columns, probably because this type of column has a matrix made of a steel fiber material. In our experience, using the Positive Selection Columns (which contain spherical metal beads) for negative selection results in less DC trapping

despite this type of column having a smaller pore size (30 μm) than the Depletion columns (100 μm). However, the DC purity obtained this way may be slightly lower, possibly because this type of column has a lower magnetic strength, allowing weakly labeled cells to escape.

Cell impurity: Since DC are more likely to form aggregates and to be lost during washing when an insufficient g force is used, low DC purity is always associated with significant DC loss. In addition, owing to the long procedures involved in DC separation in the cold (i.e., on ice), identifying DC morphologically can be more difficult, especially for small DC. Leaving the cells in the counting chamber or slides at room temperature for 15 min before counting may help. Alternatively, immunostaining or FACS analysis of the cells using antibody cell markers gives a better idea of cell purity.

13. Usually, from one lymphadenectomized, cannulated rat, between 0.5 and 2×10^6 DC can be obtained in a 20 h lymph collection. A high purity of DC (> 90% by FACS staining) can be achieved by combining the NycoPrep and MACS (negative selection) methods. The purities and yields of the L-DC obtained very much depend on the methods used and the handling of cells. Cell loss may be significant, especially when using the MACS separation procedure. DC purity can be checked by FACS using anti-DC markers, such as OX62 and OX6 (Class II^{hi}), or by their morphological characteristics on slides or cytospin slides.
14. As alternatives to immunomagnetic separation DC can be further enriched by rosetting or the use of Dynal beads. These methods have been used for separation of DC ***(21)***, and although we now routinely use MACS, rosetting may give higher recoveries. Positive selection by rosetting or Dynal beads has the disadvantage that the SRBC or beads remain attached to the DC. In the case of rosetting, DC can be recovered by lysis of the SRBC, but we are not aware of a method of removing beads. ("Detachabead" is not applicable.)
15. We find that direct injection of the spleen prior to mincing it gives higher yields of DC.
16. We use a wheel that rotates at 20–30 rpm.
17. The proportion of DC that can be isolated from any organ or tissues is uncertain. Our experience with spleen, lymph node, and small intestine has shown that a relatively small proportion is isolated but we have no quantitative data. We have compared collagenase digestion with mechanical isolation procedures and find that the yield of DC is markedly (two or three times) greater with collagenase. The method we describe is slightly more laborious than the method involving overnight adhesion, but the yields are higher and the DC are not "matured" by overnight culture, and thus are closer to in vivo DC.
18. The method we have described mainly yields immature DC. The majority of MHC class II molecules is intracellular, but they will move to the surface if given maturation signals, including overnight culture. The endocytic capacity of the cells is maximal during the first few hours after isolation but decreases rapidly with culture. If the cells are to be pulsed with antigen, this must be done soon after isolation.

References

1. Pugh, C. W., MacPherson, G. G., and Steer, H. W. (1983) Characterization of nonlymphoid cells derived from rat peripheral lymph. *J. Exp. Med.* **157,** 1758–79.
2. Mayrhofer, G., Holt, P. G., and Papdimitriou, J. M. (1986) Functional characteristics of the veiled cells in afferent lymph from the rat intestine. *Immunology* **58,** 379–387.
3. Harkiss, G., Hopkins, J., and McConnell, I. (1990) Uptake of antigen by afferent lymph dendritic cells mediated by antibody. *Eur. J. Immunol.* **20,** 2367–2373.
4. Bujdoso, R., Hopkins, J., Dutia, B. M., Young, P., McConnell, I. (1989) Characterisation of sheep afferent lymph dendritic cells and their role in antigen carriage. *J. Exp. Med.* **170,** 1285–1302.
5. Bujdoso, R., Harkiss, G., Hopkins, J., and McConnell, I. (1990) Afferent lymph dendritic cells: a model for antigen capture and presentation in vivo. *Int. Rev. Immunol.* **6,** 177–86.
6. Howard, C. J., Sopp, P., Brownlie, J., Parsons, K. R., Kwong, L. S., and Collins, R. A. (1996) Afferent lymph veiled cells stimulate proliferative responses in allogeneic CD4+ and CD8+ T cells but not gamma delta TCR+ T cells. *Immunology* **88,** 558–64.
7. Howard, C. J., Sopp, P., Brownlie, J., Kwong, L. S., Parsons, K. R., and Taylor, G. (1997) Identification of two distinct populations of dendritic cells in afferent lymph that vary in their ability to stimulate T cells. *J. Immunol.* **159,** 5372–5382.
8. Matsuno, K., Ezaki, T., Kudo, S., and Uehara, Y. (1996) A life stage of particle-laden rat dendritic cells in vivo: their terminal division, active phagocytosis, and translocation from the liver to the draining lymph. *J. Exp. Med.* **183,** 1865-1878.
9. Kudo, S., Matsuno, K., Ezaki, T., and Ogawa, M. (1997) A novel migration pathway for rat dendritic cells from the blood: hepatic sinusoids-lymph translocation. *J. Exp. Med.* **185,** 777–784.
10. Gowans, J. L. (1959) The recirculation of lymphocytes from blood to lymph in the rat. *J. Physiol.* **146,** 54.
11. Bollman, J. L., Cain, J. C., and Grindley, J. H. (1948) Techniques for the collection of lymph from the liver, small intestine or thoracic duct of the rat. *J. Lab. Clin. Med.* **33,** 1349.
12. Steinman, R. M., Cohn, Z. A. (1973) Identification of a novel cell type in peripheral lymphoid organs of mice. I. Morphology, quantitation, tissue distribution. *J. Exp. Med.* **137,** 1142–1162.
13. Klinkert, W. E. F., LaBadie, J. H., and Bowers, W. E. (1982) Accessory and stimulating properites of dendritic cells and macrophages isolated from various rat tissues. *J. Exp. Med.* **156,** 1–19.
14. Metlay, J. P., Witmer-Pack, M. D., Agger, R., Crowley, M. T., Lawless, D., and Steinman, R. M. (1990) The distinct leukocyte integrins of mouse spleen dendritic cells as identified with new hamster monoclonal antibodies. *J. Exp. Med.* **171**, 1753–1771.
15. Inaba, K., Pack, M., Inaba, M., Sakuta, H., Isdell, F., and Steinman, R. M. (1997) High levels of a major histocompatibility complex II-self peptide complex on dendritic cells from the T cell areas of lymph nodes. *J. Exp. Med.* **186,** 665–672.

16. Schuler, G., and Steinman, R. M. (1985) Murine epidermal Langerhans cells mature into potent immunostimulatory dendritic cells in vitro. *J. Exp. Med.* **161,** 526–546.
17. Austyn, J. M., Hankins, D. F., Larsen, C. P., Morris, P. J., Rao, A. S., Roake, J. A. (1994) Isolation and characterization of dendritic cells from mouse heart and kidney. *J. Immunol.* **152**, 2401–2410.
18. MacPherson, G. G. (1989) Properties of lymph-borne (veiled) dendritic cells in culture. I. Modulation of phenotype, survival and function: partial dependence on GM-CSF. *Immunology* **68,** 102–107.
19. Leenen, P. J., Radosevic, K., Voerman, J. S., et al. (1998) Heterogeneity of mouse spleen dendritic cells: in vivo phagocytic activity, expression of macrophage markers, and subpopulation turnover. *J. Immunol.* **160**, 2166–2173.
20. Gyure, L. and Hall, J. (1990) A quick method for obtaining lymph borne dendritic macrophages from rats. *J. Immunol. Methods.* **131,** 49–53.
21. Liu, L. M., Zhang, M., Jenkins, C., and MacPherson, G. G. (1998) Dendritic cell heterogeneity in vivo: Two functionally different dendritic cell populations in rat intestinal lymph can be distinguished by CD4 expression. *J. Immunol.* **161,** 1146–1155.

5

Isolation, Enrichment, and Culture of Murine Epidermal Langerhans Cells

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1. Introduction

Langerhans cells are the epidermal variant of the dendritic cell system ***(1–5)***. They were—unknowingly, though—the first dendritic cell to be described: In 1868 Paul Langerhans published his observations of a dendritically shaped cell in the human epidermis ***(6)***. Until the early 1990s (i.e., the advent of methods for mass production of dendritic cells from blood or bone marrow) Langerhans cells served as a prototype dendritic cell. The basis of our current knowledge on the maturation of dendritic cells stems from experiments with epidermal Langerhans cells ***(7–9)***.

Still, Langerhans cells remain interesting to the researcher. First, Langerhans cells are of importance in many dermatologic diseases and their role in these processes needs to be elucidated. A typical example is contact hypersensitivity ***(10,11)***. Second, Langerhans cells may serve as a model to learn about the heterogeneity of dendritic cell populations. A recent example is the finding of Caux's group that "non-Langerhans dendritic cells" derived from CD14+/CD1a– precursors in cord blood have different functions as compared to "Langerhans type dendritic cells" derived from CD14-/CD1a+ precursors in cord blood ***(12)***. Third, isolated Langerhans cells represent probably the "cleanest" experimental system to study features of immature vs mature dendritic cells. In other words, in populations of freshly isolated Langerhans cells, the great majority of Langerhans cells are immature (> 95%); after short-term

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culture, the great majority (> 95%) show all signs of maturity. This line of distinction between immature and mature appears to be more blurred with populations of dendritic cells from other organs such as spleen or bone marrow ***(13).***

We have previously outlined in some detail the methods to procure dendritic cells ***(14–17)***. Here we will concentrate on mouse Langerhans cells and present our updated protocols in great detail. The standard approach to isolate Langerhans cells from the epidermis is by enzymatic digestion (trypsin) of keratinocyte–keratinocyte and keratinocyte–Langerhans cell adhesion. This results in a single-cell suspension containing a small percentage only (1–3%) of Langerhans cells. These suspensions can be enriched for Langerhans cells by the methods to be described below. Alternatively, the bulk suspension may be cultured for few days and Langerhans cells may be enriched thereafter. These steps will be described here.

2. Materials

2.1. The Preparation of Epidermal Cell Suspensions

1. Two pairs of thin but strong, straight forceps with rounded tips (anatomical type, to tear ears apart).
2. Two pairs of thin, curved, and pointed forceps (to pull epidermal sheets off the dermis).
3. Flat bottom tea strainer with handle to fit into a 100 mm Petri dish (e.g., "cell dissociation sieve" CD-1, Sigma, St. Louis, MO).
4. Nylon gauze with a mesh size of about 40 μm (NITEX 3-325-44; Tetko, Elmsford, NY). Alternatively, nylon gauze with a wide range of mesh sizes may be obtained from suppliers for the graphic arts.
5. Tissue culture 100 mm dishes (e.g. Falcon, Oxnard, CA; cat. no. 3003).
6. Bacteriological 100 mm Petri dishes (e.g., Falcon; cat. no. 1029).
7. A normal sterilizable glass funnel that fits onto a 50 mL centrifuge tube.
8. Trypsin: 2.5% commercial stock, aliquoted and frozen at –20°C; we prefer trypsin from Sera-Lab, Crawley Down, UK (cat. no. 59-22777). With regard to batch-to-batch variability of enzymatic activity (*see* **Note 1**).
9. Salines: phosphate-buffered saline (PBS) and Hank's balanced salt solution (HBSS), both without calcium and magnesium salts.
10. Cytotoxicity medium: for complement-mediated cytotoxicity use RPMI-1640 containing 25 m*M* HEPES buffer and 0.3% bovine serum albumin (BSA), pH 7.2. Generally, pH has to be adjusted with 1 *N* HCl or NaOH.
11. Complement: rabbit Low-Tox-M complement from Cedarlane Laboratories, Hornby, ON, Canada works best in our opinion.
12. Deoxyribonuclease (DNAse I) (Boehringer-Mannheim, Mannheim, Germany; cat. no. 104 159). Prepare a stock solution of 5 mg/mL in PBS, sterile filter, and store at 4°C for up to 3 mo.

2.2. Dense BSA Columns

1. Bovine serum albumin (BSA; Bovuminar Cohn fraction V powder, Intergen, Purchase, NY; cat. no. 3220-75, available in Europe from Biomex Ges.m.b.H., Mannheim, Germany). Put 186 mL PBS, 65 mL double-distilled water, and 29 mL 1 *N* NaOH into a 2-L glass beaker. Do not splash! Carefully sprinkle 106 g of albumin powder onto the surface of the solution. Cover beaker with aluminum foil and leave to dissolve at 4°C overnight. Do not attempt to speed the dissolution by stirring! All the powder will dissolve during the night. Next morning check the density (1,080 g/mL) of the resulting brown BSA solution by means of its refractive index (1,355–1,365). We use a simple Abbé refractometer. (If you don't have one in your lab, walk over to the physicists. This device is part of their standard equipment.) If too dense, add some PBS (approximately 5 mL to reduce index by 0.0005); if too light, add some more BSA powder (approx 1.5 g to increase index by 0.0005). Such a small amount of BSA will dissolve instantly upon stirring. Sterilize by filtration through a combination of a coarse prefilter and a 0.45 μm filter. The solution takes a long time to go through the filter and you will need several filters. One 250 or 500 mL filter will do for only about 100 mL. Store in sterile 50 mL tubes at 4°C for up to 3 mo. For reasons not known, the particular source of BSA as indicated above is critical! (It is even more critical for the preparation of spleen dendritic cells ***[18].***)
2. Ultra-Clear centrifuge tubes (Beckman Instruments, Palo Alto, CA; cat. no. 344085). These tubes need not be sterilized. It is sufficient to rinse them with 70% ethanol and place them upside down for drying. Standard 15 mL polypropylene tubes are also suitable. It is important that the tubes be clear in order to see properly the interphase of cells after centrifugation.

2.3. Antibodies Used for Further Enrichment of Langerhans Cells

1. Hybridoma culture supernatants:
    a. Mouse IgM anti-Thy-1 / CD90 (ATCC-TIB99).
    b. Rat IgG2b anti-I-$A^{b,d}$ / clone B21-2 (TIB229).
    c. Mouse IgG2a anti-I-$E^{k,d}$ / clone 14-4-4S (HB32).
    d. Mouse IgG2a, anti-I-A^{d} / clone MK-D6 (HB3).

    Hybridoma cells can be obtained from the American Type Culture Collection (ATCC), Manassas, VA. Where two clones with similar reactivity are mentioned, they are mixed for use. For panning, mAb HB32 (anti-I-E) gives better yields than mAb B21-2 (anti-I-A).
2. Additional reagents for panning:
    a. AffiniPure Goat anti-rat IgG (H+L) (Jackson Immunoresearch, West Grove, PA; cat. no. 112-005-003)
    b. Goat anti-mouse IgG+IgM (Tago, Burlingame, CA; cat. no. 4153).
    c. Rat gamma globulin (Jackson; cat. no.012-000-002).
    d. Mouse gamma globulin (Jackson; cat.no. 015-000-002).

2.4. Cytokines

These cytokines are only needed when highly enriched Langerhans cells are to be cultured (*see* below).

1. Recombinant murine granulocyte/macrophage colony-stimulating factor (GM-CSF; e.g., from Immunex Corporation, Seattle, WA; specific activity 4×10^7U/mg) is used at a final concentration of 500 U/mL.
2. Recombinant murine tumor necrosis factor-α (TNF-α; e.g., from Bender Co., Vienna, Austria; specific activity 2.6×10^7U/mg) is used at a final concentration of 125 U/mL.

2.5. Culture Medium

RPMI-1640 supplemented with 5 or 10% fetal calf serum (FCS), 200 m*M* L-glutamine, 50 μ*M* 2-mercaptoethanol, and 20 μg/mL gentamicin. Antifungal antibiotics such as Fungizone™ are not needed. Source and batches of FCS are not critical.

3. Methods

3.1. Preparation of an Epidermal Cell Suspension

1. Prepare the following necessary utensils beforehand. (a) two bacteriological 100 mm Petri dishes, containing 9 mL and 6 mL, respectively, of HBSS and gentamicin sulfate (50 μg /mL final concentration); (b) one Falcon 100 mm tissue culture dish containing 36 mL of culture medium.
2. Kill mice with CO_2 (dry ice added to the cage), and cut the ears off at their base. Usually, 30 mice per run are sacrificed (*see* **Note 2**).
3. In a tissue culture hood, rinse the ears briefly twice in two changes of 70% ethanol (in 100 mm Petri dishes), place on sterile gauze in a Petri dish and allow to airdry for approx 20–30 min.
4. Split each ear into ventral and dorsal halves using the strong forceps. The ventral halves are thicker because the ear cartilage adheres to them.
5. Place the ear halves separately into the two Petri dishes with HBSS previously prepared: ventral halves onto 6 mL HBSS, dorsal halves onto 9 mL HBSS. It is important that the ear halves be placed dermal side down and that they float and do not sink.
6. Add 2.5% trypsin stock solution to the two dishes: 4 mL to the ventral halves, 1.5 mL to the dorsal halves. This results in final concentrations of 1% and 0.33% trypsin, respectively. Incubate at 37°C. In the meantime place the tea strainer in the dish containing the 36 mL of culture medium.
7. After 30 min of incubation in trypsin, remove the *dorsal* (thin) ear halves from the 37°C incubator. Leave the ventral halves in the incubator, as they are thicker and require a longer trypsinization time.

8. Carefully aspirate the trypsin solution with a Pasteur pipet.
9. Peel off the epidermal sheets from the underlying dermis. This is best achieved by using one fine, curved forceps to hold the ear half down to the bottom of the Petri dish. With the other curved forceps, the epidermis is grasped at the edge of the ear half and the epidermal sheet is carefully peeled off. The extent of trypsinization is optimal when the sheets can be pulled off in one piece.
10. Carefully put the sheets onto the culture medium dermal side down in the tissue culture dish with the tea strainer until about three-quarters of the surface area of the solution is covered. (If the sheets are too crowded, they will stick to the sides of the tea strainer upon shaking.) The sheets will readily spread and float.
11. Shake the tea strainer for 3 min. Don't overdo it! More is not better! If the sheets are shaken for too long, the upper epidermal layers will also dissociate and the suspensions will contain a large percentage of polygonal stratum granulosum or even stratum corneum keratinocytes. This should be avoided! It is best to move the handle up and down repeatedly, thus rocking the sieve rather than lifting it. This prevents the sieve from being inadvertently raised above the fluid level, which would cause foaming. A "cloud" of basal-layer cells being released into the medium will be seen.
12. Remove the spent remainders of the sheets, and repeat until all sheets are processed.
13. Remove the dish with the ventral (thick) ear halves from the 37°C incubator and process them similar to the dorsal ones. Working up the dorsal halves takes about 30 min. This means that the ventral halves will have been trypsinized for about 60 min by that time, which is optimal. The ventral ear halves contain an uneven area that usually prevents one from removing the epidermal sheets in one piece. It is best to immobilize the ear half with one forcep at the edge opposite the bumpy area, and to grasp the epidermis there using a second pair of forceps. The epidermis of one half of the flat ear region can usually be pulled off in one piece. Grasp again and remove the other half. Do not try to remove the epidermis from the small bumpy area. This cannot be done unless the ear halves are overtrypsinized or one scrapes the epidermis off, which would result in dermal contamination.
14. After removing the strainer, pipet the epidermal cell suspension up and down several times using a 5 or 10 mL pipet. This breaks up most residual cell aggregates.
15. As the epidermal cell suspension is transferred to a 50 mL centrifuge tube, it should be filtered through nylon mesh to remove residual clumps and debris. It helps to stick the nylon mesh into a sterile glass funnel that is placed on top of the 50 mL tube.
16. Centrifuge the epidermal cell suspension at 300*g* (1200 rpm on most tabletop centrifuges) for 10 min, and wash twice in culture medium. Do not centrifuge faster! (*see* **Note 3**)
17. At this point the cells may be either put in culture (about 15 - 20 x 10^6 viable epidermal cells per 100 mm tissue culture Petri dish in 10 mL of culture medium) or processed further in order to enrich the Langerhans cells (*see* **Notes 4** and **5**).

3.2. Enrichment of Freshly Isolated Langerhans Cells

3.2.1. Complement-Mediated lysis ("Thy-1 kill")

Pre-enrichment of freshly isolated LC is achieved by depleting the majority of keratinocytes as well as dendritic epidermal T cells ***(19,20)*** by complement-mediated lysis (*see* **Note 6**).

1. In a 50 mL polypropylene tube, resuspend 100–150 × 10^6 epidermal cells (the expected yield from 60 ears) in 3 mL of hybridoma culture supernatant of mAb anti-Thy-1.
2. Add 10 mL of a sterile-filtered solution consisting of 8.5 mL cytotoxicity medium, 1 mL of reconstituted complement, and 0.5 mL of DNase stock solution. This comes to a final dilution of complement of 1:12.
3. After incubation for 1 h at 37°C in a shaking water bath, the resulting epidermal cell suspension is washed twice with cold PBS. The viability of this cell suspension is very low (10–20% only!).

3.2.2. "Trypsin Trick"

This simple procedure removes most dead cells and results in viable (> 90%) epidermal cell suspensions containing about 15% (range 10–28%) LC, ideally suited as a starting population for a further enrichment step such as panning.

1. Treat cells for 10 min at 37°C with 0.125% trypsin and 80 μg/mL DNase in PBS at a cell concentration 1–2 × 10^6 viable cells per mL. This is generally done in a 50 mL tube.
2. To stop the digestion, add an equal volume of the FCS-containing culture medium (don't fill the tube to the very top!) and centrifuge the cells for 10 min at 4°C, at 200*g* (1000 rpm on most conventional tabletop centrifuges) with brakes off. This centrifugation step is very critical! The low speed depletes the suspension of most dead cells and cell ghosts.

3.2.3. Mismatched Panning

For final enrichment by "mismatched panning" ***(15)*** (*see* **Notes 7** and **8**). Petri dishes are prepared, which, in contrast to published panning techniques ***(21,22)***, have been coated with goat anti-Ig antibodies that are mismatched with regard to species specificity. This means that for *panning of LC treated with mouse mAb, goat anti-rat Ig is used and for panning of LC treated with rat mAb, goat anti-mouse Ig is used.*

1. Coating of Petri dishes.
   a. To coat bacteriological Petri dishes (100 mm), dilute 40 μg of goat anti-Ig in 10 mL PBS (*see* **Note 9**).
   b. Incubate dishes with this solution for 1 h at room temperature.
   c. Rinse with several changes of PBS. Coated Petri dishes may be kept in PBS at 4°C for 1–2 d.

2. Labeling of epidermal cells with anti-MHC class II mAbs:
   a. Incubate pre-enriched epidermal cells for 30 min at 4°C with culture supernatants from anti-MHC class II mAbs (anti-I-$E^{k,d}$ / HB32 mixed with anti-I-A^{d} / MK-D6) or anti-I-$A^{b,d}$. The final concentration of epidermal cells should be 2×10^6/mL; supernatants may be diluted 1:5 with culture medium.
3. Adherence of Langerhans cells to coated dishes*:*
   a. After two washes with culture medium, load 10 - 30 x 10^6 cells onto one "mismatched" anti-Ig-coated dish in 10ml of culture medium, and incubate for 20 min at room temperature. Place dishes in a still corner of your lab. Avoid vibrations!
   b. Check for adherence of the cells to the dishes at this point by phase contrast microscopy. If necessary, place dishes at 37°C for another 10 min.
4. Removal of non-Langerhans cells*:*
   a. Gently rinse nonadherent cells off the dish with prewarmed (!) PBS. This is best done with a 5 mL pipet (preferably a short one) and a pipet aid. Slowly dispense the warm PBS from the pipet at the side of the dish and let it flow down over the inclined dish.
   b. Repeat several times and monitor complete removal of nonadherent cells under the phase contrast microscope (**Fig.1**).
   c. Even though freshly isolated Langerhans cells do not display a very pronounced morphology, the experienced eye will soon be able to distinguish the slightly nobby-looking Langerhans cells from keratinocytes with their smooth circumferences. At this point the numbers of adherent Langerhans cells may be determined by means of a calibrated grid. The more one washes, the better the degree of enrichment will be; on the other hand, more washes also mean more cell losses. An example for how far washes should go is given in **Fig.1**. There is no ideal solution to the experimenter's dilemma—"the better purity, the worse cell yields, and vice versa." This is particularly important with the described method: sometimes one can spot adherent Langerhans cells that have one or two keratinocytes still attached to them. If one washes these conjugates away—for the sake of maximal purity—one obviously loses Langerhans cells. A hint that may occasionally come in handy: don't discard the first few rinses containing most non-adherent cells (for an explanation *see* **Note 4**).
5. Detachment of Langerhans cells*:*
   a. Finally, release adherent cells (i.e., Langerhans cells) by adding excess amounts of gamma globulin matched to the antibody bound to the dish: *In the case of anti-rat Ig-coated dishes, use rat gamma globulin; in the case of anti-mouse Ig-coated dishes, apply mouse gamma globulin* (**Fig. 2**). Incubate dishes with either reagent at a concentration of 0.5 mg/mL in culture medium: add 10 mL of ice-cold gamma globulin solution to each dish and incubate for 10 min at room temperature. Owing to the higher affinity of the plate-bound anti-Ig to the *matched* gamma globulins, there will be an effective competition for the binding of Langerhans cells to the dish.

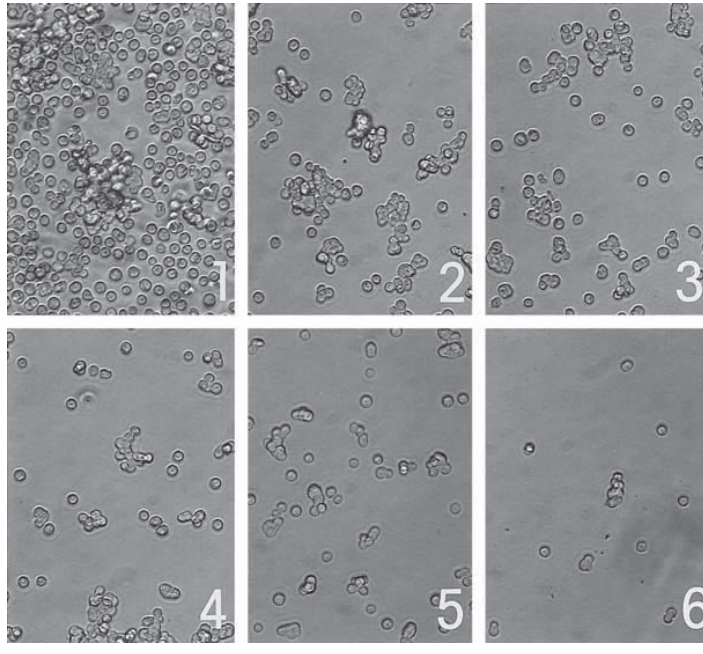

Fig. 1. *Monitoring of rinsing progress under phase contrast.* The first picture **(1)** shows a pre-enriched population of epidermal cells applied to an antibody-coated Petri dish. Subsequent pictures (2–6) are spaced by 2–3 rinses (i.e., one 5 mL pipet of warm PBS each). Detachment of these cells will result in a highly enriched population of Langerhans cells with yields as described in **Note 3.**

b. The Langerhans cells can easily be dislodged by gentle pipeting, producing a highly viable and highly enriched LC population. To reduce extensive cell losses collect the detached LC in 15 mL polypropylene tubes (Falcon; cat. no. 2097) that have been pre-incubated with culture medium. The first centrifugation should be done at 460*g* (1500 rpm on most tabletop centrifuges) (*see* **Note 10**).

For more information about the enrichment of Langerhans cells, *see* **Notes 11–15**.

3.3. Culture of Freshly Isolated Langerhans Cells

1. Primary epidermal cell suspensions may be cultured in bulk for 2–3 d at a density of 15×10^6 to at most 20×10^6 epidermal cells per 100 mm tissue culture dish in 10 mL of culture medium.
2. Alternatively, pre-enriched populations of Langerhans cells may be plated at the same cell densities.

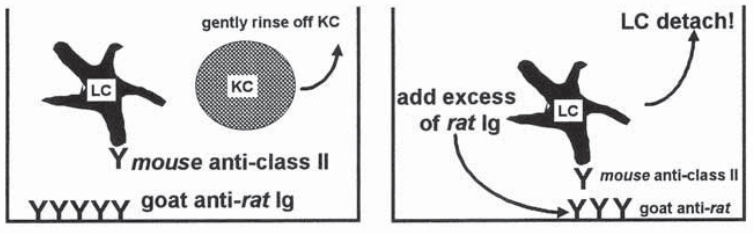

Fig. 2. *Schematic demonstration of "mismatched" panning*. The **left panel** shows the situation when a pre-enriched epidermal cell suspension, where Langerhans cells have been tagged with a mAb against MHC class II, is placed into an antibody-coated dish. The **right panel** schematizes how the adherent Langerhans cells can be detached by adding an excess amount of a competing Ig. LC, Langerhans cells; KC, keratinocytes.

3. During culture keratinocytes form a dense, contiguous monolayer of cells. This is an important criterion for the viability of keratinocytes and Langerhans cells. When no monolayer forms, one can anticipate low yields of cultured Langerhans cells. It takes a good phase contrast to see the monolayer. It can be better appreciated after having washed off the adherent cells. So, at the end of the culture period, keep the dishes and check them!
4. Upon close and patient inspection under a phase contrast microscope one can also spot occasional "veiled" Langerhans cells on d 2 and 3 of culture. They are either lying on top of spread keratinocytes or they are floating freely.
5. When freshly isolated Langerhans cells, highly enriched by panning, are cultured (typically, 1×10^5 per well of a 24-well tissue culture plate), it is necessary to add GM-CSF ***(22,23)*** or TNF-α ***(24)***, in order to ensure survival of the cells during the 3 d culture (around 50% of LC plated on d 0) (*see* **Note 16**). Note that TNF-α by itself leads to a disturbed maturation of Langerhans cells ***(24,25)***.
6. If Langerhans cells survive without exogenously added cytokines, this can be taken as an indication that the panning procedure was insufficient (< 75%), leaving enough residual contaminating keratinocytes to produce the necessary cytokines. Here, too, it is important always to monitor the cultures by phase contrast!

3.4. Enrichment of Cultured Langerhans Cells

1. On d 2 or 3 of bulk epidermal cell culture, collect the nonadherent cells from the dishes. At this point viability of cell populations will be poor. Yet, Langerhans cells can easily be made out in the haemocytometer by their very pronounced hairy shape.
2. Collect cells in a 50 mL tube, spin down, pool in a 15 mL polypropylene tube and centrifuge again.

3. Carefully aspirate the supernatant, as close to the cell pellet as possible. This is important, because too much residual fluid could influence the density of the BSA solution.
4. Resuspend the well-drained cell pellet in dense bovine serum albumin (BSA) solution. Use 5 mL BSA for the cells recovered from two or three 100 mm culture dishes. Avoid bubbling and foaming!
5. Transfer 5 mL portions to the centrifuge tubes. Overlay with 2 mL Ca^{++} and Mg^{++}-free HBSS. (If Beckman Ultra-Clear tubes are used, they may be covered with the sterile lid of a 15 mL centrifuge tube.)
6. Spin to equilibrium in a rotor with swing-out bucket for 15 min at 4°C and 10,000*g* with slow acceleration and the brakes turned off (e.g., Sorvall Superspeed RC5B).
7. Collect the low-density fraction (all of the overlaid medium and the upper 1 mL of dense BSA). It helps to look against a light source when aspirating the interphase with a Pasteur pipet. You can monitor nicely how you suck up the cells, like with a vacuum cleaner, leaving behind a mirror-like clean interphase devoid of cells. (If your hands are shaking, plug the Pasteur pipet into a gently working electrical pipeting aid ([e.g., Pipetus].)
8. Pool the two interfaces into one 15 mL tube.
9. Fill the tube with RPMI-1640 medium, mix well (!), and spin at 460*g* (1500 rpm on most tabletop centrifuges) for the first wash.
10. Resuspend in culture medium and spin at 300*g* (1200 rpm on most tabletop centrifuges) for the second wash. Check the pellet after the first centrifuge run lest you suck off your Langerhans cells inadvertently: The reason is that if you have not mixed well enough, a second interphase may have formed a few millimeters above the pellet (*see* **Note 17**).

3.5. Identification of Langerhans Cells by Phenotype

Fortunately, the epidermis is an easy tissue when it comes to identifying Langerhans cells. In the mouse, MHC class II expression is a reliable marker: No other cell within the normal murine epidermis expresses class II molecules. Freshly isolated Langerhans cells also express CD32, sialoadhesin (F4/80), and—more importantly—the lectin-like receptor DEC-205 (recognized by mAb NLDC-145 ***([26])***. Cultured Langerhans cells display very high levels of class II on their surfaces; moreover, they express costimulatory molecules CD80 and CD86 *(27)*. For MHC class II staining of panned cells, it suffices to incubate the enriched populations with secondary reagents only because the primary antibody (i.e., anti-MHC class II) is already on the cells **(Fig. 3)**.

One of the most useful antibodies for the identification of Langerhans cells recognizes a Birbeck granule-associated protein of human Langerhans cells ***(28)***. Unfortunately, it does not cross-react with murine Langerhans cells. New antibodies recognizing the mouse homolog of LC are being developed and will soon be valuable tools for LC reseach ***(28a)***.

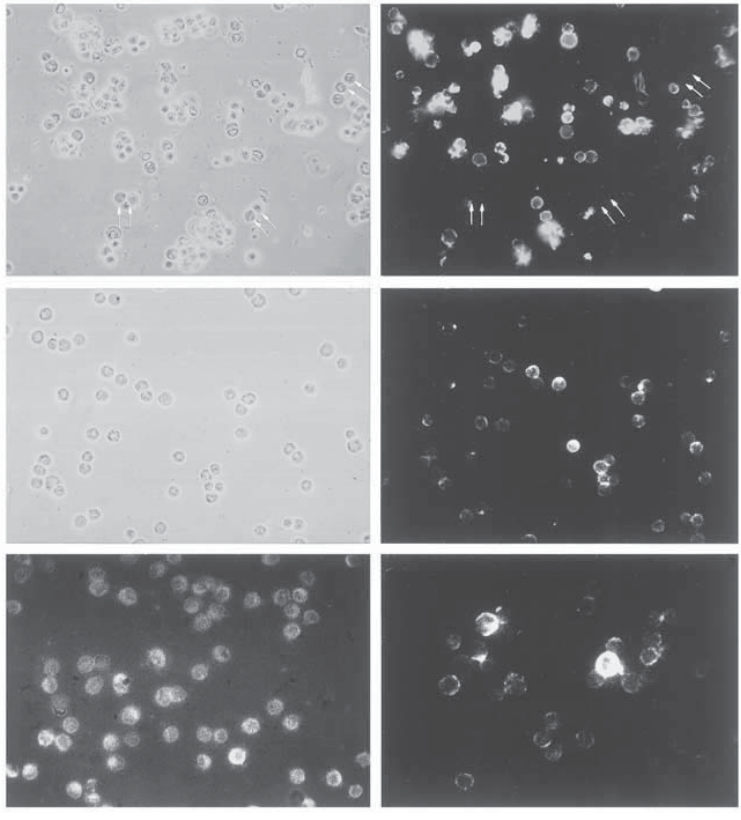

Fig.3. Immunofluorescence pictures of highly enriched populations of Langerhans cells obtained by "mismatched" panning. The top panel shows a suboptimal enrichement of freshly isolated LC; some keratinocytes (arrows) are still visible. *Middle panel* represents an optimal experiment. On the right side of the bottom panel an example for LC heterogeneity in freshly isolated populations is shown: One strongly MHC class II expressing cell stands out against the majority of less positive LC. Bottom left depicts a highly enriched population of cultured LC. MHC class II fluorescence and phase contrast are shown simultaneously.

3.6.Characterization of Langerhans Cells by Function

Although our panning method uses MHC class II molecules for the selection of LC, it does not appear to disturb the antigen presenting functions of the

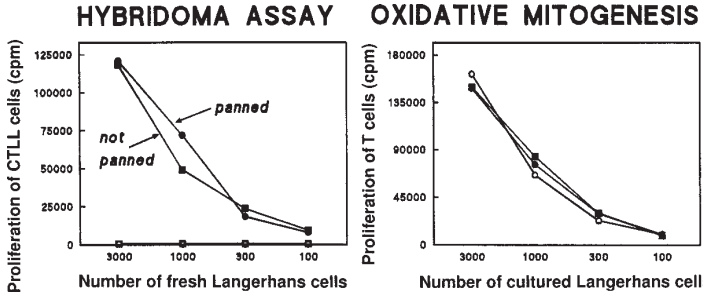

Fig. 4. *Functional properties.* Freshly isolated Langerhans cells were tested in an assay that measures antigen processing capacity (myoglobin-peptide-specific T-T hybridoma assay; left) — a feature highly developed in immature dendritic cells but not in mature ones. Cultured Langerhans cells were tested in an assay that measures T-cell sensitizing capacity (oxidative mitogenesis; right) - a feature highly developed in mature dendritic cells but not in immature ones. Equal numbers of Langerhans cells, as determined by MHC class II expression, were plated. Fresh Langerhans cells were either panned using mAb HB-32 (closed circles) or were used as pre-enriched populations as described (closed squares). Open symbols represent controls without myoglobin. Cultured Langerhans cells were either panned using mAb HB32 (closed circles), B21-2 (open circles) or were pre-enriched by floatation on dense BSA columns (closed squares). Note that functional capacity is unchanged by the antibody treatment of Langerhans cells.

cells. We have shown that both processing capacity for native protein antigens and sensitizing capacity for resting T lymphocytes are not impaired in panned LC as compared to LC that had been obtained without occupying the class II MHC molecules with antibody ***(15)*** (**Fig. 4**).

4. Notes

1. *Enzymatic activity of trypsin.* Standard tissue culture reagents including FCS may be obtained from many different manufacturers with no differences in results. An exception to this is trypsin, where batch-to-batch variability may be of importance. We found that batches of "aggressive" trypsin may prevent adhesion of LC to the antibody-coated dishes. Also, it is important not to add too much trypsin. If ear halves are overtrypsinized, sheets can be removed very easily, but intraepidermal cell contacts are so loosened that many basal epidermal cells including the LC located therein are lost. The concentrations of trypsin stated here should not be regarded as absolute, and may have to be optimized for a particular lot of enzyme.

Table 1
Flow Diagram of Procedure and Cell Yields and Enrichment Percentages

Experimental step	Enrichment of Langerhans cells[a]	Yield of Langerhans cells[a,b]
Trypsinization		
→ primary epidermal cell suspension	*1–3%*	*100%*
↓		
Thy-1 depletion and "trypsin trick"		
→ preenriched epidermal cell suspension	*10–25%*	*80–100%*
↓		
"Mismatched Panning"		
→ highly enriched epidermal cell suspension	*85–98%*	*40–60%*

[a]Data are from Koch et al. ***(15)***.
[b]These figures indicate numbers of Langerhans cells in the enriched fractions expressed as percentage of Langerhans cells present in the original epidermal cell suspension (100%).

2. *Langerhans cells from body wall skin.* By using body wall skin instead of ears, the number of mice can be reduced. In principle, the same technique is applied. It must be mentioned, though, that the isolation of LC from the body wall is more tricky and time consuming, needs more experience, and is therefore not used routinely.
3. *Centrifugations*. For all centrifugations it is recommended to use accelerations and brake settings in the middle range. This consideration is particularly important when small numbers of cells have to be handled and cell losses need to be minimized.
4. *Cell yields of freshly isolated Langerhans cells.* Depending on the mouse strain, (3–5 × 10^6 primary epidermal cells (viability > 80%), containing 1.4–3% LC (as monitored by staining for class II MHC antigens) can be obtained per mouse. Yields depend on the mouse strain, since the ear size as well as the LC density varies. After panning 1.0 –2.5 × 10^4 LC/ear can be obtained. This corresponds to a recovery of 40 - 60% of the LC that had been present in the primary epidermal cell suspension (**Table 1**). A general rule of cellular separation methods applies also here: the lower the starting cell number, the higher in proportion are cell losses.
5. *Pre-enrichment vs purification.* Preenriched (10–15%) fresh LC rather than highly enriched LC (> 90%) suffice for many experimental purposes. For instance, the antigen-processing capacity can be measured in such populations without interference from the majority population of keratinocytes ***(29)***. Pre-enriched populations are also well suited as starting populations for other enrichment methods such as fluorescent cell sorting.
6. *Thy-1+dendritic epidermal cells—dendritic epidermal T cells (DETC).* These gamma/delta TCR-expressing cells are abundant in C57BL/6 or C3H mice; in

BALB/c they are scarce ***(19,20)***. The step of complement-mediated lysis of keratinocytes by means of anti-Thy-1 mAbs removes the population from the suspensions. If epidermal cell populations are not subjected to the lysis procedure, however, one has to be aware of the presence of this cell type.

7. *Antibodies.* A critical precondition for successful panning is the choice of appropriate anti-Ig antibodies (anti-mouse Ig, anti-rat Ig). Different batches or products of different manufacturers may have variably strong binding affinities for the Ig of the "mismatched" species. Antibodies that cross-react sufficiently well must be chosen. For this reason mouse anti-rat Ig or rat anti-mouse Ig antibodies must not be used. With regard to primary antibodies, it is of note that one may also choose use mAb other than anti-MHC class II, e.g., anti-CD45 or anti-CD1a (for human Langerhans cells).
8. *"Mismatched" vs "matched" panning.* With the conventional approach (i.e., "matched" panning) one coats epidermal cells with, for example, a *rat* mAb anti-MHC class II and pulls out the labeled cells (i.e. Langerhans cells) by means of anti-*rat* Ig bound to the surface of the Petri dishes. This brings about problems with detachment of bound cells and is therefore not recommended when suspended populations of highly enriched Langerhans cells are desired. However, the "matched" technique may be useful for purposes of molecular biology. This is particularly true for cultured Langerhans cells that express large amounts of MHC class II on their surfaces and therefore stick very tightly to the panning dishes. This allows a thorough rinsing of the plates and therefore a very high degree of enrichment (close to 100%). Adherent Langerhans cells can easily be enumerated by means of a calibrated grid under the phase contrast microscope. They need not (and in most cases cannot!) be detached from the plates. Rather, they can be lysed directly on the Petri dish and be processed further for molecular biology.
9. *Tissue culture plastic.* The Petri dishes used for panning sometimes have defects in their surfaces. Sometimes, sharply delineated areas on one dish or whole dishes cannot be coated with antibody. This problem is invisible, and will only be detected during the panning procedure: no cells will adhere. It is therefore wise to always have some extra precoated dishes in reserve.
10. *Cell yields of cultured Langerhans cells.* Flotation of cultured epidermal cell suspensions on dense BSA columns yields populations where Langerhans cells are pre-enriched to 40–75%. This may be followed—if necessary—by mismatched panning as described. In that case, populations consist of more than 95% highly viable LC. $3.6 \pm 0.4 \times 10^4$ cultured LC [range $(3.0–4.5) \times 10^4$ LC] may be expected per mouse ear ***(15)***. This corresponds to a recovery of 50–75% of all LC that had been present in the primary epidermal cell suspension on day 0 of culture.
11. *Evaluation of other enrichment procedures.* The two most widespread and successful approaches to enrich cells are magnetic cell sorting (MACS™) and fluorescent cell sorting (FACS). The panning method described here is the least expensive of all approaches. As compared to FACS, it has the advantage of being less time-

consuming, particularly when it comes to larger cell numbers. For this and other reasons (gentle detachment of antibodies from cell surface) mismatched panning is less strenuous to the selected cells. As was pointed out in the description of experimental steps, keratinocytes sometimes stick to adherent Langerhans cells on the antibody-coated dishes. This phenomenon can be monitored directly under phase contrast and the extent of washing can be adjusted accordingly. This kind of monitoring is not possible with FACS and immunomagnetic methods. With regard to "antibody loading" of selected Langerhans cells panning, FACS and magnetic methods are alike: In each method Langerhans cells still carry the primary (anti-MHC class II or other) mAb on their surface. As mentioned earlier, this does not interfere with their immunostimulatory function, though.

12. *Comparison with "crawl-out" Langerhans cells.* A simple method to obtain cutaneous dendritic cells has been described by Larsen ***(39)***. It is based on the property of Langerhans cells to emigrate out of their epidermal environment into the culture medium. The main differences to the classical technique of Langerhans cell preparation, as outlined here, are as follows: First, the emigration technique is much easier! Second, the emigration technique yields populations of mature dendritic cells. The classical trypsinization approach allows the experimentor to choose and/or to compare immature and mature Langerhans cells. Third, standard emigration cultures of whole ear halves yield mixed populations of epidermal Langerhans cells and dermal dendritic cells ***(35)***. These two types of dendritic cells may differ in certain respects ***(12,36)***. This has not yet been studied in the mouse, however. The problem can be solved by setting up emigration cultures from dermis-free epidermal sheets prepared by dispase ***(35)***. And finally, we have evidence that the state of maturation may be different depending on whether Langerhans cells were allowed to mature in a suspension culture (as described here) or in an in vivo environment such as in the skin organ cultures as described in *ref.* **39**. Some features of maturation such as the disappearance of invariant chain expression of the loss of Birbeck granules seem to lag behind in populations of dendritic cells obtained from skin organ cultures ***(37)***.
13. *Heterogeneity of Langerhans cell populations.* Although we claim in the Introduction that Langerhans cells represent the experimental system with the sharpest separation of immature and mature dendritic cells, this statement has to be taken with a grain of salt. Populations of freshly isolated Langerhans cells contain a small subset (always < 5%) of apparently mature Langerhans cells ***(23,34)***. These cells are highly class II-positive, and they can also be found *in situ* on epidermal sheets ***(34)***. Inversely, populations of cultured Langerhans cells contain small subsets (< 5%) of immature Langerhans cells, as defined by the expression of the MHC class II-associated invariant chain. We have previously identified these "stragglers" and held them responsible for the residual capacity to process native protein antigens in populations of mature dendritic cells ***(29)***.
14. *Maturational status of Langerhans cell populations.* Typically, dendritic cells have been regarded as either immature or mature, depending on their functional

and phenotypical repertoire ***(7)***. Recently, Pierre et al. ***(30)*** have refined this scheme on the basis of important observations made with murine bone marrow-derived dendritic cells. It became clear that what we had often considered as immature dendritic cells in fact contained two stages of development: an "early" stage, characterized by intracellularly located MHC class II molecules (in "MIIC's"), and an "intermediate" stage, identified by abundant biosynthesis of MHC molecules. Freshly isolated Langerhans cells correspond to the "intermediate" type of dendritic cell. It was previously shown, that they actively synthesize MHC class II ***(31–33)***. Isolated populations of "early" Langerhans cells are not accessible for experimental purposes. All attempts to isolate them from their *in situ* environment result in a triggering of the maturation process. **Table 2** schematizes the interrelationship of the two terminologies that describe dendritic cell development. In bone marrow dendritic cells the transition from "early" to "intermediate" occurs quite rapidly within few hours ***(30)***. In the light of these data the possibility must be considered that freshly isolated Langerhans cells may differ in their maturational stage depending on the experimental procedure applied. Highly enriched Langerhans cells may be slightly more advanced in their maturation process (i.e. less immature) than Langerhans cells within primary epidermal cell suspensions. The reason for this is that the former populations have experienced longer periods at 37°C (complement-mediated lysis, "trypsin trick"). Apart from some unpublished observations this issue has not been systematically studied, although should be kept in mind.

15. *Loss of subpopulations with gradients.* This method avoids the use of density gradients for the enrichment of freshly isolated Langerhans cells. It can therefore be assumed that pre-existing subsets of Langerhans cells (e.g., Langerhans cells of different maturation state) are not inadvertently selected or depleted. This danger is inherent to all gradient approaches.
16. *Cytokines.* Human GM-CSF does not work in the murine system. The murine cytokine must be used. If there is no need to culture highly enriched Langerhans cells in a defined cytokine mix, it is recommended that keratinocyte-conditioned medium be used for this purpose. It contains the necessary cytokines to ensure survival and maturation in optimal quantities and relative proportions ***(22,23)***. Supernatants of confluent monolayers of keratinocytes cultured from day 3 (after removal of Langerhans cell-containing non-adherent cells) to day 5 are used as conditioned medium.
17. *Adherence properties of Langerhans cells.* When pre-enriched or highly enriched populations of Langerhans cells are used for short-term time course studies, one should be aware that during the first few hours (approx 12 h) of culture Langerhans cells readhere, preferably to keratinocytes but also to plastic surfaces (Koch et al., manuscript in preparation). This makes it mandatory to always monitor the actual content of Langerhans cells in such populations and not rely on initially determined values!

Table 2
Maturational Status of Murine Langerhans Cells

	Langerhans cells *in situ*	Freshly isolated Langerhans cells	Cultured Langerhans cells
Conventional classification (7)	immature		mature
*Classification by Pierre et al. (**30**)*	early	intermediate	late
Cell surface MHC class II expression	very little—mostly localized intracellularly in MIIC's	substantial[a]	very much[a]

[a]It should be emphasized here, that the trypsinization procedure does not substantially affect MHC class II expression of both freshly isolated and cultured Langerhans cells ***(8,38)***.

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References

1. Moll, H. (ed.) (1995) *The immune functions of epidermal Langerhans cells*. R.G.Landes Company / Springer-Verlag. Austin.
2. Schuler, G. (ed.) (1991) *Epidermal Langerhans cells.* CRC Press Inc., Boca Raton, FL.
3. Steinman, R.M. (1991) The dendritic cell system and its role in immunogenicity. *Annu. Rev. Immunol.*, **9,** 271–296.
4. Maurer, D. and Stingl, G. (1998) Dendritic cells in the context of skin immunology. In *Dendritic cells: biology and clinical applications*, Lotze, M. T. and Thomson, A. W. (eds.) Academic Press, London.
5. Banchereau, J. and Steinman, R.M. (1998) Dendritic cells and the control of immunity. *Nature*, **392,** 245–252.
6. Langerhans, P. (1868) Über die Nerven der menschlichen Haut. *Virchows Arch.[A]*, **44,** 325–337.
7. Romani, N., Witmer-Pack, M., Crowley, M., et al. (1991) Langerhans cells as immature dendritic cells. In *Epidermal Langerhans cells*, Schuler, G. (ed.) CRC Press Inc., Boca Raton, FL, pp. 191–216.
8. Schuler, G. and Steinman, R.M. (1985) Murine epidermal Langerhans cells mature into potent immunostimulatory dendritic cells in vitro. *J. Exp. Med.*, **161,** 526–546.

9. Romani, N., Koide, S., Crowley, M., et al. (1989) Presentation of exogenous protein antigens by dendritic cells to T cell clones: intact protein is presented best by immature epidermal Langerhans cells. *J. Exp. Med.*, **169,** 1169–1178.
10. Lappin, M.B., Kimber, I., and Norval, M. (1996) The role of dendritic cells in cutaneous immunity. *Arch. Dermatol. Res.* **288,** 109–121.
11. Knop, J. and Enk, A.H. (1995) Cellular and molecular mechanisms in the induction phase of contact sensitivity. *Int. Arch. Allergy Immunol.*, **107,** 231–232.
12. Caux, C., Massacrier, C., Vanbervliet, B., et al. (1997) $CD34^+$ hematopoietic progenitors from human cord blood differentiate along two independent dendritic cell pathways in response to granulocyte-macrophage colony-stimulating factor plus tumor necrosis factor α .2. Functional analysis. *Blood*, **90,** 1458–1470.
13. Inaba, K., Inaba, M., et al. (1992) Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. *J. Exp. Med.* **176,** 1693–1702.
14. Schuler, G. and Koch, F. (1991) Enrichment of epidermal Langerhans cells. In *Epidermal Langerhans cells*, Schuler, G. (ed.) CRC Press Inc., Boca Raton, FL., pp. 139–157.
15. Koch, F., Kämpgen, E., Schuler, G. and Romani, N. (1992) Effective enrichment of murine epidermal Langerhans cells by a modified— "mismatched"— panning technique. *J. Invest. Dermatol.* **99,** 803–807.
16. Romani, N., Bhardwaj, N., Pope, M., et al. (1997) Dendritic cells. In *Weir's Handbook of Experimental Immunology*, Herzenberg, L.A., Weir, D.M., Herzenberg, L. and Blackwell, C. (eds.) Blackwell Science, Oxford, pp. 156.1–156.14.
17. Schuler, G., Lutz, M. B., Bender, A., Thurner, B., Röder, C. and Romani, N. (1998) A guide to the isolation and propagation of dendritic cells. In *Dendritic cells: biology and clinical applications*, Lotze, M. T. and Thomson, A. W. (eds.) Academic Press, London.
18. Inaba, K., Swiggard, W. J., Steinman, R. M., Romani, N. and Schuler, G. (1998) Isolation of dendritic cells. In *current protocols in immunology*, Coligan, J. E., Kruisbeek, A. M., Margulies, D. H., Shevach, E. M. and Strober, W. (eds.) Wiley, New York, pp. 3.7.1–3.7.15.
19. Tschachler, E., Schuler, G., Hutterer, J., Leibl, H., Wolff, K., and Stingl, G. (1983) Expression of Thy-1 antigen by murine epidermal cells. *J. Invest. Dermatol.*, **81,** 282–285.
20. Bergstresser, P. R., Tigelaar, R. E., Dees, J. H., and Streilein, J. W. (1983) Thy-1 antigen bearing dendritic cells populate murine epidermis. *J. Invest. Dermatol.*, **81,** 286–288.
21. Wood, G. S., Kosek, J., Butcher, E. C. and Morhenn, V. B. (1985) Enrichment of murine and human Langerhans cells with solid phase immunoabsorption using pan-leukocyte monoclonal antibodies. *J. Invest. Dermatol.,* **84,** 37–40.
22. Witmer-Pack, M. D., Olivier, W., Valinsky, J., Schuler, G., and Steinman, R. M. (1987) Granulocyte/macrophage colony-stimulating factor is essential for the viability and function of cultured murine epidermal Langerhans cells. *J. Exp. Med.*, **166,** 1484–1498.

23. Heufler, C., Koch, F., and Schuler, G. (1988) Granulocyte-macrophage colony-stimulating factor and interleukin-1 mediate the maturation of murine epidermal Langerhans cells into potent immunostimulatory dendritic cells. *J. Exp. Med.*, **167,** 700–705.
24. Koch, F., Heufler, C., Kämpgen, E., Schneeweiss, D., Böck, G., and Schuler, G. (1990) Tumor necrosis factor alpha maintains the viability of murine epidermal Langerhans cells in culture but in contrast to granulocyte/macrophage colony-stimulating factor without inducing their functional maturation. *J. Exp. Med.* **171,** 159–171.
25. Koch, F., Kämpgen, E., and Trockenbacher, B. (1995) TNFα interrupts antigen-presenting function of Langerhans cells by two mechanisms: loss of immunogenic peptides and impairment of antigen-independent T cell clustering. *Adv. Exp. Med. Biol.*, **378,** 207–209.
26. Kraal, G., Breel, M., Janse, M. and Bruin, G. (1986) Langerhans' cells, veiled cells, and interdigitating cells in the mouse recognized by a monoclonal antibody. *J. Exp. Med.* **163,** 981–997.
27. Inaba, K., Witmer-Pack, M., Inaba, M., et al. (1994) The tissue distribution of the B7-2 costimulator in mice: abundant expression on dendritic cells in situ and during maturation in vitro. *J. Exp. Med.*, **180,** 1849–1860.
28. Kashihara, M., Ueda, M., Horiguchi, Y., Furukawa, F., Hanaoka, M., and Imamura, S. (1986) A monoclonal antibody specifically reactive to human Langerhans cells. *J. Invest. Dermatol.*, **87,** 602–612.
28a. Valladeau, J., Ravel, O., Dezutter-Dambuyant, C., et al. (2000) Langerin, a novel C-type lectin specific to Langerhans cells, is an endocytic receptor that induces the formation of Birbeck granules. *Immunity* **12,** 71–81.
29. Koch, F., Trockenbacher, B., Kämpgen, E., et al. (1995) Antigen processing in populations of mature murine dendritic cells is caused by subsets of incompletely matured cells. *J. Immunol.* **155,** 93–100.
30. Pierre, P., Turley, S. J., Gatti, E., et al. (1997) Developmental regulation of MHC class II transport in mouse dendritic cells. *Nature*, **388,** 787–792.
31. Kämpgen, E., Koch, N., Koch, F., et al. (1991) Class II major histocompatibility complex molecules of murine dendritic cells: synthesis, sialylation of invariant chain, and antigen processing capacity are down-regulated upon culture. *Proc. Natl. Acad. Sci. USA*, **88,** 3014–3018.
32. Puré, E., Inaba, K., Crowley, M. T., et al. (1990) Antigen processing by epidermal Langerhans cells correlates with the level of biosynthesis of major histocompatibility complex class II molecules and expression of invariant chain. *J. Exp. Med.*, **172,** 1459–1469.
33. Becker, D., Reske-Kunz, A.B., Knop, J. and Reske, K. (1991) Biochemical properties of MHC class II molecules endogenously synthesized and expressed by mouse Langerhans cells. *Eur.J. Immunol.*, **21,** 1213.
34. Romani, N., Fritsch, P., and Schuler, G. (1991) Identification and phenotype of epidermal Langerhans cells. In *Epidermal Langerhans cells*, Schuler, G. (ed.). CRC Press, Boca Raton, FL., pp. 49–86.
35. Ortner, U., Inaba, K., Koch, F., et al. (1996) An improved isolation method for murine migratory cutaneous dendritic cells. *J. Immunol. Methods,* **193,** 71–79.

36. Lenz, A., Heine, M., Schuler, G., and Romani, N. (1993) Human and murine dermis contain dendritic cells. Isolation by means of a novel method and phenotypical and functional characterization. *J. Clin. Invest.*, **92,** 2587–2596.
37. Weinlich, G., Heine, M., Stössel, H., et al. (1998) Entry into afferent lymphatics and maturation in situ of migrating cutaneous dendritic cells. *J. Invest. Dermatol.*, **110,** 441–448.
38. Witmer-Pack, M. D., Valinsky, J., Olivier, W., and Steinman, R. M. (1988) Quantitation of surface antigens on cultured murine epidermal Langerhans cells: rapid and selective increase in the level of surface MHC products. *J. Invest. Dermatol.*, **90,** 387–394.
39. Larsen C. P., Steinman, R. M., Witmer-Pack, M., Hankins, D. F., Morris, P. J. and Austyn, J. M. (1990) Migration and maturation of Langerhans cells in skin transplants and explants. *J. Exp. Med.* **172,** 1483–1493.

6

Murine Intestinal Dendritic Cell Isolation

Paul Pavli

1. Introduction

This chapter describes techniques for the isolation of dendritic cell-enriched single-cell suspensions from the lamina propria (LP) of both small and large intestine and from Peyer's patches (PP). This technique can also be used to obtain intestinal macrophage-enriched populations. The characterization of an assay system, the allogeneic mixed leukocyte reaction (MLR), is also described.

Intestinal dendritic cells resemble the lymphoid dendritic cell first described by Steinman and Cohn (1973) ***(1)***, in their surface phenotype (Ia-positive, absence of typical macrophage, T-cell, and B-cell markers), their physical properties (low density, weakly or nonadherent, nonphagocytic) and their cytological and ultrastructural features (absence of secondary lysosomes, pleiomorphic nucleus). Intestinal macrophages can be differentiated from dendritic cells on the basis of morphology, function (macrophages are phagocytic, adherent overnight in culture, and suppress MLR stimulation), and the expression of Class II MHC antigens (dendritic cells express very high levels). The intestinal MLR stimulatory cells had features of dendritic cells ***(2)***.

2. Materials

1. Mice. In the development of these methods, C57BL/6 and BALB/c mice of both sexes aged between 8–16 wk were studied under specific pathogen-free (SPF) conditions.

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2. Media:
   a. Calcium- and magnesium-free Hank's balanced salt solution (CMF-HBSS) supplemented with penicillin (final concentration 100 U/mL) and gentamycin (60 U/mL). Where indicated below, EDTA was added (Fluka, Buchs, Switzerland), to a final concentration of 0.75 m*M*.
   b. RPMI 1640 supplemented with 2 m*M* L-glutamine (Sigma, St Louis, MO), penicillin (100 U/mL), gentamycin (60 U/mL), and 10% heat-inactivated fetal calf serum (FCS) (Flow Laboratories, Melbourne, Victoria).
   c. For cell culture experiments, 0.01 m*M* 2-mercaptoethanol (2ME) (BDH Chemicals Pty Ltd, Sydney, NSW) was added`.
3. Surgical instruments.
4. Wheaton stirring flask with sterile magnetic flea.
5. Enzyme cocktail: 1.2 U/mL Dispase II (Boehringer-Mannheim, Tutzing, FRG), 10 U/mL collagenase CLSPA (Cooper Biomedical, Malvern, PA), and 5 U/mL DNase Type II (Calbiochem, Behring Diagnostics, La Jolla, CA).
   To make 20 mL of enzyme cocktail: 10 mL 2.4 U/mL Dispase II in CMF-HBSS plus 1mL 200 U/mL collagenase in RPMI plus 0.2 mL 500U/mL DNase in RPMI plus RPMI/10% FCS to 20 mL final volume.
6. 90–120 mm glass Petri dishes siliconized using Coatasil (Ajax Chemicals, Sydney, NSW).
7. Plastic bacteriological grade 90 mm Petri dishes.
8. Rabbit anti-rat heavy and light chain IgG (RAR) (Nordic, Tilburg, The Netherlands).
9. Mitomycin C (Sigma, St Louis, MO) or an irradiator.
10. Round-bottom 96-well plates (Linbro Flow Laboratories, McLean, VA).
11. Tritiated thymidine (Amersham Australia, Surry Hills, NSW or New England Nuclear, Boston, MA.).
12. Dynatech CH-103 cell harvester or similar.
13. Glass fiber discs (Whatman, Maidstone, UK).
14. Scintillant (0.5% 2,5-diphenyloxazole (PPO) in xylene) and liquid scintillation counter.
15. Poly-L-lysine (Sigma, St Louis, MO).
16. Cytocentrifuge slides.
17. Biotinylated sheep anti-rat immunoglobulin (Amersham, Amersham, UK).
18. Avidin-biotin-peroxidase complex (Vector Labs., Burlingame, CA).
19. 3,3'-diaminobenzidine (Sigma, St Louis, MO).
20. Imidazole (BDH Chemicals, Poole, UK).
21. Hydrogen peroxide.
22. Mayer's hematoxylin (BDH, Poole, UK).
23. Fluorescein isothiocyanate-labeled sheep anti-rat immunoglobulin (Silenus, Melbourne, Australia) or 1/100 fluorescein-conjugated affinity-purified $F(ab')_2$ fragment goat anti-rat IgG (heavy and light chain specific) (Cappel, Cooper Biomedical, Cochranville, PA).

3. Methods

3.1. Tissue Disaggregation

See **Notes 1** and **2**.

1. Dissect the small intestine or colon from the mice and gently express and discard the luminal contents.
2. Carefully strip the mesentery and serosal tissues from the muscle layers using curved forceps.
3. Where indicated, remove the Peyer's patches under a dissecting microscope using curved scissors.
4. Split the intestine lengthwise and cut into 1 cm segments.
5. Remove remaining luminal contents by washing the tissue for 20–30 s in CMF-HBSS using vigorous agitation in a closed container.
6. Place the intestines in a Wheaton stirring flask with a sterile magnetic flea and wash gently for 2–2.5 h in CMF-HBSS with EDTA at 37°C. During this time, change the medium every 10–15 min until no increase in particulate matter (predominantly epithelial cells) is visible in the supernatant (*see* **Note 3**).
7. Wash for 5 min in RPMI to inactivate the EDTA.
8. Chop the tissue finely using scissors. **Steps 4–8** inclusive can be excluded if dissected Peyer's patches are being disaggregated.
9. Weigh tissues and add to the enzyme cocktail. Maximal yields are obtained when < 500 mg tissue is digested with 20 mL enzyme mixture. Incubate for 2–3 h at 37°C under 5% CO_2 with occasional gentle agitation of Petri dishes.
10. Pipet the digested tissue through a sterile, fine mesh stainless steel sieve to disrupt it mechanically. Use CMF-HBSS at 4°C.
11. Pass the cell suspension through six layers of cotton gauze to remove undigested tissue. Gently flush the gauze two or three times using CMF-HBSS at 4°C and squeeze carefully to remove the remaining cells.
12. Wash the filtered cells three times in CMF-HBSS at 4°C. (If necessary, the cells can be pelletted in RPMI 1640 containing 20% FCS and kept overnight at 4°C for use the next day without loss of antigen-presenting activity.) *See* **Note 4** for expected cell yields.

3.2. Removal and Enrichment of Intestinal Macrophages

Attempts to enrich for MLR stimulatory cells were unsuccessful until macrophages were removed from the cell suspension; this resulted in a significant increase in the activity of the remaining nonadherent lamina propria cells.

1. Prepare fibronectin-coated gelatinized flasks ***(3)***: Incubate tissue culture flasks at 20°C with 1% gelatin in sterile water for 30–60 min. Human serum from healthy donors can be used as a source of fibronectin. Allow the blood to clot at 37°C, then centrifuge at 600*g* for 30 min at room temperature. Remove the supernatant

and store at 4°C. Incubate the gelatinized flasks with the supernatant for 30-60 min at 37°C, then wash three times with PBS before applying the cell suspension. The serum can be used as a source of fibronectin four or five times without loss of effectiveness.

2. Incubate the cell suspension at a concentration of (5–10) × 10^6/mL for 2–3 h on fibronectin-coated gelatinized flasks.
3. Remove the nonadherent cells by washing gently three times using RPMI containing 10% FCS. Pellet nonadherent cells and further incubate the adherent cells overnight. Remove nonadherent cells after overnight incubation by washing with RPMI at 37°C and pool with the pelletted nonadherent cells.
4. Adherent cells (macrophage-enriched) can be harvested by incubating with 10 m*M* EDTA in RPMI with 10% FCS at 4°C for 10–15 min then shake vigorously or use a rubber policeman to scrape them off. Wash cells three times in culture medium at 4°C. *See* **Note 5** for further information regarding intestinal macrophages.

3.3. Enrichment of Dendritic Cells

1. Layer nonadherent lamina propria or Peyer's patch cells at concentrations of (10–20) × 10^6/mL onto 2–3 ml Nycodenz [(density < 1.068 g/mL) Nyegaard, Oslo, Norway] and centrifuge at 600*g* for 15 min at 20°C.
2. Wash cells at the interface three times in RPMI and resuspend in RPMI containing 10% FCS for further experiments.
3. Further enrichment can be obtained by positive or negative selection using monoclonal antibodies and cell sorting or magnetic bead techniques ***(4)***. *See* **Note 7** for further information about cell yields and characteristics of intestinal DC.

3.4. Isolation of Mesenteric Lymph Node Cells for use as Responders in the MLR

1. Remove mesenteric lymph nodes from BALB/c mice and push through a fine mesh stainless steel sieve to obtain a single cell suspension. Wash, count and assess viability.
2. Deplete cells bearing Class II MHC molecules by incubating with a monoclonal antibody directed against murine Class II MHC antigens (TIB120) and use the technique of panning (*see* **Subheading 3.4.1.,** and **Notes 6** and **7**).

3.4.1. Panning to Remove Lymph Node Cells Expressing Particular Antigens (5).

1. Pretreat bacteriological grade 90-mm Petri dishes with 5 mL 1/1000 rabbit anti-rat heavy and light chain IgG (RAR), in phosphate-buffered saline (PBS) for 45 min at 4°C. Remove the unbound antibody by washing four times in PBS.
2. Incubate cells with a saturating amount of the monoclonal antibody for 30 min at 4°C. Wash three times, then remove the antibody-binding cells by panning.
3. Add (20–30) × 10^6 of the labeled cells suspended in 5 mL PBS/5% FCS to the RAR antibody-coated plates.
4. Keep plates at 4°C for 75 min and remove the nonadherent cells by gentle agitation and aspiration.

5. Centrifuge, then reapply to a second RAR-coated dish for another 75 min at 4°C. Remove the remaining nonadherent cells, wash, count and use immediately or pellet and keep overnight at 4°C.

3.5. Mixed Leukocyte Reaction

See **Note 8**.

1. Prevent cell proliferation in the stimulator population in one of the following ways :
   a. Pretreatment with mitomycin C: Incubate 5×10^6 cells/mL with mitomycin C at a final concentration of 25 mg/mL for 45 min at 37°C, then wash three times.
   b. Exposure to 2500–3000 Rads irradiation (minimizes macrophage losses).
2. Incubate varying numbers of stimulator cells with a constant number of responder MLN cells (2×10^5) in round-bottom 96-well plates in a total volume of 0.2 mL culture medium.
3. After 4 d, add 1 μCi tritiated thymidine to each well and continue the incubation for a further 16 h. Freeze–thaw the contents of the wells and harvest the cells onto glass fiber discs using cell harvester. Add 5 mL scintillant [0.5% 2,5-diphenyloxazole (PPO) in xylene] and perform the assay on a Packard TriCarb K60 scintillation counter or similar machine.

3.6. Immunocytochemistry

1. Incubate 10^4 cells in RPMI with 50% FCS on poly-L-lysine-coated glass slides (0.1% poly-L-lysine for 10 min then air-dried).
2. Air-dry cytocentrifuge slides overnight and block with horse serum (four drops in 10 mL PBS) for 30 min.
3. Add primary antibodies and incubate at 20°C for 30 min; wash three times in PBS. Add appropriate positive and negative isotype controls.
4. Add biotinylated sheep anti-rat immunoglobulin (1/200) for 30 min. Wash three times in PBS.
5. Add avidin-biotin-peroxidase complex for 1 h, then wash three times in PBS.
6. Develop the slides for 10 min with 0.5 mg/mL 3,3'-diaminobenzidine, 10 m*M* imidazole, and 0.3% hydrogen peroxide in PBS (pH 7.3).
7. Lightly counterstain the slides in Mayer's hematoxylin, wash, air-dry, and mount.

3.7. Cytofluorometry

1. Suspend cells at a concentration of $(2–10) \times 10^6$/mL in a solution containing the primary antibody for 30 min at 4°C; wash three times in PBS.
2. Resuspend at a concentration of 2×10^7/ml in 1/100 fluorescein isothiocyanate-labeled sheep anti-rat immunoglobulin or 1/100 fluorescein-conjugated affinity-purified $F(ab')_2$ fragment goat anti-rat IgG (heavy and light chain specific); wash three times.
3. Analyze and/or sort.

4. Notes

1. In developing this technique for obtaining lamina propria cell suspensions, many variables were examined including the use of mucolytic agents, e.g., dithiothrietol

(DTT), the type and concentration of enzymes, the length of incubation, the receptacle used for disaggregation, the medium used, the presence or absence of FCS, and the amount of tissue digested in a given volume of enzyme mixture. Data from 40 experiments was analyzed to calculate cell yields in relation to the type and concentrations of enzymes and the length of incubation. Factors critical to the successful application of the isolation procedure included: careful dissection of the intestines to ensure removal of blood vessels and fatty tissue; frequent washing in low concentrations of EDTA, the inclusion of DNase (to prevent cell clumping) and FCS (presumably to inhibit cytotoxic proteases *[6]*); the use of Petri dishes (rather than a stirring bar, which was associated with poor viability); and siliconization to prevent loss of adherent cells during the enzymatic digestion. One of the most important factors was the amount of tissue digested (P.G. Holt., personal communication). When more than 500 mg tissue was digested in 20 ml enzyme mixture, cell yields and viability dropped considerably.

2. Initial studies on immune responses in Peyer's patches failed to demonstrate accessory cell activity in preparations obtained by mechanical dissociation ***(7)***, so that it was concluded that Peyer's patches were deficient in functional accessory cells. Similar observations were made from the study of lung-derived cell suspensions (reviewed in ***8***). With the development of techniques for the enzymatic dissociation of tissue, it was clear that this deficiency was due to the failure of mechanical methods to release accessory cells from the connective tissue stroma ***(9)***.

   However, the use of enzymatic methods in tissue disaggregation is also associated with particular problems. For example, trypsin treatment, results in the loss of cell surface proteins ***(10)*** and impaired cellular cytotoxicity ***(11)***. Other enzymes such as pronase and papain have variable effects depending on their concentration and duration of treatment ***(12)***. Generally, the impairment of cellular function is completely reversible with time in culture ***(13)***.

   In spite of these possible adverse effects, enzymatic treatment of murine Peyer's patch with the neutral protease, Dispase, yielded cell suspensions that were capable of accessory cell activity in vitro. Furthermore, Dispase treatment of murine splenic dendritic cells did not alter their stimulatory activity ***(9)***. Treatment with collagenase generally has little effect on cellular function ***(14)*** or phenotype ***(15)***, although occasional batches may be overtly toxic ***(8)***. Since broad-spectrum proteases and/or mixtures of enzymes are more efficient for tissue disruption than single enzymes alone ***(16)***, it was decided to examine the use of multiple enzymes and the combination was found to give higher yields.

3. Effective removal of the epithelial monolayer by washing with EDTA was confirmed by histological examination of the specimens on several occasions.

4. Disaggregation of intact small intestinal mucosa resulted in yields of $17 \pm 4 \times 10^6$ cells per mouse (mean $\pm$ standard deviation, $n = 204$ mice) with $78 \pm 16\%$ viability as determined by Trypan blue exclusion. When the lamina propria and Peyer's patches were disaggregated separately, the average yield was $11.5 \pm 4 \times 10^6$/mouse ($n = 56$) for Peyer's patches and $7 \pm 3 \times 10^6$/mouse

(n = 59) for the lamina propria. The yield from the colon was 7×10^6 cells/mouse (n=18). There are many factors affecting the yield of cells using a particular disaggregation technique. Examples include the age and strain, but possibly the most important is antigen-exposure. This may affect both the size and the cellular composition of Peyer's patches ***(18–20)***. The use of Peyer's patches from SPF-mice resulted in lower yields when directly compared with mice housed in a conventional environment (e.g., ***21)*** and greater yields were obtained following inoculation with a specific microorganism ***(22)***. The yields of cells may also differ between different SPF facilities. For example, lamina propria yields were $7.0 \pm 3.0 \times 10^6$/mouse vs $3.0 \pm 1.3 \times 10^6$/mouse and Peyer's patch yields $11.5 \pm 4.0 \times 10^6$/mouse vs $7.2 \pm 1.9 \times 10^6$/mouse at Institutions A and B, respectively. These data supported the visual observation that the number and size of Peyer's patches was substantially greater at Institution A than at B. The final yields described here are comparable with other published results, although methods reporting greater yields have also been published (reviewed in ***17***). One important difference between this method and that of van der Heijden and Stok ***(17)*** is that the final proportion of epithelial cells was very low (< 5%) using this method compared to approx 25% overall in their study. Epithelial cells co-purify with other low density cells and cause cell losses by clumping, whereas van der Heijden and Stok ***(17)*** were concerned with plaque-forming cell assays, which presumably are unaffected by epithelial cells.

5. The yields of adherent cells from the intact small intestinal mucosa, the non-Peyer's patch lamina propria, and the Peyer's patch were 3%, 10%, and < 0.1%, respectively. Yields from the colonic lamina propria were also 10%. Viability was > 90% (Trypan blue exclusion). The proportion of adherent cells that were macrophages as judged by morphology was > 90% from all tissues. They had an oval nucleus, basophilic cytoplasm, and numerous inclusions giving a "foamy" appearance. An average of 91% phagocytosed fluorescent latex beads ***(2)***.

   Using depletion and reconstitution experiments, intestinal macrophages were shown to have suppressive activity in the MLR. Although staining weakly for Pgp-1, they had no detectable F4/80 antigen and were 45–55% (average 47%) positive for Ia. Other markers that could not be detected on intestinal macrophages were 2.4G2 (anti-F_cR) and 33D1 (anti-dendritic cell). These observations were confirmed and extended using flow cytometry. When compared to peritoneal macrophages, intestinal macrophages expressed very low levels of the macrophage-specific markers, F4/80, 2.4G2 (the F_c receptor), and M1/70 (the $C3b_i$ receptor) ***(2)***. The low level of F4/80 antigen expression did not appear to be due to the enzyme digestion, since peritoneal macrophage expression of F4/80 antigen was unaffected by the enzyme cocktail.
6. The yield of cells harvested at the Nycodenz interface was 2.7% of the starting cell number from both lamina propria and Peyer's patches. These cells had 20-50x greater MLR-stimulatory activity than the initial, unfractionated lamina propria cell suspensions. Dendritic cells from the lamina propria or Peyer's patches were not distinguishable in terms of the number of cells required to induce a maximal

MLR. In experiments in which cells from small and large intestine of the same animals were compared the MLR-stimulating activity was not distinguishable *(2)*.

Morphologically, they are large cells and have oval, irregular, or pleiomorphic nuclei and a basophilic cytoplasm. Characterization is best done by phase-contrast microscopy, immunofluorescence, or immunocytochemistry, where they can be identified by their morphology and the expression of Class II MHC antigens. They expressed very high levels of Class II MHC proteins with low, but detectable, levels of F4/80, Pgp-1, and the dendritic cell specific marker, 33D1. Other markers, in particular, surface immunoglobulin, were negative. The majority of the remaining cells in the intestinal low density fractions (20–35% of the total cell number at cytofluorometry) were Thy-1^+. The low density cells from the lamina propria of either the small or large intestine were greatly enriched for MLR-stimulatory activity ***(2)***. When studied by electron microscopy, the cells exhibited the cytological features described previously for lymphoid dendritic cells. The nucleus was irregular with a peripheral rim of heterochromatin and small nucleoli. The cytoplasm contained well-developed mitochondria and scattered smooth vesicles but no evidence of phagolysosomes. The surface of the cells observed with the scanning electron microscope showed characteristic cytoplasmic processes or veils often observed in cells from afferent lymphatics.

7. Yields from mesenteric lymph nodes were 39×10^6 cells/mouse. Following panning to remove cells expressing Class II MHC antigens (TIB 120), the average yield of mesenteric node cells was $52 \pm 7\%$ of the starting number.
8. Untreated or nylon-wool treated MLN cells give unacceptably high background levels of stimulation reaching a peak of 5,000–20,000 cpm between d 6 and 7 of culture. Cytofluorometric analysis of the MHC-II-depleted population demonstrated a homogenous population of T lymphocytes with $CD4^+$:$CD8^+$ = 70:30. The background levels of proliferation are always < 1,000 cpm and generally between 200–300 cpm. The time course of the MLR was determined using both unfractionated spleen and lamina propria cell suspensions as stimulators. Maximum levels of proliferation were comparable and occurred between 96–120 h of culture.

   When compared to splenic dendritic cells, lamina propria dendritic cells had similar MLR stimulatory activity. In contrast, tissue dendritic cells in other sites e.g., Langerhans cells of the epidermis, are weak stimulators of the MLR upon initial isolation ***(23)*** but in the presence of granulocyte/ macrophage-colony stimulating factor (GM-CSF) and IL-1, in vitro rapidly mature into potent MLR stimulators ***(24,25)***. The relative functional maturity of tissue dendritic cells obtained by the methods described above probably reflects the differences in maturation resulting from different tissue microenvironments and the time taken in the isolation process. Studies of rat intestinal dendritic cells showed that freshly isolated dendritic cells stimulated a moderately strong MLR and that they became more potent with overnight culture ***(26)***.

References

1. Steinman, R. M. and Cohn, Z. A. (1973) Identification of a novel cell type in peripheral lymphoid organs of mice. I. Morphology, quantitation, tissue distribution. *J. Exp. Med.* **137,** 1142–1162.
2. Pavli, P., Woodhams, C., Doe, W. F., Hume, D. A. (1990) Isolation and characterisation of antigen-presenting dendritic cells from the mouse intestinal lamina propria. *Immunology* **70,** 40–47.
3. Freundlich, B. and Avdalovic, N. (1983) Use of gelatin/plasma-coated flasks for isolating human peripheral blood monocytes. *J. Immunol. Methods* **62,** 31–37.
4. Ruedl, C., Rieser C, Boeck G, Wick G, Wolf H. (1996) Phenotypic and functional characterisation of $CD11c^+$ dendritic cell population in mouse Peyer's patches. *Eur. J. Immunol.* **26,** 1801–1806.
5. Wysocki, L. J. and Sato, V. L. (1978) "Panning" for lymphocytes: A method for cell selection. *Proc. Natl. Acad. Sci. U S A* **75,** 2844–2848.
6. Holt, P. G., Degebrodt, A., Venaille, T., et al. (1985) Preparation of interstitial lung cells by enzymatic digestion of tissue slices: Preliminary characterization by morphology and performance in functional assays. *Immunology* **54,** 139–147.
7. Kagnoff, M. F. and Campbell, S. (1974) Functional characteristics of Peyer's patch lymphoid cells. I. Induction of humoral antibody and cell-mediated allograft reactions. *J. Exp. Med.* **139,** 398–406.
8. Holt, P. G., Degebrodt, A., Venaille, T., et al. (1985) Preparation of interstitial lung cells by enzymatic digestion of tissue slices: preliminary characterization by morphology and performance in functional assays. *Immunology* **54,** 139–147
9. Spalding, D. M., Koopman, W. J., Eldridge, J. H., McGhee, J. R., and Steinman, R. M. (1983) Accessory cells in murine Peyer's patch. I. Identification and enrichment of a functional dendritic cell. *J. Exp. Med.* **157,** 1646–1659
10. Bland, P. W., Richens, E. R., Britton, D. C., and Lloyd, J. V. (1979) Isolation and purification of human large bowel mucosal lymphoid cells: Effect of separation technique on functional characteristics. *Gut* **20,** 1037–1046.
11. Rolley, R. T. and Marchalonis, J. J. (1972) Release and assay of antigen-binding immunoglobulin from the surfaces of lymphocytes of unsensitized mice. *Transplantation* **14,** 734–741.
12. Shorter, R. G., McGill, D. B. and Bahn, R. C. (1984) Cytotoxicity of mononuclear cells for autologous colonic epithelial cells in colonic diseases. *Gastroenterology* **86,** 13–22
13. Crofton, R. W., Cochrane, C., and McClelland, D. B. L. (1978) Preparation of lymphoid cells from small specimens of human gastrointestinal mucosa. *Gut* **19,** 898–906.
14. Gibson, P. R., Hermanowicz, A., Verhaar, H. J. J., Ferguson, D. J. P., Bernal, A. L. and Jewell, D. P. (1985) Isolation of intestinal mononuclear cells - factors released which affect lymphocyte viability and function. *Gut* **26,** 60–68.

15. Holt, P. G., Robinson, B. W. S., Reid, M., et al. (1986) Extraction of immune and inflammatory cells from human lung parenchyma: Evaluation of an enzymatic digestion procedure. *Clin. Exp. Immunol.* **66,** 188–200.
16. Russell SW, Doe WF, Hoskins RG and Cochrane CG. (1976) Inflammatory cells in solid murine neoplasms. 1. Tumor disaggregation and identification of constituent inflammatory cells. *Int J Cancer* **18,** 322-330.
17. van der Heijden, P. J. and Stok, W. (1987) Improved procedure for the isolation of functionally active lymphoid cells from the murine intestine. *J. Immunol. Methods* **103,** 161–167.
18. Crabbe, P. A., Nash, D. R., Bazin, H., Eyssen, H. and Heremans, J. F. (1970) Immunohistochemical observations on lymphoid tissues from conventional and germ-free mice. *Laborat Invest.* **22,** 448–457.
19. Pollard, M. and Sharon, N. (1970) Responses of the Peyer's patches in germ-free mice to antigenic stimulation. *Infect Immunity* **2,** 96–100.
20. Smith, M. W., James, S. P. and Tivey, D. R. (1987) M cell numbers increase after transfer of specific-pathogen free (SPF) mice to a normal animal house environment. *Am. J. Pathol.* **128,** 385–389.
21. MacDonald, T. T. and Carter, P. B. (1982) Isolation and functional characteristics of adherent phagocytic cells from mouse Peyer's patches. *Immunology* **45,** 769–774.
22. Carlson, J. R., Heyworth, M. F. and Owen, R. L. (1986) Response of Peyer's patch lymphocyte subsets to Giardia muris infection in Balb/c mice. *Cell Immunol* **97,** 44-58
23. Inaba, K., Schuler, G, Witmer, M. D., Valinksy, J., Atassi, B. and Steinman, R. M. (1986) Immunologic properties of purified epidermal Langerhans cells. Distinct requirements for stimulation of unprimed and sensitized T lymphocytes. *J. Exp. Med.* **164,** 605–613
24. Heufler, C., Koch, F. and Schuler, G. (1988) Granulocyte/ macrophage colony-stimulating factor and interleukin 1 mediate the maturation of murine epidermal Langerhans cells into potent immunostimulatory dendritic cells. *J. Exp. Med.* **167,** 700–705
25. Witmer-Pack, M. D., Olivier, W., Valinsky J., Schuler, G., and Steinman, R. M. (1987) Granulocyte/macrophage colony-stimulating factor is essential for the viability and function of cultured murine epidermal Langerhans cells. *J. Exp. Med.* **166,** 1484–498
26. Liu, L.-M., and MacPherson, G. G. (1995) Rat intestinal dendritic cells: immunostimulatory potency and phenotypic characterization. *Immunology* **85,** 88–93

7

Identification and Isolation of Rodent Lung Dendritic Cells

Philip A. Stumbles, Jennifer Thomas, and Patrick G. Holt

1. Introduction

Dendritic cells (DC) are a trace cell population found in most tissues that display potent antigen acquisition and presentation capabilities and are unique among antigen-presenting-cell types in their ability to activate unprimed T cells ***(1)***. DC are particularly prominent at the mucosal surfaces of the lung and gastrointestinal tract, where they are thought to play a critical role in the regulation of immune responses to environmental antigens ***(2–4)***.

Respiratory tract DC (RTDC) populations are found in the airway epithelium and underlying mucosa, where they form an integrated network of cells. Significant populations are also found within the lung parenchyma and the nasal turbinates ***(5)***. Studies in rats (which appear to resemble humans most closely in terms of DC distribution) have indicated that this network of epithelial RTDC develops slowly after birth from MHC class II negative precursors arriving from the bone marrow. Cell numbers and MHC class II expression slowly increases with time, presumably in response to environmental antigens, reaching adult-equivalent levels at 4–6 wk of age ***(5)***. Whether this epithelial network exists in humans, however, and the rate at which it develops from birth still remain unclear.

A unique feature of the airway intraepithelial population of RTDC is their rapid rate of turnover. Under steady-state conditions, these cells display turnover rates in the order of 2–3 d which is markedly more rapid than the lung parenchymal population (7–10 d) or epidermal populations (> 21 d) ***(6)***. This

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turnover rate can be further increased by exposure to inflammatory stimuli including bacteria, virus, and also inert protein *(7)*. This unique responsiveness of epithelial RTDC to inflammation highlights the importance of these cells in antigen surveillance at this site.

DC have been shown to have a complex life history, with evidence suggesting that DC at nonlymphoid sites are poor stimulators of T-cell responses but highly specialized for antigen uptake and processing *(1)*. In response to maturation stimuli, however, these cells will then enter the afferent lymph and migrate to draining secondary lymphoid organs, where they act as potent T-cell stimulators. This pattern of DC maturation also appears to apply to lung and airway mucosal DC. Freshly isolated RTDC express low to moderate levels of MHC class II and are relatively poor stimulators of naive T-cell responses, although these cells show high levels of endocytic activity in vitro and acquire soluble antigen in vivo *(8)* (P. Stumbles et al., manuscript in preparation). In response to the DC maturation factor granulocyte macrophage-colory stimulating factor (GM-CSF), however, marked increases in the expression of MHC class II and T cell stimulating activity are observed and this is associated with a decrease in endocytic activity (P. Stumbles et al., manuscript in preparation). This maturation process appears to be typical of DC of nonlymphoid origin and is thought to limit the potential of these cells to induce local tissue inflammation *(1)*.

RTDC have been shown to express a range of surface markers typical of most tissue DC populations. As mentioned previously, these cells exhibit low to moderate levels of MHC class II and as well express variable levels of other markers such as CD1, FcR, integrins, and Birbeck granules *(9–12)*. Other markers such as CD4, CD11a, CD80, and CD86 are also expressed at varying levels on resident lung DC populations *(13,14)*. In the rat, the DC specific β-integrin OX62 has been described on a subset of airway epithelial DC *(5)*, whereas lung DC in the mouse express NLDC-145, a marker of interdigitating DC *(10)*.

As mentioned previously, the majority of experimental work with lung-derived DC has been performed in the rat as the distribution of DC in this species appears to resemble humans more closely than does the mouse. Although OX62 can be used as a marker of subsets of RTDC, the expression of this antigen appears to be lost on single-cell suspensions of lung tissue, presumably as a result of the isolation procedure. Thus, the following sections describe protocols primarily for the isolation and purification of RTDC based on the unique expression of MHC class II by these cells following careful removal of other non-DC, MHC class II expressing cells that are found in lung cell preparations. These cells show all the properties of immature RTDC in terms of antigen acquisition and T-cell stimulation and are responsive to maturation factors such as GM-CSF and are thus suitable for a variety of functional studies. Although designed primarily for the isolation of rat RTDC, these

protocols can be adapted for the isolation of mouse cells using the suggested modifications.

2. Materials

2.1. Animals

Animals to be used as lung donors should be between 13 and 16 wk of age to achieve optimal recoveries. We routinely use male PVG ($RT1^c$), BN ($RT1^n$), or WAG ($RT1^u$) strain rats, with the BN strain giving the greatest recoveries.

2.2. General Reagents

1. Sodium pentobarbitone solution (325 mg/mL).
2. Bovine serum albumin (BSA) (Sigma Chemicals, MO). Store at –20°C as a 10% w/v solution in PBS.
3. Fetal calf serum (FCS) (TRACE Biosciences, Melbourne, Australia). Heat-inactivate at 56°C for 30 min and store at –20°C.
4. Collagenase A (Boehringer Mannheim, Mannheim, Germany). Store desiccated at –20°C.
5. Deoxyribonuclease I (DNAse I; Sigma Chemicals). Store desiccated at –20°C.
6. Mouse recombinant granulocyte macrophage-colony stimulating factor (rGM-CSF) (Biosource International, CA). Make a 1 μg/mL stock solution in PBS-BSA and store at –20°C.
7. Ficoll-hypaque solution: 1.077 g/mL, 280 mOsm (LymphoPrep™; Nycomed Pharma, Oslo, Norway).
8. Cotton-wool filter—5 mL syringe barrel plugged with cotton wool and autoclaved.
9. Nylon-wool columns (*see* **Note 1**).

2.3. General Buffers and Media

1. Dulbecco's A+B (DAB).
2. DAB containing 0.2% w/v BSA (DAB-BSA).
3. Phosphate buffered saline (PBS).
4. PBS containing 0.2% w/v BSA (PBS-BSA).
5. Digestion buffer: 10 mL DAB-BSA containing between 0.4 and 1.0 U/mL Collagenase A (*see* **Note 2**) and 1 mg/mL DNAse I. Make fresh on day of use.
6. RPMI containing L-glutamine (Gibco-BRL, City NY) supplemented with 5% FCS (R-5).
7. Glucose-potassium-sodium (GKN) buffer: 11 m*M* D-glucose, 5.5 m*M* KCl, 137 m*M* NaCl, 25 m*M* Na_2HPO_4, 5.5 m*M* NaH_2PO_4. Filter-sterilize and store at 4°C. (*see* **Note 3**).
8. GKN containing 0.2% w/v BSA (GKN-BSA).
9. GKN containing 5% FCS (G-5) prepared on day of use.
10. GKN containing 50% FCS (G-50) prepared on day of use.

2.4. Monoclonal Antibodies and Fluorescence Staining Reagents

The following reagents are used for the isolation of rat RTDC by flow cytometric and magnetic bead sorting. The Medical Research Council "OX" range of mouse anti-rat monoclonal antibodies are obtainable through the European Collection of Animal Cell Cultures or are available commercially from Serotec, Kidlington, UK. They are used as tissue culture supernatants unless otherwise indicated.

1. Monoclonal Antibodies
   a. OX6 - mouse anti-rat MHC class II directly conjugated to FITC (OX6-FITC).
   b. OX12- mouse anti-rat immunoglobulin *kappa* chain for labeling B cells.
   c. OX19 - mouse anti-rat CD5 for labeling T cells.
   d. OX21 - mouse anti-human C3b inactivator as isotype control.

   (For isolation of mouse RTDC, *see* **Note 4**).
2. Fluorescence Staining Reagents
   a. Purified mouse IgG1 conjugated to FITC (IgG1-FITC) (Dako, Denmark). Use at a final concentration of 5 μg/mL.
   b. Polyclonal goat anti-mouse IgG conjugated to phycoerythrin (GAM-PE) (Dako).
   c. Normal mouse serum (NMS): heat inactivated and stored at –20°C.

2.5. Reagents for Magnetic Bead Sorting of RTDC

The following additional reagents are required for MACS® microbead (Miltenyi Biotec, Germany) separation of RTDC (*see* **Note 5**):

1. MACS® microbeads coated with goat antimouse IgG.
2. Separation columns: BS separation columns for negative selection; RS^+ separation columns for positive selection.
3. Cell-sorting magnet.

3. Methods

3.1. Preparation of Lung Tissue

3.1.1. Removal of Lungs

1. Anethetize animal and euthenize by sodium pentobarbitone overdose.
2. Sever the abdominal aorta and blot with tissue paper.
3. Cut around the diaphragm and remove the rib cage.
4. Perfuse the heart with PBS until the lungs have turned white and are free of blood.
5. Clamp and excise the heart after removing the thin cardiac film.
6. Grasp the trachea with forceps, cut it as close to the head as possible and then cut carefully around the area until the lungs and trachea can be lifted free.
7. Cut off the trachea, leaving 2–3 mm above the lungs for manipulation.

3.1.2. Enzymatic Digestion of Lung Tissue

1. Slice lung tissue into 2 mm slices using a McIlwain tissue chopper (Mickle Laboratory Engineering, Surrey, UK).
2. Place 1 (BN) or 1.5 (PVG, WAG) chopped lungs into 40 mL of digestion buffer in a 50 mL sterile conical flask.
3. Incubate for 90 min at 37°C in a shaking water bath.
4. Disrupt tissue with a plastic transfer pipet or wide-bore Pasteur pipet until most of the larger pieces of tissue have dispersed.
5. Pass the digest mixture through a cotton-wool filter to remove tissue debris. Rinse the flask and filter to maximize cell recoveries.

3.1.3. Ficoll-Hypaque Purification and Nylon-Wool Elution

Perform the following steps at room temperature.

1. Calculate cell numbers and viability by Trypan Blue exclusion.
2. For every 1×10^8 viable cells, wash once in G-5 and resuspend in 5 mL of G-5.
3. Pre-coat a 25 mL v-bottom universal tube with FCS and add 8 mL of ficoll-hypaque.
4. Carefully overlay cell suspension onto ficoll-hypaque cushion and centrifuge at 500*g* for 10 min at room temperature with brake off. Do not overload ficoll cushion—use a maximum of 1×10^8 cells/8 mL ficoll-hypaque.
5. Collect viable cells from the ficoll-hypaque interface along with 90% of the ficoll solution without disturbing the pellet and transfer to a fresh tube.
6 Dilute with an equal volume of G-5 and centrifuge at 800*g* for 10 min.
7. Wash once and resuspend in G-5, count cells and resuspend to 1×10^8/mL
8. Load 1 mL of cell suspension onto a nylon-wool column and wash with 1.5 mL G-5 (stop-cock open) (*see* **Note 1**).
9. Close stopcock and incubate for 45 min at 37°C, 5% CO_2.
10. Elute nonadherent cells with 25 mL of G-5 warmed to 37°C.
11. Pellet cells and resuspend in GKN-BSA.

3.2. Isolation of RTDC by Flow Cytometry

3.2.1. Labeling of Cell Surface Antigens

1. For every 1×10^8 viable, nylon-wool eluted cells, resuspend in 1 mL each of OX12 and OX19 mAb tissue culture supernatants to label B cells and T cells. Take an aliquot of 5×10^5 cells and label with OX21 mAb as an isotype control.
2. Incubate for 15 min at 4°C and wash once in GKN-BSA.
3. Resuspend in 1 mL of a 1:400 solution of GAM-PE in GKN-BSA.
4. Incubate as above and wash once in GKN-BSA at 4°C.
5. Resuspend in a 10% v/v solution of NMS and incubate for 10 min on ice.
6. Resuspend in 1 mL of a 5 μg/mL solution of OX6-FITC in GKN-BSA containing 10% NMS. Add 5 μg/mL of IgG1-FITC in GKN-BSA to control cells.
7. Incubate for 15 min at 4°C and wash once in GKN-BSA at 4°C.
8. Pellet cells and resuspend to 1×10^7 cells/mL in G-5.

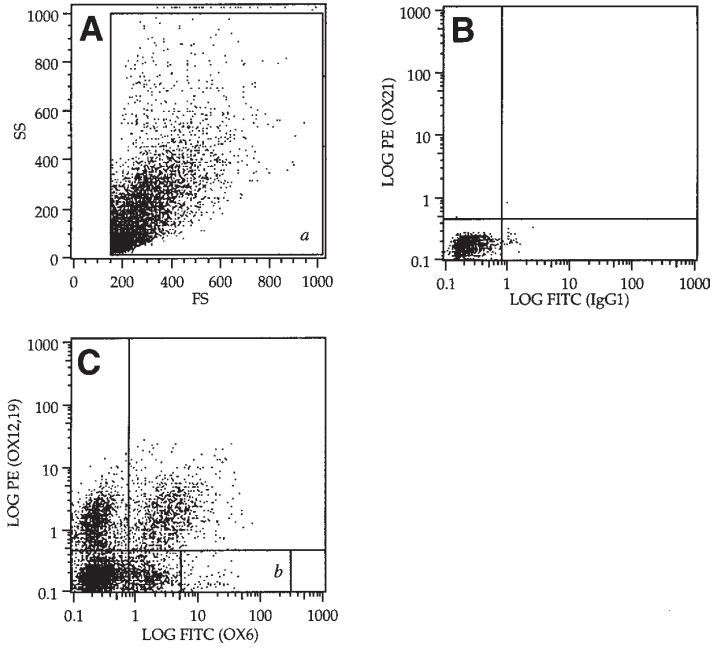

Fig. 1. Pre-sort analysis regions for separation of rat RTDC by flow cytometry. (**A**) Forward scatter and side scatter analysis of ungated whole lung digest. (**B**) Background FITC and PE staining with control antibodies (IgG1-FITC and OX21+GAM-PE) after gating on region *a*. (**C**) OX6-FITC and OX12, OX19, + GAM-PE staining of whole lung digest gated on region *a*. Note positioning of sort region *b*.

3.2.2. Cell Sorting

1. Run control cells through the cytometer and adjust forward and side angle light scatter high voltage and gain settings so that the majority of cells are detected.
2. Set a forward scatter discriminator at channel 100 and draw a gating region (region *a*) around all viable cells (**Fig. 1A**).
3. Create a two-color histogram of log FL1 (FITC) vs log FL2 (PE) gated on region *a*. Rerun control cells, adjust high-voltage settings and set quadrant regions around the negative population (**Fig. 1B**).
4. Run a sample of positively labeled cells and set FL1 and FL2 compensation levels.
5. Draw a sorting region around the OX12, $OX19^{-}$, $OX6^{high}$ dendritic cell population (**Fig 1C**, region *b* and *see* **Note 6**).
6. Sort $OX6^{high}$ dendritic cells into borosilicate glass tubes containing 2–3 mL of G-50, keeping cells at 4°C (*see* **Note 7**).
7. Pellet cells by centrifugation and wash once in R-5.

8. Check cell numbers and viability by Trypan Blue exclusion and resuspend to desired concentration in R-5.

3.3. Isolation of RTDC by Magnetic Bead Separation

1. For every 1×10^8 viable nylon-wool-eluted cells, resuspend in 1 mL OX12 mAb tissue culture supernatant to label B cells. These will represent approx 5–10% of the total cell population.
2. Incubate for 15 min at 4ºC and wash once in GKN-BSA.
3. For every $1x10^7$ positively labeled cells (i.e. approx 1×10^8 total cells), resuspend in 80 µL of GKN-BSA, and add 20 µL of anti-mouse IgG coated MACS® beads.
4. Incubate for 15 min at 4°C, wash once and resuspend to 2.5×10^7 cells in 1 mL GKN-BSA.
5. Magnetize a MACS® BS depletion column and attach a three-way stopcock with a 21-g needle on the 180° port and a 10 mL syringe filled with cold GKN-BSA on the 90° port.
6. With the stopcock open to the 180º port, load 2.5×10^7 cells and wash into column with 1.5ml GKN-BSA.
7. Close the stopcock and allow 30 s for the positively labeled cells to adhere.
8. Open the stopcock to the 90° port and back-flush the column with 1 mL GKN-BSA using the 10 mL syringe.
9. Allow the cells to reenter the column and wash with 1 mL of GKN-BSA.
10. Repeat **steps 7–9** four times.
11. Replace the 21-g needle with a 19-g needle and elute non adherent cells with 20mL of G-5.
12. Repeat the above process on any remaining nondepleted cells.
13. Pellet nonadherent cells, combine all fractions, and resuspend in 1 mL OX6 mAb tissue culture supernatant.
14. Incubate for 15 min at 4°C and wash once with GKN-BSA.
15. Resuspend in 80 µL of GKN-BSA plus 20 µL of MACS® beads and incubate for 15 min at 4°C.
16. Wash once and resuspend in 200 µL of GKN-BSA.
17. Magnetize a MACS® RS positive selection column and load the cell suspension.
18. Recycle the column effluent according to the manufacturer's instructions to maximize recovery of positively labeled cells, rinse the column with 3 mL of GKN-BSA and remove from magnet.
19. Elute and collect adherent cells using the plunger provided according to the manufacturer's instructions.
20. Wash cells twice in GKN-BSA and determine OX6 purity by flow cytometry (*see* **Note 8**).

3.4. In Vitro Culture

1. Wash cells twice in R-5 and resuspend to 1×10^6 cells/mL in R-5 warmed to 37°C.
2. Add 10 ng/mL of rGM-CSF and transfer cells to a 10 mL v-bottom tube (*see* **Note 9**).

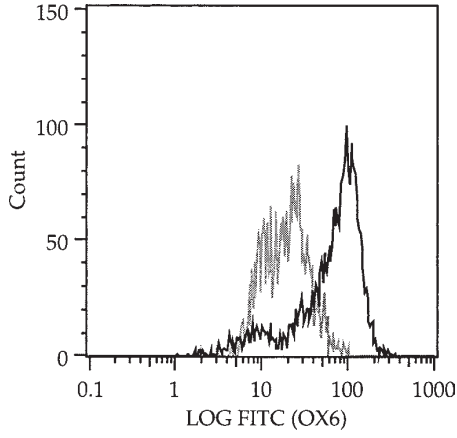

Fig. 2. Surface MHC class II expression of freshly isolated (gray line) or GM-CSF exposed (black line) RTDC sorted by the flow cytometric protocol.

3. Incubate cells for 24 to 48 h at 37°C in 5% CO_2, 90% humidity with the tube cap loosened.
4. Check cell viability and relabel an aliquot of cells with OX6-FITC and confirm up-regulation of surface MHC class II expression by flow cytometry. A 10-fold increase in the intensity of expression of MHC class II is typically observed at this stage (**Fig. 2** and **Notes 10** and **11**).

4. Notes

1. To prepare nylon-wool columns, weigh out 1.2 g of nylon wool (Type 200L, Robbins Scientific, Sunnyvale, CA) and tease fibers apart with fingers until a uniform density is achieved. Pack loosely into a 10 mL hypodermic syringe barrel, package, and autoclave. Prior to use, load column with G-5 and incubate at 37°C for 45 min to pre-wet nylon wool.
2. Correct working concentrations of Collagenase A must be determined for each batch of enzyme. Batches of enzyme vary considerably in terms Unit activity required for optimal cell recovery and viability, and this should be determined prior to use.
3. GKN is an all-purpose cell-handling buffer that is particularly suited to the handling of dendriform cell types (M. Puklavec, Oxford University, personal communication).
4. Several mAbs to mouse dendritic cells have been produced and can be used to directly label and sort mouse DC. Mouse lung DC have been shown to express the DEC-205 antigen recognized by the NLDC-145 mAb ***(10)***. In addition, detailed use of the N418 hamster mAb to sort mouse DC has been published ***(15)***, although whether the integrin recognized by this mAb is expressed on lung DC

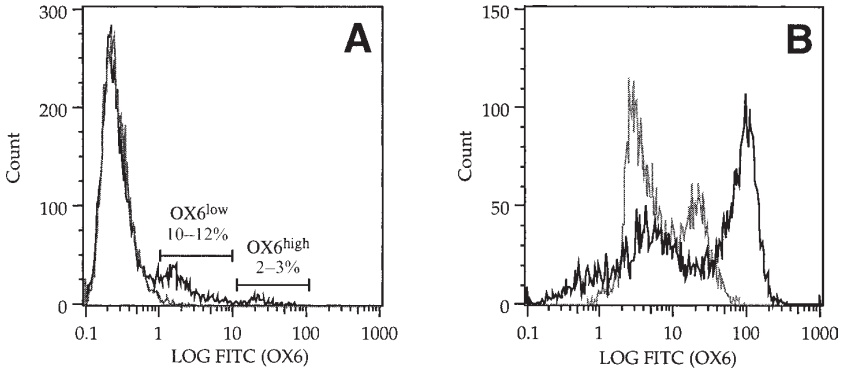

Fig. 3. Surface MHC class II expression of RTDC sorted by the magnetic bead protocol. (**A**) Pre-sort lung digest showing $OX6^{low}$ and $OX6^{high}$ populations. (**B**) OX6 labelling of freshly isolated (gray line) or GM-CSF exposed (black line) RTDC. Note the increased heterogeneity of OX6 expression.

has not been determined. A third mAb, termed 33D1, also specifically recognizes DC in mouse spleen and lymph node ***(16)*** and may also be useful for the direct isolation of lung DC. The isolation and sorting protocols described in this chapter can be adapted for use in mouse, although unlike rat DC, mouse DC are adherent and thus should not be passaged over nylon wool.

5. We use MACS® beads in preference to Dynabeads™ (Dynal, Oslo, Norway) due to their smaller size and thus potentially less interference in antigen presentation assays.
6. Flow cytometric analysis of macrophage depleted, OX12, $OX19^{-}$ $OX6^{+}$ lung digest cells reveals a bimodal expression for MHC class II, with the $OX6^{high}$ and $OX6^{low}$ populations representing approx 2–3% and 10–12% of total lung digests, respectively (**Fig. 3A**). The majority of $OX6^{high}$ cells show a dendriform morphology, express CD86 (but not CD80), are actively endocytic, and upregulate surface MHC class II expression and both in vitro and in vivo antigen-presenting capacity in response to GM-CSF. These cells thus show characteristics representative of dendritic cells isolated from other tissue sites ***(1)***. In contrast, less than 50% of $OX6^{low}$ lung digest cells are endocytic, and the majority of these do not express CD86. Although a small proportion do upregulate MHC class II in response to GM-CSF, we generally exclude these cells from our sorting regions and collect only the OX12, $OX19^{-}$ $OX6^{high}$ population, although this will exclude a possibly more immature population of cells that may reside within the $OX6^{low}$ subset.
7. Best cell recoveries and purities will be obtained if cells are sorted at a low cell concentration ($< 1 \times 10^7$/mL) in order to reduce coincidence aborts. If preferable, an enrichment sort can be run initially at a high data rate with coincidence abort OFF, followed by a second high-purity sort at a low data rate with coinci-

dence abort ON; however, this will result in reduced cell recoveries. Keep cells cold during the sort, and collect into borosilicate glass tubes containing 2 mL of G-50.

8. A factor to consider when using magnetic bead sorting is the inability to separate $OX6^{high}$ vs $OX6^{low}$ populations (*see* **Fig. 3A** and **Note 5**). Thus, purification of RTDC by magnetic bead sorting will result in a more heterogeneous population of cells consisting of high and low MHC class II-expressing cells. A 24-h culture in GM-CSF will enrich for MHC class II^{high} RTDC (**Fig. 3B**) and these cells are fully functional in antigen presentation assays.
9. We typically culture purified RTDC in 10 mL tubes to avoid cell losses during postculture washes and use a maximum of 1×10^6 cells in 1 mL /tube. GM-CSF is required for survival of RTDC in culture; however, additional factors such as TNFα, bacterial LPS, and CD40-ligand may also influence DC maturation and may be added together with GM-CSF ***(17)***.
10. Postculture analysis of the degree of up-regulation of MHC class II induced by GM-CSF should be performed prior to the use of RTDC in presentation assays. Cell viabilities after culture in GM-CSF will be greater for $OX6^{high}$ cells sorted by flow cytometry compared with the mixed $OX6^{high+low}$ cells sorted by magnetic beads (approx 70% and 30% viable, respectively) as a high proportion of $OX6^{low}$ cells do not respond to GM-CSF.
11. In the case of magnetically sorted cells, we use propridium iodide labeling after GM-CSF culture to exclude dead cells for flow cytometry analysis and include 5% NMS when relabeling with OX6-FITC to block any free antimouse IgG binding sites associated with beads remaining on the cell surface. Include a ficoll-hypaque purification step to remove dead cells prior to use.

References

1. Steinman, R. M. (1991). The dendritic cell system and its role in immunogenicity. *Ann. Rev. Immunol.* **9,** 271–296.
2. Holt, P. G., Schon-Hegrad, M. A. and Oliver, J. (1988). MHC class II antigen-bearing dendritic cells in pulmonary tissues of the rat. Regulation of antigen presentation activity by endogenous macrophage populations. *J. Exp. Med.* **167,** 262–274.
3. Holt, P. G., Schon-Hegrad, M. A., Phillips, M. J. and McMenamin, P.G. (1989). Ia-positive dendritic cells form a tightly meshed network within the human airway epithelium. *Clin. Exp. Allergy* **19,** 597–601.
4. Pavli, P., Hume, D. A., Van De Pol, E. and Doe, W.F. (1993). Dendritic cells, the major antigen-presenting cells of the human colonic lamina propria. *Immunology* **78,** 132–141.
5. Nelson, D. J., McMenamin, C., McWilliam, A. S., Brenan, M. and Holt, P. G. (1994). Development of the airway intraepithelial dendritic cell network in the rat from class II major histocompatibility (Ia)-negative precursors: differential regula-

tion of Ia expression at different levels of the respiratory tract. *J. Exp. Med.* **179,** 203–212.
6. Holt, P. G., Haining, S., Nelson, D. J. and Sedgwick, J. D. (1994). Origin and steady state turnover of class II MHC-bearing dendritic cells in the epithelium of the conducting airways. *J. Immunol.* **153,** 256–261.
7. McWilliam, A. S., Nelson, D., Thomas, J. A. and Holt, P. G. (1994). Rapid dendritic cell recruitment is a hallmark of the acute inflammatory response at mucosal surfaces. *J. Exp. Med.* **179,** 1331–336.
8. Holt, P. G., Oliver, J., McMenamin, C. and Schon-Hegrad, M.. (1992). Studies on the surface phenotype and functions of dendritic cells in parenchymal lung tissue. *Immunology* **75,** 582–587.
9. Kradin, R. L., McCarthy, K. M. Xia, W. J., Lazarus, D. and Schneeberger, E. E. (1991). Accessory cells of the lung. I. Interferon-gamma increases Ia+ dendritic cells in the lung without augmenting their accessory activities. *Am. J. Respir. Cell Mol. Biol.* **4,** 210–218.
10. Pollard, A. M., and Lipscomb, M. F. (1990). Characterization of murine lung dendritic cells: similarities to Langerhans cells and thymic dendritic cells. *J. Exp. Med.* **172,**159–167.
11. Sertl, K., Takemura, T., Tschachler, E., Ferrans, V. J., Kaliner, M. A. and Shevac, E. M. (1986). Dendritic cells with antigen-presenting capability reside in airway epithelium, lung parenchyma and visceral pleura. *J. Exp. Med.* **163,** 436–451.
12. Soler, P., Moreau, A., Basset, F. and Hance, A. J. (1989). Cigarette smoking-induced changes in the number and differentiated state of pulmonary dendritic cells/Langerhan's cells. *Am. Rev. Respir. Dis.* **139,** 1112–1117.
13. Schon-Hegrad, M. A., Oliver, J., McMenamin, P. G. and Holt, P. G. (1991). Studies on the density, distribution, and surface phenotype of intraepithelial class II major histocompatibility complex antigen (Ia)-bearing dendritic cells (DC) in the conducting airways. *J. Exp. Med.* **173,** 1345–1356.
14. Masten, B. J., Yates, J. L., Pollard Koga, A. M., and Lipscomb, M. F. (1997). Characterization of accessory molecules in murine lung dendritic cell function: roles for CD80, CD86, CD54, and CD40L. *Am. J. Respir. Cell Mol. Biol.* **16,** 335–342.
15. Crowley, M. T., Inaba, K., Witmer-Pack, M. D., Gezelter, S., and Steinman, R. M. (1990). Use of the fluorescence activated cell sorter to enrich dendritic cells from mouse spleen. *J. Immunol. Methods.* **133,** 55–66.
16. Nussenzweig, M. C., Steinman, R. M., Witmer, M. D., and Gutchinov, B. (1982). A monoclonal antibody specific for mouse dendritic cells. *Proc. Natl. Acad. Sci. USA* **79,** 161–165.
17. Cella, M., Scheidegger, D., Palmer-Lehmann, K., Lane, P., Lanzavecchia, A., and Alber, G. (1996). Ligation of CD40 on dendritic cells triggers production of high levels of interleukin-12 and enhances T cell stimulatory capacity: T-T help via APC activation. *J. Exp. Med.* **184,** 747–752.

8

Isolation and Propagation of Mouse Liver-Derived Dendritic Cells

Lina Lu, Maureen L. Drakes, and Angus W. Thomson

1. Introduction

Dendritic cells (DC) are highly specialized antigen-presenting cells (APC) derived from precursors within the bone marrow (BM). They are distributed ubiquitously throughout the body, and are few in number ***(1)***. They are classified as lymphoid-related or myeloid DC depending on their developmental lineage. Lymphoid-related DC develop from very immature T-cell precursors or BM progenitors, which are also the source of future natural killer cells and B cells ***(2,3)***. In vitro, these lymphoid-derived DC can be generated in the absence of granulocyte macrophage colony stimulating factor (GM-CSF), and are $CD8^+$ and Fas ligand$^+$ ($CD95L^+$). In the presence of GM-CSF, DC develop directly from myeloid committed precursors, which also give rise to monocytes and granulocytes, or from myelomonocytic cells, which are also precursors of monocytes ***(4,5)***. DC can be propagated from progenitors in BM ***(6)***, blood ***(7)***, or secondary lymphoid tissues ***(8)***. In addition, peripheral blood mononuclear cells (PBMC) can develop into DC-like cells if cultured in the presence of GM-CSF and interleukin-4 (IL-4) ***(9,10)***.

Other properties of DC include the ability to take up, process, and present antigen (Ag) and their capacity to migrate from peripheral sites to lymphoid tissues (11). Myeloid-derived DC associate with antigen-specific T-lymphocytes in lymphoid tissue, and are responsible for initiating and directing specific T lymphocyte responses.

Very low numbers of DC are found in non-lymphoid tissues. For example, the liver interstitial leukocyte population comprises approx 1% DC that are

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located mainly in the portal triads. Anti-MHC class II antibody staining of mouse livers reveals MHC class II positive cells primarily in the periportal area and surrounding central veins, with few cells scattered throughout the parenchyma ***(12,13)***. These cells exhibit a dendritiform morphology. DC are classified mainly by their low buoyant density, presence of DC-restricted surface antigens (DEC-205, CD11c, and 33D1 in the mouse), MHC class II staining, and the presence of prominent dendrites, eccentric multilobed nucleus, and the absence of prominent cytoplasmic granules.

When freshly isolated from the liver, DC like those from other nonlymphoid organs, are adherent , have a phenotype distinct from that of "mature" lymphoid tissue DC, and can process protein-containing antigens. They lack critical costimulatory molecules (B7-1[CD80], B7-2[CD86], and CD40) and cannot initiate proliferative responses in naive T cells. After overnight culture, these liver-DC become mature, are nonadherent, and exhibit low buoyant density. The methods developed for the isolation and purification of liver DC take advantage of these properties.

The mouse liver is a hematopoietic organ, and contains stem cells and the precursors and progenitors of DC. To obtain proliferating DC progenitors or more mature DC, the non-parenchymal cells (NPC) are grown in GM-CSF with or without IL-4, respectively. Propagation of DC from the liver, unlike the BM or spleen, results in cells with an immature phenotype and function, properties that may be related to the relative immunologic privilege of the transplanted liver ***(14)***.

In order to propagate DC from liver, NPC are first isolated after collagenase digestion of the perfused organ. The procedures outlined below describe the isolation of NPC from normal mouse livers without impairment of expression of surface molecules and without loss of DC precursors. The very low numbers of DC obtained from normal mouse liver can be amplified if the animals are pretreated with the recently cloned hematopoietic growth factor, Flt3 ligand ***(15,16)***. Large numbers of DC at different maturation stages obtained by this method will allow more detailed and extensive studies on the biology of these cells, and their possible roles in immune reactivity and tolerance.

2. Materials

2.1. Culture Medium

All cells are cultured in RPMI-1640 (Gibco-BRL; Grand Island, NY) supplemented with 10% heat-inactivated fetal calf serum (FCS; Tissue Culture Biologicals; Tulare, CA), 2 m*M*/mL L-glutamine, 0.1 m*M*/mL nonessential amino acids, 1 mM/ml sodium pyruvate, 20 mM 2-ME, and antibiotics (100 U/mL penicillin, 100 mg/mL streptomycin). This is referred to subsequently as RPMI-1640 complete medium.

2.2. Cells and Reagents

1. Mice; obtained from the appropriate animal housing facility and used between 6 and 8 wk of age.
2. Collagenase (type IV; Sigma; St. Louis, MO) solution (1 mg/mL in Hank's balanced salt solution [HBSS]).
3. Percoll (Sigma. St. Louis, MO), used at a relative density of 1.079 in RPMI-1640 supplemented with 5% FCS.
4. Flt3 Ligand (Flt3L; CHO cell-derived—a kind gift from Immunex, Seattle, WA); administered at a dose of 10 μg/day ip in 200 mL HBSS, for nine consecutive days. Untreated mice receive 200 mL HBSS, or are uninjected.
5. Metrizamide (grade 1, approx 99% pure; Sigma); 14.5% w/v in RPMI-1640 complete medium.
6. Thymidine; [^{3}H]TdR (NEN; Boston, MA) 1 μCi per well in 10 mL volume.
7. All cultures are incubated in a 5% CO_2 humidified incubator.
8. Tissue culture plates (24-well flat bottom and 96-well round bottom plates).
9. Type I collagen (Sigma)-coated plates. 24-well plates are left overnight (18 h) in the refrigerator (4°C) with 250 ml collagen in HBSS at 100 ng/mL. The solution is poured off the plates; they are allowed to dry, and given a light wash in RPMI-1640 complete medium before use.

3. Methods

3.1. Isolation of Nonparenchymal Cells (NPC) from Liver Tissue

1. Anesthetize mice with metofane and swab with 70% alcohol.
2. Make an abdominal mid-line incision.
3. Insert a 22-g catheter (J & J Medical, Inc., Arlington, TX) into the inferior vena cava.
4. Attach a syringe containing 30 mL ice-cold HBSS (Mediatech Cellgro) to the catheter and slowly inject 2 mL HBSS.
5. Use a pair of fine scissors to cut the portal vein, and then continue to inject the remaining HBSS over a period of 3 min.
6. Inject 1 mL ice-cold collagenase solution.
7. Excise the liver immediately, and tease it apart using a pair of forceps and a cell scraper.
8. Add 30 mL collagenase solution per 2–3 livers, and place the liver suspension in a bottle with a stirring bar.
9. Digest the liver at 37°C for 30 min in a water bath, using constant stirring.
10. Filter the digested tissue suspension through 0.1 mm sterile nylon mesh.
11. Wash the cell suspension twice in cold-HBSS (400*g*, 5 min) (*see* **Notes 1** and **2**).
12. Resuspend the cells in 7 mL sterile Percoll solution, and centrifuge at 4°C for 10 min at 39,000*g* using an SS 34 rotor in an ultracentrifuge.
13. Discard the top layer of cells, containing hepatocytes and hepatocyte fragments.
14. Collect the cell suspension between the upper and lower (erythrocyte) layer, and

wash twice in RPMI-1640 complete medium. This fraction constitutes the liver NPC population (*see* **Note 3**)

3.2. Isolation and Purification of DC from Liver NPC

1. Culture freshly-isolated liver NPC in 25 cm^2 tissue culture flasks for 18 h at 37°C in a 5% CO_2 humidified incubator.
2. Remove nonadherent cells, wash (400*g*, 5 min), and resuspend them in 8 mL RPMI-1640 complete medium.
3. Layer this onto 2 mL 14.5 % (w/v) metrizamide solution in a 15 mL conical tube.
4. Centrifuge the cells at 600*g* for 15 min at room temperature (20°C).
6. Collect the low-density DC-enriched interface layer carefully with a disposable pasteur pipet.
5. Wash cells twice in RPMI-1640 complete medium.
7. Prepare cytospins of these cells, referred to as enriched DC, and stain with Giemsa to determine purity (*see* **Note 4**).

3.3. Culture of Liver-Derived DC

1. Plate 1 mL of liver NPC in each well of a 24-well plate at a concentration of 2×10^6/ mL in RPMI-1640 complete medium, supplemented with 4 ng/mL recombinant mouse GM-CSF.
2. Culture at 37°C in a humidified 5% CO_2 incubator, and after 48 h, remove the medium and floating cells from the wells without disturbing clusters of proliferating adherent cells. This process is referred to as "selecting" (*see* **Note 5**).
3. Add 0.5 ml fresh GM-CSF (4 ng/ml) - containing RPMI-1640 complete medium to each well.
4. Spin down the mixture removed from the wells (400*g*, 5 min) so that the cells are pelleted. Add 0.5 mL of the supernatant thus obtained to each well, bringing the total volume of each well back up to 1 mL.
5. Reincubate and feed the cultures after another 48 h by removing 0.5 mL medium from the surface of each well (discard this medium), and adding back an equivalent amount of fresh GM-CSF- supplemented medium (*see* **Note 6**).
6. Repeat this procedure every 48 h as the growth of clusters attached to a layer of adherent cells is being established.
7. Continue to culture cells for 8-10 days after which time many dendritic-shaped cells are released from the clusters and float in the medium (*see* **Note 7**).
8. Tap plates lightly, and with a pasteur pipet, gently remove all of the medium from the wells, thus removing the floating cells which are mostly DC (*see* **Note 8**).

3.4. Maturation of Liver-Derived DC with IL-4

1. Culture NPC in RPMI-1640 complete medium supplemented with GM-CSF (4 ng/mL) in the presence and absence of recombinant interleukin-4 (IL-4), 1000 U/mL.
2. Harvest floating cells, which are enriched for DC at d 7–10 (*see* **Note 9**).

3.5. Maturation of Liver-Derived DC in the Presence of Type I Collagen

When d 7 GM-CSF-stimulated immature liver DC are transferred to culture plates precoated with type-1 collagen (an extracellular matrix protein with which DC are spatially associated in normal liver) and maintained for three more days in the presence of GM-CSF, cell proliferation can be observed. The nonadherent cells on the collagen-coated plates show marked upregulation in the expression of MHC class II and the DC markers DEC 205, 33D1, and CD11c. After exposure to collagen, the liver-derived DC show a marked reduction in phagocytic activity, which is associated with immature DC (determined by uptake of opsonized sheep erythrocytes). The GM-CSF-propagated liver DC, which express low MHC class II antigen, are poor stimulators of allogeneic, naive T cells, compared with spleen-derived DC propagated under the same conditions that express higher levels of surface MHC class II antigen. Type-I collagen-exposed DC, however, become potent allogeneic stimulators in MLR assay, although not as potent as spleen cell-derived DC. These liver-derived DC home after injection subcutaneously in the footpad, almost exclusively to T cell dependent areas of allogeneic recipients' spleens.

1. Harvest immature DC from cultures with GM-CSF alone at d 7.
2. Transfer to type I collagen-coated plates for another 3 d in the continued presence of GM-CSF.
3. Harvest cells released into the culture medium as the DC enriched fraction and perform flow cytometric analysis and MLR.

3.6. Liver DC from Flt3 L-Treated Mice

The study of liver DC is restricted by the low yield of DC isolated or propagated from normal liver tissue. The yield of liver DC can be dramatically increased by in-vivo treatment with Flt3L. Flt3L is a recently cloned hematopoietic growth factor, the cognate ligand for Flt-3, a receptor-type II tyrosine kinase that is expressed on $CD34^+$ stem cells. Flt3L administration is highly effective in mobilizing stem and progenitor cells in vivo, and results in their accumulation in tissue ***(15–18)***.

3.6.1 Isolation of Liver DC

1. Inject mice for 9 d with Flt3 L, 10 μg/day, ip.
2. Sacrifice mice and isolate the NPC fraction as described in **Subheading 3.1.** (*see* **Note 10**).
3. Culture the freshly isolated NPC overnight and derive the enriched DC fraction as described in **Subheading 3.2.** (*see* **Note 11**). These isolated liver DC are short lived and are not maintained in culture beyond d 3, even in the presence of GM-CSF.

3.6.2 Culture of Liver DC

1. Culture NPC in GM-CSF, as described previously. **Subheading 3.3**.
2. On day 2, select as described in **Subheading 3.3.**
3. Continue cultures as described in *3.3* and harvest cells on d 4–5 (*see* **Note 12**).

3.7. Immunophenotypic Analysis of Liver NPC and Enriched DC

1. Adjust cells to a concentration of 2×10^6 /mL.
2. Perform flow cytometric analysis using 100 µL cells and 10 µL appropriate monoclonal antibody.

3.8. Stimulatory Activity of Hepatic NPC and Enriched DC

1. Perform a mixed leukocyte reaction (MLR) in a 96-well round bottom plate, using serial dilutions of irradiated NPC or enriched DC as stimulators (S) and purified allogeneic T cells as responders (R), with an initial S:R ratio of 1:2.
2. Use syngeneic spleen cells as negative control stimulators, and allogeneic spleen cells as positive control stimulators.
3. Incubate for 72 hr, pulsing with ^{3}H thymidine for 18 hr before harvesting.

4. Notes

1. All cell washes in these procedures are done at 4°C (unless specified) to prevent adherence of DC to tubes at higher temperature.
2. After digestion of livers, it is important to pour off the supernatant thoroughly after the first wash, leaving only the cell pellet. This step removes as many hepatocytes as possible.
3. This method should yield $5–10 \times 10^6$ NPC from a normal adult mouse liver, with less than 5% hepatocyte contamination by microscopic examination ***(14)*** **(Fig. 1.)**. As determined by flow cytometric analysis, freshly-isolated hepatic NPC are strongly positive for the leukocyte-common antigen (CD45), but are MHC class II^{dim}. They stain both for lymphoid markers (CD3$^+$, B220^{low}, and HSA^{high}) and the macrophage cell surface antigen, F4/80; they also express the following receptors/adhesins: CD11b (MAC-la unit), CD44 (Pgp-1), and CD32 (FcγRII). Expression of the DC-restricted marker DEC-205 (NLDC 145) is also detected, but that of 33D1 and CD11c is low. A large proportion (40–50%) of the isolated liver NPC is B220$^+$. These cells account largely for the MHC class II$^+$ population (13). Within the total population of liver NPC, Thy1.2$^+$ T cells are present in smaller numbers (20%) compared with B220$^+$ cells. Within the population of small cells, a subpopulation of cells bearing the DC-restricted marker DEC 205 can be identified. Gating for larger-sized cells, a population of DEC-205$^+$ cells can also be found. The stimulatory activity of freshly isolated liver NPC is similar to that of fresh spleen cells, and results in the proliferation of naive T cells in a primary mixed leukocyte culture (MLR). However, when an excess of liver NPC stimulator cells over responder cells is used, a progressive reduction in the MLR is observed. This reduction does not occur when spleen cells are used as

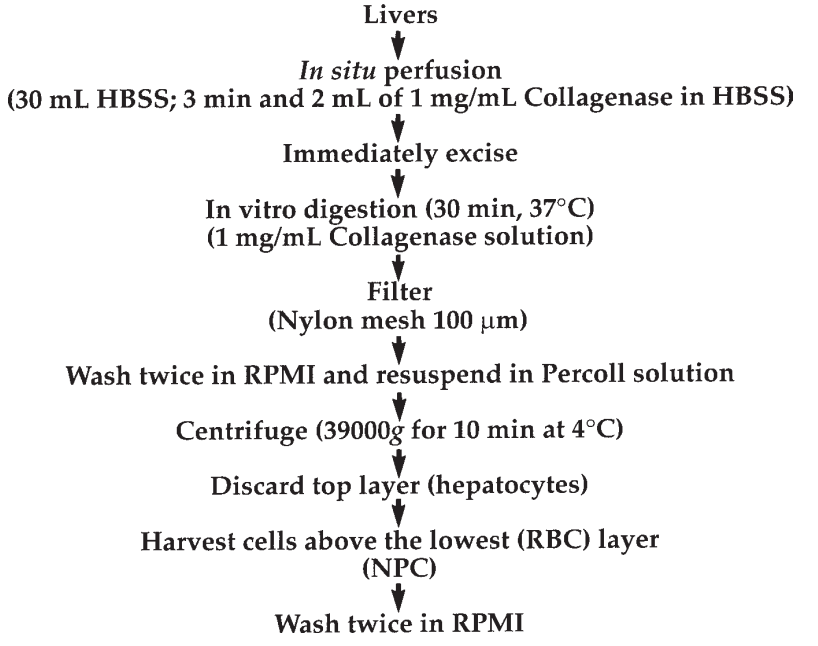

Fig. 1. Flow plan for the isolation of liver NPC.

stimulators. The underlying mechanism is unclear, but may be related to nitric oxide production by the DC.

4. This fraction of enriched DC should consist of approx 40–50% mononuclear cells with the features of DC: irregular-shape, eccentric nuclei, abundant cytoplasm, few cytoplasmic granules, and prominent spiky, veil- or sheet-like cytoplasmic projections (**Fig. 2**) (**13**). Their cell surface MHC class II expression is markedly upregulated *(2)*. They are Thy1.2$^-$, CD3$^-$, CD4$^-$, CD8a$^-$, B220$^-$, HSA$^+$, CD32$^-$, F4/80$^-$, CD44^{dim}, and show moderate expression of CD11b and DEC-205 (NLDC 145), but are 33D1^{dim} *(1)*. These phenotypic features are similar to those of immature mouse DC isolated from other nonlymphoid tissues *(19–21)*. Compared with freshly-isolated liver NPC or spleen cells, the liver-enriched DC after overnight culture are highly active in allostimulation (approx 3–4-fold more potent) (**Fig. 3**).
5. The process of selecting removes the nonadherent granulocytes, macrophages, and lymphocytes, leaving a population of cells rich in DC precursors on the plates. These will then develop into cells with a high purity (60–85%) DC progenitors.
6. After selecting, cells are fed every two days. This replenishes necessary nutrients and GM-CSF for the growth of DC.
7. Microscopically, the cells released from proliferating aggregates of GM-CSF-stimulated liver NPC display typical morphological features of DC. Many cells

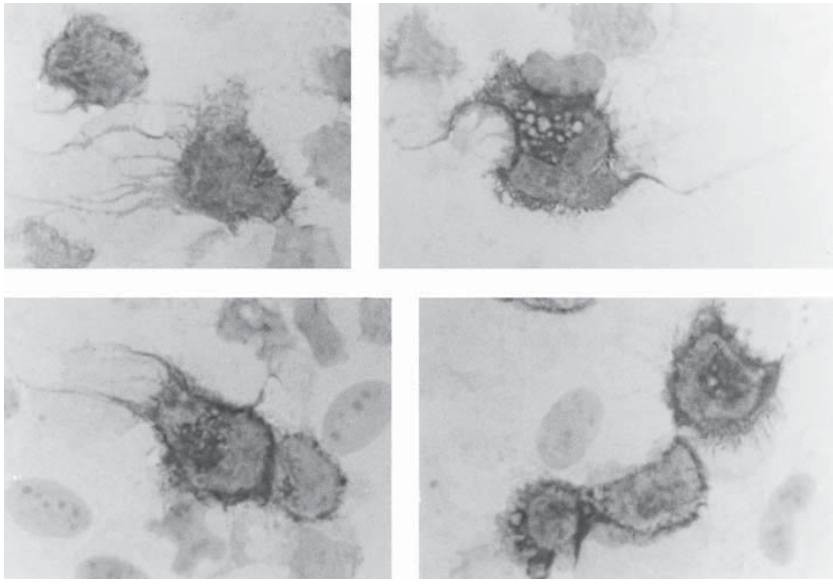

Fig. 2. Strongly MHC class II^+ cells with distinct dendritic morphology present in the nonadherent, low density fraction recovered from overnight-cultured B10.BR ($H\text{-}2^k$) mouse liver NPC. Cells were stained using anti-$I\text{-}E^k$ mAb, and avidin-biotin-peroxidase procedure. (× 1000). *Reproduced from Woo et al 1994[13] with the permission of the publishers.*

have irregular shape, eccentric nuclei, and numerous "veil"-like cytoplasmic dendrites. Flow cytometric analysis reveals that the propagated liver DC strongly express DC-associated surface antigens, including CD45, heat stable antigen, ICAM-1(CD 54), CD11b (MAC-1α), and CD44. In addition, staining of weak to moderate intensity is observed for the mouse DC-restricted markers DEC 205, 33D1, CD11c, and for F4/80 and FcγRII. The liver-derived, GM-CSF-stimulated cells express only a low level of MHC class II surface antigen molecule when compared with GM-CSF-stimulated spleen cells propagated under the same conditions. The intensity of MHC class II expression cannot be increased on these liver-derived DC by increasing the concentration of GM-CSF and/or by extending the period of culture for up to 4 wk. The low intensity of MHC class II expression on the liver-derived cell population suggests that these proliferating cells, though possessing several surface markers indicative of developing DC, are still at a phenotypically immature stage of differentiation. Further culture in GM-CSF, together with TNF-α, IFN-γ, or LPS, or culture on a "feeder layer" of irradiated, syngeneic spleen cells, does not affect significantly the expression of

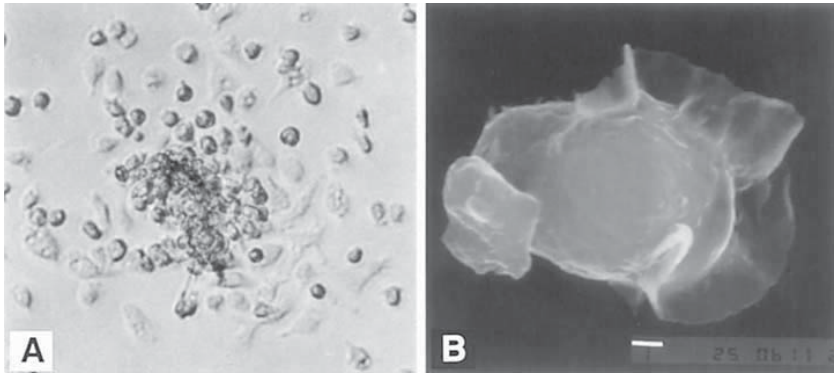

Fig. 3**.** Illustration of the development and isolation of normal mouse liver-derived DC progenitors in liquid cultures supplemented with GM-CSF. (**A**), an early aggregate of proliferating, putative DC progenitors attached to strongly-adherent macrophages, and showing typical, loosely-adherent cells that are released from the aggregates. (**B**), transmission electron micrographs of loosely-adherent cells exhibiting DC morphology released from proliferating aggregates of GM-CSF-stimulated liver-derived cells 9 d after initiation of the culture. *Reproduced from Lu et al. 1994[14] with the permission of the publishers.*

cell surface MHC class II on these "immature" liver-derived DC. They exhibit low stimulatory capacity for allogeneic T cells in MLR and thus they exhibit both the phenotype and function of immature DC ***(14)***.

8. By this method, approximately 2–5 × 10^6 DC per mouse liver can be obtained from cultures at about day 7-10.
9. Compared to cultures grown in GM-CSF alone, those maintained in the presence of IL-4 have upregulated DC restricted markers, DEC-205 and CD11c, as well as increased surface expression of MHC class II molecules, and of costimulatory molecules CD40, CD80, and CD86. The stimulatory function of these cells for T cells in MLR is increased accordingly.
10. These NPC stain 15–20% $CD11c^+$ and DEC-205^+ and have moderate allostimulatory capacity in MLR.
11. The numbers of DC isolated after overnight culture from liver NPC of Flt3 L treated mice increases 100–200-fold, compared with normal ***(17,18)***. Lower doses of Flt3 L or shorter periods of cytokine administration may be sufficient, depending on requirements. Overnight culture in RPMI-1640 complete medium yields approximately 50-70% $CD11c^+$ or DEC-205^+ cells, with high MHC class II, high CD40, CD80, and CD86, as well as high allostimulatory capacity in MLR.
12. DC are generated from GM-CSF-stimulated NPC of Flt3 L treated mice between d 4–6, compared with d 7–10 for those from untreated mice. On average, approx

10-fold increased numbers of DC can be obtained by this method, compared with those from untreated mice. Compared with normal livers, culture of liver NPC from Flt3L-treated mice in GM-CSF results in the earlier formation of proliferating cell clusters, and release of nonadherent cells (DC progenitors) ***(16)***. These findings suggest that Flt3L treatment stimulates both generation and maturation of liver DC. The surface phenotype of DC propagated from GM-CSF cultures has been detailed previously in **Note 7**. Low buoyant density cells harvested from livers of Flt3L-treated (d 4–5 in culture) and normal mice (d 6–8 in culture) exhibit a similar phenotype, which is also characteristic of DC progenitors . These cells exhibit moderate levels of DEC 205, but low CD40, CD80, and CD86 (costimulatory molecules), low/moderate MHC class II, and intercellular adhesion molecule-1 (CD54; ICAM-1), high MAC-1, and high Fcγ RII (CD32). Upregulation of DEC-205, CD40, CD80, and CD86, as well as MHC class II is seen if the cultures are grown in GM-CSF and IL-4. This is accompanied by a decrease in MAC-1 and CD32 expression. MLR shows cells with low allostimulatory capacity for T cells for those DC generated in GM-CSF, and this is increased if cells are grown in GM-CSF and IL-4 ***(16)***.

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References

1. Steinman, R. M. (1991) The dendritic cell system and its role in immunogenicity. *Annu. Rev. Immunol.* **9,** 271–296.
2. Ardavin, D., Wu, L., Li, C.-L., and Shortman, K. (1993) Thymic dendritic cells and T cells develop simultaneously in the thymus from a common precursor population. *Nature (Lond.)* **362,** 761–763.
3. Galy, A., Travis, M., Cen, D., and Chen, B. (1995) Human T, B, natural killer, and dendritic cells arise from a common bone marrow progenitor cell subset. *Immunity* **3,** 459–473.
4. Inaba, K., Inaba, M., Deguchi, M., et al. (1993) Granulocytes, macrophages, and dendritic cells arise from a common major histocompatibility complex class II-negative progenitor in mouse bone marrow. *Proc. Natl. Acad. Sci. USA* **90,** 3038–3042.
5. Banchereau, J. and Steinman, R. M. (1998) Dendritic cells and the control of immunity. *Nature* **392,** 245–252.
6. Inaba, K., Inaba, M., Romani, N., et al. (1992) Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. *J. Exp. Med.* **176,** 1693–1702.
7. Inaba, K., Steinman, R.M., Pack, M.W., et al. (1992) Identification of proliferating dendritic cell precursors in mouse blood. *J. Exp. Med.* **175,** 1157–1167.

8. Lu, L., Hsieh, M., Oriss, T. B., et al. (1995) Generation of dendritic cells from mouse spleen cell cultures in response to GM-CSF: immunophenotypic and functional analyses. *Immunology* **84,** 127–134.
9. Sallusto, F., and Lanzavecchia, A. (1994) Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor α. *J. Exp. Med.* **179,**1109–1118.
10. Romani, N., Gruner, S., Brang, D., et al. (1994) Proliferating dendritic cell progenitors in human blood. *J. Exp. Med.* **180,** 83–93.
11. Hart, D.N.J. (1997) Dendritic cells: Unique leukocyte populations which control the primary immune response. *Blood.* **90,** 3245–3287.
12. Witmer-Pack, M. D., Crowley, M. T., Inaba, K., and Steinman, R. M. (1993) Macrophages, but not dendritic cells, accumulate colloidal carbon following administration *in situ. J. Cell Sci.* **105,** 965–973.
13. Woo, J., Lu, L., Rao, A. S., Li, Y., Subbotin, V., Starzl, T. E., and Thomson, A. W. (1994) Isolation, phenotype, and allostimulatory activity of mouse liver dendritic cells. *Transplantation* **58**, 484–491.
14. Lu, L., Woo, J., Rao, A. S., et al. (1994) Propagation of dendritic cell progenitors from normal mouse liver using GM-CSF and their maturational development in the presence of type-1 collagen. *J. Exp. Med.* **179,** 1823–1834.
15. Maraskovsky, E., Brasel, K., Teepe, et al. (1996) Dramatic increase in the numbers of functionally mature dendritic cells in Flt3 Ligand-treated mice: Multiple dendritic cell subpopulations identified. *J. Exp. Med.* **184,** 1953–1962.
16. Drakes, M. L., Lu, L., Subbotin, V., and Thomson, A. W. (1997) In vivo administration of *flt3* ligand markedly stimulates generation of dendritic cell progenitors from mouse liver. *J. Immunol.* **159,** 4268–4278.
17. Qian, S., Lu, L., Fu, F., et al. (1998) Donor pretreatment with FLT-3 ligand augments anti-donor CTL, NK and LAK cell activities within liver allografts and alters the pattern of intragraft apoptotic activity. *Transplantation* **65,** 1590–1598.
18. Steptoe R. J., Fu, F., Li, W.,et al. (1998) Augmentation of dendritic cells in murine organ donors—by treatment with flt3 ligand alters the balance between transplant tolerance and immunity. *J. Immunol.* **159,** 5483–5491.
19. Schuler, G., and Steinman, R. M. (1985) Murine epidermal Langerhans cells mature into potent immunostimulatory dendritic cells in vitro. *J. Exp. Med.* **161**: 526–535.
20. Austyn, J. M., Hankins, D. F., Larsen, C. P., Morris, P. J., Rao, A. S., and Roake, J. A. (1994) Isolation and characterization of dendritic cells from mouse heart and kidney. *J. Immunol.* **152,** 2401–2410.
21. Pollard, A. M., and Lipscomb, M. F. (1990) Characterization of murine lung dendritic cells: similarities to Langerhans cells and thymic dendritic cells. *J. Exp. Med.* **172,** 159–167.

II

Isolation and Characterization of Human Dendritic Cells

9

Identification and Immunophenotypic Analyses of Peripheral Blood Dendritic Cells

Stephen P. Robinson

1. Introduction

Dendritic cells (DC) form a complex network of cells that are distributed throughout the body and whose primary function is the stimulation of antigen-specific immune responses ***(1)***. DC are derived from $CD34^+$ progenitor cells located in the bone marrow ***(2)*** and undergo a complex process of maturation as they migrate first to peripheral tissues and then to the secondary lymphoid tissue. The vascular tree provides an ideal system for the distribution of DC from where DC were first isolated in 1982 ***(3)***. It is presumed, although not proven, that these peripheral blood DC (PBDC) are migrating from the bone marrow to peripheral tissues. More recent studies have demonstrated that at least two subsets PBDC exist that have distinct phenotypic ***(4,5)***, functional ***(6–9),*** and developmental ***(10)*** characteristics. These two subpopulations of PBDC are characterized by an absence of surface markers for other lineages and the phenotypes $CD11c^-$ $CD2^-$ $CD13^-$ $CD33^{DIM}$ $HLA\text{-}DR^{++}$ and $CD11c^+$ $CD2^+$ $CD13^-$ $CD33^{Bright}$ $HLA\text{-}DR^{+++}$ respectively ***(5,6,10)***. The cellular morphology of these PBDC subsets differs from one another and from that of mature DC in that the $CD11c^-$ population possesses a lymphoid appearance and the $CD11c^+$ population possesses a monocytoid morphology ***(5,10)***. Furthermore, both subsets lack expression of both the Langerhans cell marker CD1a and the DC maturation marker CD83 and express only low levels of adhesion and costimulatory molecules CD80, CD86, CD40 suggesting that

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these cells are relatively immature DC. However, when cultured, both populations develop into cells with typical dendritic cell morphology that express high levels of adhesion and costimulatory molecules and that possess potent allostimulatory function ***(5,6,10)***.

If these two populations of cells are isolated from peripheral blood and are allowed to mature in vitro, it may be demonstrated that they belong to distinct developmental pathways ***(10)***. The $CD11c^-$ PBDC mature into cells with the phenotype $CD83^+CD1a^-CD4^+$ and do not develop expression of myeloid markers or CD11c throughout their development. In contrast, the $CD11c^+$ PBDC retain expression of the myeloid markers CD13 and CD33 and develop the phenotype $CD83^+CD1a^+CD4^-$. The $CD11c^+$ but not the $CD11c^-$ PBDC express the receptor for macrophage-colony-stimulating factor (M-CSF) and are capable of developing into macrophages if cultured in M-CSF. These distinct developmental properties are reflected in the differing functional specializations of the two subsets of cells. Although both populations possess potent allostimulatory capacity, only the $CD11c^+$ population demonstrates significant ability to take up antigen ***(10)***. Both populations of DC may secrete IL-12 and IL-10 *(unpublished observations)* but the $CD11c^-$ population produces large quantities of interferon (IFN) ***(7,8)*** and has recently been designated as the natural IFN producing cell of peripheral blood. It therefore seems that the $CD11c^-$ subset of DC is also involved in the generation of innate immune responses to viral infections and tumors. There has also been some suggestion that the $CD11c^-$ PBDC may favor the development of Th2 type immune responses whilst the $CD11c^+$ PBDC stimulate Th1 type responses ***(8,9)***.

The foregoing data support the hypothesis that the two subsets of PBDC represent distinct lineages of antigen-presenting cells. The $CD11c^-$ subsets bears a striking resemblance to plasmacytoid cell precursors of DC that may be identified adjacent to the high endothelial venules (HEV) of lymph nodes ***(11)***. Given that the $CD11c^-$ DC express high levels of CD62L ***(10,12)***, the ligand that mediates homing of naive T cells to the lymph node (HEV), it is possible that the $CD11c^-$ DC are the immediate blood precursors of lymph node plasmacytoid cells and do not migrate via the peripheral tissues and afferent lymph ***(10,11,12)***. In contrast, the $CD11c^+$ PBDC resemble the common precursor to monocytes and DC that may be identified in ex vivo culture systems ***(13,14)***. This subset of PBDC is therefore likely to migrate into the peripheral tissues where its endocytic capacity enables it to take up antigen prior to migration to the lymph nodes.

Cells with the characteristic features of DC may also be derived from other precursor cells that are present in peripheral blood. Thus, $CD34^+$ cells that exist as a trace population in normal adult blood, have the potential to develop into DC if cultured in GM-CSF and TNFα ***(15)***. The differential expression of

the cutaneous leukocyte antigen (CLA) on $CD34^+$ cells further identifies a subset of $CD34^+$ CLA^+ cells that gives rise to Langerhans cell type DC and $CD34^+$ CLA^- cells that give rise to dermal type DC ***(16)***. A further subset of circulating $CD34^+$ cells identified by their coexpression of CD14 is also capable of migrating through endothelium and giving rise to Langerhans cells under the influence of GM-CSF alone ***(17)***. It is also well established that the blood monocyte is capable of differentiating into cells with the properties of DC following culture in GM-CSF+IL-4+TNFα ***(18,19)*** or following migration through endothelial monolayers ***(20)***.

It remains to be established which of these DC precursor cells that are present in peripheral blood represent physiological precursors to DC in vivo. Furthermore, given the array of DC precursors in peripheral blood, care must be taken when using the term peripheral blood DC. This chapter focuses on the $CD11c^-$ and $CD11c^+$ subsets of PBDC and describes how they may be identified and enumerated in peripheral blood and their surface phenotype determined using three color flow cytometry. The method requires only small volumes of blood and is therefore applicable to the study of these cells in patient populations. These cells may be further studied following their purification as described in Chapter 10 of this book.

2. Materials

1. FACS buffer: phosphate-buffered saline (PBS) (Sigma, UK) supplemented by 5 m*M* EDTA (Sigma, UK), 10% fetal calf serum (FCS) (Sigma, UK,) and 0.1% sodium azide.
2. Medium. RPMI 1640 Dutch modification (Sigma, UK.) supplemented by 10% FCS, 100units/ml penicillin, 100 μg/mL streptomycin, and 100 μg/mL glutamine (Sigma UK).
3. Ficoll (Pharmacia, Sweden).
4. Heparin 25,000 U/mL.
5. Monoclonal antibodies. PE conjugated mouse anti-human-CD3 (clone SK7), -CD11c (clone KB90), -CD123 (clone 9F5), -CD14 (clone MϕP9), -CD16 (clone B73.1), -CD19 (clone 4G7), -CD34 (clone 8G12), PerCP conjugated mouse anti-human-HLA-DR (L243) (all Becton Dickinson), FITC conjugated mouse anti-human-CD11c (clone KB90) (Dako), -HLA-DR (clone L243) (Becton Dickinson). The following isotype control antibodies: PE conjugated mouse IgG_1 and IgG_{2b}, PerCP conjugated mouse IgG_{2a}, and FITC conjugated mouse IgG_1. Additional FITC conjugated mouse anti-human antibodies and isotype control antibodies should be obtained as required.
6. 50 mL Falcon tubes, 6 mL FACS tubes, and 10 mL syringes.
7. FACScan flow cytometer (Becton Dickinson).
8. CellQuest flow cytomter Analyses Software.

3. Methods

3.1. Preparation and Labeling of Cells

1. Collect 10–20 mL of peripheral blood from volunteer donors or patients into heparin 1000 U/mL (*see* **Note 1**).
2. Place the blood in a 50 mL Falcon tube and dilute with an equal volume of cold medium and mix gently (*see* **Note 2**).
3. Place 10ml of Ficoll in two 50 mL Falcon tubes. Carefully layer up to 20 mL of the peripheral blood/medium over the Ficoll taking care not to disturb the interface (*see* **Note 3**).
4. Centrifuge at 600*g* for 20 min at 4°C with no brake on the centrifuge.
5. Identify the peripheral blood mononuclear cells at the interface and carefully aspirate into a pipet.
6. Combine the harvested PBMC fractions and wash twice in 30 mL of FACS buffer.
7. Resuspend the PBMC pellet without adding buffer and count on a hemocytometer. Adjust the concentration to 25×10^6 cells/ml by adding an appropriate volume of FACS buffer (*see* **Note 4**).
8. Prepare 20 µL aliquots (equivalent to 5×10^5 cells) of PBMC in 6 mL FACS tubes and add antibodies as described in **Steps 9–11** below (*see* **Note 5**).
9. For identification and enumeration of both $CD11c^+$ and $CD11c^-$ subsets of PBDC add the following antibody combinations to the aliquots .

   Aliquot a. No antibody.
   Aliquot b. FITC conjugated anti-HLA-DR.
   Aliquot c. PE conjugated anti-CD3.
   Aliquot d. PerCP conjugated anti-HLA-DR.
   Aliquot e. PE isotype control antibody, FITC isotype control antibody, PerCP isotype control antibody.
   Aliquot f. PE conjugated anti-CD3, anti-CD14, anti-CD16, anti-CD19, anti-CD34, PerCP conjugated anti-HLA-DR, FITC conjugated anti-CD11c (*see* **Note 6**).

   With the exception of anti-CD19 (where 10 µL of antibody should be added) 5 µL of each antibody is sufficient (*see* **Note 7**). Mix the tubes well and incubate on ice for 30 min.
10. For the immunophenotypic analyses of the $CD11c^-$ subset of PBDC add antibodies as described for aliquots a.–e. in **step 9** above and additionally prepare one aliquot for each surface antigen to be studied as follows.
    Aliquot g–*x.* PE conjugated anti-CD11c, anti-CD3, anti-CD14, anti-CD16, anti-CD19, anti-CD34, PerCP conjugated anti-HLA-DR and one of a panel of FITC conjugated antibodies against the surface antigen of interest. Mix the tubes well and incubate on ice for 30 min.
11. For the immunophenotypic analyses of the $CD11c^+$ subset of PBDC add antibodies as described for aliquots a–e in **step 9** above and additionally prepare one aliquot for each surface antigen to be studied as follows.

Aliquot g–*x*. PE conjugated anti-CD123, anti-CD3, anti-CD14, anti-CD16, anti-CD19, anti-CD34, PerCP conjugated anti-HLA-DR and one of a panel of FITC conjugated antibodies against the surface antigen of interest. Mix the tubes well and incubate on ice for 30 min.

12. Wash each aliquot twice in 5 mL of FACS buffer using a 400*g* centrifugation for 5 min.
13. Resuspend the cells in 1 mL of FACS buffer for immediate analyses on the flow cytometer (*see* **Note 8**).

3.2. Acquisition of Flow Cytometric Data

1. Identify the lymphocyte and monocyte populations on the flow cytometer using the forward scatter (FSC)/side scatter plots (SSC). Adjust the gain in the FSC and SSC channels so that the cell populations are centralized on the dot plot and draw a gate around the cells that excludes debris and platelets (**Fig. 1A**) (R1) (*see* **Note 9**). All subsequent acquisition and analyses on the flow cytometer should be performed on this region of cells.
2. Using aliquot e adjust the gain in each channel on the cytometer so that the mean fluorescence intensity of the isotype control is < 10 in each channel.
3. Using aliquots b–d) in turn, compensate the cytometer appropriately (*see* **Note 10**).
4. Using aliquot f identify the PBDC as the PE negative (lineage negative), PerCP positive (HLA-DR positive) population of cells on a dot plot (**Fig. 1B**) (R2) (*see* **Note 11**). If the PBDC are readily identified, acquire sufficient events on the flow cytometer from each aliquot of cells and store data for later analyses (*see* **Note 12**).
5. Pass each aliquot through the cytometer and acquire sufficient events for later analyses.

3.3. Analyses of Flow Cytometric Data

1. To enumerate the total number of PBDC use data acquired from aliquot f. Create a dot plot of PE labelling against PerCP labeling and identify the PBDC as the PE negative PerCP positive population of cells (**Fig. 1B**) (*see* **Note 11**).
2. Determine the percentage of PBMC that are PBDC by drawing a region around the PBDC (**Fig. 1B**) (R2) (*see* **Note 13**).
3. To identify and enumerate the $CD11c^-$ and $CD11c^+$ subsets of PBDC use data collected from aliquot f. Identify the total PBDC population as described in **Steps 1** and **2** above. Create a dot plot of FITC (CD11c) vs PerCP (HLA-DR) labeling for the cells falling within R2 only (**Fig. 2B**). Identify the two populations of PBDC, draw regions around either and determine the percentage of $CD11c^-$ and $CD11c^+$ PBDC (*see* **Note 14**).
4. To determine the surface phenotype of the $CD11c^-$ PBDC subset use aliquots g.–*x* labeled as described in **step 10, Subheading 3.1**. Use aliquot g to identify the $CD11c^-$ PBDC in a dot plot of PE vs PerCP as the PE negative PerCP positive cells. Draw a region around this population of cells. For each aliquot g–*x* generate a histogram plot of FITC staining for the cells falling within this region

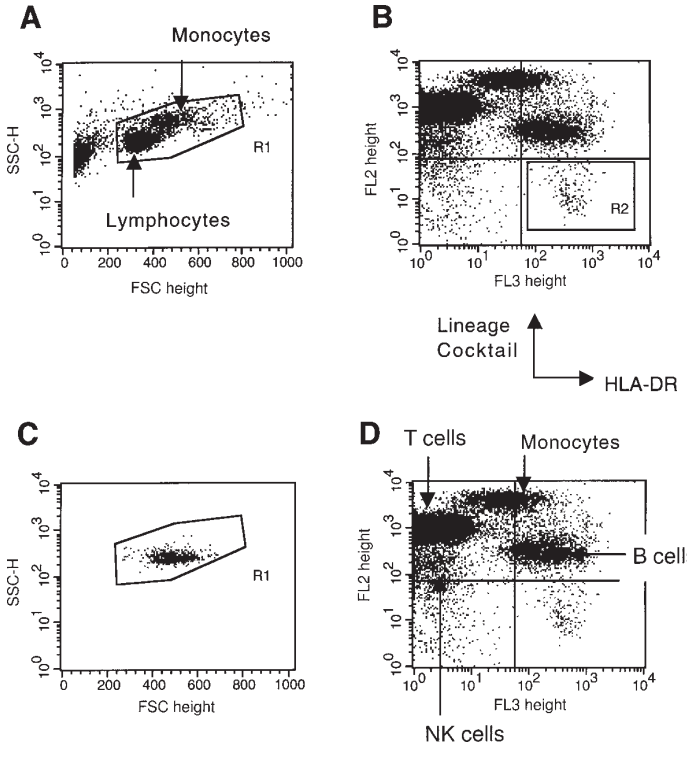

Fig. 1. Flow cytometry demonstrates the presence of DC among PBMC. (**A**) Scatter plot showing the Forward Scatter (FSC) (*x*-axis) and Side Scatter (SSC) (*y*-axis) properties of PBMC (R1). (**B**) PE conjugated lineage cocktail vs PerCP conjugated HLA-DR labelling of PBMC (R1) reveals the presence of lineage negative HLA-DR$^+$ DC (R2). (**C**) Back gating of the DC in R2 reveals their FSC/SSC properties. (**D**) This two color labeling method permits the simultaneous identification of T cells, B cells, monocytes, and NK cells.

(*see* **Note 15**). Staining with the FITC conjugated antibodies should be compared to background staining (negative control) with isotype control antibodies (*see* **Note 16**).

5. To determine the surface phenotype of the CD11c$^+$ PBDC subset use aliquots g–*x* labeled as described in **Subheading 3.1., step 11**. Use aliquot g to identify the CD11c$^+$ PBDC in a dot plot of PE vs PerCP as the PE negative PerCP positive cells. Draw a region around this population of cells. For each aliquot g–*x* generate a histogram plot of FITC staining for the cells falling within this region. Staining with the FITC conjugated antibodies should be compared to background staining (negative control) with isotype control antibodies (*see* **Note 16**).

4. Notes

1. Blood should be collected according to local institutional guidelines, appropriate local ethical committee approval, and patient consent. Healthy donors and patients with normal blood counts need only donate 10–20 mL of blood in order to both enumerate PBDC and to determine the surface expression of 10–15 different antigens on each PBDC subset. For patients with lower blood counts, a larger volume of blood will be required.
2. The cells should be processed at 4°C in order to reduce any cell activation and alterations in surface phenotype. Both medium and FACS buffer should be kept at 4°C.
3. Using 50 mL tubes for Ficoll gradients generates 1×10^6 PBMC from 1ml of blood. By using several 10–15 mL tubes, 1.5×10^6 PBMC may be obtained from each 1 mL of blood thereby enhancing recovery of cells. However, in order to achieve these yields it is important to create a clean interface when layering blood over Ficoll and to ensure that all the PBMC are aspirated from the interface (including those that adhere to the side of the tube).
4. It is important to concentrate the cells so that the final total volume during labeling is minimized. This facilitates a reduction in the amount of each antibody used and saturation of all antibody binding sites.
5. In healthy donors and patients with normal blood counts, 5×10^5 PBMC is equivalent to 5000 PBDC. These figures may be affected by certain disease states and therapies. If smaller numbers of DC are studied, the data derived may lack the required statistical power.
6. The PE conjugated lineage cocktail of anti-CD3, -CD14, -CD16, -CD19, -CD34, and PerCP conjugated anti-HLA-DR readily identifies PBDC in healthy controls. Data from our own laboratory has shown that if anti-CD34 and anti-CD16 are not included, then the lineage$^-$ HLA-DR$^+$ region is contaminated by CD34$^+$HLA-DR$^+$ progenitors and CD14$^-$CD16$^+$HLA-DR$^+$ monocytes, respectively (S. Robinson, PhD thesis, 1999).
7. The antibodies used in this method were titrated in order to determine the minimum volume of antibody required to block all antibody-binding sites on the cells (as determined by the MFI on the flow cytometer). The amount varies with the number of cells being stained and the final volume that cells are being stained in. Use of other antibodies will require similar titration.
8. For practical purposes the cells may be fixed and stored at 4°C in the dark for up to 3 d prior to analyses without affecting the results.
9. The lymphocyte and monocyte populations are readily identified on FSC/SSC plots. These cells are over 99% viable as determined by propidium iodide staining. The PBDC are located between the lymphocytes and monocytes (**Fig. 1C**), but overlap both of these populations on the FSC/SSC plots.
10. It is very important to appropriately compensate the flow cytometer when using two- and three-color analyses otherwise both false negative and false positive results may be obtained. A full description of compensating a flow cytometer is beyond the scope of this chapter. However, the operator should be experienced in the use of a flow cytometer or have access to a skilled flow cytometrist.

11. In almost all healthy subjects studied, the PBDC are readily identified as a discrete population of cells in the lower right quadrant of a lineage cocktail vs HLA-DR dot plot (**Fig. 1B**). In practice the PBDC are not truly lineage negative as they show some labeling with the lineage cocktail of antibodies above background levels. This is due to a low level expression of CD34 on both subsets of PBDC and a low level expression of CD14 on the $CD11c^+$ subset of PBDC. However, this degree of labeling does not prevent discrimination between the lineage positive cells and the PBDC.
12. In order to enumerate the PBDC as a percentage of total PBMC data from approx 1×10^5 PBMC should be collected of which approximately $1x10^3$ will be PBDC. This is necessary due to the inherent variability of data when studying rare events. In order to derive a figure for the absolute number of PBDC circulating in blood one may relate this percentage to the absolute number of PBMC derived from the sample of blood. This however will be an underestimate of absolute cell numbers owing to the loss of cells during processing. An alternative is to perform the labeling on whole blood, lyse the red cells (with commercially available reagents such as QPrep), and then analyze the total peripheral blood white cell population by flow cytometry. Labeling of whole blood reduces cell losses during processing and will also minimize in vitro-induced changes of the surface phenotype as there is no centrifugation step. When collecting data from aliquots labeled for phenotypic analyses, it is advisable to gate on the PBDC in a lineage cocktail vs HLA-DR plot and then only collect data on approx 5000 PBDC from this gate (equivalent to 5×10^5 PBMC). If data are collected on all the PBMC, the resulting data files are massive and severely impede the subsequent analyses.
13. The CellQuest program readily provides quantitative and qualitative data on any population or region of cells identified on a dot plot.
14. The two populations of PBDC are readily identified using three-color flow cytometry. By gating on the PBDC (**Fig. 2A**), (R2) their expression of FITC conjugated CD11c (or FITC CD33) can be examined in either histogram plots (**Fig. 2A**) or in dot plots (**Fig. 2B**). Either method readily identifies two distinct populations of cells and their relative quantities may be determined using the CellQuest Analyses program.
15. By including PE conjugated anti-CD11c in the lineage cocktail, the $CD11c^+$ subset of DC are removed from the $lineage^-$ $HLA\text{-}DR^+$ quadrant of the dot plot, thereby leaving only the $CD11c^-$ PBDC in this region. Conversely, by including PE conjugated anti-CD123 in the lineage cocktail, the $CD11c^-$ subset of DC are removed from the $lineage^-$ $HLA\text{-}DR^+$ quadrant of the dot plot leaving only the $CD11c^+$ subset of PBDC in this region. The surface phenotype of the two subsets of PBDC may therefore be determined by analyzing in turn the FITC labeling of the $lineage^-$ $HLA\text{-}DR^+$ cells. In most healthy control subjects, the two subsets of PBDC may actually be observed as two distinct (or partially overlapping) populations of cells within the $lineage^-$ $HLA\text{-}DR^+$ region. When this occurs, it is then

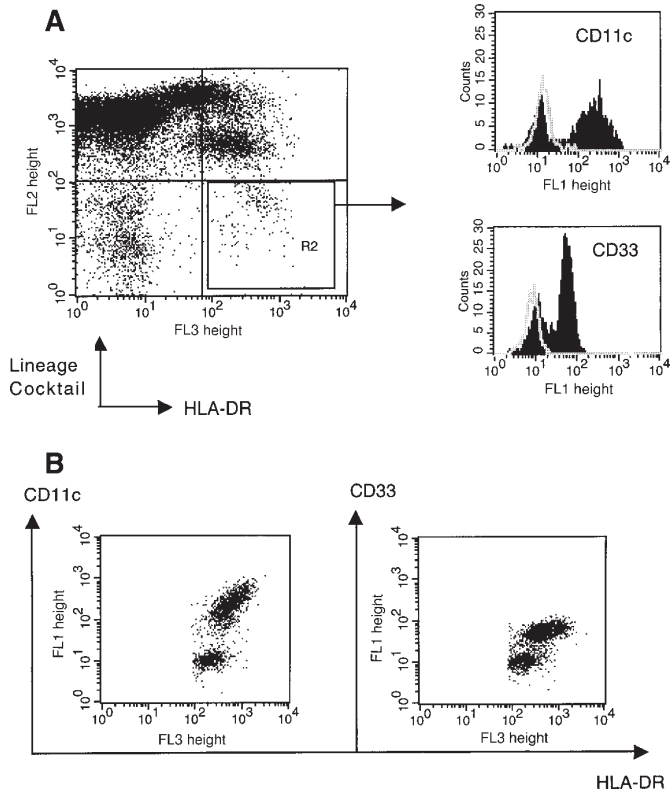

Fig. 2. (**A**) Flow cytometry scatter plot showing the fluorescence characteristics of PBMC labeled with the PE conjugated cocktail of antibodies to lineage markers (*y*-axis) and PerCP conjugated anti-HLA-DR (*x*-axis). The PBDC are identified as the lineage negative HLA-DR positive population of cells (R2). Third-color analyses of cells in R2 demonstrates two distinct populations with respect to labeling for CD11c or CD33. (**B**) Dot plots of cells in R2 labeled with either FITC CD11c of FITC CD33 also demonstrate the presence of two populations of cells.

possible to analyze the surface phenotype of the two subsets in the same aliquot of cells, thereby reducing antibody usage.

16. For phenotypic studies the ideal negative control is PBDC labeled with isotype control antibodies. If this is required, then label a separate aliquot of cells with the PE-conjugated cocktail of antibodies against lineage markers, PerCP conjugated anti-HLA-DR and FITC conjugated isotype control antibody. The PBDC may be identified in the PE vs PerCP dot plot and their labeling with isotype control antibody determined using the third color.

References

1. Banchereau, J. and Steinman, R. M. (1998) Dendritic cells and the control of immunity. *NatureI* **392,** 245–252.
2. Reid, C. D., Stackpoole, A., Meager, A. and Tikerpae, J. (1992) Interactions of tumour necrosis factor with granulocyte-macrophage colony-stimulating factor and other cytokines in the regulation of dendritic cell growth in vitro from early bipotent CD34+ progenitors in human bone marrow. *J. of Immunol.,* **149,** 2681–2688.
3. van Voorhis, W. C., Hair, L. S., Steinman, R. M. and Kaplan, G. (1982) Human dendritic cells. Enrichment and characterisation from peripheral blood. *J. Exper. Med.,* **155,** 1172–1187.
4. O'Doherty, U., Peng, M., Gezelter, S., et al. (1994) Human blood contains two subsets of dendritic cells, one immunologically mature and the other immature. *Immunol.*, **82,** 487–493.
5. Thomas, R. and Lipsky, P. E. (1994) Human peripheral blood dendritic cell subsets. Isolation and characterisation of precursor and mature antigen-presenting cells. *J. Immunol.*, **153,** 4016–4028.
6. Takamizawa, M., Rivas, A., Fagnoni, F., et al. (1997) Dendritic cells that process and present nominal antigens to naïve T lymphocytes are derived from $CD2^+$ precursors. *J. Immunol.,* **158,** 2134–2142.
7. Siegal, F. P., Kadowaki, N., Shodell, M., et al. (1999) The nature of the principal Type 1 interferon producing cells in human blood. *Science* **284,** 1835–1837.
8. Cella, M., Jarrossay, D., Facchetti, F., et al. (1999) Plasmacytoid monocytes migrate to inflamed lymph nodes and produce large amounts of type 1 interferon. *Nat. Med.* **5,** 919–923.
9. Rissoan, M. C., Soumelis, V., Kadowaki, N., et al. (1999) Reciprocal control of T helper cell dendritic cell differentiation. *Science* **283,** 1183–1186.
10. Robinson, S. P., Davies, D., English, N., Patterson, S., Knight, S. C., and Reid, C. D. L. (1999) Human peripheral blood contains two distinct lineages of dendritic cell. *Eur. J. of Immunol.,* **29,** 2769–2778.
11. Grouard, G., Rissoan, M.-C., Filgueira, L., Durand, I., Banchereau, J. and Liu, Y.-J. (1997) The enigmatic plasmacytoid T cells develop into dendritic cells with interleukin (IL)-3 and CD40-ligand. *J. of Exp. Med.,* **185,** 1101–1111.
12. Olweus, J., BitMansour, A., Warnke, R., et al. (1997) Dendritic cell ontogeny: A human dendritic cell lineage of myeloid origin. *Proc. Nat. Acad. Sci. USA* **94,** 12551–12556.
13. Caux, C., Vanbervliet, B., Massacrier, C., et al. (1996) $CD34^+$ haematopoietic progenitors from human cord blood differentiate along two independent dendritic cell pathways in response to GM-CSF + TNFα. *J. of Exp. Med.,* **184,** 695–706.
14. Szabolcs, P., Avigan, D., Gezelter, S., et al. (1996). Dendritic cells and macrophages can mature independently from a human bone marrow-derived, post-colony-forming unit intermediate. *Blood,* **87,** 4520–4530.
15. Bernhard, H., Disis, M. L., Heimfeld, S., Hand, S., Gralow, J. R. and Cheever, M. A. (1995) Generation of immunostimulatory dendritic cells from human CD34+

haematopoietic progenitor cells of the bone marrow and peripheral blood. *Cancer Res.*, **55,** 1099–1104.
16. Strunk, D., Egger, C., Leitner, G., Hanau, D. and Stingl, G. (1997) A skin homing molecule defines the Langerhans cell progenitor in human blood. *J. Exp. Med.,* **185,** 1131–1136.
17. Ferrero, E., Bondanza, A., Leone, B. E., Manici, S., Poggi, A. and Zocchi, M. R. (1998) $CD14^+$ $CD34^+$ peripheral blood mononuclear cells migrate across endothelium and give rise to immunostimulatory dendritic cells. *J. Immunol.,* **160,** 2675–2683.
18. Sallusto, F. and Lanzavecchia, A. (1994) Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumour necrosis factor alpha. *J. Exp. Med.,* **179,** 1109–1108.
19. Pickl, W. F., Majdic, O., Kohl, P., et al. (1996) Molecular and functional characteristics of dendritic cells generated from highly purified $CD14^+$ peripheral blood monocytes. *J. Immunol.,* **157,** 3850–3859.
20. Randolph, G. J., Beaulieu, S., Steinman, R. M. and Muller, W. A. (1998) Differentiation of monocytes into dendritic cells in a model of transendothelial trafficking. *Science* **282,** 480–483.

10

Purification of Dendritic Cells from Peripheral Blood

Steven Patterson, Aaron Rae, and Heather Donaghy

1. Introduction

It is well established that dendritic cells (DC) exhibit different phenotypes and functions as they progress down the developmental pathway toward interdigitating DC that stimulate T cells in the secondary lymphoid tissue *(1)*. In the blood, two populations of DC can be identified based on the expression of the β-intergrin, CD11c. For most individuals, the proportion of DC that are $CD11c^+$ ranges from 30% to 70%. The $CD11c^-$ population was originally proposed to be the precursor of the $CD11c^+$ cells, but there is now good evidence that they constitute a distinct population of DC that have a separate developmental pathway *(2,3)*. The $CD11c^+$ blood DC give rise to the Langerhans cells located in the epidermal layers of the skin and mucosal tissue, where their function is to capture and process antigens from invading pathogens for presentation. Dermal and interstitial DC are also derived from $CD11c^+$ blood DC. $CD11c^-$ DC, on the other hand, are thought to migrate directly to secondary lymphoid tissue and not to take up residence in the tissues. $CD11c^+$ and $CD11c^-$ DC were claimed to stimulate Th1 and Th2 types of immune responses, respectively, and hence were termed DC1 and DC2 *(2)*. However, other reports suggest that both cell populations can stimulate Th1 and Th2 responses *(4)*. The role of the $CD11c^-$ DC is currently unclear but they are potent producers of interferon-α *(4,5)* and could therefore be important in antiviral immunity. Elucidating the

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function of these cells and comparing them with the $CD11c^+$ population will be a major aim of DC research groups in the future. There is thus a need for techniques to purify these two cell populations.

Human blood DC constitute less than 1% of the mononuclear cell population and lack a specific cell membrane marker that would facilitate their purification. These factors have made their purification difficult and hampered progress in DC research. The discovery that large numbers of dermal type DC could be generated from $CD14^+$ monocytes by culturing in granulocyte macrophage colony-stimulating factor (GM-CSF) and interleukin-4 (IL-4) ***(6)*** has led to an explosion in DC research. Although this discovery represents an important advance in DC technology, care must be taken in extrapolating findings made from these in vitro generated cells to the in vivo situation. It is therefore important that freshly isolated DC are studied in parallel with cells derived in vitro. Furthermore, there are currently no procedures available to generate $CD11c^-$ DC in vitro and direct isolation from blood or lymphoid tissue is the only way to obtain these cells. We describe in this chapter a procedure to isolate the two populations of DC from buffy coats.

HIV infection is characterized by infection and loss of $CD4^+$ T cells. There is now evidence that blood DC and mucosal Langerhans cells are also targets for HIV. Investigations into the role of DC in the pathology of HIV infection are facilitated by an ability to purify DC directly from peripheral blood of infected patients. The procedure for separating DC from buffy coats utilizes a fluorescent activated cell sorter (FACS), but, owing to safety considerations, this technique is unsuitable for samples from HIV-infected patients. To overcome this problem, a method employing immunomagnetic beads to purify blood DC was developed. A mixed population of $CD11c^+$ and $CD11c^-$ DC can be isolated from 40–50 mL of patient blood. To date we have been unable to separate satisfactorily the two DC subpopulations, this is mainly due to the very small numbers of cells remaining after depletion of the non-DC leukocytes.

Buffy coats yield between 800 and 1300×10^6 mononuclear cells. It would be prohibitively expensive, with respect to antibody reagents, and require excessively long sorting sessions to separate DC directly by FACS. Therefore the aim of the procedure described is to deplete non-DC from buffy coat peripheral blood mononuclear cells (PBMC) as economically as possible to leave a DC-enriched fraction containing less than 100×10^6 cells, which can be purified by FACS. The DC are identified by the absence of labeling with a cocktail of mononuclear-cell lineage-specific antibodies and expression of MHC class II. These cells are then differentiated into two subpopulations based on staining for CD11c (**Fig. 1**). A similar approach is used on the HIV patient samples with a final purification using magnetic beads. There is a significant

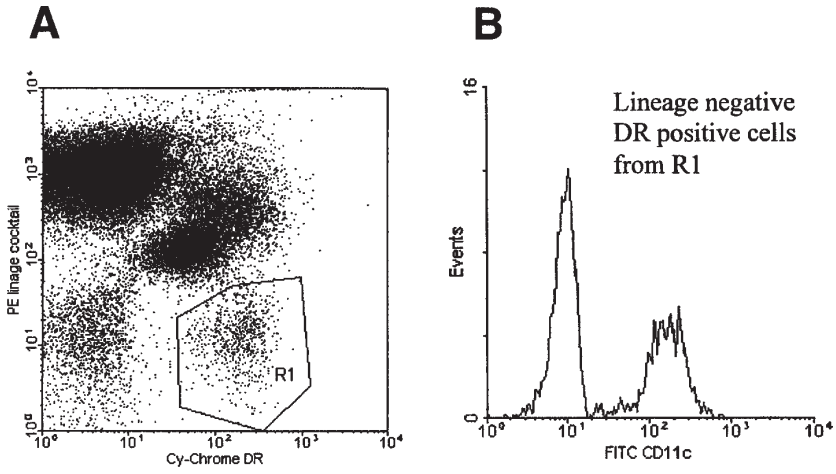

Fig. 1. Identification of CD11c$^+$ and CD11c$^-$ DC in peripheral blood. This FACS profile (A) shows freshly separated human PBMC labeled with PE-conjugated anti-CD3, CD14, CD16, and CD19 and Cy-Chrome-conjugated anti- HLA-DR. DC (shown in the R1 gated region) are identified by lack of labeling with the cocktail of PE conjugated antibodies and labeling for HLA-DR with the Cy-Chrome-conjugated antibody. The DC from R1 in (A) are differentiated into two subpopulations by labelling with a FITC-labeled anti CD11c reagent (B).

loss of cells, particularly the CD11c$^-$ fraction, during the procedure, with a typical buffy coat separation yielding up to 10^6 CD11c$^+$ DC and 2-4 x 10^5 CD11c$^-$ DC. The cells obtained are effective in stimulating the mixed leukocyte reaction and in the presence of appropriate cytokines and can be maintained in culture for 1–2 wk.

In both the buffy coat and patient blood separation procedures the majority of small lymphocytes are depleted from a PBMC preparation by centifugation over a percoll gradient. A DC-enriched fraction is formed at the percoll interface, and negligible numbers of DC are found in the small lymphocyte pellet (**Fig. 2**). Mononuclear-cell lineage specific-antibodies and immunomagnetic beads are then used to further deplete the non-DC from the DC-enriched fraction. Buffy coat DC are finally purified by FACS. Cells purified from the blood of HIV-infected patients are subjected to two rounds of immunomagnetic bead depletion, which removes 95% of the lineage positive cells leaving a mixture of DC and basophils. DC are finally selected using immunomagnetic beads after labeling for MHC class II.

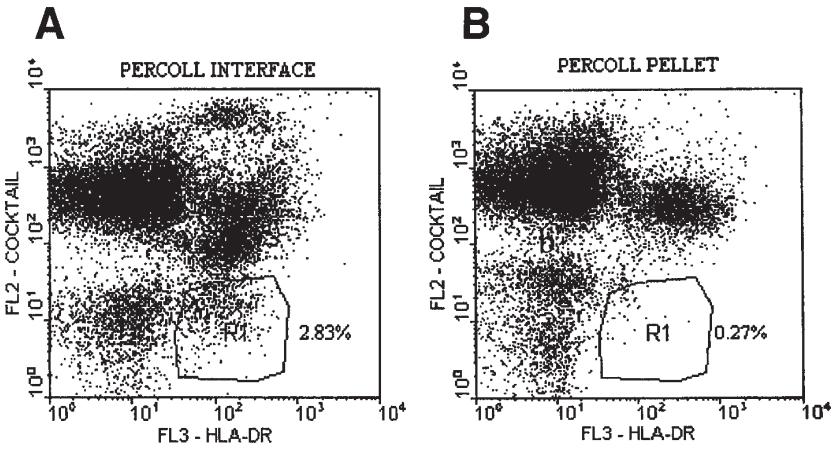

Fig. 2. The first step in the purification of DC from peripheral blood mononuclear cells is to separate the majority of the small lymphocytes from monocyte/DC population populations by centrifugation over a 50% percoll gradient. DC, identified as lineage cocktail negative HLA-DR positive cells, are retained at the Percoll interface (**A**) and are essentially absent from the pelleted cells at the bottom of the tube (**B**).

2. Materials

2.1. Culture Medium

After isolation, purified DC are cultured in RPMI-1640 bicarbonate-buffered medium (Sigma-Aldridge Poole, Dorset, UK; cat. no. R 7638) containing:

1. 10% heat-inactivated fetal calf serum (FCS) (Sigma F 9665).
2. 2 m*M* L-glutamine (Sigma G 7513).
3. Penicillin (100 U/mL) and streptomycin (0.1 mg/mL) solution (Sigma P 0781).
4. $CD11c^+$ DC are cultured in medium containing 100 ng/mL recombinant GM-CSF (R&D Systems 215-GM-005) with or without 50 U/mL recombinant tumor necrosis factor α (TNFα) (R&D Systems 210-TA-010).
5. $CD11c^-$ DC are cultured in medium containing 10 ng/mL recombinant IL-3 (R&D Systems 203-IL-010).

2.2. Isolation Medium and Gradients

1. **Subheading 2.1.**, **steps 2** and **3**. HEPES-buffered RPMI-1640 (Sigma R 5886) containing penicillin, streptomycin, and L-glutamine (concentrations as above), and 2% FCS (RPMI-HEPES-FCS) is used during the isolation procedure.

2. The following stock solutions are used to make a 50% percoll gradient: Percoll mix solution made by adding 60 mL of 10× concentrate PBS (Sigma D 1408) to 176ml of sterile distilled water. 70% percoll solution made by mixing 145 mL of percoll (Sigma P 4937) with 85 mL of percoll mix solution. Hank's balanced salt solution (HBSS) (Sigma H 9269). Prepare a 50% percoll solution by mixing 107 mL of 70% percoll with 43 mL HBSS.

2.3. Cells and Reagents

1. Buffy coats are obtained from the local blood transfusion service.
2. To make the procedure economically viable it is recommended that hybidoma cultures are established and supernatants harvested to provide monoclonal antibody reagents for cell depletions. Antibodies that label T, B, and monocytic cells are required. Hybridomas producing these antibodies are available from The American Tissue Culture Collection (ATCC). Anti-CD3 hybridomas, CRL-8001, HB-231, HB-10166; anti-CD14 hybridomas HB246, HB247, HB11363, HB11364, HB-11637, TIB-228; anti-CD20 hybridomas HB-9110, HB-9303, HB-9645, HB-11388. Hybridomas against CD16 or CD56 would also be useful, but are not available from tissue-culture-collection agencies.
3. Magnetic beads conjugated to an anti-pan mouse IgG (Dynal, 110.23) Immunomagnetic beads manufactured by PerSeptive Biosystems (cat. no. 4340 G; Framlington, MA; UK distributor Metachem Diagnostics Ltd. Piddington, Northampton, UK) may be used as an alternative.
4. Magnet (Dynal MPC-1). If beads from PerSeptive Systems are used, then a magnet that holds 50 mL tubes will be required; suitable magnets may be obtained from Polysciences Inc. (cat. no. 84102S; Warrington, PA, UK distributor Metachem Diagnostics Ltd.)
5. Phycoerythrin (PE) conjugated antibodies to human CD3, CD14, CD16, CD34, (Pharmingen, CD3, 30105X; CD14, 30545X; CD16 30625X; CD34, 34375) and PE conjugated anti-human CD19 from Becton Dickinson (34909). Cy-Chrome-conjugated anti-human HLA-DR antibody (Pharmingen 34238). Fluorescein conjugated anti-human CD11c antibody (Dako, F0713). Unconjugated antibodies; anti-human CD16 (Pharmingen 30621A) anti-human CD19 (Pharmingen 30661A), and anti-human HLA-DR (Pharmingen 34231A)
6. Depletion buffer (DB): PBS containing 4% FCS and 5 m*M* EDTA.

3. Methods

Both of the purification procedures described use magnetic beads. Either 4.5 μm diameter beads from Dynal or 1 μm diameter beads from PerSeptive Biosystems may be used. The labeling procedures are slightly different for the two types of beads. To illustrate this, DC purification from buffy coat and HIV patient blood will be described using Dynal and PerSeptive Biosystems beads, respectively.

3.1. Purification of CD11c$^+$ and CD11$^-$ DC from Buffy Coats.

Owing to screening for hepatitis B and C and for HIV, buffy coats are not released from the local blood transfusion service until the evening of the day that the samples are donated. The first two steps in the isolation procedure can be performed and then the cells stored overnight at 4ºC in RPMI-HEPES-FCS. Alternatively, blood may be collected from the transfusion service the next day.

1. Dilute blood with an equal volume of HEPES-buffered RPMI and separate the PBMC fraction by centrifugation over histopaque (Sigma H-8889) at 600 *g* for 20 min at 20°C with the centrifuge brake off.
2. Harvest the interface cells, resuspend in 50 mL of RPMI-HEPES-FCS and centrifuge for 12 min at 150*g*. Resuspend cells in 50 mL of RPMI-HEPES-FCS (*see* **Note 1**).
3. Add 4 mL of 50% percoll solution to five centrifuge tubes and carefully overlay each with 10 mL of cell suspension, taking care not to disturb the interface. Centrifuge at 300*g* for 20 min at 20°C with the brake off.
4. Harvest the cells at the interface, resuspend in 50 mL of DB, and centrifuge at 150*g* for 12 min at 20°C.
5. Pour off the supernatant and resuspend the cells in the residual buffer. Label the cells with the hydridoma supernatants (i.e., anti-CD3, CD14, and CD20) using 1 mL of supernatant per 10^8 cells (*see* **Note 2**) and incubate for 30 min at 4°C with gentle shaking in a 50 mL plastic centrifuge tube.
6. Add DB to bring the volume up to 50 mL and then centrifuge for 7 min at 350*g*.
7. Pour off the supernatant, resuspend the cells in the residual buffer, and repeat **step 6** (*see* **Note 3**).
8. Resuspend the cells in DB at 1–2 × 10^7 cells/ml.
9. Dynal beads are supplied at a concentration of 4 × 10^8 beads/mL and require washing before use. For cell depletion, use beads at a ratio of 2 beads/cell (*see* **Note 4**). Add the required number of beads to a 10 or 15 mL conical centrifuge tube and dilute to 10^8 beads/mL with DB. Place on the magnet for 2–3 min and then remove the supernatant. Remove the beads from the magnet and resuspend in the same volume of DB. Repeat the washing procedure once more and then add the labeled cell suspension to the beads and incubate for 20 min at 4°C on a slow rotator.
10. Place the cell–bead suspension in the magnet for 2–3 min and remove the unlabeled cells, which contain the DC population.
11. Wash the bead-labeled cells twice using the same volume of DB to remove any DC that have become nonspecifically associated with the magnetic beads and pool these fractions with the unlabeled cells.
12. Centrifuge for 7 min at 350*g*, remove the supernatant, and resuspend in the residual liquid.
13. Label the DC enriched fraction for 30 min on ice with the following fluorochrome conjugated antibodies: FITC-anti-CD11c, cychrome-anti-HLA-DR, PE-anti-

CD3, PE-anti-CD14, PE-anti-CD16, PE-anti-CD19, and PE-anti-CD34. Use 5 µL of each antibody per 2×10^6 cells.

14. Wash cells with PBS and resuspend cells in PBS at approximately 2-3x 10^6 cells per mL. The cells are now ready for cell sorting.
15. Treat FACS cell collection tubes overnight with 5% BSA in PBS (*see* **Note 5**).
16. Prior to collecting sorted cells, remove BSA solution from collection tubes and replace with 1 mL of HEPES RPMI medium containing 2% FCS.
17. Resuspend cells to be sorted in sterile PBS at a concentration of $2–3 \times 10^6$ cells/mL. Filter the cells through a 70 µm cell strainer to produce a single- cell suspension. There should be no free protein present (e.g., FCS) in the cells to be sorted.
18. Sort cells on a Becton Dickinson Vantage™ Cell Sorter equipped with a 448 nm argon-ion laser. A trained operator should carry out the cell sorting.
19. Use the following filters to produce optimal fluorescence signals for the fluorochromes used: FITC 530/30 BP; PE 575/26 BP; Cy-Chrome 660/20 BP.
20. Sterilize tubing and surfaces with 70% ethanol for 1 h. Sterilize the sheath tank with disinfectant. Add sterile PBS to the sterilized sheath tank. Attach a 0.22 µm Millipore filter to the sheath tank before reattaching to the machine.
21. Cool the sorter to 4°C prior to sorting.
22. Align the sorter using Fluoresbrite 3.0 µm beads (Polysciences, cat no. 17155).
23. Establish correct compensation settings using single color FITC, PE, and Cy-Chrome positive controls.
24. Gate cells on Forward vs Side Scatter, Cy-Chrome positive, PE negative, and split into FITC negative and positive subpopulations (i.e., $CD11c^-$ and $CD11c^+$ DC) (**Fig. 3**).
25. When 1.2×10^6 cells have been collected in either collection tube, it should be removed, placed immediately on ice, and replaced with a new BSA- coated collection tube (*see* **Note 6**).

3.2. Purification of the Total DC Population from the Blood of HIV-infected Patients.

All procedures should be performed in a designated category 3 laboratory by appropriately trained staff.

1. Perform **steps 1–7 in subheading 3.1.** for purification of DC from buffy coats.
2. Resuspend cells in DB at a concentration of 10^6 cells/mL in a 10 or 15 mL centrifuge tube.
3. Two cycles of bead depletion are used. The first depletion employs 200 beads/cell and the second 100 beads/cell. Beads (PerSeptive Biosystems) are supplied at a concentration of 5×10^8/mL and should be washed three times in DB. A 10 min period is required for the particles to migrate to the side of the tube adjacent to the magnet (*see* **Note 7**).
4. Add the labeled cells to the beads and incubate for 20–30 min on ice. Gently shake the tube every 5 min to ensure the cells and beads stay in suspension.
5. Place the cell suspension in the magnet for 10 min and remove unbound cells.
6. Centrifuge unbound cells for 7 min at 350*g*.

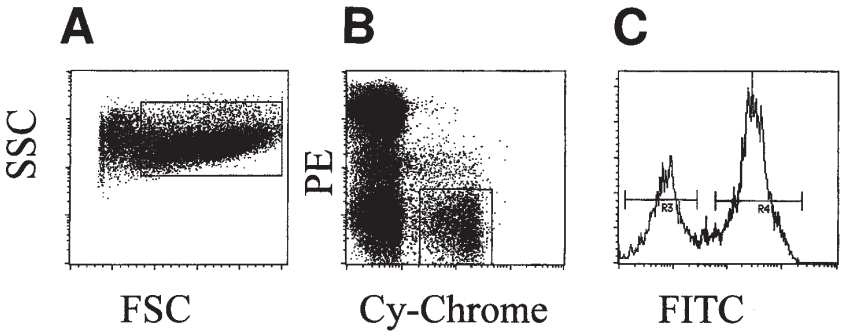

Fig. 3. FACS profile of cells immediately prior to sorting showing the gates used to sort cells. (A) FSC vs SSC profile (B) PE cocktail vs Cy-Chrome HLA-DR. The DC are identified as the PE negative Cy-Chrome positive cells. The $CD11c^-$ and $CD11c^+$ subpopulations of DC may be identified by their differential labeling with the FITC-anti CD11c (c R3 and R4, respectively) and collected.

7. Resuspend the pelleted cells at a concentration of 10^6 cells/mL and label with washed beads at a ratio of 100 beads/cell. Repeat steps 4–6. The purification of the lineage negative cells can be monitored by flow cytometry (**Fig. 4**).
8. Label the DC enriched fraction with anti HLA-DR, using 10 μL of antibody per 10^6 cells, for 20–30 min on ice.
9. Wash twice with DB.
10. Incubate labeled cells with anti-mouse immunoglobulin conjugated magnetic beads at a ratio of 50 beads/cell for 20 min at 4°C resuspending the cells every 5 min.
11. Place on magnet for 10 min, then remove supernatant containing unlabeled cells.
12. Wash the cells twice with DB.
13. Resuspend beads with attached beads in 400 μL of DNA extraction buffer (*see* **Note 8**).

4. Notes

1. The aim of this low-speed centrifugation is to remove excessive numbers of platelets, which significantly slow down acquisition of cells during cell sorting. A rather loose pellet of cells is formed, so care must be taken when pouring off the supernatant.
2. The amount of antibody used will obviously depend on its titer. It is suggested that the antibodies be titrated before use. The amount of antibody advocated in the method described is based on the observation that optimal staining of 10^6 PBMC was obtained with 10 μL of hybridoma supernatant.
3. It is important to thoroughly remove the hybridoma antibody by using large volumes in these washes. Any remaining antibody will absorb the anti-mouse conju-

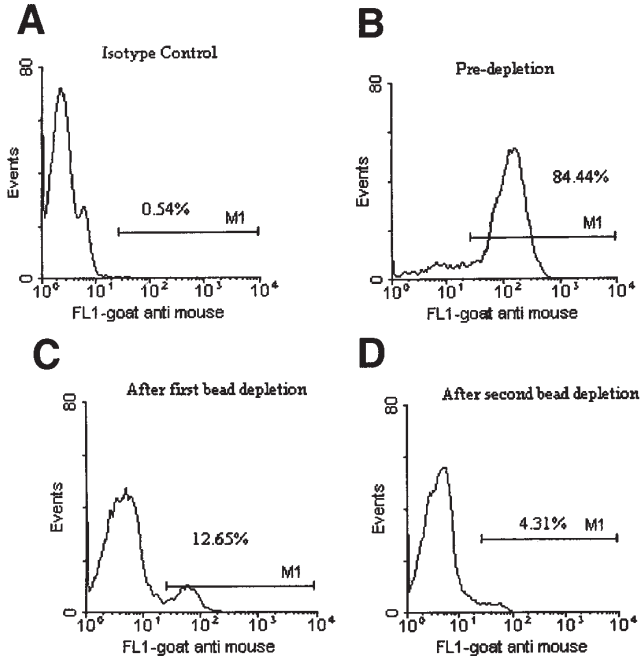

Fig. 4. Depletion of antibody cocktail labeled non-DC fraction with immunomagnetic beads supplied by PerSeptive Biosystems. (A) Isotype control labelled cells. (B) Labeled cells before depletion. (C) Labeled cells after first depletion. (D) Labeled cells after second depletion.

gated beads used in the next step thereby reducing the effectiveness of the magnetic bead depletion step.

4. The manufacturers recommend 4 beads/cell, but, as the cells will be finally purified by FACS, it is not necessary to remove all labeled cells. The main aim of this depletion is to remove as many non-DC as economically as possible, thus reducing the amounts of expensive flurochrome labeled reagents required for cell sorting.
5. During prolonged sorting experiments charges may develop in the collecting tubes and a significant percentage of sorted cells may be lost due to their adherence to the side of the tubes. This loss may be reduced by coating the collecting tubes in BSA.
6. The purity of the sorted DC populations may be checked by running a small aliquot of the collected cells back through the sorter. The collected DC are on average 94% lineage cocktail negative, HLA-DR positive. Contamination of either collected subset of DC by the other subset is less than 1%. It is important to confirm the purity, as a variety of technical problems with the cell sorter may lead to significant contamination of the collected DC.

7. If beads from PerSeptive Biosystems are used for buffy coat purifications, a bead to cell ratio of 50 should be used. It will be necessary to use 50 mL tubes to which no more than 40 mL of cell suspension should be added. It takes a longer period on the magnet, about 15 min, to deplete labeled cell in these larger tubes.
8. If cells are required for functional studies the DC may be selected using a Dynal bead which uses DNA to link the antibody and bead (CELLection™ Pan Mouse IgG Kit product no 115.19). The beads are then released from the cells by treating with Dnase.

References

1. Banchereau, J. and Steinman, R. M. (1998) Dendritic cells and the control of immunity. *Nature* **392,** 245–252.
2. Rissoan, M. C., Soumelis, V., and Kadowaki, N. E. A. (1999) Reciprocal control of T helper cell and dendritic cell differentiation. *Science* **283,** 1183–1186.
3. Robinson, S. P., Patterson, S., English, N. E., Davies, D., Knight, S. C. and Reid, C. D. L. (1999). Human peripheral blood contains two distinct lineages of dendritic cells. *Euro. J. Immunol.* **29,** 2769–2778.
4. Cella, M., Jarrossay, D., Facchetti, F., et al. (1999) Plasmacytoid monocytes migrate to inflamed lymph nodes and produce large amounts of type I interferon. *Nat. Med.* **5,** 919–923.
5. Siegal, F. P., Kadowaki, N., Shodell, M., et al. (1999) The nature of the principal type 1 interferon-producing cells in human blood. *Science* **284,** 1835–1837.
6. Sallusto, F. and Lanzavecchia, A. (1994) Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and down-regulated by tumor necrosis factor alpha. *J. Exp. Med.* **179,** 1109–1118.

11

Isolation of Human Tonsillar Dendritic Cells

Paul U. Cameron and Gianna Stent

1. Introduction

1.1. Tonsils as a Source of Human Lymphoid Dendritic Cells

Tonsillectomy remains a frequently performed operation in developed countries ensuring that tonsils are the most readily available source of human lymphoid tissue and an easily accessible source of dendritic cells (DC). Tonsil lymphoid tissue also provides a source of the different DC that are resident within the B- and T-cell microenvironments. Although an alternative model for follicular dendritic cell (FDC) ontogeny has been proposed ***(1)*** the FDC within tonsil B cell areas probably develop *in situ* from mesenchymal precursors ***(2)***. Whatever their origin, the phenotype and function of FDC ***(3)*** seem to be unrelated to the bone-marrow-derived DC that are the subject of these protocols. The precise relationship between the distinct sub-populations of the bone-marrow-derived DC within the tonsil is still not clear (*see ref.* ***4*** for review).

The early identification of bone marrow derived DC as populations distinct from monocyte/macrophages was based on studies in lymphoid tissue by Steinman and Cohn ***(5)***. Based on the physical properties and expression of surface marker on DC identified in subsequent work, DC were enriched from tonsils by Hart and McKenzie ***(6)***. The tonsil mononuclear cells were depleted of contaminating non-DC by a series of steps including rosetting with neuraminidase-treated sheep erythrocytes, density gradient separation after 1–2 d culture, and final enrichment by cell sorting. A population of potent T-cell stimulatory cells that expressed high levels of major histocompatibility complex (MHC), but did not express CD11c was isolated by these methods.

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Since then, it has become apparent from studies of the expression of surface markers in situ *(7,8)* that the commonly used isolation methods select for only a subset of the tonsil DC and that the DC cultured during the isolation procedure have different levels of expression of many of the important surface markers including costimulatory molecules *(8,9)*. It has also become apparent that DC may arise from distinct lineages *(4)*, although in humans all DC subpopulations may derive from a common myeloid precursor *(10)*. Both of the two main subsets of DC and their precurors are represented in the tonsil and may be isolated as uncultured cells using isolation methods that employ selective depletion and FACS sorting with monoclonal antibodies against lineage specific markers.

1.2. "Lymphoid" and "Myeloid" DC Populations

Two populations of DC have been isolated from mouse and characterized by differences in expression of CD8 *(11)* and by differences in ability to expand T cells *(12)*. Although originally identified in the thymus as a population derived from progenitors giving rise to lymphocytes and DC, the "lymphoid" DC are also found in peripheral lymphoid tissue where they exist in separate anatomical locations *(13)*. In spleen, the lymphoid DC appear to correspond to the interdigitating DC located within the T-cell areas and the "myeloid" lineage DC correspond to the "marginal" DC that occupy a more peripheral location and migrate into the T-cell areas during exposure to stimuli such as lipopolysaccharides (LPS) *(14)*.

There may be corresponding, functionally distinct subpopulations of DC in humans. The lineage negative $CD11c^-$ $HLA\text{-}DR^+$ populations isolated without culture from human blood *(15,16)* and human tonsil *(8)* have a plasmacytoid morphology *(8)* and appear as immature precursors that develop into typical DC after culture *(8,15,17)*. They differ from the $CD11c^+$ DC in survival after isolation, as well as in the expression of IL-3Rα (CD123) *(10)* and their dependence on IL-3 for survival *(8)*. A T-cell precursor population expressing low levels of CD2 may exist within this plasmacytoid cell population in blood *(18)*.

1.3. Phenotype of Tonsil DC

Tonsil DC identified in tissue section and as isolated cells have been designated interdigitating DC (IDC), located within the parafollicular T-cell areas; germinal center DC (GCDC), located with T-cells-within the B cell rich follicles; and plasmacytoid cell DC precursors, located around high endothelial venules. Most of the recent studies of DC in different disease processes have used DC isolated by cell sorting after negative selection to remove the contaminating B, T, and NK cells and monocyte/macrophages. Commercial antibodies designed to enumerate the lineage negative $CD11c^-$ $CD123^+$ and lineage

negative CD11c$^+$ DC subpopulations have recently become available (Lineage cocktail, CD11c, CD123, HLA-DR, Becton Dickinson Immunocytometry Systems, San Jose, CA). The use of these commercial antibodies for preparative procedures using negative selection from large numbers of tonsil mononuclear cells is impractical for many research laboratories.

Appropriate selection of a few critical monoclonal antibodies allows the removal of contaminating populations and the isolation of highly enriched and sorted DC populations at modest cost. The CD11c$^-$ cells include the IDC and are represented as populations in tonsil and blood that can be distinguished from the CD11c$^+$ DC by expression of CD45RA and IL3-R and lack of expression of CD11c, CD13, and CD33 as well as low level expression of CD11b. There have been no markers specific for DC, although IL-3R (CD123) seems to enrich for the CD11c$^-$ subpopulation of DC and has been used as the basis for positive selection ***(10)***. Other markers such as CD83 and CMRF-44 are expressed at high levels on cultured DC but poorly on directly isolated uncultured cells ***(19,20)***.

1.4. Changes During Culture that Affect the Isolation of Tonsil DC

The freshly isolated CD11c$^-$ plasmacytoid cells from tonsils do not express costimulatory molecules and are poorly stimulatory in an mixed-lymphocyte-reaction (MLR) response ***(8,10)***. These cells rapidly die by apoptosis unless provided with growth factors such as IL-3 and maturational factors such as CD40 ligand. These cells as differentiated DC may be isolated after culture of the tonsil leukocytes ***(6,21)***. The CD11c$^-$, cells whether isolated directly and cultured or isolated from cultured tonsil cells remain CD45RA$^+$ and CD11c$^-$ but express high levels of CD40 and costimulatory molecules. Both the freshly isolated CD11c$^-$ cells cultured with conditioned medium or IL-3 and CD40 ligand, and the cultured CD11c$^-$ cells have a typical DC morphology and are potent stimulators of T cells ***(8,9)***.

The initial methods for isolating DC used methods of isolation that included 1–2 d of culture ***(6,21)***. These cultured DC undergo homotypic interactions to form DC clusters as well as clustering tightly with memory CD4$^+$ T cells. This clustering has been particularly important in studies of HIV-1 where clusters are the critical sites for viral replication ***(22,23)***. For such studies sorting is required to isolate pure DC populations and differences in DC purity and inclusion of DC T-cell clusters may have contributed to the differences in reported infectivity of DC ***(24)***.

Earlier isolation methods also included monoclonal antibodies to CD11b as a marker for non-DC myeloid cells and NK cells ***(6,21)***. The expression of CD11b is variable on DC and increases with culture. This resulted in the effective exclusion of the CD11c$^+$ DC population from the preparations. The cur-

rent protocol is designed to isolate both the $CD11c^-$ and the $CD11c^+$ populations of DC as uncultured cells. A minimal lineage cocktail, including CD3, CD14, CD16, CD19, CD21, is used. Although this may not exclude all possible contaminants, the use of three-color sorting with HLA-DR and CD11c effectively excludes contaminants such as basophils (lineage negative, $CD123^+$ and $HLA\text{-}DR^-$) and NK cells (DR^- $CD11c^+$). Inclusion of CD11b in the depletion cocktail excludes granulocytes, NK, and macrophages but also removes $CD11c^+$ DC.

2. Materials

2.1. Mechanical and Enzymatic Disruption of Tonsils

1. Tonsils can be obtained from individuals undergoing elective tonsillectomies. Usually the donor will be a child or adolescent, and surgery is performed when symptoms of acute tonsillitis have abated, but acute disease should be specified as an exclusion criterion. Collect the tonsils into sterile 50 mL sample pots containing 250 μg/mL gentamicin (Delta West Ltd., Bentley, WA) and store at 4°C or on ice until processed. Tonsil samples should be used as soon as possible after collection but should be processed on the day of collection.
2. RPMI 1640. Endotoxin-free medium should be used throughout the procedure.
3. Dexoyribonucleases (cat. no. DPRF, Worthington Biomedical Corporation, Freehold, NJ).
4. Collagenase (Type II, cat. no. CLS-2, Worthington Biomedical Corporation).
5. Sterile filtered isotonic 100 m*M* EDTA. Prepare 500 m*M* EDTA by disolving 18.6g of $EDTA{\cdot}2H_2O$ in 80 mL distilled water. Add up to 2g of NaOH. Reduce the pH to 7.2 and make up to 100 mL with water. Autoclave and store at room temperature. Make up working solution by diluting stock solution 1 in 5 with pyrogen-free water. Filter-sterilize the working solution using a 0.2 μm filter (Schleisher and Schule).
6. Screen-printing nylon mesh, 70 μm pore size. This can be obtained from silk screen suppliers. Alternatives include polyester printing screen mesh and plankton netting (available at many fishing supply outlets). Although the cells are only briefly in contact with the mesh, it is worth checking for any toxic effect of the material chosen. Plankton netting but not nylon and polyester printing mesh from local suppliers reduced T-cell proliferative responses. Cut the mesh into approx 10 cm squares. Put squares into a 1 L beaker and wash with several changes of pyrogen-free water over a few hours. Cover the beaker with aluminum foil and autoclave. Store dry at room temperature.

2.2. Solutions

1. Phosphate-buffered saline (PBS) without Mg and Ca. Make 5 L of PBS by disolving 40g NaCl, 1 g KCl, 7.2 g Na_2HPO_4, and 1.2 g KH_2PO_4 in 4.5 L of pyrogen-free water. Adjust pH to 7.4 if necessary, make to 5 L, aliquot, and sterilize by autoclaving. 1× PBS may be stored at 4°C for months.

2. FACS wash. Add sterile isotonic EDTA (100 m*M*) to a final concentration of 0.5mM EDTA, and heat-inactivated human serum to a final concentration of 1% to sterile PBS (2.5 mL EDTA and 5 mL of NHS to 500 mL PBS). FACS wash can be stored at 4°C for 1–2 wk.

2.3. Density Gradient Separation

Make up metizamide (Nycomed AS, Oslo, Norway) density medium of desired density as detailed below (*see* **Notes 1** and **2**).

1. Preparation of stock metrizamide solution. Weigh a 100 mL volumetric flask and add 23.4 g of metrizamide (Nycomed AS). Fill to 100 mL with pyrogen free water, dissolve, and allow to equilibrate at 4°C. You will need to top up the water as the metrizamide dissolves and reaches 4°C. Reweigh the flask, substract the weight of the empty flask, and determine the density of the metrizamide by dividing by the volume (100 mL). e.g.,

Empty flask	57.126 g
Flask + dissolved metrizamide (4°C)	169.453 g
Dissolved metrizamide (4°C)	112.33 g
Density (4100)	1.1233

2. Filter metrizamide stock through 0.2 mm filters and aliquot into 15 mL tubes in 5 mL and 2.5 mL aliquots. Make three 5 mL aliquots for every one 2.5 mL aliquot.
3. Use the following formulas to determine the dilution factor required to attain aliquots of metrizamide with densities of 1.040, 1.065, and 1.077, starting with volumes of 5 and 2.5 mL of stock metrizamide.

   For 5 mL volumes of stock: Density = 5×[x - y]/[y - 1.006]

   For 2.5ml volumes of stock: Density = 2.5×[x - y]/[y - 1.006]

   where x = metrizamide stock density; y = density required. For example to make up 1.040, 1.065, and 1.077 densities with stock metrizamide with a density of 1.1233 g/mL

Density required	vol metrizamide	stock vol wash to add	FACS total vol	No. aliquots required	Approx vol density gradient
1.040	2.5 mL	6 mL	8.6 mL	1	1.5
1.065	5 mL	4.9 mL	9.9 mL	2	1.7
1.077	5 mL	3.2 mL	8.2 mL	1	1.4

Use cold FACS wash for dilutions and keep metrizamide on ice until required.

2.4. Sorting Reagents

1. Hybridoma supernatants against lineage specific markers. See **Table 1** for a complete list of hybridomas and commercial antibodies used in the isolation protocol. Titrate hybridoma supernatants against PBMCs to determine optimal dilutions.
2. Anti-mouse immunoglobulin (Ig) magnetic beads. Goat anti-mouse Ig microbeads (2 mL, cat. no. 484.01, Miltenyi Biotec, Bergisch Gladback, Germany).

2.5. Sorting Equipment

1. MACS, VarioMACS, or SuperMACS (Miltenyi Biotec, Bergisch Gladback, Germany).
2. MACS columns, AS, BS, or CS. (All available from Miltenyi Biotec.)
3. Fluoresence-activated cell sorting. Flow cytometer with high-speed-cell sorting capacity. e.g., FACStar plus (Becton Dickinson, Mountain View, CA) or similar.

2.6. Cell Culture

1. RH10 tissue culture medium. Prepare by adding to RPMI-1640, 10% heat inactivated human serum, 25 μg/mL gentamicin, 10 mM HEPES buffer, 2 m*M* glutamine.
2. Cytokines: IL-3, GM-CSF (kind gifts from Dr. A. Lopez. Commercial suppliers: R&D, Schering Plough).
3. Monocyte-conditioned medium (MCM). Generate supernatant by FcR panning of fresh PBMCs onto IgG-coated plates *(17)*. Make 5 mg/mL stock solution of human gamma globulin (Cappell chromatographically purified immunoglobulin, cat. no 0001-0910) by dissolving 100 mg into 20 mL of sterile ddH_2O. Add 5 mL of Ig solution to 100 mm bacteriological Petri dishes to cover the bottom of the plate. Allow to sit for 5 min and transfer the solution to a second and subsequent plates as necessary. The IgG solution should be kept sterile and may be reused after storage at 4°C. Wash the pans twice with PBS and add cells ($5–10 \times 10^7$) in RH10 medium. Unused pans may be stored after adding 5mls PBS for up to 5 d at 4°C but are best used on the day of preparation. Incubate cells on plates for 2 h at 37°C in the incubator and wash off nonadherent cells with warm PBS. Culture the FcR^+ cells in RH10 and collect the supernatant after 24 h. Store supernatants at –20°C.

3. Methods

3.1. Mechanical and Enzymatic Disruption of Tonsils

1. Divide each tonsil into four quarters using a scalpel and forceps. Remove the lymphoid tissue by teasing away from the epithelial and connective tissue capsule using the scalpel. Discard the epithelial cell layer and capsule.

Table 1. Sources for Antibodies and Hybridomas Used for Isolation of DC

Marker	Clone	Subclass	Source	cat. no.
Generated In House From Hybridomas				
CD3	OKT3	IgG2a	ATCC[a]	CRL8001
CD11b	OKM1	IgG2b	ATCC	CRL8026
CD14	3C10	IgG2b	ATCC	TIB228
CD16	3G8	IgM	ATCC	HB9114
	HNK2	IgG2a	Prof I. McKenzie, Austin Research Institute, Heidelberg, Vic. Australia. ATCC	
CD19	FMC63	IgG2a	Prof. H. Zola Flinders Medical Centre, Adelaide, S.A. Australia	
CD21	CD21	IgG2a	ATCC	HB135
Commercial Antibodies				
HLA-DR/PerCP	BDIS, San Jose, CA[b]			347364
CD11c/PE	BDIS, San Jose, CA			347637
CD14/FITC	BDIS, San Jose, CA			347493
CD3/PE	BDIS, San Jose, CA			347347
CD4/FITC	BDIS, San Jose, CA			340133
CD123	BDIS, San Jose, CA			340545
Anti-mouse IgG/FITC	Silenus, Hawthorn, Australia			DDAF
Anti-mouse IgG/PE	Silenus, Hawthorn, Australia			985052005

[a]ATCC: American Type Culture Collection
[b]BDIS: Becton Dickinson Immunocytometry Systems

2. Prepare 10–15 mL per tonsil of RPMI containing DNase 25 μg/mL and collagenase II 2 mg/mL and sterilize by passing through a 0.2 mm filter (Schleisher and Schule).
3. Cut up any large tissue fragments and resupend the cells and tissue in the medium in a 50ml polypropylene conical tube.
4. Digest the tissue at 30–37°C by placing the tube in a waterbath (a 500–1000 mL beaker containing water from a 37°C waterbath will suffice) and by repeatedly aspirating the cell suspension through a transfer pipet for 20 min. Avoid bubbling the cell suspension (*see* **Note 3**). Start with a pipet with a large orifice and progressively decrease the diameter of the pipet opening as the tissue is digested. This can be achieved by progressing through succesive graduated plastic transfer pipet (cat. no. 222–20S, Samco Scientific Incorporated, San Fernando, CA) that have been cut further down to produce pipet with smaller orifices. For maximal yield of $CD11c^+$ DC do a second round of digestion of residual fragments using fresh RPMI–DNase–collagenase.

3.2. Single-Cell Suspensions

1. After 20 min of digestion, a homogeneous mixture of cells should be present. Add isotonic EDTA to a 10 mM final concentration ([one-tenth volume of isotonic (100 mM) EDTA stock]) to the cell suspension and mix well.
2. Continue to pipet using an intact transfer pipet for a further 5 min on ice.
3. Fill tubes to 40 mL with cold FACS wash and sieve carefully through the nylon mesh filters that have been folded into a sterile funnel or into a fresh 50 mL tube.
4. Fill the tube to 50 mL with cold FACS wash and perform a cell count. Pellet cells at 300g for 10 min at 4°C, using low brake. Take care to remove virtually all of the FACS wash from the cell pellet.
5. Keep pellet on ice. All subsequent procedures should be carried out under cold conditions, i.e., use cold solutions, centrifuge at 4°C, and keep cell suspensions and pellets on ice when not in use.

3.3. Density Gradient Separation

1. Prepare metrizamide gradients by dividing the 1.077 working solution of metrizamide equally into 10 mL centrifuge tubes (approx 2 mL per tube). Keep on ice.
2. Carefully layer equal volumes of the 1.065 working solution over the 1.077 gradient.
3. Resuspend the pelleted cells using a 1.065 working solution of metrizamide and carefully layer over the 1.065 gradients at 5–10 × 10^7 cells per gradient (*see* **Note 3**). Then layer 1.04 over the cell suspension, and finally layer approximately 2 mL of RPMI media.
4. Centrifuge at 1600*g* for 10 min at 4°C with no brake.
5. Remove and discard the media and the cells at the 1.040 interface. (This interface contains some FDC and epithelial cells.)
6. There will be an interface at both the top and bottom of the fraction that initially contained the loaded cells. The DC are distributed in the both upper and lower 1.065 interfaces. Collect both fractions from each of the gradients and pool into 50 mL centrifuge tubes.
7. Fill centrifuge tubes with FACS wash, count cells, and pellet at 600*g* for 10 min at 4°C with a low brake. Do not load more than 15 mL of cell suspension from the gradient into each 50 mL tube.
8. If lymphocytes are required, then pool the high density cells from the interface above the 1.077 metrizamide.

3.4. Enrichment of DC by Negative Selection and MACS Sorting

1. Transfer approx 4 × 10^8 low-density cells to a 14 mL polypropylene centrifuge tube, pellet the cells, and resuspend in 2.5 mL of a cocktail of hybridomas (anti-CD3, CD14, CD16, CD19, CD21).
2. Incubate for 45 min on ice.
3. Pellet cells by centrifugation and wash once with cold FACS wash. Count cells and process approx 2 × 10^8 cells for MACS sorting.

4. Wash cells once more in FACS wash and resuspend the cell pellet in 100 mL of goat anti-mouse MACS beads, incubate at 4°C for 10 minutes (*see* **Note 4**).
5. Add 5 mL of anti-mouse FITC (or PE, depending on the directly conjugated antibodies to be used for FACS sorting).
6. Wash once with cold FACS wash, resuspend with 2 mL FACS wash, and load onto a freshly prepared MACS "C" column. Smaller columns can also be used for lower cell numbers, but it is important not to overload the column. (AS column = 3×10^7, BS Column = 10^8, CS column= 2×10^8 cells.)
7. Follow the manufacturer's instruction for MACS sorting; connect the column (CS) to the stopcock and 10 mL hypodermic syringe prefilled with cold FACS wash. Wash the column with cold FACS wash, 10 times the column vol (55 mL). Place the column in the magnet, attach a 21-gauge needle, and add the resuspended low-density cells to the column.
8. Wash the cells through the column while collecting the MACS negative cells. Collect about 35 mL and then perform a single back-flush and collect an additional 15 mL of MACS negative cells.
9. Pellet the cells immediately after collection, in a cold centrifuge.
10. Resuspend cells in a small volume of FACS wash and perform a cell count.
11. If required, MACS+ cells may be recovered from the column by flushing with cold FACS wash after removing the column from the magnet. If MACS columns are to be re-used commence cleaning procedure immediately (*see* **Note 5**).

3.5. FACS Sorting to Select Specific Cell Populations

The DC-enriched MACS-negative population can be further sorted into specific subpopulations based on CD11c expression.

1. Pellet MACS-negative cells.
2. Block binding of directly conjugated antibodies to the secondary antibody or MACS beads present on the indirectly stained cells by incubation in 40 μL of 10% normal mouse sera, for 10 min on ice.
3. Do not wash out the mouse sera but add 20 μL anti-CD11c-PE and 20 μL DR-PerCP and label for 45 min on ice (*see* **Note 6**).
4. Wash once and resuspend in 2 mL of cold FACS wash. Keep on ice until ready to sort.
5. Using a FACS Star Plus or equivalent sorter, the two DC populations are sorted based on the following criteria: $CD11c^-$ DC are Cocktail-FITC negative, HLA-DR-PerCP positive, CD11c-PE negative. $CD11c^+$ DC are cocktail-FITC negative; HLA-DR-PerCP positive; CD11c-PE positive (*see* **Fig. 1**).

3.6. Culture of Isolated Cells

Without cytokines the CD11c positive and negative DC survive better when cultured at 10^6/mL or greater. Culture 10^5 cells per well in 100–200 μL of RH10 media containing relevant cytokines in 96 well "U" bottom plates. Culture CD11c+ cells in RH10 media containing 200 U/mL GM-CSF (or 30% monocyte culture media, MCM). Culture CD11c negative plasmacytoid DC in

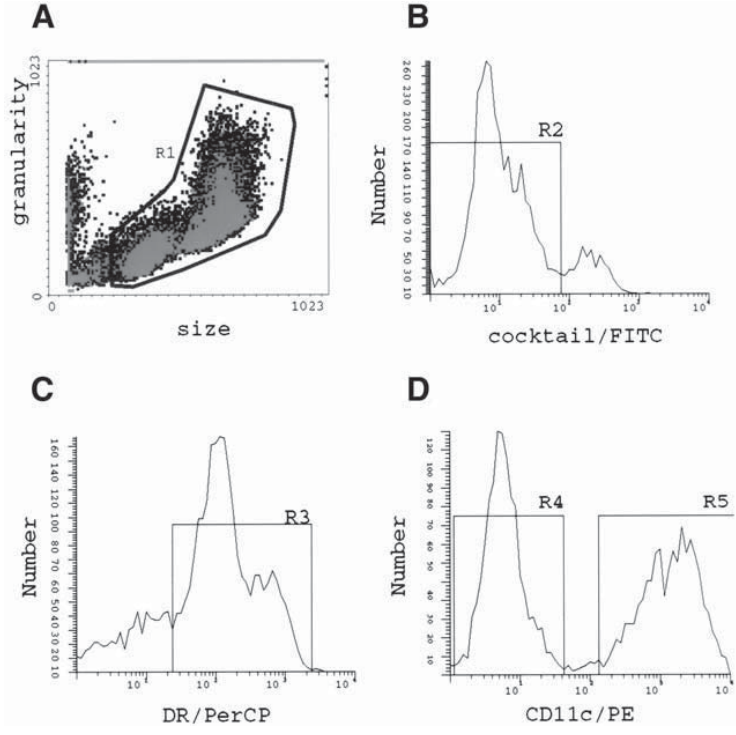

Fig. 1. Sorting profiles of tonsil DC populations. The MACS- fraction was gated according to size vs granularity profiles (R1) **(A)**. DC were then gated as the (**B**) cocktail-FITC negative (R2) and (**C**) HLA-DR-PerCP positive (R3) cells which fall within region 1 (R1). DC subpopulations were sorted as either (**D**) CD11c negative (R4) or CD11c positive (R5).

RH10 media plus 30% MCM and 1ng/ml IL-3. IL3 allows survival; however, the addition of CD40L expressing cells to the CD11c-negative DC is required for maturation of these cells ***(8)***. Addition of MCM also results in up-regulation of costimulator expression. It is necessary to titrate the granulocyte/macrophage colony-stimulating factor (GM-CSF) and IL-3 in each system, as the optimal dose required for cell survival may depend on the source of cytokines.

3.7. Checking the Purity and Phenotype of Sorted DC

The purity of the specific DC populations can be determined by labeling with a direct antibody that has the same fluorochrome as that used for the lineage specific markers. CD11b is useful for the CD11c- DC, because it will

detect most NK, granulocyte and macrophage contamination. CD68 may be used to detect contamination of the $CD11c^+$ cells with tissue macrophages; however, labeling of permeabilized cells or cells on cytospins is required for this cytoplasmic antigen. The requirement for two- or three color sorting to isolate pure populations of tonsil DC means that it is difficult to perform phenotyping on the sorted $CD11c^+$ and $CD11c^-$ DC, which will be labeled with HLA-DR or HLA-DR and CD11c. If extensive phenotyping is required, the mixed DC population should be sorted as lineage negative cells. This will result in unlabeled DC that can be used to study expression of specific markers. Phenotyping can then be performed in two or three colors using direct or indirect labeling followed by blocking with mouse serum and direct CD11c and HLA-DR labeling. The expression of the specific marker on the $CD11c^+$ and $CD11c^-$ DC can then be determined.

3.8 Purification of Cultured DC

The limitations of sorting cultured tonsil cells are detailed in **Subheading 1.4.** Lower yields of DC are obtained but the population of cells recovered is mature in that they express high levels of CD40, CD80, and CD86 and are potent T-cell stimulators. If a source of potent stimulatory DC is required, these may be isolated from cultured tonsil cells as previously described ***(6,21)***.

1. Isolate tonsil cells by collagenase DNase digestion as described above (*see* **Suheading 3.1.** and **3.2.**).
2. Separate low density cells by metrizamide density gradient (*see* **Suheading 3.3.**).
3. Culture low density cells overnight at $3\text{-}5x10^6$ /ml in RH10.
4. Harvest cells and load onto a metrizamide 1.065 density gradient.
5. MACS sort for cocktail negative cells (*see* **Subheading 3.4.**).
6. FACS sort for lineage cocktail negative HLA-DR+ cells (*see* **Subheading 3.5.**).

3.9. Purification of Other Lymph Node Cell Populations

The preceding methods may be adapted to purify $CD11c^-$ IDC, $CD11c^+$ germinal center DC, tonsillar macrophages, follicular DC, and tonsillar lymphocytes (*see* **Notes 7–11**).

4. Notes

1. Nycodenz (Nycomed AS, Oslo Norway) may be used instead of metrizamide and may have some advantage in having reduced metabolic effects on the myeloid cells ***(25)***. The density of the solutions of metrizamide or nycodenz required can be determined empirically using step density columns.
2. Testing density gradients. The metrizamide or nycodenz density gradients can be tested by loading tonsil mononuclear cells onto the top of a step gradient formed by layering several different densities (for example, 1.077, 1.070, 1.0675, 1.065, 1.0625, 1.040 metrizamide solutions). Harvest the cells at each interface and analyze each cell population for DC by FACS analysis using cocktail-FITC, HLA-

DR-PerCP, and CD11c-PE. Select density gradients to maximize the enrichment and yield of DC.

3. The maximal recovery of DC from tonsil preparations requires the minimization of loss during the various steps of the isolation procedure. In particular, the following items are important to:

    1. Adequately dissociate the tissue during the enzymatic disgestion. Frequent agitation of the tonsil tissue by pipeting up and down through a transfer pipet facilitates tissue disruption and isolation of single cells.
    2. Avoid bubbling the cell suspension as this will reduce cell viability.
    3. EDTA reduces divalent cations and further reduces the formation of clusters, which can be a significant source of loss during subsequent filtration and negative selection.
    4. After EDTA treatment it is important to maintain the cells at low temperature.
    5. Use sufficient density gradients to allow clear separation of cells. Overloading of gradients reduces the enrichment obtained in the DC enriched fractions by trapping high-density cells at the 1.065 interface.

4. One of the major disadvantages of negative selection using magnetic beads is the relatively high cost of the reagents when used at the recommended concentrations required for negative selection (10 beads per cell for dynal beads, 20 μL/10^7 cells in 100 μL of FACS wash for MACS beads). These concentrations are optimal for efficient depletion in a single round of sorting when there is no further processing. The use of subsequent sorting by FACS reduces the requirement to presort enrichment rather than highly efficient negative selection. Accordingly, we have found that the MACS bead volumes can be reduced to as few as 30–50 μL per 10^8 cells with only a modest reduction in presort purity. If reduced bead to cell ratios are used, the bead concentration should be maintained by reducing the volume of FACS wash to maintain the recommended volume ratio of 1:5 for beads to cell suspension.
5. Although the manufacturer originally supplied MACS columns as multiple-use columns, they are now only available as single use items. These columns can be reused if they are treated as originally recommended by Miltenyi for the multiuse columns. The protocol for cleaning and storing MACS columns includes:

    a. After washing positive cells from the column, immediately wash well with PBS (at least six column volumes).
    b. Wash well with double-distilled or pyrogen-free water. Connect the three way valve to the house vaccum and wash by aspirating 100–200 mL of water.
    c. Wash the column through with 70% ethanol.
    d. Airdry by leaving on the vaccum in a sterile hood until the column is completely dry.
    e. Store at 4°C with dessicant.
    f. When setting up the column for reuse fill from the bottom using a syringe of 70% ethanol attached to the side arm of the three-way tap, being careful to avoid introducing bubbles into the column.

g. Remove any alcohol from the system by replacing the syringe with one containing cold FACS wash solution and equilibrating the column by running through at least 10 column volumes of FACS wash. Be careful to avoid running the column dry and introducing air into the white spacer at the top of the column, as this will reduce the flow rates and reduce yeilds and cell purity.

Columns can be reused up to 10 times, but obtaining consistent results requires careful attention to the cleaning protocol, the storage conditions and the priming of a reused column. Single-use columns were introduced to eliminate some of the common problems associated with reusing columns including:

a. The cell suspension loaded onto the column is not filtered through 70 μm mesh and cell clumps or debris are loaded onto the column.
b. The columns are allowed to dry out before washing out cells and protein-containing solutions.
c. The columns are not dry when stored.
d. Alcohol is not adequately removed from the column during the loading procedure and affects cell viability.

6. These isolation protocol uses HLA-DR as a marker to select for DC. This does reduce the stimulatory function of DC. This can be minimized by titrating the concentration of HLA-DR to the minimum required to identifiy the HLA-DR+ cells. Alternatives include the use of addition markers during negative selection to obtain sorted unlabeled cells. For example pure populations of CD11c- DC can be obtained by sorting with a cocktail that includes CD11c, CD11b, CD56, and CD34, as well as the standard lineage markers (**Subheading 3.4.**). Sorting for the cocktail-negative cells will result in a population of unlabeled cells that can be used in functional studies.
7. Purification of IDC. *In situ* IDC express high levels of CD40 and also CD80 and CD86 ***(7)***, but only a small number of the freshly isolated CD11c- HLA-DR+ lineage negative cells express CD80, CD86, and high levels of CD40 ***(8)***. Most of the lineage negative CD11c- HLA-DR+ cells are immature and precursor plasmacytoid cells and only a small number are the mature IDC. Sorting for IDC requires processing of all the tonsil mononuclear cells and exhaustive negative selection for the lineage negative cells. The HLA-DR+ cells can then be sorted by expression of CD11c and CD40.
8. Purification of germinal center DC. The standard protocol is designed to isolate all tonsil DC and precurors within the CD11c- and CD11c+ populations. Germinal center DC have the CD11c+ phenotype and will be contained within that population. They may be sorted separately using the protocol used by Grouard et al. ***(7)***. Ensure complete digestion of tonsil tissue by increasing the collagenase to 5 mg/mL and by two rounds of collagenase/DNase digestion. Deplete low-density cells of lineage-specific markers (CD3, CD14, CD19, CD20) by negative selection and sort germinal center DC by three colour sorting as cocktail-negative (CD3, CD34, CD16, CD20, CD1a), CD11c+, CD4+ cells.

9. Purification of tonsillar macrophages. Both tonsil macrophages and DC are maximally enriched in the 1.065 density fraction of a metrizamide gradient. To obtain reasonable yields of both populations, the low- density cells must be processed together to allow final separation of the two populations by sorting. Tissue macrophages can be isolated using the preceding MACS and FACS isolation protocol by varying the antibodies used. For the MACS sort use a cocktail comprising anti-CD3, 19, 21, and 16 as described above (**Subheading 3.4.**) but omit CD14. Label the MACS negative population with cocktail-FITC, HLA-DR-PerCP, CD11c-PE, and CD14-FITC. Macrophages can be selected by FACS as the large cells (FSC high) expressing CD14-FITC, DR-PerCP, and CD11c-PE.
10. Purification of FDC. The 1.040 interface of the metrizamide density gradient is composed of epithelial cells and FDC and this fraction may be used as a source of FDC; however, the numbers of these cells is small and this method is only useful as a source of autologous cells to compare to other DC populations. To improve yield, higher-density fractions should be included in the gradient; however, as density is increased, there will be some contamination with DC, macrophages, and low-density lymphocytes.
11. Purification of tonsillar lymphocytes. Lymphocytes can be sorted by FACS directly from the high density cells. For negative selection use HLA-DR and a cocktail of antibodies to B cells, macrophages, and NK cells. CD4 and CD8 cells may be selected by positive selection using anti-CD4-FITC or anti-CD8-FITC and CD3-PE, but such cells will be activated during culture because of the CD3 crosslinking.

References

1. Szakal, A. K., Kapasi, Z. F., Haley, S. T. and Tew, J. G. (1995). A theory of follicular dendritic cell origin. *Curr. Top. Microbiol. Immunol.* **201,** 1–13.
2. Liu, Y., Grouard, G., De Bouteiller, O. and Banchereau, J. (1996). Follicular dendritic cells and germinal centers. *Int. Rev. Cytology* **166,** 139–179.
3. Schriever, F., Freedman, A.S., Freeman, G., Messner, E., Lee, G., Daley, J. and Nadler, L. M. (1989). Isolated Follicular dendritic cells display a unique antigenic phenotype. *J. Exp. Med.* **169,** 2043–2058.
4. Shortman, K. & Caux, C. (1997). Dendritic cell development: Multiple pathways to nature's adjuvants. *Stem Cells* **15,** 409–419.
5. Steinman, R. M. and Cohn, Z. A. (1973). Identification of a novel cell type in peripheral lymphoid organs of mice. I. Morphology, quantitation and tissue distribution. *J. Exp. Med.* **137,** 1142
6. Hart, D. N. J., and McKenzie, J. L. (1988). Isolation and characterization of human tonsil dendritic cells. *J. Exp. Med.* **168,** 157–170.
7. Grouard, G., Durand, I., Filgueria, L., Banchereau, J. and Liu, Y. (1996). Dendritic cells capable of stimulating T cells in germinal centres. *Nature* **384,** 364–367.
8. Grouard, G., Rissoan, M., Filgueira, L., Durand, I., Banchereau, J. and Liu, Y. (1997). The enigmatic plasmacytoid T cells develop into dendritic cells with interleukin (IL)-3 and CD40-Ligand. *J. Exp. Med.* **185,** 1101–1111.

9. Hart, D. N. J., Starling, G. C., Calder, V. L. and Fernando, N.S. (1993). B7/BB1 is a leucocyte differentiation antigen on human dendritic cells induced by activation. *Immunology* **79,** 616–620.
10. Olweus, J., Bitmansour, A., Warnke, R., Thompson, P. A., Carballido, J., Picker, L. J. and Lundjohansen, F. (1997). Dendritic cell ontogeny - a human dendritic cell lineage of myeloid origin. *Proc. Natl. Acad. Sci. USA.* **94,** 12,551–12,556.
11. Vremec, D., Zorbas, M., Scollay, R., Saunders, D.J., Ardavin, C.J., Wu, L. & Shortman, K. (1992). The surface phenotype of dendritic cells purified from mouse thymus and spleen: Investigation of CD8 expression by a subpopulation of dendritic cells. *J. Exp. Med.* **176,** 47–58.
12. Suss, G. and Shortman, K. (1996). A subclass of dendritic cells kills CD4 T cells via Fas/Fas-ligand-induced apoptosis. *J. Exp. Med.* **183,** 1789–1796.
13. Vremec, D. & Shortman, K. (1997). Dendritic cell subtypes in mouse lymhoid organs. Cross-correlation of surface markers, changes with incubation, and differences among thymus spleen and lymph nodes. *J. Immunology* **159,** 565–573.
14. Desmedt, T., Pajak, B., Muraille, E., Lespagnard, L., Heinen, E., Debaetselier, P., Urbain, J., Leo, O. and Moser, M. (1996). Regulation of dendritic cell numbers and maturation by lipopolysaccharide in vivo. *J. Exp. Med.* **184,** 1413–1424.
15. O'Doherty, U., Peng, M., Gezelter, S., Swiggard, W. J., Betjes, M., Bhardwaj, N. and Steinman, R.M. (1994). Human blood contains two subsets of dendritic cells, one immunologically mature, and the other immature. *Immunology* **82,** 487–493.
16. Thomas, R. and Lipsky, P. E. (1994). Human peripheral blood dendritic cell subsets. Isolation and characterization of precursor and mature antigen-presenting cells. *J. Immunol.* **153,** 4016–4028.
17. O'Doherty, U., Pang, M., Steinman, R. M., Cameron, P. U., Kopeloff, I., Swiggard, W., Pope, M. & Bhardwaj, N. (1993). Dendritic cells freshly isolated from human blood express CD4 and mature into typical immunostimulatory dendritic cells after culture in monocyte-conditioned medium. *J. Exp. Med.* **178,** 1067–1078.
18. Bruno, L., Res, P., Dessing, M., Cella, M. and Spits, H. (1997). Identification of a committed T cell precursor population in adult human peripheral blood. *J. Exp. Med.* **185,** 875–884.
19. Zhou, L. J., Schwarting, R., Smith, H. M. and Tedder, T. F. (1992). A novel cell-surface molecule expressed by human interdigitating reticulum cells, Langerhans cells, and activated lymphocytes is a new member of the Ig superfamily. *J. Immunol.* **149,** 735–742.
20. Fearnley, D. B., McLellan, A. D., Mannering, S. I., Hock, B. D. and Hart, D. N. J. (1997). Isolation of human blood dendritic cells using the CRMF-44 monoclonal antibody - implications for studies on antigen- presenting cell function and immunotherapy. *Blood* **89,** 3708–3716.
21. Cameron, P. U., Lowe, M. G., Sotzik, F., Coughlan, A., Crowe, S. M. and Shortman, K. (1996). Interactions of macrophage-tropic and non-macrophage-tropic isolates of HIV-1 with thymic and tonsillar dendritic cells in vitro. *J. Exp. Med.* **183,** 805–811.

22. Pope, M., Betjes, M., Romani, N., Hirmand, H., Cameron, P. U., Hoffman, L., Gezelter, S., Schuler, G. and Steinman, R. M. (1994). Conjugates of dendritic cells and memory T lymphocytes from skin facilitate productive infection with human immunodeficiency virus. *Cell* **78,** 389–398.
23. Cameron, P. U., Pope, M., Gezelter, S., Barker, J. M. and Steinman, R. M. (1994). Infection and apoptotic cell death of CD4+ T Cells during an immune response to HIV-1-pulsed dendritic cells. *AIDS Res. Hum. Retroviruses* **10,** 61–71.
24. Cameron, P., Pope, M., Granelli-Piperno, A. and Steinman, R. M. (1996). Dendritic cells and the replication of HIV-1. *J. Leukocyte Biol.* **59,** 158–171.
25. McLellan, A. D., Starling, G. C. and Hart, D. N. J. (1995). Isolation of human blood dendritic cells by discontinuous Nycodenz gradient centrifugation. *J. Immunol. Methods* **184,** 81–89.

12

Isolation of Dendritic Cells from Human Afferent Lymph

Christoph U. Brand, Nikhil Yawalkar, Robert E. Hunger, and Lasse R. Braathen

1. Introduction

The skin acts as a mechanical, physicochemical, and immunological control and defense system. The efficient operation of the skin immune system involves cytokine production and adhesion molecule expression by both infiltrating and resident cutaneous cells, and thus depends upon a system of cell homing, based on interactions between cell-surface properties and soluble mediators.

Inflammatory skin diseases demonstrate dermal and epidermal accumulation of leukocytes. There is general agreement that immunocompetent cells arrive from the blood, leaving the dermal postcapillary venules to enter the extravascular tissue of the dermis and epidermis. The lymphatic system with its vessels allows the clearance of protein and fluid from the tissue and provides the exit pathway for immunocompetent cells from the tissue. These functions play an important role in regulation of cell hydration and osmosis, as well as in immunologic responses ***(1)***. The organization of the skin lymphatics is similar to that of the vascular network with upper and lower dermal plexuses. Starting in the papillary dermis the lymphatics drain the adjacent tissue in a one-way direction toward the regional lymph nodes.

In the lower leg in normal individuals there are no anastomoses between the subfascial and epifascial lymphatic trunks ***(2)***. Thus, microsurgical cannulation of an epifascial peripheral lymph vessel of the medial superficial trunk on the lower leg permits the isolation of lymph that is derived exclusively from the skin over the medial and upper part of the foot ***(3)*** (**Fig. 1**). Because the compo-

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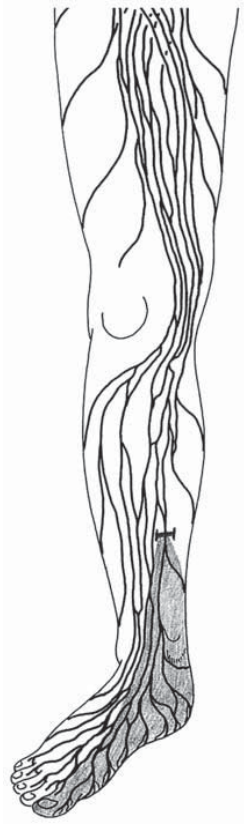

Fig. 1. The anatomic distribution of the superficial lymphatic drainage system of the leg.

sition of the afferent lymph in all probability reflects processes in the drained tissue area, investigation of such lymph may permit the study of cells and soluble mediators that are trafficking from the skin to the regional lymph nodes.

Dendritic cells (DC) constitute a widely distributed system of antigen-presenting cells, which are found in nonlymphoid tissue ("tissue dendritic cells"), in the circulation (blood, afferent lymph), and in lymphoid organs ***(4)***. The main function of dendritic cells in the skin is to take up and process antigen for subsequent presentation to T cells in the regional lymph nodes ***(5)***. The maturation state of DC varies according to their location in different cutaneous compartments, i.e., epidermis, dermis, or afferent lymph of the skin. Consequently,

they possess different morphological, phenotypic, and functional properties when isolated from these distinct sites. The lymph cannulation system described in this chapter enables the isolation of skin-derived DC that are migrating to regional lymph nodes so that they may be analyzed. The investigation of these cells in human afferent skin lymph is a unique opportunity to learn about the local immunity and especially about the induction of an immune response.

2. Materials

1. Microsurgery equipment consisting of an operation microscope as well as instruments suitable for microsurgery.
2. Specially prepared polyethylene tube (Clay Adams P60, Division of Becton Dickinson and Co, Parsippany, NJ) of about 30 cm length. This tube is drawn out at one end to a thin tip by holding it over a little flame.
3. Lidocaine 1%.
4. Methylene blue.
5. Thread 4-0 for closure of the wound and resorbable thread 6-0 for fixation of the catheter to the lymph vessel.
6. Steri strip.
7. Adhesive tape.
8. Elastic bandage.
9. Hibitane 0.1% for disinfection of the polyethylene tubes.
   0.9% NaCl .
11. Liquemin N (Roche Pharma AG, Reinach, Switzerland).
12. 10 mL sterile plastic vial (Greiner, Langenthal, Switzerland).
13. Hank's balanced salt solution (HBSS).
14. HBSS with 5% fetal calf serum (HBSS/5% FCS).
15. 12 × 75 mm round bottom polystyrene test tubes (e.g., Falcon tubes, Becton Dickinson, Lincoln Park, NJ, cat. no. 2054)
16. FITC-conjugated anti-CD1a antibody (clone HI149) (Pharmingen).
17. Unconjugated anti-CD1a antibody (clone HI149) (Pharmingen).
18. Antibodies for depletion of non-DC populations: anti-CD3 (clone HIT3a), anti-CD56 (clone B159), anti-CD19 (clone B43) (all from Pharmingen).
19. FACScan flow cytometer (Becton Dickinson).
20. FACS-Vantage cell sorter (Becton Dickinson).
21. Dynabeads M-450 sheep anti-mouse magnetic beads (Dynal, Norway).
22. Magnetic particle concentrator (Dynal).
23. Bidirectional sample mixer (Dynal).

3. Methods

3.1. Cannulation of Lymph Vessels

1. Place the leg to be operated on in a slight external rotation.

2. Shave and disinfect the lower leg.
3. Inject methylene-blue distal to the site of lymph cannulation (*see* **Note 1**).
4. Apply a field block with Lidocaine 1% (without addition of a vasoconstrictor) proximal to the site of incision.
5. Make a superficial incision of approx 2 cm on the anteromedial part of the leg about 15 cm above the ankle (*see* **Note 2**).
6. Expose the lymph vessel using a nontraumatic technique. If methylene blue has been injected, the lymphatic vessels can be detected by their blue staining.
7. Expose the vessel for a length of 1–2 cm and dissect free from fat and fibrous tissue.
8. Make a small incision in the top exposed wall of the lymph vessel.
9. Insert the tip of a polyethylene tube into the lymph vessel in a distal direction (*see* **Note 3**). Fix the tube with two ligatures around the lymph vessel and tape to the skin outside of the wound.
10. Close the wound and immerse external end of the polyethylene tube in 1 mL 0.9 % NaCl with 20 IU Liquemin added in a 10 mL sterile plastic vial (**Fig. 2**).
11. Tape the vial to the leg and then cover the whole lower leg and vial with an elastic bandage.
12. Collect lymph twice daily using a sterile technique (*see* **Notes 4–6**).

3.2. Identification of Lymph Cells by Flow Cytometry

1. Centrifuge the collected lymph at 300*g* for 5 min and discard the supernatant.
2. Wash the cells twice in phosphate-buffered saline prior to further use.
3. Resuspend the cells in 0.5-2ml HBSS/5%FCS and determine the cell count.
4. Wash the cells once by adding 5 ml HBSS/5%FCS and centrifuge at 300*g* for 5 min.
5. Resuspend lymph cells in HBSS/5% FCS at a concentration of between 500,000 to 5,000,000 cells/mL.
6. Add 100 mL of the cell suspension to each 6 mL Falcon tube.
7. Add an appropriate concentration of fluorochrome conjugated antibody to each tube and incubate on ice for 30 min (*see* **Note 7**).
8. Add 5 mL HBSS (without serum) and centrifuge at 300*g* for 5 min and discard supernatant.
9. Resuspend cells in 0.3–0.5 mL HBSS containing 0.5% formaldehyde (*see* **Note 8**).
10. Acquire and analyze the stained cells on a FACScan flow cytometer.

3.3. Purification of Dendritic Cells using ImmunoMagnetic Beads

DC may be purified from the collected afferent lymph by either immunomagnetic bead selection or cell sorting on the flow cytometer. Two methods of DC purification employing immunomagnetic beads are described below.

3.3.1. Positive Selection of CD1a$^+$ Dendritic Cells

1. Wash and count the lymph cells as described in **Subheading 3.2 steps 1–3.**

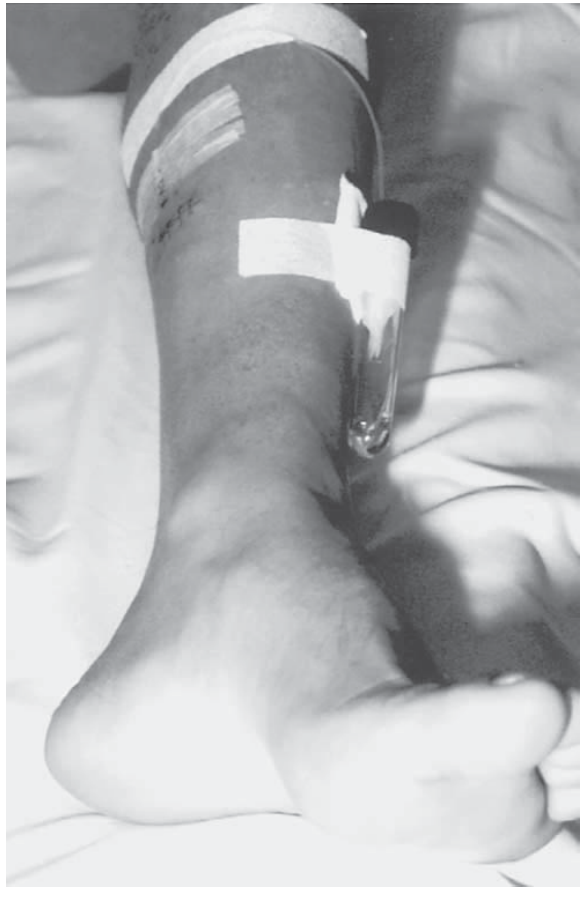

Fig. 2. The installed lymph cannulation system.

2. Resuspend the cells at a concentration of 1×10^7 cells/ml in HBSS/5% FCS and add anti-CD1a antibody according to manufacturer's guidelines (*see* **Note 7**).
3. Incubate on ice for 30 min.
4. Wash the cells in HBSS/5% FCS (at least five times the volume of anti-CD1a used) and centrifuge at 500*g* for 5 min.
5. Discard the supernatant and resuspend the cells.
6. Repeat **steps 4** and **5** twice.
7. Resuspend cells at a concentration of 1×10^7 cells per ml.
8. Add washed Dynabeads to a final concentration of $1x10^7$ beads/mL.
9. Incubate for 30 min at 4°C on a bidirectional sample mixer.

10. Resuspend cells and then place sample tube in a magnetic particle concentrator (MPC) and discard the supernatant.
11. Remove the sample tube from the MPC and resuspend the cell–magnetic bead conjugates in 5 mL of HBSS/5% FCS and centrifuge at 300*g* for 5 min.
12. Discard the supernatant and resuspend the cells in HBSS/5%FCS. The positively selected DC are now ready for further analyses (*see* **Note 9** and **10**).

3.3.2. Negative Selection of Lineage Negative Dendritic Cells

1. Wash and count the lymph cells as described in **Subheading 3.2 steps 1-3**.
2. Resuspend the cells at a concentration of 1×10^7 cells per mL in HBSS/5% FCS and add appropriate amounts of the antibodies anti-CD3, anti-CD56, and anti-CD19 (*see* **Note 7**).
3. Incubate on ice for 30 min.
4. Wash the cells in HBSS/5% FCS (at least five times the volume of antibodies used) and centrifuge at 500*g* for 5 min.
5. Discard the supernatant and resuspend the cells.
6. Repeat **steps 4** and **5** twice.
7. Resuspend cells at a final concentration of 1×10^7 per ml.
8. Add washed Dynabeads to a final concentration of $2x10^7$ beads per ml.
9. Incubate for 30 minutes at 4°C on a bidirectional sample mixer.
10. Resuspend cells and then place sample tube in an MPC and collect the supernatant that is enriched for DC.
11. Centrifuge the supernatant for 5 min at 300*g* and then discard the supernatant.
12. Resuspend the DC enriched pellet cells in HBSS/5%FCS. The negatively selected DC are now ready for further analyses (*see* **Note 9**).

4. Notes

1. Lymph vessels may be better visualized by injecting methylene-blue intracutaneously distal to the site of the incision and lymph cannulation. If methylene-blue injection is used for easier detection of the lymph vessel, a wash out phase of at least 2 or 3 d should be allowed so that the isolated cells are not damaged by the dye. However, with experience, lymph vessels can be detected even without injection of methylene blue.
2. In this location the lymph vessels drain the skin of the medial and upper part of the foot, and no anastomoses between the subfascial and the epifascial lymphatic trunks are present ***(2).***
3. Even in the case of a successful lymphcannulation, the outcome of the experiment can be hampered by thrombus formation in the catheter. Thus, before insertion into the lymph vessel, the polyethylene tube is rinsed with highly concentrated Liquemin saline solution to prevent thrombus formation. Furthermore, during the operation contamination of the polyethylene tip with blood should also be avoided. During insertion, the lumen of the polyethylene tip should not be occluded either by pressure of the pair of tweezers or by the ligature tied around the lymph vessel.

4. With this installation the volunteers are able to perform normal daily activities and lymph may be collected over a period of 1–3 wk. Since we started the lymphcannulation experiments in 1990, more than 100 volunteers have been cannulated. Cannulation of the tiny lymphatics is rather difficult, thus, only about 70% of the volunteers can be successfully cannulated ***(6)***.
5. Postoperatively, a minimal lymph flow is crucial in order to keep the draining system open. Because the lymph flow increases during walking (7–8), the volunteers have to walk for at least half an hour after completion of the operation as well as after each lymph sampling. When time course analyses are performed, it is crucial to ask the volunteers to perform the same daily activities during the study. As documented previously, the total output of cells in individual lymph samples varies substantially among the volunteers (6). Thus, throughout the experiments, the individual cell counts can easily vary from 10^2 to 6×10^5 cell/h. Because the dendritic cells constitute 2–8% of the lymph cell population, experiments dealing with this population are only possible in volunteers with a high cell output. The technique of lymphcannulation allows kinetic studies on skin immunity over periods up to 3 wk. However, owing to the significant interindividual variations in the different measured lymph values, the interpretation of the lymphcannulation results has to focus mainly on time courses and patterns (6).
6. During the experiments, especially when lymph flow is low, fibrin clots are formed at the end of the tube. These clots are easily removed by a sterile needle or forceps.
7. The amount of antibody indicated by the manufacturer may be sufficient, but to get the best results it is often necessary to titrate the antibody concentration. Afferent lymph DC may be identified by their expression of CD1a and HLA-DR and lack of expression of the lineage associated markers CD3, CD19, and CD56.
8. Formaldehyde may be omitted if the cells are analyzed immediately on a flow cytometer.
9. For some experiments the $CD1a^+$ DC will have to be detached from the Dynabeads. The DETACHaBEAD system available from DYNAL is suitable for this purpose.
10. The sampling periods required to collect sufficient lymph for analysis range from 8 to more than 10 h. Furthermore, because the temperature of the lymph collected into vials fixed directly to the skin is about 28°C, some of the collected cells will die whereas others will be activated. These factors have to be taken into account when analyzing the collected cells.

References

1. Ryan, R. J. , Mortimer, P. S., and Jones, R. L. (1986) Lymphatics of the skin. Neglected but important. *Int. J. Dermatol.* **7,** 411–419.
2. Pflug, J. J. , and Calnan J. S. (1955) The normal anatomy of the lymphatic system in the human leg. *Br. J. Surg.* **58,** 757.

3. Brand, C. U., Hunziker, T., and Braathen, L.R. (1992) Isolation of human skin derived lymph. *Arch. Derm. Res.* **284,** 123–126.
4. Teunissen, M. B. (1992) Dynamic nature and function of epidermal Langerhans cells in vivo and in vitro: a review with emphasis on human Langerhans cells. *J. Histochem.* **24,** 697–716.
5. Romani, N. and Schuler, G. (1992) The immunologic properties of epidermal Langerhans cells as a part of the dendritic cell system. *Springer Semin. Immunopathol.* **13,** 265–279.
6. Brand, C. U., and Braathen L. R. (1996) Investigation of skin derived lymph: an approach to get insight into the skin immune system. *Dermatology.* **193,** 283–288.
7. Engeset, A., Sokolowski, J., and Olszewski, W. L. (1977) Variation in output of leukocytes and erythrocytes in human peripheral lymph during rest and activity. *Lymphology* **10,** 198–203.
8. Olszewski, W. L., Engeset, A. , and Sokolowski, J. (1977) Lymph flow and protein in the normal male leg during lying, getting up, and walking. *Lymphology* **10,** 178–183.

13

Isolation of Human Skin Dendritic Cells by In Vitro Migration

Cornelia D. Richters, Matthias J. Hoekstra, John S. du Pont, Robert W. Kreis, and Eduard W. A. Kamperdijk

1. Introduction

In the human skin, various types of antigen-presenting cells (APC) are present. In the epidermis, they are identified ultrastructurally as Langerhans cells (LC) by the presence of Birbeck granules. LC are considered to belong to the family of dendritic cells (DC) that are important for the initiation of immune responses ***(1)***. In the dermis, macrophages and DC are present ***(2,3)***. The expression of CD1a molecules can be used to identify DC in the skin ***(4,5)***, because macrophages do not express this marker. In vivo, these skin DC are supposed to take up antigens penetrating in the skin. Thereafter, they migrate via the afferent lymphatics into the draining lymph nodes, where a T-cell response can be initiated ***(6,7)***. During migration, the DC mature into potent APC. Besides an increase in MHC class II expression, adhesion ***(8,9)*** and B7 co-stimulatory molecules ***(10)*** are up-regulated. Most research on skin DC has been carried out with cells isolated from enzyme digested skin ***(8–10)***. In this chapter, we describe a method to obtain DC from human skin without enzymes, by making use of their migratory capacities. The cells migrate "spontaneously" out of the skin during culture. Characterization of the cells shows that mature DC are obtained with a marker expression not influenced by enzymes.

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2. Materials

2.1. Tissue

Normal human skin is the best to use, for example, obtained from healthy women undergoing corrective plastic surgery. However, cadaveric skin obtained within 8 h after death can also be used.

2.2. Culture Medium

RPMI 1640, supplemented with 100 μg/mL streptomycin, 100 μg/mL penicillin, 2 m*M* glutamine, and 10% heat-inactivated fetal calf serum, all from Gibco, Scotland.

2.3. Reagents and Cells

1. Sterile phosphate-buffered saline (PBS).
2. Plastic Petri dishes for cell culture (Greiner).
3. Monodur gauze (100 μm; Stokvis & Smits B.V., IJmuiden, The Netherlands).
4. Dynal beads coated with goat anti-mouse IgG (M-450, Dynal, Oslo, Norway).
5. Monoclonal antibodies (Mabs) to characterize the migrating cells: anti-CD1a, CD1b, CD3, CD14, CD54, CD 58, CD80, CD86, anti HLA-DR (*see* also **Subheading 3.2.**). Anti-CD3 and CD14 Mabs can be used to deplete contaminating cells.
6. Secondary antibodies used to stain positive cells.
7. Recombinant human TNF-α (Genzyme, Cambridge, MA).
8. Allogeneic T cells isolated from human buffy coats (*see* **Subheading 3.4.**). These T cells are used in the mixed lymphocyte reaction with the isolated skin DC in order to determine their alloantigen presenting capacities.
9. 96-well microtiter plates (Greiner).
10. β-Mercapto-ethanol (Sigma).
11. ^{3}H-thymidine (25 Ci/mmol, 1 μCi/well, Amersham, UK).

3. Methods

3.1. Culture of Split Skin, Isolation of Migrated Cells

1. First rinse the thinly cut (0.3–0.4 mm, *see* **Note 1**) skin pieces in sterile phosphate buffered saline (PBS) and then put in culture medium.
2. Culture the skin with the dermal site down in plastic Petri dishes at 37°C, in a humidified atmosphere with 5% CO_2 in air. Because the skin is very thin, it will float on the medium. Sometimes contamination of bacteria or fungi may occur (*see* **Note 2**).
3. At different timepoints, for example, after 24 h or 48 h, harvest the medium containing "spontaneously" migrated cells. Depending on the purpose of the experiment, the skin DC can be harvested every day, placing the tissue into fresh medium or all together after longer culture periods.

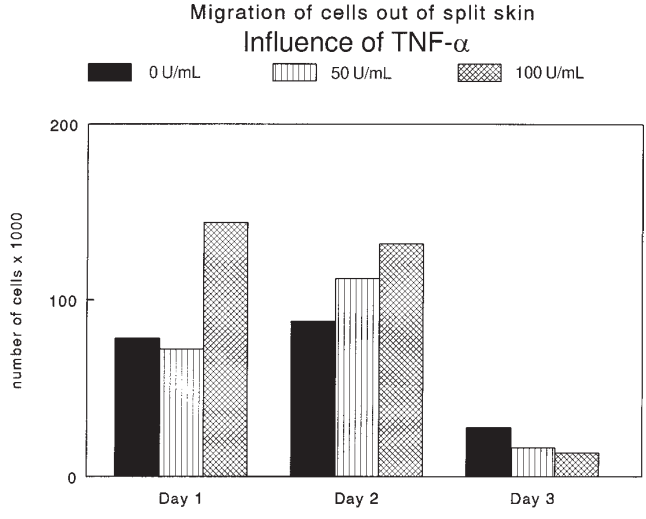

Fig. 1. One representative experiment out of four is shown.

4. Sieve the collected medium through monodur gauze to remove hairs and debris.
5. Spin the cells down and washed twice in culture medium.
6. Count the cells in a Bürker chamber and determine the viability by trypan blue exclusion.
7. Viability of the isolated cells is usually > 90%. The number of harvested cells can vary (*see* **Note 3**). Most cells are obtained in the first days of culture, up to 1.5×10^5 cells/10 cm^2 tissue. After longer culture periods (> 3 d), degeneration of the skin starts and keratinocytes will appear in the harvested medium. The cells can be used directly for phenotyping or functional assays.
8. Characterization of the cells on cytospins by using the antibodies and the sensitive staining procedure described in **Subheading 3.2.** shows that percentages of $CD1a^+$ DC in these cell populations range from 55 to 80% (**Table 1**). Other migrating cells are nonadherent macrophages and T cells (*see* **Note 4**). Further purification can be done by negative selection using goat anti-mouse coated magnetic Dynabeads after incubation with mouse anti-human antibodies against contaminating cells ($CD3^+$ T cells and $CD14^+$ macrophages).
9. Not all $CD1a^+$ cells leave the skin during culture; there are still some $CD1a^+$ cells present in cryosections of the skin after 5 d of culture, both in the epidermis and dermis.

3.2. Immunophenotyping

1. To analyze the migrated cells, the following MoAbs are useful:
   OKT6 (against CD1a, ATCC), IOT6b, (against CD1b which is expressed on dermal DC *[3,5]*, Immunotech, France), 3C10 (against CD14, ATCC), and Leu 4

Table 1
Immunophenotype of the Isolated Migrated Cells.[a]

Specificity	d 1 cells	d 2 cells	d 3 cells
CD1a	60–80	50–65	40–55
CD1b	26–30	13–16	10–13
CD3	16–40	17–45	23–45
CD14	4–9	3–10	2–9
HLA-DR/DQ	75–85	50–70	43–60

[a]The percentages are shown as a range (data from 12 experiments). Control slides, in which the specific Mab was replaced by an irrelevant antibody of the same isotype, or was omitted, did not show any reactivity. A minimum of 200 cells were examined for the presence of the different epitopes.

(against CD3, Becton Dickinson). For the detection of molecules important for the stimulation of T cells, Mabs against MHC class II molecules (9.3F10, ATCC), adhesion molecules (CD58, LFA-3 and CD54, ICAM-1, CLB, Amsterdam, The Netherlands), and B7.1 and B7.2 (Pharmingen, San Diego, CA) can be used.

2. A sensitive method to stain the cells on cytospins is the indirect APAAP method described by Cordell et al. ***(11)***.
   a. Fix the cytospins in acetone.
   b. Incubate with the specific Mab.
   c. Use rabbit-anti-mouse IgG (Dakopatts, Denmark) as the second step and APAAP immune complexes (consisting of alkaline phosphatase and monoclonal antialkaline phosphatase, Dakopatts) as the third step.
   d. Stain positive cells with a substrate containing Naphthol AS-BI phosphate (Sigma) and New Fuchsine (Gurr, BDH Ltd., Poole, UK).
   e. Add levamisole to block endogenous alkaline phosphatase activity.
3. The immunophenotype of the migrated cell population is described in **Table 1**. The majority of the cells are $CD1a^+$ DC. The number of T- cells ranges from 20–40% but there are only few nonadherent CD14-positive macrophages present (5–10%). At the bottom of the Petri dishes, some adherent macrophages can be observed. A proportion of the $CD1a^+$ cells are also positive for CD1b (*see* **Note 5**).
4. Besides a high MHC class II expression (**Figs. 2** and **3B**), the migrated cells show a high expression of adhesion and co-stimulatory molecules, indicating that by using this method mature DC are obtained (**Fig. 3**). Maturation is probably influenced by locally produced cytokines such as IL-1, TNF-α, or GM-CSF ***(14–16)***.

3.3. Electronmicroscopy

1. To study the ultrastructure of the isolated cells and for the detection of Birbeck granules:

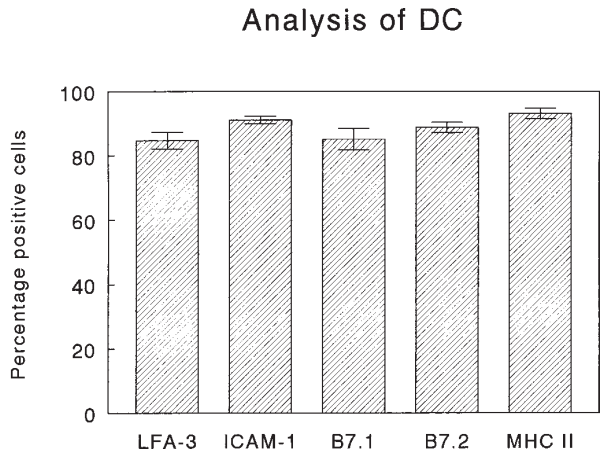

Fig. 2. Migrated skin DC show a mature phenotype.

a. Fix the isolated cells in 1.5% glutaraldehyde in Na-cacodylate (0.1 *M*, pH 7.4) for at least 1 h at room temperature.
b. Post-fix the cells in 1% OsO_4 (also in Na-cacodylate) for 1 h at 4°C.
c. Pellet in soft-agar.
d. After dehydration and embedding in araldite epon mixture (1:2), prepare ultrathin sections using an ultramicrotome.
e. Stain with lead citrate and uranylacetate ***(12)***.

2. The percentage of cells bearing Birbeck granules is only 15–20% on the first day of culture, but increases up to 40% during the following days of culture. Furthermore, most cells are irregularly shaped and show cytoplasmic veils (**Fig. 3A**).
3. To study expression of MHC class II molecules at the ultrastructural level:

a. Fix the cells in 2% paraformaldehyde and 0.2% glutaraldehyde in PBS at room temperature.
b. After 1 h, pellet the cells and resuspend in 2% paraformaldehyde at 4°C.
c. Infuse with 2.3 *M* sucrose.
d. Freeze the samples in liquid nitrogen.
e. Prepare ultrathin cryosections as described by Liou et al. ***(13)***.
f. Label with polyclonal antiserum against MHC class II molecules (a gift of Dr. H.L. Ploegh) and visualize expression using colloidal gold particles coupled to protein A.

This labeling procedure shows that the surface of the cells is strongly positive for MHC class II, a feature of mature DC (**Fig. 3B**).

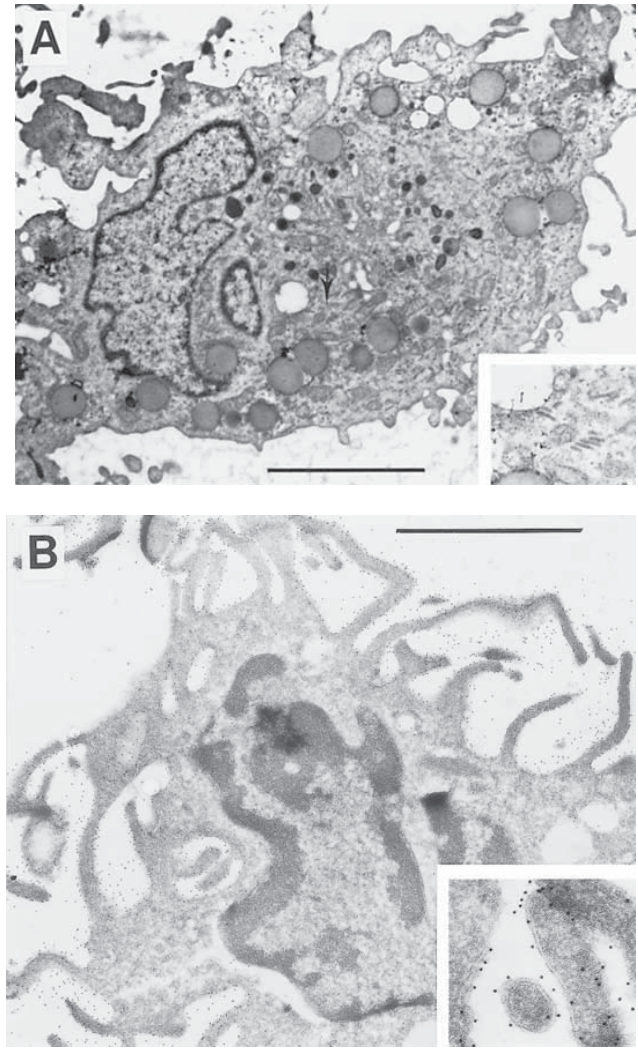

Fig. 3(**A**). Electronmicrograph of a LC migrated out of skin after 48 h of culture. Birbeck granules are present (inset) (**B**). Ultrathin cryosection of a skin DC labeled with anti-MHC class II antibodies and 10 nm gold protein-A. The plasma membrane shows a high expression of MHC class II as shown by the presence of dark grains (gold particles) but positive vesicles in the cytoplasm are also present. Bar: 2 mm. The veils are also positive for MHC class II, inset. Bar: 500 nm.

3.4. Functional Capacities of the Migrated Cells

1. An easy test to measure functional capacities of the CD1a$^+$ DC isolated by this migration method is the allogeneic mixed leukocyte reaction (MLR).
2. The MLR can be carried out as follows:
   a. Isolate T cells from human buffy coats by rosetting with neuraminidase-treated sheep erythrocytes.
   b. Separate rosettes by centrifugation on a density gradient (Lymphoprep, Nycomed, Oslo, Norway).
   c. Lyse erythrocytes with NH_4Cl.
   d. If necessary, the T cells can be cryo-preserved in medium containing 10% DMSO and 50% FSC, in liquid nitrogen until use. It is also possible to store migrated cells in this way.
   e. After irradiation with 2000 rad of ^{37}Cs gamma radiation, co-culture the isolated migrated DC for 6 d with the allogeneic responder T cells in round-bottomed 96-well microtiter plates (Nunc, Denmark), in a volume of 200 µL culture medium containing 50 µ*M* β-mercapto-ethanol.
   f. Sixteen hours before cell harvesting, add ^3H-thymidine to each well.
   g. Measure responses by means of the incorporation of the isotope, determined by a liquid scintillation counter.
   h. As the migrated cells are mature, high responses are induced, at a ratio of 1 skin DC to 100 allogeneic T cells.

See **Note 6** for discussion of potential uses of these techniques for studying immunosuppressive agents.

4. Notes

1. It is very important that the skin is thinly cut (0.3–0.4 mm). This can best be done using a dermatome. Otherwise, the skin can be fixed on a rubber plate and then cut thinly with a scalpel blade, but this technique needs practice.
2. The skin can be rinsed for half an hour in medium containing fungizone (2.5 µg/mL Amphotericin B, Bristol-Meyers Squibb) before the culture. To prevent bacterial overgrowth, gentamycin (50 µg/mL, Centafarm) can be used instead of penicillin/streptomycin.
3. Probably the thickness of the skin plays a role in the number of cells that can be obtained. The factors responsible for the "spontaneous" migration of the cells out of the skin into the medium are still unknown. Cytokines such as IL-1 and TNF-α may be responsible, produced by the keratinocytes in response to excision of the skin. When TNF-α is added to the medium, migration of cells is enhanced, as shown in **Fig. 1**.
4. Some T cells are clustered with the CD1a$^+$ DC. 21–38% of DC are clustered with one to four of the co-migrating T cells. The immunological significance of these clusters is not clear yet. In earlier studies ***(17)*** we observed that the cells in these clusters did not proliferate. It is not possible to separate the DC and T cells using PBS with 1 m*M* EDTA or by vigorous pipeting.

5. Some of the CD1a$^+$ cells are also positive for CD1b. Furthermore, not all DC-like cells bear Birbeck granules. Together, this indicates that cells migrate both from the epidermis and the dermis during culture. It is possible to separate the epidermis from the dermis before culture using trypsin or dispase but these enzymes may alter the phenotype and function of the cells. Furthermore, the dermal DC also possess strong antigen presenting capacities ***(3,5)*** in vitro. Thus these cells may play a role similar to the epidermal LC in vivo. In many experiments it will not be necessary to study the epidermal and dermal DC separately. The method described here has the advantage that the relevant DC of both compartments can be studied together. The method results in a selection of the most actively migrating skin DC that probably represents the cells migrating in vivo after application of antigen on the skin.
6. The technique is very suitable to test the effects of immunosuppressive agents such as UVB ***(18)*** or glucocorticoids on migration and function of resident skin DC, because the entrance of new DC from the circulation is not possible in this method. To examine the effects of immunosuppressives on the migration of cells out of the skin, the tissue can be divided into pieces of equal size. One part is cultured in normal medium, the other part in medium in which the test agent is present.

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References

1. Steinman, R. M. (1990) The dendritic cell system and its role in immunogenicity. *Annu. Rev. Immunol.* **9,** 271–296.
2. Richters, C. D., Hoekstra, M. J., van Baare, J., Du Pont, J. S., Hoefsmit, E. C., and M., Kamperdijk, E. W. A. (1994) Isolation and characterization of migratory human skin dendritic cells. *Clin. Exp. Immunol.* **98,** 330–336.
3. Richters, C. D., Hoekstra, M. J., van Baare, J., Du Pont, J. S., Hoefsmit, E. C . M., Kamperdijk, E.W.A. (1995) Migratory properties and functional capacities of human skin dendritic cells. *Br. J. Dermatol.* **133,** 721–727.
4. Murphy, G. F., Bhan, A. K. Sato, S., Mihm M. C., and Harrist, T. J. (1981) A new immunologic marker for human Langerhans cells. *N. Engl. J. Med.* **304,** 791–792.
5. Meunier, L., Gonzalez-Ramos, A., and Cooper K.D. (1993) Heterogeneous populations of class II MHC+ cells in human dermal cell suspensions. Identification of a small subset responsible for potent dermal antigen-presenting cell activity with features analogues to Langerhans cells. *J. Immunol.* **151,** 4067–4080.

6. Silberberg-Sinakin, I., Thorbecke, G. J., Baer, R. L., and Berezowsky, V. (1976) Antigen bearing Langerhans cells in skin, dermal lymphatics and in lymph nodes. *Cell Immunol.* **25,** 137–141.
7. Hoefsmit, E. C. M., Duijvestijn, A. M., and Kamperdijk, E. W. A. (1982) Relation between Langerhans cells, veiled cells and interdigitating cells. *Immunobiol.* **161,** 225–265.
8. Teunissen, M. B. M., Wormmeester, J., Krieg, S. R.,et al. (1990) Human epidermal Langerhans cells undergo profound morphological and phenotypical changes during in vitro culture. *J. Invest. Dermatol.* **94,** 166–173.
9. Romani, N., Lenz, A., Glessel, H., et al. (1989) Cultured Langerhans cells resemble lymphoid dendritic cells in phenotype and function. *J. Invest. Dermatol.* **93,** 600–609.
10. Symington, F. W., Brady, W., and Linsley, P. S. (1993) Expression and function of B7 on human epidermal Langerhans cells. *J. Immunol.* **150,** 1286–1295.
11. Cordell, J. L., Faline, B., Erber, W. N., et al. (1984) Immunoenzymatic labeling of monoclonal antibodies using immune complexes of alkaline phosphatase and monoclonal anti-alkaline phosphatase (APAAP complexes). *J. Histochem. Cytochem.* **32,** 219–229.
12. Pease, D. C. (ed.) (1964) *Histological techniques for electron microscopy.* Academic Press, New York.
13. Liou, W., Geuze, H. J., and Slot, J. W. (1996) Improving structural integrity of cryosections for immunogold labeling. *Histochem. Cell. Biol.* **106,** 41–58.
14. Sallusto, F., Cella, M., Danielli, C., and Lanzavecchia, A. (1995) Dendritic cells use macropinocytosis and the mannose receptor to concentrate macromolecules in the MHC class II compartment: downregulation by cytokines and bacterial products. *J. Exp. Med.* **182,** 389–400.
15. Witmer-Pack, M. D., Olivier, W., Valinsky, J., Schuler, G., and Steinman, R. M. (1987) Granulocyte/macrophage colony stimulating factor is essential for the viability and function of cultured murine epidermal Langerhans cells. *J. Exp. Med.* **166,** 1484–1498.
16. Heufler, C., Koch, F., and Schuler, G. (1988) Granulocyte/macrophage colony-stimulating factor and interleukin 1 mediate the maturation of murine epidermal Langerhans cells into potent immunostimulatory dendritic cells. *J. Exp. Med.* **167,** 700–705.
17. Richters, C. D., Hoekstra, M. J., Hoefsmit, E. C. M., Kamperdijk, E. W. A. (1995) Phenotype of cells migrated from human skin explants. *Adv. Exp. Med. Biol.* **378,** 247–251.
18. Richters, C. D., Reits, E. A. J., van Pelt, A. M., et al. (1996) Effect of low dose UVB irradiation on the migratory properties and functional capacities of human skin dendritic cells. *Clin. Exp. Immunol.* **104,** 191–197.

14

Human Intestinal Dendritic Cell Isolation

Paul Pavli

1. Introduction

Cells with the morphology of veiled cells were first described in the human intestinal lamina propria in tissue obtained from patients suffering from inflammatory bowel diseases ***(1,2)***. These cells were found in greater numbers in inflamed bowel than in normal controls. The isolation of dendritic (DC) cells from human intestinal tissue is associated with many of the problems encountered in the mouse, for example, low cell numbers and the paucity of specific cell surface markers. Particular problems are encountered using human tissues. For example, human DC are fibronectin-adherent, at least in the short term ***(3)***, so this property does not permit their separation from macrophages. In addition, the function and viability of human peripheral blood dendritic cells appears to be sensitive to the toxic effects of complement ***(4)***. The purest populations of human DC are obtained by sophisticated techniques, including cell sorting by negative selection using a broad range of monoclonal antibodies ***(5,6)***.

This chapter describes a method for the partial enrichment and characterization of human colonic DC. It involves isolation of cells from intestinal mucosal tissue and rigorous depletion of mononuclear phagocytes. Particular importance was placed on the depletion of mononuclear phagocytes because of the demonstration of a suppressive effect of mouse intestinal lamina propria macrophages, and the observation that cell populations need to be depleted of monocytes to see the rapid development of large cell aggregates in the human mixed leukocyte reaction (MLR) ***(7)***.

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2. Materials

1. Media:
   a. CMF-HBSS: Calcium- and magnesium-free Hank's balanced salt solution supplemented with penicillin (final concentration, 100 U/mL) and gentamycin (50 mg/mL).
   b. RPMI 1640 with 10% FCS.
2. Wheaton flasks.
3. Digestion enzyme mixture: RPMI 1640 (Flow Labs, Australia) containing 10% heat-inactivated FCS (CSL Melbourne), 2 U/ml purified collagenase (CLSPA type, Worthington Biochemical Corp, Freehold, NJ), 5 U/mL DNase II (Calbiochem, San Diego, CA.),100 IU/mL penicillin, 50 μg/mL gentamycin, 100 U/mL nystatin, 20 mM HEPES, 2 mM glutamine.
4. Sterile surgical gauze.
5. Buchner funnel.
6. Ficoll-Paque (Pharmacia Fine Chemicals AB, Uppsala, Sweden).
7. 0.1% trypan blue.
8. Cytocenrifuge (Shandon).
9. Diff-Quick stain.
10. Bacteriological-grade plastic Petri dishes.
11. Normal human γ-globulin (CSL, Melbourne).
12. Carbonyl iron powder (Sigma, St Louis, MO).
13. Dynal magnet (Dynal AS, Oslo, Norway).
14. Nycodenz monocytes density separation medium (density 1.068 g/mL) (Nyegaard).
15. Biotinylated sheep anti-mouse immunoglobulin (Amersham, Amersham, UK).
16. L243 hybridoma cell line (anti-class II MHC).
17. Affinity-purified rabbit anti-mouse immunoglobulin G (Cappel, Cochranville, PA).
18. Complement (Pel-Freez, Rogers, AR or Cedar Lane, Hornby, Ontario).
19. Dimethylsulfoxide (DMSO) (Malinckrodt, Paris, KY).
20. Round-bottomed 96-well plates (Linbro Flow Labs, McLean, VA).
21. Tritiated thymidine (Amersham, Surry Hills, NSW).
22. Glass-fiber discs (Whatman, Maidstone, UK).
23. Nonaqueous scintillant (0.5% 2,5-diphenyloxazole in xylene).

3. Methods

3.1 Disaggregation of Intestinal Mucosa (8–10)

1. Obtain full thickness specimens of mucosa, place in large specimen containers without fixatives, and transport to the laboratory in ice-cold HBSS. Tissue disaggregation should be initiated as soon as possible after resection.
2. Dissect the strips of mucosa (2–3 cm × 0.5 cm) from the muscular layer and incubate with continuous stirring in Wheaton flasks in CMF-HBSS containing 20 m*M* HEPES (pH 7.4) and 0.75 m*M* EDTA at 37°C for 60 min.

3. Wash the tissue in CMF-HBSS with EDTA for 30 min periods until there is no increase in particulate matter between washes (four or five washes).
4. Wash the tissue once in CMF-HBSS without EDTA.
5. Mince the tissue finely (2 × 2 mm) with crossed scalpel blades and incubate overnight with gentle stirring in the digestion enzyme mix.
6. Allow the undigested tissue fragments to settle and filter the digest through four layers of surgical gauze supported in a sterile Buchner funnel.
7. Wash the cells (400*g*, 10 min at 4°C) and resuspend in RPMI 1640 with 10% FCS.
8. Layer the cells on to a Ficoll-Paque density gradient (1.077 g/mL) and spin at 400*g* for 30 min at 4°C to isolate lamina propria mononuclear cells and remove red blood cells, neutrophils, and debris.
9. Harvest interface cells and wash twice. Assess viability by the exclusion of 0.1% trypan blue (*see* **Note 1**).
10. Prepare cytospin slides using 1×10^5 cells in a Shandon cytocentrifuge (50*g* 5 min) followed by fixation in methanol and staining in Diff-Quik.

3.2. Removal of Macrophages

Effective removal of macrophages requires at least two procedures (*see* **Notes 2–4**). The use of human γ-globulin to immobilize the macrophages permits their harvesting and use in subsequent experiments. Adherence to plastic does not remove as great a proportion of macrophages, whereas adherence to fibronectin results in populations of macrophages containing significant numbers of DC. Macrophages not adherent to γ-globulin-coated dishes are then depleted by carbonyl iron phagocytosis. The use of carbonyl iron phagocytosis as the initial step precluded comparative studies of MLR stimulation. If performed on the third day of the isolation, this procedure was not as effective in depleting the cell suspension of macrophages.

3.2.1. Binding to Human γ-Globulin: Modification of the Technique of Young and Steinman

1. Prepare Petri dishes coated with normal human γ-globulin:
   a. Add pooled human γ-globulin (5 mL; 10 mg/mL) to 100 mm dishes.
   b. Incubate for 30 min at 20°C.
   c. Wash the dishes.
2. Add the lamina propria cell suspension at a final concentration of $5–10 \times 10^6$/mL.
3. Incubate at 37°C for between 30 min and 4 h.
4. Remove the nonadherent cells and wash the plates twice with warm (37°C) medium.
5. Harvest adherent cells by adding 10 m*M* EDTA in RPMI with 10% FCS. Incubate at 4°C for 10–15min then remove cells by vigorous shaking or by scraping with a rubber policeman.
6. Wash the nonadherent cells and treat with carbonyl iron (**Subheading 3.2.2.**).

3.2.2. Carbonyl Iron Phagocytosis

1. Add the lamina propria cell suspension at 2–3 × 10^7/mL to 4 mg carbonyl iron powder and mix thoroughly.
2. Incubate the cells at 37°C for 30 min with occasional mixing.
3. Place the test tube containing the cells in a Dynal magnet for 10 min at 4°C.
4. Transfer the cells in suspension to a second tube and place in the magnet for a further 10 min at 4°C.
5. Remove, wash, and count the remaining cells.

3.2.3. Nycodenz Separation

1. Incubate the cells remaining after carbonyl iron treatment in siliconized dishes at 5–10 × 10^6 cells/mL in RPMI containing 5% heat-inactivated pooled human AB serum overnight.
2. Remove, wash, then resuspend the cells at 5–10 × 10^6 cells/mL.
3. Underlay with Nycodenz monocytes (density 1.068 g/mL) and centrifuge at 600*g* for 20 min at 20°C.
4. Wash the low-density (DC-enriched) cells and high-density (T- cell enriched) cells twice, count, and assess viability.

3.3. Immunocytochemistry See Note 5.

The methods are as described in chapter 6, using mouse monoclonal antibodies directed against human cell-surface antigens and biotinylated sheep anti-mouse immunoglobulin. To inactivate endogenous peroxidase activity, treat some air-dried slides with 0.1% (v/v) hydrogen peroxide in methanol for 30 min at 20°C. Rehydrate the slides with decreasing concentrations of ethanol in water (90%, 70%, 50%) before blocking. The methods are otherwise unchanged.

3.4. Flow Cytometry See Note 6.

Use directly labeled monoclonal antibodies against human cell surface antigens or unlabeled antibodies followed by fluorochrome-conjugated affinity-purified anti-mouse immunoglogulins.

3.5. Mixed Leukocyte Reaction (MLR)

The functional activity of enriched DC can be assessed in an MLR.

3.5.1. Isolation of Responder Cells

1. Obtain buffy coat.
2. Dilute the buffy coat 1:2 with HBSS and underlay with Ficoll-Paque.
3. Centrifuge the cells at 600*g* for 30 min at 4°C.
4. Harvest the interface and wash twice.

5. Resuspend the cells at 10–20 × 10^6/mL in the supernatant of the L243 hybridoma cell line (anti-class II MHC) for 30 min at 4°C, wash three times, and remove the antibody-labeled cells by panning or complement lysis (*see* **Note 7**).
   a. Panning: pretreat bacteriological grade 90 mm Petri dishes with 5 mL 1/1000 affinity-purified rabbit anti-mouse immunoglobulin G (RAM) in PBS for 45 min at 4°C. Remove the unbound antibody by washing four times in PBS. Incubate cells with a saturating amount of the monoclonal antibody for 30 min at 4°C. Wash three times, then remove the antibody-binding cells by adding 20–30 × 10^6 of the labeled cells suspended in 5 mL PBS/5% FCS to the RAM antibody-coated plates.
   b. Complement lysis: resuspend the peripheral blood mononuclear cells prelabeled with primary antibody at a final concentration of 10^7/ml and add complement at a final dilution of 1:5. Incubate the cells for 30min at 37°C, wash, layer over Ficoll-Paque to remove dead cells (600*g* at 20°C for 20 min), and wash three times.
6. Responder cells may be frozen in RPMI containing 20% FCS and 20% DMSO and kept in liquid nitrogen until use. Thaw on the day of use.

*3.5.2. MLR Assay (*See ***Note 8****)*

1. Perform cultures in triplicate in round-bottomed 96-well plates.
2. Incubate a constant number of responder cells (generally 2 × 10^5/well) with varying numbers of irradiated stimulators from the colon (2500 rads).
3. Carry out the reaction in a total volume of 0.2 mL of medium supplemented with 5% AB serum.
4. After 4 d, add 1 μCi tritiated thymidine to each well and continue the incubation for a further 16 h.
5. Freeze-thaw the plates and harvest the cells onto glass-fiber disks. Add nonaqueous scintillant (5 mL/vial) and perform liquid scintillation counting.

4. Notes

1. Cell yields after Ficoll-Paque were 15 ± 12 × 10^6/g tissue. Recovery represented 69 ± 15% of the number applied. The amount of tissue obtained from surgical specimens ranged from 5 to 21 g. The final yield of DC-enriched populations was always < 2% ***(11)***.
2. The differentiation of DC from intestinal macrophages is difficult because of their shared properties including fibronectin-adherence (dendritic cells weakly), low density, and the expression of class II MHC antigens (DC > macrophages). The distinction can be made using several criteria: macrophages have a characteristic morphology with oval or round nuclei and basophilic cytoplasm; the nuclei of dendritic cells were oval or pleiomorphic whereas the cytoplasm contained only small vesicles; when cytospin preparations were examined, the majority of the macrophages expressed the antigen labeled by the antibody, 25F9. (This marker was not readily detectable at flow cytometry because it labels a

predominantly intracellular, rather than a cell-surface, antigen ***[12]***.) This antigen was not detectable on DC using either method. Third, macrophages could be distinguished from DC by their electron microscopic features; and finally, DC were not adherent to human γ-globulin and were not phagocytic.

3. Initial attempts to enrich for MLR stimulatory cells assessed overnight adherence to fibronectin-coated gelatinized flasks and density gradient centrifugation (Nycodenz monocytes and Percoll at various densities). It was evident that this method did not allow the effective separation of human colonic macrophages from DC. Examination of the cytospin preparations and electron micrographs showed that the low density, non-adherent cells contained low, but significant (5–10%), numbers of macrophages. Similarly, the fibronectin-adherent cell fraction contained dendritic cells.
4. Each of the techniques described in the **Methods Section** was used in different sequences and combinations to try to obtain populations of cells maximally enriched for DC with minimal macrophage contamination. The procedures were monitored for their effectiveness in removing macrophages and enriching for DC mainly by examining cytospin preparations and immunocytochemical slides (assessing the proportion of class II MHC^+ or $25F9^+$ cells, see below). In later experiments, flow cytometry was also used. To date, the best isolation technique is as described above.
5. *Immunocytochemistry findings.* Cells with dendritic cell morphology were labeled with the antibody to class II MHC antigens. The monoclonal antibody, Leu-M5, directed against the p150,95 antigen (CD11c) weakly labeled DC in three of eight experiments in which it was used. None of the other antibodies used stained DC. The majority (> 90%) of fibronectin- or plastic-adherent cells with the morphology of classical macrophages had detectable class II MHC antigens when studied with immunocytochemistry. The mature macrophage marker, 25F9, also labeled virtually all the macrophages. The other macrophage markers used, OKM1 and Leu-M5, were either not detectable on intestinal macrophages or present on small numbers (< 5%) only. Immunohistochemical studies of intestinal macrophages ***(12)*** and immunocytochemistry of populations of macrophages isolated from the colon ***(8)*** demonstrated similar findings.
6. *Flow cytometry findings.*The low-density nonadherent nonphagocytic cells consisted of two populations when analyzed using forward and side scatter characteristics. When fluorescence was plotted against size or forward scatter, it was apparent that the highly fluorescent cells were spread over a wide size range. In addition, almost all the remaining small cells expressed intermediate levels of class II MHC determinants. Analysis of a gated population of large cells demonstrated high-level expression of class II MHC antigens, with no detectable macrophage, or T- or B-cell marker expression. This population comprised 40% of the total cell number. When the smaller population was analyzed, the cells expressed T cell markers (OKT3 and OKT4) with intermediate and high levels of class II MHC antigens. Markers expressed on macrophages and B cells were undetectable or expressed only on small numbers of cells.

When the human γ-globulin-adherent cells were analyzed using forward and side scatter, two populations were demonstrated. The larger cells expressed high levels of class II MHC antigens but did not have detectable macrophage, T-, or B-cell antigens. These cells had much higher levels of background fluorescence (nonspecific primary and secondary antibody staining and autofluorescence) than the smaller cells. The population of smaller cells consisted of mainly T and B cells and comprised < 25% of the total cell number.

7. The peripheral blood responder lymphocytes consisted of a homogenous population of T cells with a $CD4^+$:$CD8^+$ ratio = 70:30. There was no detectable expression of class II MHC antigens on the cells treated with L243 and either complement lysis or panning.
8. In the initial experiments using fibronectin adherence, greater MLR stimulatory activity was always found in the low density, non-adherent cells. There was, however, significant activity in the adherent fraction. This suggested either that there was significant contamination with DC (as suggested by their presence in cytospin and immunocytochemical slides) and/or that macrophages had MLR stimulatory activity. In order to differentiate between these two possibilities, the effect of adding macrophages to DC-enriched populations was studied and the activities of the separate populations was directly compared.

   When added to DC-enriched populations, human colonic macrophages neither inhibited nor potentiated MLR stimulation, either in the presence or absence of indomethacin.

   The direct comparison of DC-enriched populations (40% dendritic cells) with macrophage-enriched populations (>75% macrophages), nonadherent colonic lamina propria cells (< 1% dendritic cells) and high-density cells (< 5% dendritic cells) showed greatly enhanced MLR stimulatory activity in the DC population. A small cross-contamination with DC can explain the weak activity of the macrophage-enriched population but the converse does not hold true. The levels of maximal proliferation and stimulator:response curves of the DC-enriched populations are comparable to those using purified populations of human tonsillar dendritic cells ***(5)***. Therefore, dendritic cells are the major stimulators of the MLR in the human colonic lamina propria. Intestinal macrophages, in spite of expressing high levels of class II MHC antigens, have no effect on MLR stimulation in vitro.

References

1. Wilders, M. M., Drexhage, H. A., Kokje, M., Verspaget, H. W., and Meuwissen, S. G. (1984) Peripolesis followed by cytotoxicity in chronic idiopathic inflammatory bowel disease. *Clin. Exp. Immunol.* **57,** 4–620.
2. Wilders, M. M., Drexhage, H. A., Kokje, M., Verspaget, H. W., and Meuwissen, S. G. M. (1984) Veiled cells in chronic idiopathic inflammatory bowel disease. *Clin. Exp. Immunol.* **55,** 377–387.
3. Gaudernack, G. and Bjercke, S. (1985) Dendritic cells and monocytes as accessory cells in T cell responses in man. I. Phenotypic analysis of dendritic cells and monocytes. *Scand. J. Immunol.* **21,** 493–500.

4. Young, J. W. and Steinman, R. M. (1988) Accessory cell requirements for the mixed-leukocyte reaction and polyclonal mitogens, as studied with a new technique for enriching blood dendritic cells. *Cell Immunol.* **111,** 167–182.
5. Hart, D. N. and McKenzie, J. L. (1988) Isolation and characterization of human tonsil dendritic cells. *J. Exp. Med.* **168,** 157–170.
6. McKenzie, J. L, Prickett, T. C. R., and Hart, D. N. J. (1989) Human dendritic cells stimulate allogeneic T cells in the absence of interleukin 1. *Immunology* **67,** 290–297.
7. Flechner, E. R., Freudenthal, P. S., Kaplan, G., and Steinman, R. M. (1988) Antigen-specific T lymphocytes efficiently cluster with dendritic cells in the human primary mixed leucocyte reaction. *Cell Immunol.* **111,** 183–195.
8. Golder, J. P. and Doe, W. F. (1983) Isolation and preliminary characterization of human intestinal macrophages. *Gastroenterology* **84,** 795–802.
9. Bookman, M. A. and Bull, D. M. (1979) Characteristics of isolated intestinal mucosal lymphoid cells in inflammatory bowel disease. *Gastroenterology* **77,** 503–510.
10. Bull, D. M. and Bookman, M. A. (1977) Isolation and functional characterization of human intestinal mucosal lymphoid cells. *J. Clin. Invest.* **59,** 966–974.
11. Pavli, P., van de Pol, E., Hume, D. A., and Doe, W. F. (1993) Dendritic cells, not macrophages, are the major antigen-presenting cells of the human colonic lamina propria. *Immunology* **78,** 132–141.
12. Hume, D. A., Allan, W., Hogan, P. G., and Doe, W. F. (1987) Immunohistochemical characterization of macrophages in human liver and gastrointestinal tract: Expression of CD4, HLA-DR, OKM1 and the mature macrophage marker, 25F9, in normal and diseased tissue. *J. Leuk. Biol.* **42,** 474–484.

15

Isolation of Human Lung Dendritic Cells

Michel M. van den Heuvel, Carin E. G. Havenith, and Robert H. J. Beelen

1. Introduction

In the lung several cell types are capable of presenting antigen to T cells. The dendritic cells (DC) are the most potent antigen-presenting cell. DC form a rare cell population in the lung and early studies were hampered because scarce cell populations are seldom easy to isolate. Besides, this cell is phenotypically heterogeneous depending on its localization, differentiation, or activation status. This chapter will elaborate on the heterogeneity that has to be taken into consideration when studying and isolating lung DC. Furthermore, techniques for the isolation of lung DC from human parenchymal lung tissue and bronchoalveolar lavage fluid (BAL) are described.

1.1. Dendritic Cell Maturation

DC are bone-marrow derived antigen-presenting cells that enter the lung via the blood. Although the mechanism is not completely understood, it is thought that the myeloid precursor cells enter the lung where they mature into DC. There are at least two precursors described in the peripheral blood monocytes and $CD34^+$ hematopoietic progenitor cells. In addition, it has been reported that cells from the lymphoid lineage can develop into potent DC ***(1)***. In the presence of certain cytokines, precursor cells develop into DC. In vitro studies showed that cytokines such as interleukin (IL)-4, granulocyte/macrophage colony-stimulating factor (GM-CSF), stem cell factor (SCF), and tumor necrosis factor (TNF)-α are involved in this process ***(2)***.

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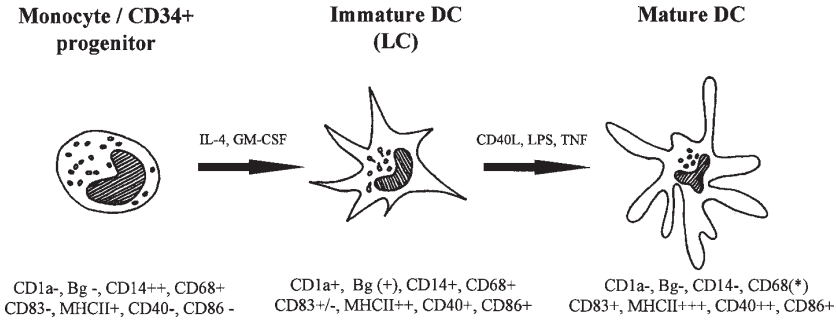

Fig. 1 (DC development) Shown is a schematic drawing of the DC development from its blood precursors. LC = Langerhans cell; Bg = Birbeck granule; – negative; +/– low to negative expression; + positive; ++/+++ strongly positive; (+) positivity under some circumstances induced; (*) spot localization.

In vitro the maturation process can be followed by studying the expression of characteristic markers (**Fig. 1**). Upon incubation with GM-CSF and IL-4, monocytes develop into immature DC that express high levels of CD1a, major histocompatibility complex (MHC) II, mannose receptor, and co-stimulatory molecules such as CD40 and CD86 ***(3–5)***. DC developed from $CD34^+$ progenitor cells can have Birbeck granules (Bg) as well. Bg are characteristic for the Langerhans cells (LC), the in vivo representative of the immature type DC ***(6,7)***. Immature cells have an excellent antigen uptake and processing machinery. Final maturation is induced by proinflammatory cytokines and T-cell contact. This process can be mimicked in vitro by adding stimuli such as CD40 ligand, TNF-α, or lipopolysaccharide (LPS) to the culture. CD83 expression is strongly enhanced on the cell surface of mature DC, and the expression of co-stimulatory molecules and MHCII expression are further enhanced ***(8,9)***. The maturation results in an increased accessory potency, whereas the antigen processing capacity decreases ***(4).***

1.2. Overlapping Features of Macrophages and DC

Macrophages and DC have many similarities and are therefore not always as easy to distinguish. DC differ in their expression of several markers as shown in **Table 1**. Some of these markers can be used during isolation procedures whereas others can be used to check the purity of the obtained cells.

1. CD1a, CD83, and L25 are expressed on DC but not on macrophages ***(10,11)***. However, these markers are not expressed on all DC and the expression is not restricted to DC only; thymocytes can express CD1a and B cells can express CD83 and L25.

Table 1
Markers commonly used to separate macrophages and DC

	DC	Macrophages	Reference
MHCII	++	+	***(12,13,48)***
RFD1+ RFD7–	+	–	***(15)***
L25	+	–	***(10)***
CD68	perinuclear spot or –	granular/cytoplasm	***(16)***
Aph	perinuclear spot or –	granular/cytoplasm	***(49)***
FcR	+	++	***(18–20)***
CD14	+/–	+	***(16)***
CD1a	+/–	–	***(3,4,47)***
CD83	+/–	–	***(11)***

+/– Maturation-dependent positivity; + positive; ++ strong positivity; – negative; Aph = acid phosphatase activity; FcR = immunoglobulin Fc receptor.

2. Both the DC and macrophage express MHCII, but the antigen density is much lower on the latter ***(12–14)***.
3. Positive staining with RFD1, a monoclonal antibody that recognizes a MHCII-associated epitope, in combination with the absence of an intracellular antigen recognized by the monoclonal antibody RFD7 selects for the DC ***(15)***.
4. Also the organization of the lysosomal compartment can be used to differentiate the macrophage from the DC. Staining of the lysosomal compartment with an anti-CD68 antibody shows a characteristic perinuclear spot in DC whereas macrophages show a granular cytoplasm staining ***(16)***. This pattern is similar to the acid phosphatase staining ***(13)***. That the lysosomal organisation is not always as different has been shown in the rat, where the lysosomal marker ED1 is abundantly expressed in some of the rat lung DC subsets ***(17)***.
5. Many studies have used the differences in Fc-receptor expression to isolate DC. However, recent data suggest that many DC subsets express a variety of Fc-receptors ***(18–20)***.
6. Finally, macrophage and DC can be distinguished functionally by testing their accessory function. DC are potent inducers of T-cell proliferation and the only cells capable of inducing a primary response ***(21)***. In contrast, macrophages have only a modest accessory potency and, in the lung, even show a suppressive effect on T-cell proliferation ***(22)***.

   It can be concluded that phenotypic and functional differences can be used to separate DC and macrophages, but invariably subsets will be lost because of the many overlapping features.

1.3. DC Distribution in the Lung

DC have been identified in the airway epithelium, in the alveolar wall, in the visceral pleura, and in the epithelial lining fluid ***(23,24)***. In the airway epithe-

lium they form a tight network, ideally positioned to capture antigen ***(25)***. Airway epithelium DC are positive for CD1a and CD68 and therefore resemble the immature-type DC ***(26,27)***. Alveolar wall DC, however, are negative for CD1a and positive for CD68. These phenotypical distinctions might reflect the different stages of maturation *in situ* ***(27,28)***. Also subepithelial DC do not express CD1a. In the epithelial lining fluid DC only form a small population of about 0.1% of total cells, partly being positive for CD1a ***(29)***. In summary, DC can be identified in all the lung compartments and the different stages of development and maturation can be found *in situ* as well.

1.4. DC Turnover in the Lung

In the airway epithelium DC reside for 2–4 d ***(30)***. The immature DC capture antigen and then migrate to the draining lymph node ***(31)***. It is thought that maturation occurs during the migration ***(32)***. In the draining lymph node the mature DC will present the captured antigen to lymphocytes ***(33)***. During inflammation the number of DC in the lung is increased ***(17,34,35)***. Two hours after an inflammatory stimulus small monocyte-like DC appear. These DC progressively increase in size and dendritiform morphology over the ensuing 8 hours ***(36)***. Also the chronic inflammation caused by smoking results in a 10- to 20-fold increase in the number of DC, recognized by the presence of CD1a on their cell membrane ***(24,29)***. Almost half of these CD1a positive cells contain Bg in their cytoplasm, whereas in non-smoking individuals Bg normally are absent in epithelial lining fluid DC ***(24)***. We can conclude that the number, the phenotype, and, presumably, also the function of the lung DC strongly depend on microenvironmental factors that can be influenced by inflammatory processes.

2. Materials

2.1. Culture Medium and Reagents

1. Bacteriologic Petri dishes (Greiner, Alphen aan de Rijn, The Netherlands).
2. Bovine serum albumin (Sigma Chemical Co., St. Louis, MO).
3. Collagenase type 1 (Sigma Chemical Co.).
4. Elastase type IIA elastase (Sigma Chemical Co.).
5. Fetal calf serum (FCS) (Gibco Ltd, Pairsley, Scotland).
6. Hank's buffered salt solution (Flow Laboratories, Irvine Ayrshire, Scotland).
7. Immunoglobulin (IgG; Organon Teknika Corp., West Orange, NJ).
8. L-glutamine (Gibco Ltd.).
9. Lymphoprep (Nycomed, Oslo, Norway).
10. Metrizamide (Nycodenz: Nycomed As, Oslo, Norway).
11. Penicillin (Gibco Ltd.).
12. Phosphate-buffered saline (PBS).

13. Propidium iodide.
14. Phycoerythrin (PE)-labeled anti-CD3, -CD20, -CD56 and -CD14 antibodies.
15. RPMI 1640 (Flow Laboratories).
16. Saline (NaCl, 0.9%).
17. Streptomycin (Gibco Ltd).

2.2. Patients

DC can be isolated from lung tissue and from BAL. Most often, lung tissue is obtained from surgical specimens. It is important to register the reason for the surgical intervention and whether the patients were smokers. BAL specimen reflects the epithelial lining fluid compartment and can be obtained from many patient groups because of the relatively safe nature of the procedure *(37)*. However, lavage is generally regarded as an invasive technique. As such, informed consent should be received, and attention should be paid to the contraindications *(38)*. Approval of the local medical ethical committees is necessary. Lavage has to be performed under standardized regimen *(39)*. The lavage fluid should be pyrogen-free and isotonic: HBSS and saline can be used. The first BAL fraction (±/-50 mL), generally referred as reflecting the bronchial compartment, should not be used for the isolation procedure because of the high risk of contamination with microorganisms. The BAL is kept on ice to ensure the vitality of the recovered cells.

3. Methods

Functional studies of DC often require enrichment procedures. Classically, DC have been isolated by using their low expression of FcR, their tendency to adhere on plastic culture plates only transiently, and their high expression of MHCII. Macrophages in contrast, express high levels of FcR, adhere firmly to plastic, and have a relatively low expression of MHCII *(40)*. As mentioned earlier, recent studies on the development and maturation of DC have shown that these differences are not as contrasting as was once thought. LC do express high levels of different FcR and not all stages of DC maturation are accompanied by high levels of MHCII. Furthermore, depletion of adherent cells often requires an overnight culture step that induces DC maturation *(16)*. This might influence the experimental readout. Therefore, it is necessary to decide which type of DC will be studied before starting the isolation procedure. The next subheadings will focus on the different procedures that are required to isolate DC from fresh lung tissue as well as from BAL.

3.1. DC Isolation from Lung Tissue

1. Interstitial lung cells can be extracted from lung by digestion *(41)*. Rinse lung specimens with HBSS to remove residual blood. Cut the specimen in slices and

incubate with a mixture of type I collagenase (150 U/mL) and type IIA elastase (10 U/mL). This usually suffices to get a single-cell suspension ***(42)***.
2. Tap the cell suspension through a stainless-steel sieve and separate on a lymphoprep density gradient. The low-buoyant fraction contains 70–75% large macrophage-like cells and 20–25% lymphocytes.
3. Culture these cells in medium (e.g., RPMI) containing 10% heat-inactivated FCS, 20 m*M* L-glutamine, penicillin (50 U/mL), and streptomycin (50 μg/mL) at 37°C in a humidified 5% CO_2 atmosphere. After 1 h remove the nonadherent cells and culture the adherent cells in RPMI/FCS for another 16 h.
4. The cells that become nonadherent, and the cells that can be dislodged by gentle rinsing with HBSS, contain a fraction of potent DC. The DC purity in this nonadherent fraction is 10–20% as based on low autofluorescence. Further purification is necessary and can be done using flow cytometric cell sorting techniques (*see also* **Subheading 3.3.**).

3.2. Isolation of DC from BAL

1. Keeping BAL cells at 4°C, filter through a 55 μm gauze to remove mucus, centrifuge, and resuspend in PBS, pH 7.2, containing 0.5% bovine serum albumin and 0.45% glucose. Under normal circumstances BAL mainly consists of alveolar macrophages (80–90%). In addition to a small population of T cells, granulocytes and DC can be distinguished.
2. Separate BAL cells in an adherent and a nonadherent fraction after an overnight culture ***(43)***.
3. Prepare immunoglobulin-coated plastic bacteriologic Petri dishes by incubating in immunoglobulin (10 mg/mL in PBS) for 30 min at room temperatue.
4. Deplete the nonadherent cells of Fc-receptor positive (FcR^+) cells by panning on immunoglobulin-coated plastic bacteriologic Petri dishes
5. Separate the FcR^- population on a metrizamide 14.5% density gradient. Resuspend the FcR^- cells in complete medium at 2×10^7 cell/mL, layer on the gradient, and centrifuge at 600*g*, for 10 min at 20°C (no brakes). DC purity in the low buoyant fraction is 5–10%, based on high expression of MHCII and acid-phosphatase staining in a perinuclear spot.
6. Higher cell purities can be obtained using flow cytometric cell-sorting techniques (*see* **Subheading 3.3.**).

3.3. Flow Cytometric Cell Sorting

1. Sort DC on the basis of light scatter and autofluorescence. Select the low autofluorescent (LA) fraction to exclude the highly autofluorescent alveolar macrophages. Exclude debris and small cells (lymphocytes) on the basis of forward scatter (FSC) and granular cells, (granulocytes, macrophages) on the basis of side scatter (*see* **Notes 1–3**).
2. Alternatively, label the cells with PE-labeled antibodies to T, B, NK cells, and monocytes (CD3, CD20, CD56, and CD14, respectively) ***(45)*** and then sort the DC by excluding antibody-labeled and dead (using propridium iodide) cells (*see* **Note 4**).

4. Notes

1. DC sorted on the basis of LA from both the nonadherent fraction of lung tissue digests and from freshly isolated BAL cells are very potent accessory cells ***(29,42,44,46)***. Overall, 1×10^5 to 2.5×10^5 DC can be obtained from each lung specimen (20–30 g). In BAL, the LA fraction comprises 0.34–0.72% depending upon the smoking habits of the patient. In absolute numbers sorting yields approx 3×10^4 LA cells in nonsmokers and 3.3×10^5 LA cells in smokers with an estimated purity of 50%. The major contaminating populations in the LA fraction are macrophages and T cells.
2. The DC population recovered from BAL is rather heterogeneous containing $CD1a^+$ LC type cells (immature DC) as well as $CD1a^-$ MHC class II^{high} cells with a perinuclear acid phosphatase spot (mature DC). Sorting experiments show that the $CD1a^+$ have a higher accessory potency than the $CD1a^-$ subset ***(47)***.
3. The cell-sorting method is excellent when the macrophage population is very autofluorescent such as when cells are obtained from smokers. The epithelial lining fluid DC can be isolated without further in vitro culture steps. However, in nonsmoking individuals macrophages and DC are more difficult to separate on the basis of autofluorescence.
4. The presence of high levels of autofluorescence can make sorting of antibody-labeled cells difficult. New fluorophores such as allophycocyanin (APC) and the use of markers for positive selection of DC such as CD1a and CD83 may overcome these problems in the future.

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References

1. Ardavin, C., Wu, L., Li, C., and Shortman, K. (1993) Thymic dendritic cells and T cells develop simultaneously in the thymus from a common precursor population. *Nature* **362,** 761–763.
2. Peters, J. H., Gieseler, R., Thiele, B., and Steinbach, F. (1996) Dendritic cells: From ontogenetic orphans to myelomonocytic descendants. *Immunol. Today* **17,** 273–277.
3. Romani, N., Gruner, S., Brang, D., Kämpgen, E., Lenz, A., Trockenbacher, B., Konwalinka, G., Fritsch, P. O., Steinman, R. M., and Schuler, G. (1994) Proliferating dendritic cell progenitors in human blood. *J. Exp. Med.* **180,** 83–93.
4. Sallusto, F., and Lanzavecchia, A. (1994) Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor alpha. *J. Exp. Med.* **179,** 1109–1118.
5. Sallusto, F., Cella, M., Daniel, C., and Lanzavecchia, A. (1995) Dendritic cells use macropinocytosis and the mannose receptor to macromolecules in the major histocompatibility complex class II compartment: Downregulation by cytokines and bacterial products. *J. Exp. Med.* **182,** 389–400.

6. Strunk, D., Rappersberger, K., Egger, C., Strobl, H., Elbe, A., Maurer, D., and Stingl, G. (1996) Generation of human dendritic cells Langerhans cells from circulating CD34(+) hematopoietic progenitor cells. *Blood* **87,** 1292–1302.
7. Mackensen, A., Herbst, B., Kohler, G., Wolffvorbeck, G., Rosenthal, F. M., Veelken, H., Kulmburg, P., Schaefer, H. E., Mertelmann, R., and Lindemann, A. (1995) Delineation of the dendritic cell lineage by generating large numbers of Birbeck granule-positive Langerhans cells from human peripheral blood progenitors. *Blood* **86,** 2699–2707.
8. Romani, N., Reider, D., Heuer, M., Ebner, S., Kämpgen, E., Eibl, B., Niederwieser, D., and Schuler, G. (1996) Generation of mature dendritic cells from human blood: An improved method with special regard to clinical applicability. *J. Immunol. Methods* **196,** 137–151.
9. Cella, M., Engering, A., Pinet, V., Pieters, J., and Lanzavecchia, A. (1997) Inflammatory stimuli induce accumulation of MHC class II complexes on dendritic cells. *Nature* **388,** 782–787.
10. Ishii, Y., Takami, T., Kokai, Y., Yuasa, H., Fujimoto, J., and Takei, T. (1985) A novel human B cell antigen shared with lymphoid dendritic cells: characterization by a monoclonal antibody. *Clinical & Experimental Immunology* **61,** 624–632.
11. Zhou, L.-J., Schwarting, R., Smith, H. M., and Tedder, T. F. (1992) A novel cell-surface molecule expressed by human interdigitating reticulum cells, Langerhans cells, and activated lymphocytes is a new member of the Ig superfamily. *J. Immunol.* **149,** 735–742.
12. Steinman, R. M., and Witmer, M. D. (1978) Lymphoid dendritic cells are potent stimulators of the primary mixed leukocyte reaction in mice. *Proc. Natl. Acad. Sci. U. S. A.* **75,** 5132–5136.
13. Chestnut, R. W., Colon, S. M., and Grey, H. M. (1982) Antigen presentation by normal B cells, B cell tumors, and macrophages: Functional and biochemical comparison. *J. Immunol.* **128,** 1764–1768.
14. Kamperdijk, E. W. A., Verdaasdonk, M. A. M., and Beelen, R. H. J. (1987) Visual and functional expression of Ia-antigen on macrophages and dendritic cells in ACI/Ma1 rats. *Transplant. Proc.* **XIX,** 3024–3028.
15. Poulter, L. W., Campbell, D. A., Munro, C. S., and Janossy, G. (1986) Discrimination of human macrophages and dendritic cells by means of monoclonal antibodies. *Scand. J. Immunol.* **24,** 351–357.
16. Betjes, M. G. H., Haks, M. C., Tuk, C. W., and Beelen, R. H. J. (1991) Monoclonal antibody EBM11 (anti-CD68) discriminates between dendritic cells and macrophages after short-term culture. *Immunobiology* **183,** 79–87.
17. Schon-Hegrad, M. A., Oliver, J., McMenamin, P. G., and Holt, P. G. (1991) Studies on the densities, distribution, and surface phenotype of intraepithelial class II major histocompatibility complex antigen (Ia)-bearing dendritic cells (DC) in the conducting airways. *J. Exp. Med.* **173,** 1345–1356.
18. Fanger, N. A., Voigtlaender, D., Liu, C., Swink, S. L., Wardwell, K., Fisher, J., Graziano, R. F., Pfefferkorn, L. C., and Guyre, P. M. (1997) Characterization of expression, cytokine regulation, and effector function of the high affinity IgG

receptor gamma Ri (CD64) expressed on human blood dendritic cell. *J. Immunol.* **158,** 3090–3098.
19. Fanger, N. A., Wardwell, K., Shen, L., Tedder, T. F., and Guyre, P. M. (1996) Type I (CD64) and type II (CD32) Fc gamma receptor-mediated phagocytosis by human blood dendritic cells. *J. Immunol.* **157,** 541–548.
20. Fanger, N. A., Guyre, P. M., & Fanger, M. W. (1996) Fc receptors, in *Encyclopedia of Immunology* (Delves, P. J., and Roitt, I., eds.), Academic Press Ltd., London, in press.
21. Steinman, R. M. (1991) The dendritic cell system and its role in immunogenicity. *Ann. Rev. Immunol.* **9,** 271–296.
22. Holt, P. G. (1986) Down-regulation of immune response in the lower respiratory tract: The role of alveolar macrophages. *Clin. & Exp. Immunol.* **63,** 261–270.
23. Sertl, K., Takemura, T., Tschachler, E., Ferrans, V. J., Kaliner, M. A., and Schevach, E. M. (1986) Dendritic cells with antigen-presenting capability reside in airway epithelium, lung parenchyma, and visceral pleura. *J. Exp. Med.* **163,** 436–451.
24. Casolaro, M. A., Bernaudin, J.-F., Saltini, C., Ferrans, V. J., and Crystal, R. G. (1988) Accumulation of Langerhans' cells on the epithelial surface of the lower respiratory tract in normal subject in association with cigarette smoking. *Am. Rev. Respir. Dis.* **137,** 406–411.
25. Holt, P. G., Schon-Hegrad, M. A., Phillips, M. J., and McMenamin, P. G. (1989) Ia-positive dendritic cells form a tightly meshed network within the human airway epithelium. *Clin. & Exp. Allergy* **19,** 597–601.
26. Kinet, J.-P., Jouvin, R., Paolini, R., Numerof, R., and Scharenberg, A. (1996) IgE receptor (Fc-epsilon) and signal transduction. *Eur. Respir. J.* **22,** 116s–118s.
27. Haarst, J. M. W. v., Wit, H. J. d., Drexhage, H. A., and Hoogsteden, H. C. (1994) Distribution and immunophenotype of mononuclear phagocytes and dendritic cells in the human lung. *Am. J. Respir. Cell Mol. Biol.* **10,** 487–492.
28. Holt, P. G., and Schon-Hegrad, M. A. (1987) Localization of T-cell, macrophages and dendritic cells in rat respiratory tract tissue: Implications for immune function studies. *Immunology* **62,** 349–356.
29. Haarst, J. M. W. v., Hoogsteden, H. C., Wit, H. J. d., Verhoeven, G. T., Havenith, C. E. G., and Drexhage, H. A. (1994) Dendritic cells and their precursors isolated from human bronchoalveolar lavage: immunocytologic and functional properties. *Am. J. Respir. Cell & Mol. Biol.* **11,** 344–350.
30. Holt, P. G. (1997) Dendritic cell populations in the lung and airway wall, in *Asthma* (Barnes, P. J., Grunstein, M. M., Leff, A. R., and Woolcock, A. J., eds.), Vol. I, Lippincott-Raven Publishers, Philadelphia, pp 453–463.
31. Havenith, C. E. G., Miert, P. P. M. C. v., Breedijk, A. J., Beelen, R. H. J., and Hoefsmit, E. C. M. (1993) Migration of dendritic cells into the draining lymph nodes of the lung after intratracheal instillation. *Am. J. Respir. Cell & Mol. Biol.* **9,** 484–488.
32. Kamperdijk, E. W. A., Vugt, E. v., Richters, C. D., & Beelen, R. H. J. (1994) Morphology of dendritic cells, in *Immunopharmacology of Macrophages and*

Other Antigen-Presenting Cells (Bruijnzeel-Koomen, C. A. F. M., and Hoefsmit, E. C. M., eds.), academic press limited, London, pp 45–61.
33. Havenith, C. E. G., Miert, P. P. M. C. v., Breedijk, A. J., Beelen, R. H. J., and Hoefsmit, E. C. M. (1993) T cell priming in situ by intratracheally instilled antigen-pulsed dendritic cells. *Am. J. Respir. Cell & Mol. Biol.* **9,** 484–488.
34. Belline, A., Vittori, M., Marini, V., Ackerman, S., and Mattoli, S. (1993) Intraepithelial DC and the selective activation of Th2-like lymphocytes in patients with atopic asthma. *Chest* **102,** 997–1005.
35. Poulter, L. W., and Burke, C. (1996) Macrophages and allergic lung disease. *Immunobiology* 195, 574–87.
36. McWilliam, A. S., Nelson, D. J., Thomas, J. A., and Holt, P. G. (1994) Rapid dendritic cell recruitment is a hallmark of the acute inflammatory response at mucosal surfaces. *J. Exp. Med.* **179,** 1331–1336.
37. Klech, H., Pohl, W., and Hutter, C. (1992) Safety and side-effects of bronchoalveolar lavage. *Eur. Respir. J.* **2,** 54–57.
38. Rennard, S. I., Bauer, W., and Eckert, H. (1992) Bronchoalveolar lavage: Ethical considerations. *Eur. Respir. J.* **2,** 125–127.
39. Klech, H., Pohl, W., and European society of pneumology task group on BAL (1992) Technical recommendations and guidelines for bronchoalveolar lavage (BAL). *Eur. Respir. J.* **2,** 561–585.
40. Voorhis, W. C. v., Hair, L. S., Steinman, R. M., and Kaplan, G. (1982) Human dendritic cells. *J. Exp. Med.* **155,** 1172–1187.
41. Holt, P. G., Degebrodt, A., Venaille, T., O'Leary, C., Krska, K., Flexman, J., Farrell, H., Shellam, G., Young, P., Penhale, J., Robertson, T., and Papadimitriou, J. M. (1985) Preparation of interstitial lung cells by enzymatic digestion of tissue slices: preliminary characterization by morphology and performance in functional assays. *Immunology* **54,** 139–147.
42. Nicod, L. P., Lipscomb, M. F., Toews, G. B., and Weissler, J. C. (1989) Separation of potent and poorly functional human lung accessory cells based on autofluorescence. *J. Leukocyte Biol.* **45,** 458–465.
43. Havenith, C. E. G., Haarst, J. M. W. v., Breedijk, A. J., Betjes, M. G. H., Hoogsteden, H. C., Beelen, R. H. J., and Hoefsmit, E. C. M. (1994) Enrichment and characterization of dendritic cells from the human bronchoalveolar lavages. *Clin. & Exp. Immunol.* **96,** 339–343.
44. Nicod, L. P., and Habre, F. E. (1992) Adhesion molecules on human lung dendritic cells and their role for T-cell activation. *Am. J. Respir. Cell & Mol. Biol.* **7,** 207–213.
45. Aubry, J.-P., Power, C., Life, P., Schrader, M., & Bonnefoy, Y. (1995) Purification of human lung dendritic cells by three colour flow cytometry, in *Dendritic Cells in Fundamental and Clinical Immunology* (Banchereau, J., and Schmitt, D., eds.), Vol. 2, Plenum Press, New York, pp 89–91.
46. Havenith, C. E. G., Breedijk, A. J., Miert, P. P. M. C. v., Blijleven, N., Calame, W., Beelen, R. H. J., and Hoefsmit, E. C. M. (1993) Separation of alveolar macrophages and dendritic cells via autofluorescence: phenotypical and functional characterization. *J. Leukocyte Biol.* **53,** 504–510.

47. Haarst, J. M. v., Verhoeven, G. T., Wit, H. J. d., Hoogsteden, H. C., Debets, R., and Drexhage, H. A. (1996) CD1a+ and CD1- accessory cells from human bronchoalveolar lavage differ in allostimulatory potential and cytokine production. *Am. J. Respir. Cell & Mol. Biol.* **15,** 752–759.
48. Kamperdijk, E. W. A., Verdaasdonk, M. A. M., and Beelen, R. H. J. (1988) Expression and function of MHC class II antigens on macrophages and dendritic cells. *Adv. Exp. Med. & Biol.* **237,** 789–793.
49. Duijvenstijn, A. M., Schutte, R., Köhler, Y. G., Korn, C., and Hoefsmit, E. C. M. (1983) Characterization of the population of phagocytic cells in thymic suspension. A morphological and cytochemical study. *Cell Tissue Res.* **231,** 313–323.

16

Identification and Isolation of Synovial Dendritic Cells

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1. Introduction

In rheumatoid arthitis patients, three compartments need to be considered: peripheral blood (PB), synovial fluid (SF), and synovial tissue (ST). Dendritic cells (DC) characterized from each compartment have different properties. The methods given are based on cell sorting for isolation of cells, and flow cytometry and immunohistochemical staining for analysis of cells. Myeloid non-T cells are first enriched by density gradient centrifugation, sheep erythocyte rosetting, and, in some cases, magnetic immunodepletion. By flow cytometry, DC can then be analyzed or sorted based on two- or three-color immunofluorescence. Some variations on this basic theme are also outlined. The basic protocol for two-color immunohistochemistry of formalin-fixed ST is then given. This is based on the localization of the NFκB family member, RelB, to the nucleus of differentiated DC, and exclusion of B cells, macrophages, and follicular DC by double staining. Some variations that are particularly useful in frozen sections follow.

2. Materials

1. Phosphate-buffered saline (PBS): 8 g NaCl, 0.2 g KCl, 0.92 g Na_2HPO_4 (anhydrous), 0.2 g KH_2PO_4. Add H_2O to 1 L. Filter or autoclave sterilize and adjust to pH 7.4. For use in cell preparation, store at room temperature or at 4°C for up to 6 mo.
2. Hank's balanced salt solution (HBSS): HBSS without sodium bicarbonate is purchased from Sigma (St. Louis, MO). Bicarbonate is added on preparation of the solution: 5.4 m*M* KCl, 0.3 m*M* Na_2HPO_4, 0.4 m*M* KH_2PO_4 4.2 m*M* $NaHCO_3$, 1.3 m*M* $CaCl_2$, 0.5 m*M* $MgCl_2$, 0.6 m*M* $MgSO_4$, 137 m*M* NaCl, 0.02% phenol red (optional). Add H_2O to 1 L, adjust to pH 7.4 and filter sterilize. Store at 4°C for up to 6 mo.

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3. RPMI +PGG: To 500 mL RPMI add penicillin G (200 U/mL), gentamicin (10 mg/mL), and L-glutamine (0.3 mg/mL). Store at 4°C for up to 3 mo after the addition of L-glutamine, and check manufacturer's specifications beyond this.
4. Preparation of neuraminidase-treated sheep red blood cells (N-SRBC):
   a. Add 10 mL of sheep's blood in Alsever's solution to a 50 mL tube and add saline to 50 mL to wash.
   b. Centrifuge for 5 min at 910*g* at 10°C.
   c. Aspirate the supernatant carefully as pellet is soft. Add 40 μL of neuraminidase (Gibco-BRL, Gaithersburg, MD), add HBSS to 40 mL and mix.
   d. Incubate at 37°C in the water bath for 1 h and mix every 10 min. Alternatively, use a shaker-water bath set for gentle agitation.
   e. Fill to 50 mL with saline to wash.
   f. Centrifuge for 5 min at 910*g* at 10°C.
   g. Aspirate supernatant, top with 50 mL HBSS, and centrifuge as in **step f**. Repeat this wash step once.
   h. After last wash, resuspend in 50 mL of RPMI +PGG.
   Store at 4°C for up to 1 wk.
5. 10 × NH_4Cl: 82.9 g NH_4Cl, 10 g $KHCO_3$, 327 mg EDTA. Make up to 1 L with Millipore water. Dilute 1:10 for 1 × NH_4Cl and filter.
6. 1% and 4% paraformaldehyde: Add distilled water to 2 g paraformaldehyde (Sigma) to 45 mL. Add 50 μL 10 *M* NaOH and shake. Incubate at 65°C for 10–15 min. After the paraformaldehyde has gone into solution, remove from bath and add 5 mL 10 × PBS. Dilute to 1% or 4% with water. pH to 7.0. Store in a dark container.
7. 10 m*M* EDTA: Add 37 mg of disodium salt EDTA to 800 mL of H_2O and adjust to pH 7.5. Note that the pH is very unstable and should be checked just before use. Store at room temperature for up to 6 mo.
8. Tris-buffered saline (TBS): 7.88 g Tris-HCl, 8.8 g NaCl. Add H_2O to 1 L and adjust to pH 7.6. Store at room temperature for up to 6 mo.
9. Serum block—10% fetal calf serum (FCS) 10% swine serum in TBS: 1 mL of FCS, 1 mL of swine serum, 0.01% azide. Add TBS to 10 mL. Store at 4°C for up to 2 wk.
10. Peroxidase block: 0.5% H_2O_2 in TBS. Stable for 1 d at 4°C, light sensitive.
11. Collagenase: 100 mg collagenase (Type XI, Sigma), 5 mL HEPES buffer, 1 mL heat-inactivated FCS, 94 mL HBSS. Stir for 3 min on a magnetic stirrer, filter, and aliquot.

3. Methods

3.1. Analysis of Cells in Suspension by Flow Cytometry and Isolation by Flow Cytometric Sorting

3.1.1. Preparation of ST Cell Suspension

1. Collect fresh ST into sterile medium.
2. Gently tease the tissue into small pieces with scissors and forceps and add 1 mL collagenase.

3. Digest at 37°C for 1–2 h, mixing every 20 min, until the pieces have disintegrated.
4. Filter the suspension though 70 μm nylon mesh to remove tissue debris.
5. Wash in a 50 mL vol of phosphate-buffered saline (PBS) by centrifuging for 10 min at 410*g* at 10°C.
6. Resuspend in 20 mL 10% FCS/RPMI.

3.1.2. Preparation of DC from PB, SF, or ST Cell Suspension

3.1.2.1. Preparation of Mononuclear Cells and Erythocyte Rosette Fractions

1. When preparing cells from a fresh SF, it is necessary to prevent clumping of cells and consequent cell loss.
   a. Dilute the SF 1:2 with saline, and filter the suspension though 70 μm nylon mesh filters (Becton Dickinson, Franklin Lakes, NJ) into 50 mL tubes. Aliquot 40 mL per tube. If extensive cell clumping occurs at any stage during the cell purification procedure, repeat this filtration step.
   b. Aliquot 20 mL PB or ST cell suspension into 50 mL tubes and dilute 1:2 with saline.
2. Underlay with 10 mL Ficoll-Paque (Ficoll-diatrizoate, research grade, Pharmacia Biotech, Uppsala, Sweden).
3. Centrifuge for 45 min at 180*g*, at room temperature, with no brake (density gradient centrifugation).
4. Carefully aspirate 20 mL of supernatant from the top of the solution and discard.
5. Collect monolayer containing mononuclear cells into 50 mL tubes by gently aspirating just above the monolayer. Remove as much supernatant as possible without disturbing the red blood cell (RBC) and granulocyte pellet using this technique. Discard RBC pellet.
6. Wash cells in a 50 mL vol with saline by centrifuging for 10 min at 410*g* at 10°C. Resuspend pellet in 1 mL saline using a 1 mL pipet, disrupting any cell clumps.
7. Repeat wash step twice. Pool the resuspended cells each time so that by the final wash the cells are in a single tube. Count the cells after the second wash and resuspend after the final wash at 10^7 cells/mL.
8. Aliquot 5 mL of the cell suspension into 50 mL tubes and to each tube aliquot 2.5 mL of both FCS and N-SRBC, and mix.
9. Incubate for 10 min in a 37°C water bath.
10. Centrifuge for 10 min at 170*g* at 10°C.
11. Incubate, without resuspending the pellet, for 1 h at 4°C.
12. Gently resuspend pellet using a 10 mL pipet, and underlay with 10 mL Ficoll-Paque as in **step 2**.
13. Centrifuge for 35 min at 180*g* at room temperature, with no brake.
14. Harvest the monolayer as in **step 5**. The monolayer is the erythocyte-negative non-T-cell fraction and contains DC as well as other cells, and the pellet is the erythocyte-positive T-cell fraction. Discard the pellet if T cells are not required.
15. Wash the non-T cells in a 50 mL vol of PBS by centrifuging for 10 min at 410*g* at 10°C.

16. Add 10 mL NH_4Cl to each T-cell pellet and invert several times for approx 3–5 min. This will lyse the contaminating N-SRBC. Pool cell suspensions. If RBC contaminate the non-T-cell pellet, lyse with 10 mL NH_4Cl. Make up the vol to 50 mL with PBS and centrifuge for 6 min at 410*g* at 10°C.
17. Wash the T and non-T cells again in a 50 mL vol of PBS by centrifuging for 6 min at 410*g* at 10°C. Count the cells.

3.1.2.2. Expected Yields and Purity

From RA PB, the yield of PBMC will be approx 10^6 cells/mL of blood (i.e., lower than the expected yield of 1.5–2 × 10^6/mL from normal PB). From RA SF and ST, the yield will vary greatly between donors. If the patient's disease is active, approx 2 × 10^6 cells/mL SF would be expected. Both RA PB and SF MNC contain a greater proportion of contaminating granulocytes than normal PBMC. By flow cytometry these cells have a characteristically high side and forward scatter and are excluded from the live gate (**Fig. 1**).

3.1.2.3. Myeloid Enrichment of Non-T Cells

Non-T cells are enriched for myeloid cells by negative selection. Cells are first labeled with anti-CD16 (Leu-11b, NK cells; Becton Dickinson, San Jose, CA), anti-CD19 (Leu-12, B cells; Becton Dickinson), anti-CD56 (Leu-19, NK cells; Becton Dickinson), and OKT3 (anti-CD3, T cells; American Type Culture Collection, ATCC, Bethesda, MD) then depleted with immunomagnetic beads, using the MACS system (Miltenyi Biotech, CA). MACS reagents and columns are purchased though Becton Dickinson (*see* **Note 1**).

1. Resuspend non-T cells (prepared in **Subheading 3.1.2.1.**) in 1 mL PBS containing 1% FCS and add 5 μg/mL of each antibody: anti-CD16, -CD19, -CD56, and -CD3.
2. Incubate at room temperature for 10 min, or on ice for 1 h.
3. Wash cells once in a 50 mL vol of PBS by centrifuging at 410*g* at 10°C for 6 min. Resuspend pellet in 0.5 mL of 1% FCS PBS.
4. Add 1 μL of goat anti-mouse IgG magnetic beads for every 10^6 cells that are expected to be depleted (i.e., expect to deplete one-third of the cells) and gently shake. Do not vigorously mix the cell-microbead mixture or the weak microbead-antibody bond will be disrupted.
5. Incubate at 4°C for 15 min.
6. During the incubation, set up the MACS separator. Choose a depletion column for the appropriate number of cells being selected. For example, the AS column is suitable for up to 3 × 10^7 positive cells. Place the column in the magnetic field of the MACS separator with a three-way-stopcock. Add enough 1% FCS/PBS to fill the column and attach a flow resistor to the three-way-stopcock (25-G needle, supplied).
7. Mix cell suspension gently and, using a Pasteur pipet, apply the cell suspension to the depletion column. Allow the cells to run though but do not let the column run dry. Pass the cells though the column approx three times. Rinse with 3 col-

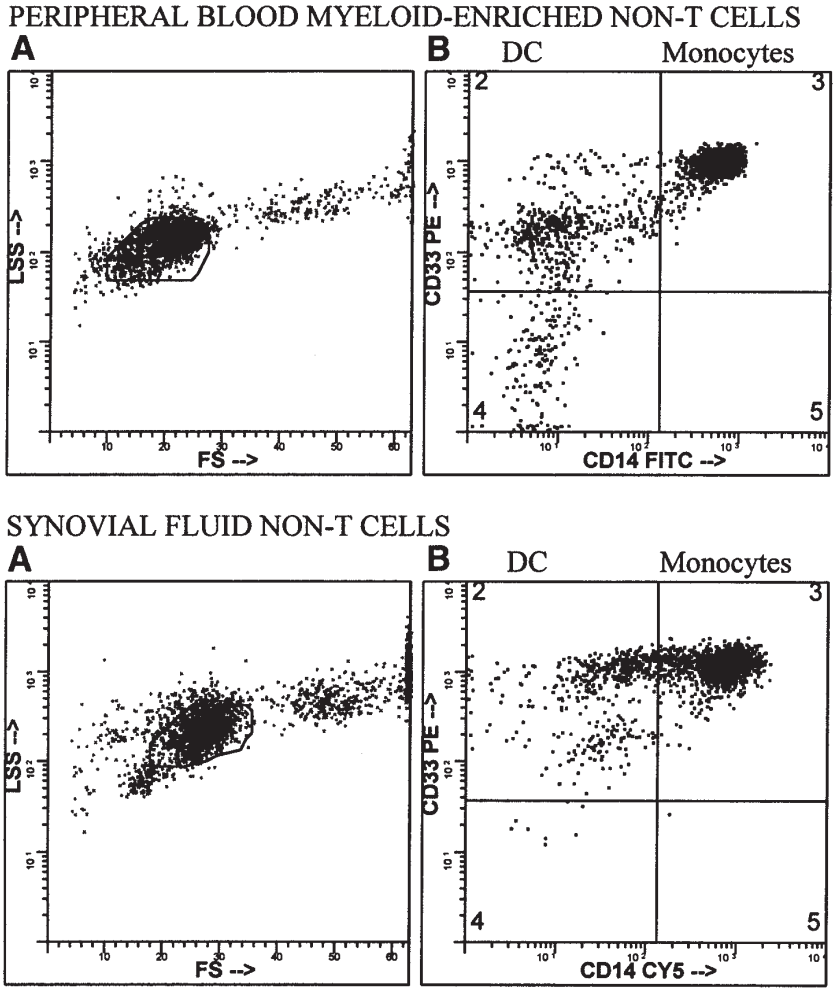

Fig. 1. Expression of myeloid antigens by PB and SF monocytes and DC. Myeloid-enriched RA PB non-T cells or SF non-T cells were stained with anti-CD33-PE and anti-CD14-FITC. (A) live cell gate, (B) DC and monocyte gates.

umn vol of 1% FCS/PBS (6 mL). Change flow resistor to a higher flow rate (23-G needle) and rinse column with approx 20 mL of 1% FCS/PBS. The myeloid-enriched cell fraction is contained in the effluent.

8. Wash in a 50 mL vol by centrifugation at 410*g* for 6 min at 10°C.
9. Resuspend in 1% FCS/PBS for staining.
10. Regenerate column as per manufacturer's instructions.

3.1.2.4. Flow Cytometric Analysis of DC

1. To the myeloid-enriched non-T cell suspension add 5 μg/mL of PE-conjugated anti-CD33 (Leu-M9, Becton Dickinson) and fluorescein (FITC)-conjugated anti-CD14 (Leu-M3, Becton Dickinson) and incubate for 30 min on ice or for 10 min at room temperature. Control tubes will contain:
   a. Negative—5 μg/mL of PE-conjugated IgG and FITC-conjugated IgG.
   b. 5 μg/mL of PE-CD33 alone.
   c. 5 μg/mL of FITC-CD14 alone.
2. Wash three times in PBS.
3. To fix, add 10 μL of 1% paraformaldehyde for 10 min at room temperature. Filter though 70 μm nylon mesh (*see* **Note 2**).
4. Set the negative gates using the control sample.
5. Set the compensation for PE and FITC by optimizing each of the single positive samples (*see* **Note 3**).
6. Run the two-color sample. Typical plots are shown in Fig. 1. DC are gated as $CD33^{+}CD14^{dim}$ cells and monocytes as $CD33^{+}CD14^{bright}$ cells for analysis or sorting.

3.1.2.5. Flow Cytometric Cell Sorting for DC

1. Resuspend the myeloid enriched non-T-cell suspension in 0.5 mL 1% FCS/PBS. Add 5 μg/mL anti-CD33-PE and anti-CD14-FITC and incubate for 30 min on ice or for 10 min at room temperature.
2. Wash in a 50 mL vol with PBS by centrifugation at 410*g* at 10°C for 6 min.
3. Resuspend cells in 10% FCS/RPMI+PGG at 10^7/mL and filter them as above in preparation for sorting.
4. Cell sorting is carried out according to the manufacturers' instructions. DC have been successfully sorted using Becton Dickinson, Coulter, and Cytomation instruments (*see* **Notes 4–7**).
5. After gating, cells are sorted and collected into 15 mL tubes containing 1 mL 10% FCS/RPMI + PGG.
6. Once collected, wash cells in a 15 mL vol of PBS by centrifugation at 410*g* for 6 min at 10°C.
7. Count the cells.
8. Variations to this basic protocol are discussed in **Note 8**.

3.1.2.6. Yields and Purity

Starting from 20–25 × 10^6 myeloid-enriched non-T cells, approx 0.5–1 × 10^6 DC and 2–3 × 10^6 monocytes can be sorted. However, yield and purity will depend on the sort parameters and the efficiency of the instrument. From 85% to 99% purity can be achieved by cell sorting. The greater the purity, the lower the yield. From RA SF non-T cells, the yields are similar to threefold greater, as many SF samples contain very few B cells and NK cells and are enriched in DC ***(1)***. For this reason, and to reduce handling of the cells, myeloid-enrichment of SF non-T cells is not routinely carried out.

3.2. Identification of DC in RA ST—Immunohistochemical Staining of Formalin-Fixed Biopsies

3.2.1. Dewaxing and Retrieval of Antigens

1. Place mounted slides at 37°C in a drying oven on a flat slide tray for a minimum of 2 h (*see* **Note 9**).
2. Transfer to a 60°C oven for 1 h (*see* **Note 10**).
3. Transfer slides to a glass slide rack and place in a research grade xylene bath for 5 min.
4. Repeat **step 3** in a fresh xylene bath.
5. Rehydration. Transfer slides to a 100% ethanol bath for 5 min.
6. Repeat **step 5** in a fresh ethanol bath.
7. Air-dry slides for 10 min and then circle section with a wax pen (PAP pen, Dako, Carpenteria, CA).
8. Repeat **step 5** in a fresh ethanol bath (*see* **Note 11**).
9. Transfer slides into a 95% ethanol bath for 5 min.
10. Transfer slides into a 70% ethanol bath for 5 min.
11. Transfer into autoclavable pots containing 10 m*M* EDTA pH 7.5 and wash for 1 min. Replace 10 m*M* EDTA pH 7.5. Loosely secure pot lids.
12. Autoclave for 10 min at 121°C
13. Allow autoclave to cool for at least 2 h, but preferably overnight before opening. Do not air vent autoclave.
14. Transfer slides into a TBS bath for at least 5 min.

3.2.2. Immunohistochemical Staining

1. Remove antigen-retrieved slides from TBS bath and dry the slide area surrounding the tissue section to remove the excess liquid (*see* **Notes 12** and **13**).
2. Using a pipet, cover the tissue section with serum block. Usually, 100 μL is sufficient when adding any reagent at any step during the staining protocol. Incubate at room temperature in a moist environment for 20 min (*see* **Note 14**).
3. Remove blocking solution by gently tapping slide onto absorbent paper.
4. Add the first primary antibody, RelB (supershift antibody, Santa Cruz Biotechnology, Santa Cruz, CA) to the sections, and the relevant control antibody (rabbit Ig, Dako) to negative control tissue sections, and incubate at room temperature for 60 min (*see* **Note 15**).
5. Wash in a TBS bath for 5 min. Dry the slide area surrounding the tissue section to remove the excess liquid.
6. Add the peroxidase block solution and incubate at room temperature for 20 min.
7. Wash in a TBS bath for 5 min. Dry the slide area surrounding the tissue section to remove the excess liquid.
8. Add the appropriate biotinylated secondary antibody (anti-rabbit Ig, Dako) and incubate at room temperature for 30 min (*see* **Note 15**).
9. Wash in a TBS bath for 5 min. Dry the slide area surrounding the tissue section to remove the excess liquid.

10. Add the streptavidin-conjugated horseradish peroxidase (HP) enzyme (Dako) and incubate at room temperature for 30 min (*see* **Note 15**).
11. Wash in a TBS bath for 5 min. Dry the slide area surrounding the tissue section to remove the excess liquid.
12. Add the second primary antibodies to individual slides. These will be anti-HLA-DR (TAL.1B5, Dako), anti-CD20 (L26, Dako), anti-CD23 (NCL-CD23-1b12, Novacastra Laboratories, Newcastle upon Tyne, UK), and anti-CD68 (PG-M1, Dako) and the appropriate control antibody (mouse Ig, Dako) to control tissue sections, and incubate at room temperature for 60 min (*see* **Note 15**).
13. Wash in a TBS bath for 5 min. Dry the slide area surrounding the tissue section to remove the excess liquid.
14. Add the appropriate biotinylated secondary antibody (anti-mouse Ig, Dako) and incubate at room temperature for 30 min (*see* **Note 15**).
15. Wash in a TBS bath for 5 min. Dry the slide area surrounding the tissue section to remove the excess liquid.
16. Add the streptavidin-conjugated alkaline phosphatase enzyme (streptavidin ABC-AP complex, Dako) and incubate at room temperature for 30 min (*see* **Note 15**).
17. Wash in a TBS bath for 5 min. Dry the slide area surrounding the tissue section to remove the excess liquid.
18. Add the diaminobenzidine (DAB, Dako) chomogen and develop brown color for 2–5 min. Check color development under the light microscope each minute (*see* **Note 16**).
19. Wash in a TBS bath for 5 min. Dry the slide area surrounding the tissue section to remove the excess liquid.
20. Add the Fast Red chromogen (Dako), and develop red color for 7–10 min. Again check color development by microscopy (*see* **Note 16**).
21. Wash in a deionized water bath for 5 min. Dry the slide area surrounding the tissue section to remove the excess liquid.
22. Counterstain for 2 min using Mayer's hematoxylin (Sigma).
23. Wash under running tap water for 5 min. Dry the slide area surrounding the tissue section to remove the excess liquid.
24. Mount and cover-slip using aqueous mounting media (Dako).
25. Variations to this basic protocol are discussed in **Note 17**.

4. Notes

4.1. Analysis of Cells in Suspension by Flow Cytometry and Isolation by Flow Cytometric Sorting

1. There are various sizes and types of MACS columns for cell separation depending on the cell number and whether cells are being negatively or positively selected, and there may be some variations in the size of the flow resistor and the volumes required to run though the column. Refer to the manufacturer's specifications for these variations. Use of the correct type and size of column and flow resistors will optimize the yield and purity of the cell preparation.

2. It is essential to filter cell clumps from the suspension before flow cytometric analysis or sorting to prevent blockages of the machine.
3. Once the two-color staining has been adequately compensated, the cytometer settings and sort protocol should be saved for routine use. There will be minimal if any variation in the compensation between donors; however, the relative proportion of DC and monocytes will vary.
4. Keep the cells cool and well mixed while sorting to prevent adherence to the sort tube. Process the cells as soon as possible after sorting to maintain viability.
5. For sterile sorting, the machine should be prerinsed with ethanol.
6. For RNA work, cells can be sorted directly into Trizol reagent (Gibco). Equal numbers of particular populations can be sorted for semiquantitative comparison, by setting the machine as required.
7. When using unconjugated mAb, background staining can be reduced by subsequent incubation with a biotinylated antibody of the appropriate species specificity, followed by streptavidin-conjugated fluorochome, rather than using fluorochome-conjugated anti-mouse antibody.
8. Variations:
   a. Three-color analysis and sorting of DC: Using an argon laser, myeloid-enriched cells or PBMC can be analyzed using fluorochomes of three different absorption/emission spectra: FITC, phycoerythin (PE), and PE/Cyanin-5. On some instruments it is possible to include a fourth fluorochome: PE/Texas Red (ECD, Coulter). We have successfully employed this technique on the Coulter Epics Elite flow cytometer (Hialeah, FL) ***(2)***. For greater flexibility, biotinylated mAb can be used and then labeled with streptavidin-conjugated fluorochomes. For low-abundance antigens, such as cytokine receptors, detection with PE or Cy5 is more sensitive than with FITC. In this way, the phenotype of gated DC and monocytes and their subpopulations can be assessed without sorting.
   b. Alternative method for DC analysis or sorting: PB or SF MNC can be stained with CD14/CD19/CD16-Cy5, HLA-DR-PE, and CD3-ECD. $CD3^{-}CD14^{-}CD19^{-}CD16^{-}HLA\text{-}DR^{+}$ DC can be analyzed using FITC-conjugated Mab. Using CD3-Cy5, this method can be adapted for three colors.
   c. DC subsets in PB and SF: Analysis of the CD33/CD14 staining of myeloid-enriched non-T cells demonstrates a number of subpopulations. First, it will be noted that both CD14 and CD33 are expressed as a spectrum from negative though dim to bright. $CD33^{bright}CD14^{bright}$ monocytes and $CD33^{bright}CD14^{dim/-}$ DC are easily distinguished and gated (**Fig. 2**). Both express CD11c. $CD33^{dim}CD14^{dim/-}$ cells constitute at least two subsets, one of which is $IL\text{-}3R\alpha^{+}CD11c^{-}$ and equivalent to "plasmacytoid $CD4^{+}$ DC" ***(3)***. The other is $CD11c^{+}IL\text{-}3R\alpha^{-}$ and expresses higher levels of CD14. Similarly, SF monocytes are activated and their CD14 expression tends to be more diffuse than in PB ***(1)***. Thus, CD11c expression can also be used to identify DC subsets. As previously reported and shown in Fig. 2, its expression is reciprocal to that of IL-3Rα ***(4)***. In our experience, however, this varies considerably among

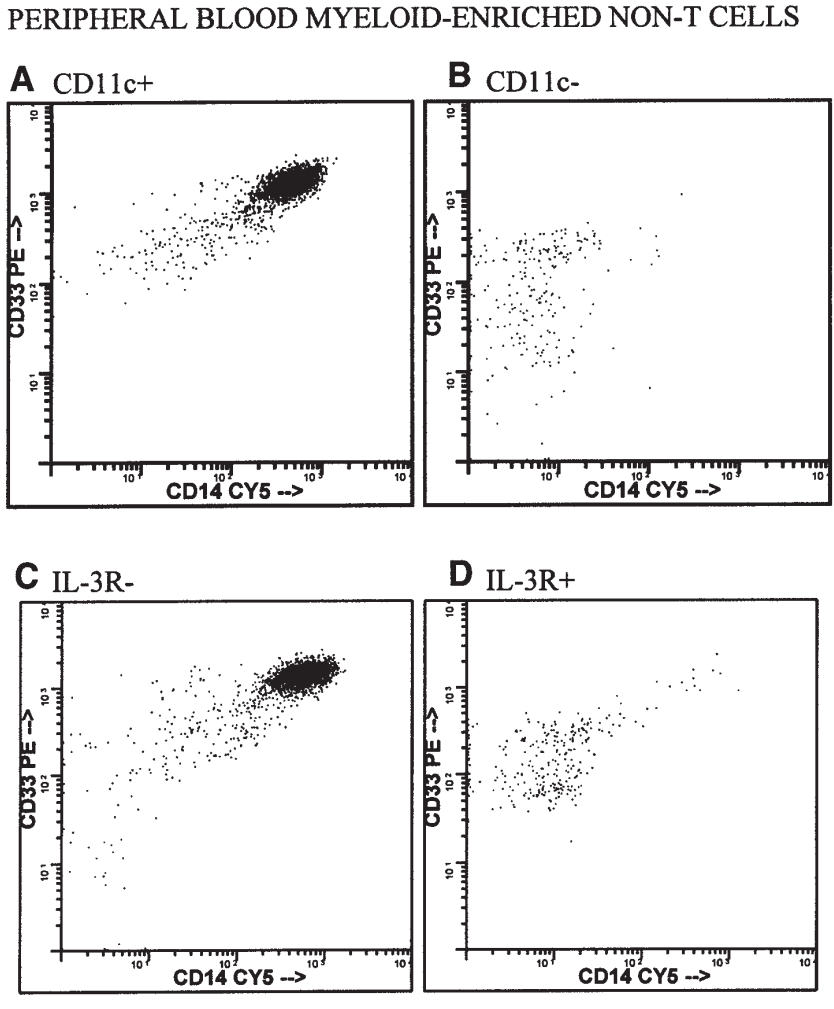

Fig. 2. IL-3R and CD11c expression by PB monocytes and DC. Myeloid-enriched PB non-T cells were stained with CD33-PE, CD14-Cy5 and either CD11c or IL-3R-FITC. Cells were gated for either **(A)** CD11c+, **(B)** CD11c–, **(C)** IL-3R– or **(D)** IL-3R+ subsets and their expression of CD33 and CD14 shown. It can be seen that CD11c and IL-3R are expressed by approximately reciprocal subsets of cells.

donors. Unlike the mouse, CD11c alone cannot be used to purify human DC, as monocytes are also $CD11c^+$ *(2)*. SF DC and monocytes are $CD11c^+$ *(2)*.

While CD11c$^+$ DC spontaneously differentiate, IL-3Rα^+ "plasmacytoid" DC require addition of IL-3 and CD40-ligand to the medium ***(4)***.The Mab CMRF-44 recognizes a lipid moiety expressed by relatively differentiated DC in PB, SF, and ST, and activated B cells ***(5)***. 20–50% of SF DC express CMRF-44. As in PB, these DC are CD33^{bright} ***(2,6)***. CD83 is a member of the Ig superfamily, and is expressed by differentiated DC cultured from PB and by skin Langerhans cells ***(7–9)***. It is expressed by neither freshly isolated PB nor SF DC. DC freshly isolated from PB or SF are round. PB and SF DC can be induced to differentiate by overnight incubation in medium in vitro, at which time they will express typical markers of differentiation and bear the characteristic dendritic morphology.

4.2. Identification of DC in RA ST— Immunohistochemical Staining of Formalin-Fixed Biopsies

9. Slides with extra grip, e.g., Superfrost/Plus (Menzel-Glaser, Germany) or Vectabond (Vector Laboratories, Burlingame, CA), should be used to prevent floating of sections during retrieval and staining. Microwave is an alternative to the autoclave ***(10)***.
10. When cutting and mounting sections, do not allow the oven temperature in **Subheading 3.2.1., step 2** to go below 58°C or above 72°C, as this may result in excess background staining on tissue sections.
11. It is important that the sections are not permitted to dry out from **Subheading 3.2.1., step 8** onward.
12. It is extremely important that the tissue sections are not permitted to dry out though the entire protocol or the staining will not be successful. All incubations should be carried out at room temperature in a moist environment. For example, sit the slides on moist paper toweling in a covered box.
13. It is necessary to be extremely gentle with the tissue sections as they are relatively weakly attached to the slides; therefore, avoid any vigorous washing techniques and use gentle pipeting methods. Biopsies containing large areas of fat float off slides more readily.
14. Background staining levels can vary between different tissues. If a tissue has high background staining this can be combated by:
    a. Increasing the length of the serum block in **Subheading 3.2.2., step 2**.
    b. Adding a serum block before the incubation with the second primary antibody in step 12.
    c. Increasing the duration and concentration of the peroxidase block solution up to 3%. This may be necessary if the biopsy contains many cells with myeloperoxidase, such as granulocytes.
    d. Increasing the concentration of the serum components of the serum block to 20%.
    e. Substitute serum block with 3% skim milk in TBS.
15. Antibody and streptavidin-conjugated enzyme dilutions (all are diluted in TBS): anti-RelB, 1:1000; rabbit Ig, 1:10000; anti-rabbit Ig biotinylated, 1:200; streptavidin-HP, 1:300; anti-HLA-DR, -CD20, -CD68, 1:50; anti-CD23, 1:40; mouse Ig, 1:200; anti-mouse Ig, 1:200. See manufacturer's specifications for the preparation and storage of the streptavidin ABC-AP and the chomogens.

16. If the color development is weak, the duration of development can be extended. In some cases, staining is more successful if the development of the DAB chomogen is carried out before the addition of the second primary antibody, i.e., **Subheading 3.2.2., step 18** followed by a TBS wash step are performed between **Subheading 3.2.2., steps 11** and **12**.
17. Variations:
    a. Staining frozen sections: The same basic protocol should be followed with the following variations: remove frozen sections from the freezer or cut fresh. Allow the slides to dry at room temperature for 1 h. Fix in acetone for 1 min. Dry for 2 min. Proceed to the block step as above. Some of the mAb will need to be retitrated on frozen tissue. Note that the morphology of formalin-fixed tissue is always superior to that of frozen tissue. On the other hand, the range of mAb available for frozen tissue is much greater. CMRF-44 and CD83 are useful additional markers for detection of DC in frozen sections ***(2,7,11)***.
    b. Staining cell cytospins: The same basic protocol should be followed with the following variations: resuspend cells at 10^6 cells/mL in PBS. Cytospin 100 μL of cells in suspension onto Vectorbond-coated slides (Vector Laboratories) using a cytospin centrifuge (Shandon, Pittsburgh, PA). Air-dry slides for a minimum of 2 h, preferably overnight, before fixation with 4% paraformaldehyde for 30 min at 37°C. (Note that toxic paraformaldehyde fumes may be generated during incubation.) Wash cytospins for 5 min in TBS and proceed with immunohistochemistry as described above. Some of the staining parameters such as protein block duration and the mAb dilutions may need to be altered to optimize staining of cell cytospins.
18. Analysis: The number of differentiated DC is calculated based on the formula: number DC = number nuclear(n) RelB+HLA–DR+cells–(number nRelB+CD20+ cells + number nRelB+CD68+ cells). The specificity of nRelB alone as a marker of differentiated DC in RA ST is approx 85% ***(12)***.

References

1. Thomas, R., Davis, L. S., and Lipsky, P. E. (1994) Rheumatoid synovium is enriched in mature antigen-presenting dendritic cells. *J. Immunol.* **152,** 2613–2623.
2. Thomas, R. and Quinn, C. (1996) Functional differentiation of dendritic cells in rheumatoid arthitis: Role of CD86 in the synovium. *J. Immunol.* **156,** 3074–3086.
3. Olweus, J., BitMansour, A., Warnke, R., Thompson, P. A., Carballido, J., Picker, L. J., and Lund-Johansen, F. (1997) Dendritic cell ontogeny: A human dendritic cell lineage of myeloid origin. *Proc. Natl. Acad. Sci. USA* **4,** 12,551–12,556.
4. Grouard, G., Rissoan, M. C., Filgueira, L., Durand, I., Banchereau, J., and Liu, Y. J. (1997) The enigmatic plasmacytoid T cells develop into dendritic cells with interleukin (IL)-3 and CD40-ligand. *J. Exp. Med.* **185,** 1101–1111.
5. Hock, B. D., Starling, G. C., Daniel, P. B., and Hart, D. N. J. (1994) Characterization of CMRF-44, a novel monoclonal antibody to an activation antigen expressed by the allostimulatory cells within peripheral blood, including dendritic cells. *Immunology* **83,** 573–581.

6. Summers, K. L., Daniel, P. B., O'Donnell, J. L., and Hart, D. N. J. (1995) Dendritic cells in synovial fluid of chonic inflammatory arthitis lack CD80 surface expression. *Clin. Exp. Immunol.* **100,** 81–89.
7. Zhou, L. J., Schwarting, R., Smith, H. M., and Tedder, T. F. (1992) A novel cell-surface molecule expressed by human interdigitating reticulum cells, Langerhans cells, and activated lymphocytes is a new member of the Ig superfamily. *J. Immunol.* **149,** 735–742.
8. Zhou, L. J. and Tedder, T. F. (1995) Human blood dendritic cells selectively express CD83, a member of the immunoglobulin superfamily. *J. Immunol.* **154,** 3821–3835.
9. Zhou, L. J. and Tedder, T. F. (1996) $CD14^+$ blood monocytes can differentiate into functionally mature $CD83^+$ dendritic cells. *Proc. Natl. Acad. Sci.* **93,** 2588–2592.
10. Boon, M. E. and Kok, L. P. (1994) Microwaves for immunohistochemistry. *Micron* **25,** 151–170.
11. Sorg, U. R., Morse, T. M., Patton, W. N., Hock, B. D., Angus, H. B., Robinson, B. A., Colls, B. M., and Hart, D. N. (1997) Hodgkin's cells express CD83, a dendritic cell lineage associated antigen. *Pathology* **29,** 294–299.
12. Pettit, A. R. MacDonald, K. P. A., O'Sullivan, B., and Thomas, R. (2000) Differentiated dendritic cells expressing nuclea RelB are predominantly located in rheumatoid synovial tissue perivascular mononuclear cell aggregates. *Arthritis Rheum.* **43,** 791–800.

III

Derivation of Animal Dendritic Cells from Progenitor Cells

17

Generation of Murine Bone-Marrow-Derived Dendritic Cells

Asher Maroof

1. Introduction

Dendritic cells (DC) are the most potent antigen-presenting cells (APC) of the immune system. Derived from stem cells in the bone marrow, DC migrate to the tissues where they become "sentinels" of the immune system. DC possess the unique ability to initiate primary T-cell responses and can also stimulate and modulate both B and T lymphocytes during an ongoing immune response ***(1–3)***.

Located in most tissues, DC are able to capture and process efficiently a wide variety of antigens. In response to danger-signaling cytokines and microbial products they up-regulate, their expression of co-stimulatory molecules, display high levels of peptide-bearing major histocompatibility complex (MHC) molecules on their cell surface and migrate to secondary lymphoid organs including the spleen and lymph nodes. In this draining lymphoid tissue they liase with and activate antigen-specific T cells ***(1)***. The capacity to activate naive as well as memory T cells is a property not shared by other APC. DC, activated by infectious agents and inflammatory products, are the mobile cells that bring together antigens and T lymphocytes in cellular clusters to enable the induction of adaptive immunity ***(4–6)***. Only a few DC are necessary to provoke a strong T-cell response ***(1–2)***.

1.1. DC Generation

Until the last decade, the paucity of DC in most tissues and the lack of specific markers have impeded the study of the DC ontogeny, phenotype, and function. On the basis of their morphology, surface phenotype, and function, they have been identified in most tissues including peripheral blood ***(7–8)***. DC

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comprise as little as 0.5% of the nucleated cells in nonlymphoid tissues, whereas lymphoid tissues contain slightly elevated numbers (≈ 1%) ***(9)***. Purification procedures involving density gradient centrifugation, adherence to glass or plastic surfaces, panning, and cell sorting have been developed to obtain significant quantities of DC ***(9,13)***. However, these purification methods are time-consuming and can induce alterations in the DC recovered. The ability to derive DC from progenitors in vitro has allowed some of these difficulties to be overcome ***(5,14–17)***. Bone marrow (BM), peripheral blood monocytes, and $CD34^+$ stem cells in cord blood preparations are three sources of such progenitor cells.

This chapter concentrates on the growth of murine DC from BM using granulocyte-macrophage colony-stimulating factor (GM-CSF) and tumor necrosis factor alpha (TNFα). A basic method is adapted from previous publications ***(19–21)***. In these BM cultures, GM-CSF alone stimulates the growth of macrophages and granulocytes as well as DC. Addition of TNFα enhances the DC maturation pathway characterized by enhanced dendritic morphology together with increased expression of MHC, adhesion, and co-stimulatory molecules ***(5,6,18)***.

2. Materials

2.1. Cell Culture Medium

1. All cell cultures are grown in complete medium:
   a. RPMI-1640 medium Dutch modification: with 1 g/L sodium bicarbonate and 20 m*M* HEPES, without L-glutamine (Sigma, cat. no. R7638) (*see* **Note 1**).
   b. 100 U/mL penicillin (Gibco) + 100 μg/mL Streptomycin (Gibco).
   c. 2 m*M* glutamine (ICN, Flow).
   d. 5×10^{-5}*M* 2-mercaptoethanol (Sigma).
   e. 10% FCS (fetal calf serum).

2.2. Cells and Reagents

1. Mice: Female CBA mice aged 6–12 wk are generally used in our laboratory.
2. 70% ethanol.
3. Lympholyte M (Cedarlane Laboratories).
4. Recombinant cytokines:
   a. Murine GM-CSF (Peprotech).
   b. Murine TNFα (Peprotech).
5. Analytical-grade metrizamide (Nygaard, Oslo, Norway): 13.7% w/v in complete medium (*see* **Note 2**).

2.3. Equipment

1. 60 mm diameter tissue culture Petri dishes.
2. 2 mL syringe with needle (0.25 mm diameter).
3. Tissue culture flasks (25 cm^2).
4. Small scissors and tweezers.

3. Methods

3.1. Isolation of Bones from Mice

1. Kill mouse by cervical dislocation.
2. Pull back skin of mouse and dissect muscle to expose the femur and tibia.
3. Use the scissors and tweezers to cut at the knee joint, slice the muscle along the femur and sever the bone from the hip joint.
4. Remove the other femur and both tibias and place all bones complete medium (*see* **Note 3**).

3.1.1. Extraction of Mononuclear Cells from Bones

1. Remove most of the muscle tissues surrounding the femurs and tibias with the scissors.
2. Place bones in 5 mL of 70% ethanol for 1 min to sterilize (*see* **Note 4**).
3. Wash bones twice in PBS and transfer to a 60 mm Petri dish containing 10 mL complete medium.
4. Cut both ends of the bones with scissors to expose the lumen and use a 2 mL syringe and 0.25 mm needle to flush out the marrow using complete medium (*see* **Note 5**).
5. Vigorously pipet marrow suspension to disintegrate clusters that may be present suspension.
6. To remove red blood cells from the cell suspension, gently layer cells over Lympholyte M (3 mL of Lympholyte M to 5 mL cell suspension) and spin at 1200*g* at room temperature (RT) for 25–30 min. Blood cells sink to the bottom of the tube whereas mononuclear cells will remain at the interface.
7. Recover the interface cells and wash once in complete medium, spinning at 500*g* for 5 min.
8. Tip off the supernatant and resuspend the pellet cells in complete medium.
9. Count cells—approx 2×10^7 mononuclear cells should be obtained from one mouse.

3.1.2. Bone Marrow Cell Culture with GM-CSF

1. At d 0, seed BM leukocytes at a concentration of 10^6 cells/mL in a 25 cm^2 culture flask containing GM-CSF at 100 U/mL (10 ng/mL) and TNFα at 50 U/mL (*see* **Notes 6–8**).
2. Feed cultures at d 3, 5, 7. and 9 without discarding any cells: swirl plates and aspirate 75% of the medium and adding back fresh medium containing GM-CSF and TNFα at appropriate concentrations.
3. Clusters of round granulocytes develop around d 3 and increasing numbers of macrophages will adhere to the plastic bottom of the flask. At d 4 large clusters of DC are visible and attached to the adherent cells (macrophages and fibroblasts) in the flask. After 5–7 d the clustering adherent cells dislodge from the surface and many typical dendritic cells are floating in the culture medium.

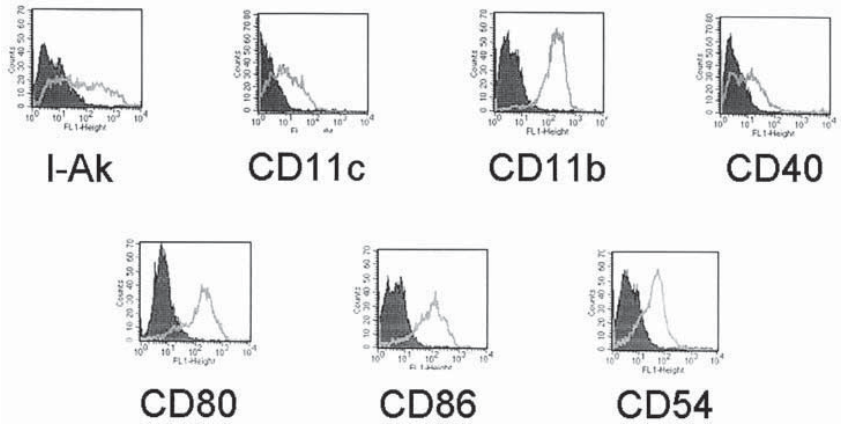

Fig. 1. Cell surface phenotype of bone-marrow-derived DC. Bone-marrow stem cells were grown in GM-CSF + TNFα for 9 d. Open histogram: antibody as indicated; solid histogram: isotype-metched control antibody.

4. After 10–12 d, isolate the low density cells (DC) by centrifugation of the nonadherent BM-derived cells at 600*g* over a 13.7% w/v metrizamide gradient at RT. The DC are at the interface (*see* **Notes 9–11**).
5. See **Figs. 1** and **2** for typical results obtained with cells grown in this way. After 10–12 d of culture, the recovered cells demonstrate a characteristic DC phenotype (**Fig. 1**) and are potent stimulators or allogeneic T cells in a mixed leukocyte reaction. (**Fig. 2**)

4. Notes

1. RPMI 1640 Dutch modification medium is suitable for all procedures during the culture of BMDC. It partly buffered with 20 m*M* HEPES, which allows for handling time outside of a CO_2 environment.
2. To obtain efficient purification of DC, metrizamide should be purchased from stated source.
3. To isolate maximal numbers of mononuclear cells from one mouse, both femurs and tibias should be removed. However, the femurs provide 80% of total BM cells.
4. 70% ethanol treatment is to reduce the chance of contamination. If there are good animal house conditions and good sterile technique practice, this step may be omitted.
5. Flushing out the marrow from the bone is achieved with greater efficiency if one side of the bone is cut instead of two. This leads to an increase in pressure within the lumen of the bone forcing more of the marrow out.
6. There is a strong correlation between the appearance of cells with dendritic morphology and the concentration of GM-CSF used to supplement the cultures. This was studied thoroughly by adding GM-CSF to the BM cultures over the range of

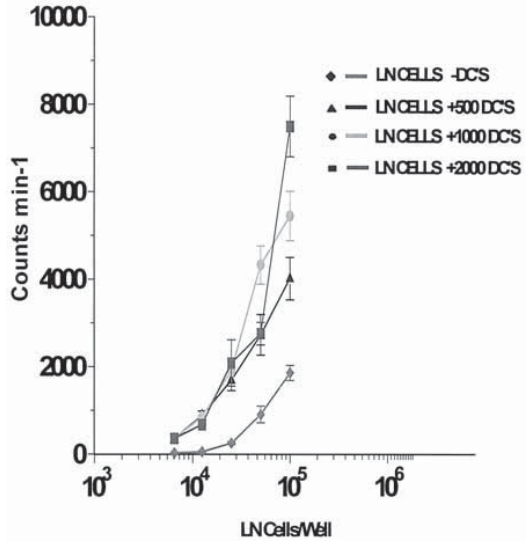

Fig. 2. Stimulation of an allogeneic mixed leukocyte reaction by bone-marrow-derived DC. Bone-marrow cells from CBA mice were grown in GM-CSF + TNFα for 9 d and then co-cultured with graded numbers of allogeneic lymph-node cells in a 20 μL hanging-drop culture system. Proliferation was assessed by uptake of ^{3}H-thymidine incorporation after 3 d of culture.

0.2–250 ng/mL and observing the morphology and expression of MHC class II (by FACS) of the developing cells. A strong increase in I-A expression was noted beginning on d 4–5 that paralleled closely the increase in DC number detected by morphology. The optimum dose of GM-CSF for the development of maximal numbers of MHC class II-positive cells was found to be 10–20 ng/mL. Under these conditions the number of total BM DC obtained by d 10 is approx 50–70% of the starting cell number.

7. Previous work on the generation of DC from mouse BM with GM-CSF employed several treatments to improve the purity of BM-DC precursors. One approach was the depletion of several non-DC precursor cells in fresh bone marrow ***(19)***. However, this may harm certain DC progenitors and/or remove subsets and developmental stages of the ill-defined DC precursors. Therefore, procedures such as depletion of T and B lymphocytes, MHC II$^+$ cells, or FcR$^+$ cells are probably best avoided.
8. Plating the cells at 10^6 per mL is the optimum seeding density as it allows for good cell–cell contact without too much overcrowding of the cells.
9. Major contaminants of the nonadherent BM-DC are granulocytes, which also respond to GM-CSF and are visible as clusters of round cells from d 2 onward.

Removing these clusters at d 2 and 4 from the culture by gentle pipeting is possible, but bears the risk of also removing some of the expanding BM-DC. However, the majority of granulocytes and B-cell contaminants are absent from the cultures by the time DC are harvested at 10–12 d making these removal procedures unnecessary.

10. This method routinely generates about 5–7 × 10^6 cells at 85–95% purity per mouse after 10 d of culture with 10–20 ng/mL (100–200 U/mL) GM-CSF and 50 U/mL TNFα. 70–80% of BM-DC have a mature phenotype after d 10 of culture.
11. Greater numbers and maturity of DC can be achieved by supplementing the cultures with additional cytokines or growth factors:
    a. *Stem cell factor (SCF).* SCF is a potent synergistic growth factor for all kinds of early and committed myeloid and lymphoid progenitor cells and can stimulate the proliferation of myeloid progenitors present in the bone marrow and spleen of mice ***(22)***.
    b. *Flt-3 ligand.* Another agent affecting stem cells, Flt-3 ligand, has been shown to enhance the frequency of both myeloid and lymphoid DC in all organs of mice after repeated injections. Adding this cytokine to BM-DC cultures induces small increase in DC numbers ***(23–24)***.
    c. *Transforming growth factor*-β. TGFβ has been found in bone marrow and fetal liver where active hematopoiesis occurs, and it is known to affect differentiation and proliferation of hematopoietic progenitors, including the differentiation of granulocytes and macrophages that is induced by GM-CSF. This cytokine is a potent immunosuppressive agent and maintains BM-DC in an immature state ***(6)***.
    d. *Interleukin-3*. Recent studies by Sata et al. demonstrated that three distinct BM-DC can be expanded from BALB/c BM cells by culture with 1) GM-CSF plus IL-3, 2) GM-CSF, IL-3 plus T helper (h)1-biasing cytokines (IL-12 and IFN-γ), or 3) GM-CSF, IL-3 plus T(h)2-biasing cytokines (IL-4). These findings suggest that T(h)1- and T(h)2-biasing cytokines, in addition to their effect on T(h) cell differentiation, may play a critical role in the functional skewing of DC ***(22,25,26)***.
    e. *Interleukin-4.* Compared with DC differentiated with GM-CSF alone, supplementation with IL-4 significantly enhances murine BMDC differentiation. Furthermore, DC cultured in GM-CSF plus IL-4 are more potent stimulators of mixed leukocyte reactions ***(5)***.
    f. *CD40-L.* CD40L is a strong inducer of full DC maturation. Cells incubated with CD40L secreted high levels of IL-12 and are very efficient at presenting alloantigen or peptide antigen in vitro. ***(5,23,27)***.
    g. *TNF-related activation-induced cytokine (TRANCE).* TRANCE increases the viability of mature myeloid DC whether generated from mouse BM or human monocytes. TRANCE does not alter adhesion and costimulatory molecules but it does make the mature DC live longer and express several cytokine genes including IL-1,6,12, and 15 ***(28)***.

References

1. Banchereau, J. and Steinman, R. M. (1998) Dendritic cells and the control of immunity. *Nature* **39,** 245–252.
2. Banchereau, J., Briere, F., Caux, C., Davoust, J., Lebecque, S., Liu, Y. J., Pulendran, B., and Palucka, K. (2000) Immunobiology of dendritic cells. *Annu. Rev. Immunol.***18,** 767–811.
3. Steinman, R. M. and Inaba, K.(1999) Myeloid dendritic cells. *J. Leukoc. Biol*. **66,** 205–208.
4. Schuhbauer, D. M., Mitchison, N. A., and Mueller, B. (2000) Interaction within clusters of dendritic cells and helper T cells during initial Th1/Th2 commitment. *Eur. J. Immunol.* **30,** 1255–1262.
5. Lardon, F., Snoeck, H. W., Berneman, Z. N., et al. (1997) Generation of dendritic cells from bone marrow progenitors using GM-CSF, TNF-alpha, and additional cytokines: antagonistic effects of IL-4 and IFN-gamma and selective involvement of TNF-alpha receptor-1. *Immunology* **91,** 553–559.
6. Yamaguchi, Y., Tsumura, H., Miwa, M., and Inaba, K. (1997) Contrasting effects of TGF-beta 1 and TNF-alpha on the development of dendritic cells from progenitors in mouse bone marrow. *Stem Cells* **15,** 144–153.
7. Nussenzweig, M. C. and Steinman, R. M. (1980) Contribution of dendritic cells to stimulation of the murine syngeneic mixed leukocyte reaction. *J. Exp. Med.* **151,** 1196–1212.
8. Steinman, R. M. and Nussenzweig, M. C. (1980) Dendritic cells: features and functions. *Immunol. Rev.* **53,** 127–147.
9. Steinman, R. M., Witmer, M. D., Nussenzweig, M. C., Chen, L. L., Schlesinger, S., and Cohn, Z. A. (1980) Dendritic cells of the mouse: identification and characterization. *J. Invest. Dermatol.* **75,** 14–16.
10. Thirdborough, S. M., London, N. J., James, R. F., and Bell, P. R. (1993) A method for the isolation of human lymphoid dendritic cells from the spleen. *Transpl. Immunol.* **1,** 143–145.
11. Ossevoort, M. A., Toes, R. E., De Bruijn, M. L., Melief, C. J., Figdor, C. G., and Kast, W. M. (1992) A rapid isolation procedure for dendritic cells from mouse spleen by centrifugal elutriation. *J. Immunol. Methods* **155,** 101–111.
12. Vremec, D., Zorbas, M., Scollay, R., et al. (1992) The surface phenotype of dendritic cells purified from mouse thymus and spleen: investigation of the CD8 expression by a subpopulation of dendritic cells. *J. Exp. Med.* **176,** 47–58.
13. Crowley, M. T., Inaba, K., Witmer-Pack, M. D., Gezelter, S., and Steinman, R. M. (1990) Use of the fluorescence activated cell sorter to enrich dendritic cells from mouse spleen. *J. Immunol. Methods* **133,** 55–66.
14. Szabolcs, P., Moore, M. A. S., and Young, J. W. (1995) Expansion of immunostimulatory dendritic cells among the myeloid progeny of human CD34 bone marrow precursors cultured with c-kit ligand, granulocyte-macrophage colony-stimulating factor, and TNFα. *J. Immunol.* **154,** 5851–5861.

15. Romani, N., Reider,D., Heuer, M., et al. (1994) Proliferating dendritic cell progenitors in human blood. *J. Exp. Med.* **180,** 83–93.
16. Romani, N., Gruner, S., Brang, D., et al. (1996) Generation of mature dendritic cells from human blood: An improved method with special regard to clinical applicability. *J. Immunol. Meth.* **196,** 137–151.
17. Sallusto, F. and Lanzavecchia, A. (1994) Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor α .*J. Exp. Med.* **179,** 1109–1118.
18. Kelleher, P. and Knight, S. C. (1998) IL-12 increases CD80 expression and the stimulatory capacity of bone marrow-derived dendritic cells. *Int. Immunol.* **10,** 749–755.
19. Inaba, K., Romani, N., Aya, H., et al. (1992) Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. *J. Exp. Med.* **176,** 1693–1702.
20. Scheicher, C., Mehlig, M., Zecher, R., Seiler, F., Hintz-Obertreis, P., and Reske, K. (1993) Recombinant GM-CSF induces in vitro differentiation of dendritic cells from mouse bone marrow. *Adv. Exp. Med. Biol.* **329,** 269–273.
21. Scheicher, C., Mehlig, M., Zecher, R., and Reske, K. (1992) Dendritic cells from mouse bone marrow: in vitro differentiation using low doses of recombinant granulocyte-macrophage colony-stimulating factor. *J. Immunol. Meth.* **154,** 253–264.
22. Feng, B., Inaba, M, Lian, Z., et al. (2000) Development of mouse dendritic cells from lineage-negative c-kit(low) pluripotent hemopoietic stem cells in vitro. *Stem Cells* **18,** 53–60.
23. Labeur, M. S., Roters, B., Pers, B., et al. (1999) Generation of tumor immunity by bone marrow-derived dendritic cells correlates with dendritic cell maturation stage. *J. Immunol.* **162,** 168–175.
24. Maraskovsky, E., Brasel, K., Teepe, M., et al. (1996) Dramatic increase in the numbers of functionally mature dendritic cells in Flt3 and ligand-treated mice: Multiple dendritic cell subpopulations identified. *J. Exp. Med.* **184,** 1953–1962.
25. Storozynsky, E., Woodward, J. G., Frelinger, J. G., and Lord, E. M. (1999) Interleukin-3 and granulocyte-macrophage colony-stimulating factor enhance the generation and function of dendritic cells. *Immunology* **97,** 138–149.
26. Sato, M., Iwakabe, K., Ohta, A., Sekimoto, M., Nakui, M., Koda, T., Kimura, S., and Nishimura, T. (2000) Functional heterogeneity among bone marrow-derived dendritic cells conditioned by T(h)1- and T(h)2-biasing cytokines for the generation of allogeneic cytotoxic T lymphocytes. *Int. Immunol.* **12,** 335–342.
27. van Kooten, C. and Banchereau, J. (2000) CD40-CD40 ligand. *J. Leukoc. Biol.* **67,** 2–17.
28. Josien, R., Li, H. L., Ingulli, E., et al. (2000) TRANCE, a tumor necrosis factor family member, enhances the longevity and adjuvant properties of dendritic cells in vivo. *J. Exp. Med.* **191,** 495–502.

18

Generation of Dendritic Cells from Rat Bone Marrow

Timothy Powell, James R. Major, and Gordon MacPherson

1. Introduction

It has long been known that tissue dendritic cells (DC) are bone-marrow-derived *(1,2)*. However, early attempts to generate DC from the bone marrow gave only low numbers, presumably because maturation and survival signals required in vivo could not be provided in vitro *(3,4)*. The availability of recombinant cytokines and the observations that DC in culture are dependent on cytokines such as granulocyte-macrophage colony-stimulating factor (GM-CSF) *(5,6)* and IL-4 *(7,8)* have revolutionized DC research. This allowed in vitro cultures of bone marrow cells to be supplemented with factors that promoted the maturation and survival of DC precursors into typical DC as judged by phenotype and function. Several years ago Inaba and colleagues reported such a method for murine bone marrow *(9)*. They showed that mouse bone marrow cells depleted of erythrocytes, B cells, and T cells and cultured with recombinant GM-CSF, after 7 d gave rise to cells that were stimulatory in the allogeneic mixed lymphocyte reaction (MLR), the "gold standard" for DC. These cells also expressed typical DC markers such as high MHC class I and MHC class II and other costimulatory molecules. Culture of DC from human bone marrow is possible but more problematic as it is difficult to obtain in large amounts *(10–12)*.

Below we report a method for rat bone marrow that generates large numbers of DC from lymphocyte and MHC class II^+-depleted bone marrow cells using the recombinant cytokines GM-CSF and IL-4.

1.1. Relation of Bone Marrow-Derived DC (BMDC) to "In Vivo" DC

It is now clear that several lineages of DC exist *(13,14)* and subpopulations of tissue DC *(15,16)* have been identified. In addition ex vivo DC are known to

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change their phenotype following culture overnight in standard media without requirement for cytokines ***(6,17–19)***. Thus, the extent to which any "DC" cultured for several days in high concentrations of cytokines are representative of tissue DC is debatable. It could be argued that culture of DC or their precursors in the presence of cytokines may represent a "danger signal" ***(20)*** and thus BMDC may not be representative of resting tissue DC. However, BMDC may relate to those DC recruited to tissues as a result of inflammation ***(17)*** (Powell and MacPherson, unpublished results).

1.2. Characteristics of BMDC

1. They are dendritic in morphology and possess long processes.
2. They express high levels of MHC class II, adhesion, and costimulatory molecules.
3. They stimulate proliferation of naive T cells in the MLR.
4. They readily endocytose macromolecules such as FITC-dextran.
5. Murine BMDC home to the T-cell areas of the spleen after intravenous administration into a naive mouse ***(21)***.

2. Materials

1. Rats. Any strain of rat can be used (*see* **Note 1**). Male rats are preferred because of their larger size. Rats should be more than 10-wk-old.
2. Surgical instruments (The Holborn Surgical Instrument Co. Margate, UK). Tweezers, surgical scissors (coarse and fine), and bone cutting forceps are required for removal of the femurs. Other long bones (tibia and humerus) also contain BM but have not been routinely used in this laboratory.
3. Washing medium (R5): RPMI 1640 (Gibco-BRL, supplied by Life Technologies Ltd., Paisley, UK.) supplemented with penicillin (50 U/mL), streptomycin (50 μg/mL), and 5% fetal bovine serum (FBS). FBS is also available from Gibco and contains < 0.1 ng/mL endotoxin (LPS) as assayed by the supplier (*see* **Note 2**).
4. Gey's solution (see Chapter 4).
5. Cell strainers. 70 μm pore size (Becton Dickinson).
6. Bone-marrow culture medium (BMM): R5 supplemented with 2 m*M* L-glutamine, 1 m*M* sodium pyruvate (Gibco), murine GM-CSF, and rat IL-4. Tissue culture supernatant (TCS) from cell lines transfected with the murine GM-CSF gene (a kind gift of Dr. D. Gray, Hammersmith Hospital, London) and the rat IL-4 gene (a kind gift of Dr. D.W. Mason, Sir William Dunn School of Pathology) are used, each at a concentration of 1%. This is equivalent to 30–50 ng/mL GM-CSF by bioassay (*see* **Note 3**) and 1500 U/mL IL-4 (*see* **Note 4**). Commercially available recombinant cytokines are alternatives (*see* **Note 5**).
7. Sheep red blood cells (SRBC) (TCS Microbiology, Botolph Claydon, UK).
8. Rabbit anti-mouse Ig, (Sigma).
9. 0.1% chromic chloride "matured" by maintaining at pH 3.0 for 3 wk or more.

10. Monoclonal antibodies: OX6 (anti-MHC class II), OX12 (Anti-Ig L chain), and OX52 (anti-T cell) either as TCS or purified antibody (Serotec).
11. Histopaque (Sigma).
12. 24-well tissue culture plates (Becton Dickinson).
13. Sterile Pasteur pipets.

3. Methods

3.1. Removal of Bone Marrow Cells (BMC) from Femurs

1. Kill rats using a rising concentration of CO_2. Death should be confirmed by exsanguination or cervical dislocation.
2. Remove the femurs by excising muscle and connective tissue using scissors. Knee joints are cut using bone cutting forceps and then the femur is removed by cutting connective tissue from the hip joint with fine scissors.
3. Remove muscle tissue from bones using a scalpel (*see* **Note 6**).
4. Immerse the bones in 70% ethanol for 1 min.
5. Lever the distal (knee joint) part of the femur off the shaft using bone cutting forceps. This also has the effect of removing some of the remaining muscle from the femur (*see* **Note 7**).
6. Remove the head of the femur using bone cutting forceps.
7. Extract the BMC from the femur by flushing R5 through the femur using a 5 mL syringe attached to a 19-G needle (*see* **Note 8**).
8. Make a single-cell suspension by pipeting with a Pasteur pipet and wash the BMC in R5.
9. Deplete the BMC of RBC using Gey's solution (*see* Chapter 4).
10. Wash the BMC twice and pass them through a cell strainer.
11. Wash the cells again in R5 and count them; yields are routinely $1–2 \times 10^8$ cells per animal (two femurs) with a viability of > 99%.

3.2. Preparation of Coated Sheep Red Blood Cells (SRBC) for Rosetting

This is a modified version of the procedure for rosetting described by Mason et al. ***(24)***. The conjugation of proteins to SRBC is inhibited by phosphate ions and hence the reaction is performed in saline and R5 medium (a source of phosphate ions) is used to quench it.

1. Wash SRBC three times with sterile saline.
2. Make up SRBC to a 5% solution in saline (*see* **Note 9**).
3. Add 50 μg/mL rabbit anti-mouse Ig followed by 50 μL/mL 0.1% chromic chloride.
4. Vortex for 1 min.
5. Stand for 10 min at room temperature.
6. Wash cells three times in R5 and resuspend as a 5% solution in R5.

3.3. Removal of Lymphocytes and MHC Class II$^+$ Cells by Rosetting

This part of the procedure is not essential but we find that it results in an increased proportion of DC in the final cultures.

1. Incubate BMC with the antibodies OX6, OX12, and OX52 at 10 μg/mL (final concentration) at 4°C for 1 h.
2. Wash cells three times with R5.
3. Resuspend the BMC pellet in the conjugated SRBC suspension.
4. Mix on a slowly rotating wheel (20–30 rpm) at 4°C for 30 min.
5. Layer cells onto Histopaque. It is important that the Histopaque is allowed to equilibrate to room temperature before use.
6. Centrifuge at 400*g* for 20 min at room temperature (*see* **Note 10**). The rosetted cells form the pellet.
7. Collect the nonrosetted cells from the interface and wash with R5.
8. Count the cells. Expected yields are between 2–3 × 10^7 per rat.

3.4. BMC Culture

1. Resuspend cells at 10^6 cells/mL in BMM.
2. Dispense the cells at 1 mL/well into 24-well plates and culture at 37°C.
3. Change the medium every 2–3 d. Remove approx 80% of the medium and replace it with fresh BMM.
4. After 6–7 d harvest the non- and semiadherent cells using a Pasteur pipet (*see* **Note 11**).

The cells will contain 40 to 80% DC as identified by MHC class II expression and an irregular outline (*see* **Note 12**).

3.5. Conclusions

Culture of rat BMC in the presence of GM-CSF and IL-4 generates large numbers of cells, many of which express phenotypic markers and functions characteristic of DC. They are able to stimulate an allogeneic MLR, and can process and present protein Ag via MHC class I and II. In our hands, however, they differ from ex vivo DC. They, but not spleen or lymph DC, can be stimulated to secrete nitric oxide, and when used as stimulators in a MLR, are weaker than spleen or lymph DC. High numbers of BMDC, but not other DC, inhibit T-cell proliferation (Powell and MacPherson, in preparation). Thus it is difficult to relate BMDC to DC present in vivo, and, given the apparent complexity of the DC system of cells, extrapolation and generalization from BMDC to other DC is fraught with danger.

4. Notes

1. Good yields of BMC and, ultimately, of BMDC have been obtained in this laboratory using the hooded (PVG), dark agouti (DA), and Lewis rats.

2. Using LPS-low or -free sera is critical as LPS has been shown to be an important maturation factor for DC grown in vitro ***(22,23)***. All reagents and media should be tested for LPS, many commercial preparations of collagen, for example, contain high levels of LPS.
3. GM-CSF was assayed by measuring proliferation of murine BMC by tritiated thymidine incorporation after 4 d culture with different amounts of TCS or purified GM-CSF. Proliferation of rat BMC was also assayed; levels of proliferation were lower than with murine BMC.
4. IL-4 was assayed by using flow cytometry to measure changes in levels of MHC class II expression on rat B cells after culture with TCS or purified IL-4. It is essential to use rat IL-4; there is almost no cross reactivity between species.
5. Recombinant rat IL-4 is available from Serotec; recombinant murine or rat GM-CSF is available from R&D Systems. Recombinant murine GM-CSF is functional in rat BM cultures at 10 ng/mL whereas rat GM-CSF has not been tested.
6. Scissors can be used initially to remove muscle. When using a scalpel, scrape from the ends of the bone toward the center.
7. If the knee joint proves difficult to remove, the shaft of the femur! can be cut with bone snips.
8. Optimal yields of BMC are obtained by flushing medium through the femur three or four times. In some strains (e.g., PVG) the marrow comes out as a plug whereas in other strains, cells come out as small clumps and more flushing is required to remove them all.
9. As a guide, use 2 mL of coated SRBC per pair of femurs. This will give a ratio of approx 3:1 SRBC:BMC.
10. It is important to centrifuge the cells with the brake off; braking leads to mixing of the fluid layers.
11. Clumps of cells appear on the floor of the wells after 4–5 d and nonadherent DC-like cells dissociate from these clumps after 6–7 d.
12. When these partially purified DC are replated some cells adhere to the base of the wells. Although it has been suggested that the adherent cells may be macrophages, in murine cultures there is evidence that they are in fact DC (R. Suri and J. M. Austyn, personal communication).

References

1. Steinman, R. M., Lustig, D. S., and Cohn, Z. A. (1974) Identification of a novel cell type in peripheral lymphoid organs of mice. III Functional properties in vivo. *J. Exp. Med.* **139,** 1431–1435.
2. Pugh, C. W., MacPherson, G. G., and Steer, H. W. (1983) Characterization of nonlymphoid cells derived from rat peripheral lymph. *J. Exp. Med.* **157,** 1758–1779.
3. Klinkert, W. E. F. (1984) Rat bone marrow precursors develop into dendritic accessory cells under the influence of a conditioned medium. *Immunobiology* **168,** 413–423.

4. Bowers, W. E. and Berkowitz, M. R. (1986) Differentiation of dendritic cells in cultures of rat bone marrow cells. *J. Exp. Med.* **163,** 872–883.
5. Witmer-Pack, M. D., Olivier, W., Valinsky, J., Schuler, G., and Steinman, R. M. (1987) Granulocyte/macrophage colony-stimulating factor is essential for the viability and function of cultured murine epidermal Langerhans cells. *J. Exp. Med.* **166,** 1484–1498.
6. MacPherson, G. G. (1989) Properties of lymph-borne (veiled) dendritic cells in culture. I. Modulation of phenotype, survival and function: partial dependence on GM-CSF. *Immunology* **68,** 102–107.
7. McKnight, A. J., Barclay, A. N., and Mason, D. W. (1991) Molecular cloning of rat interleukin 4 cDNA and analysis of the cytokine repertoire of subsets of CD4+ T cells. *Eur. J. Immunol.* **21,** 1187–1194.
8. Talmor, M., Mirza, A., Turley, S., Mellman, I., Hoffman, L. A., and Steinman, R. M. (1998) Generation or large numbers of immature and mature dendritic cells from rat bone marrow cultures. *Eur. J. Immunol.* **28,** 811–817.
9. Inaba, K., Inaba, M., Romani, N., Aya, H., Deguchi, M., Ikehara, S., Muramatsu, S., and Steinman, R.M. (1992) Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. *J. Exp. Med.* **176,** 1693–1702.
10. Young, J.W., Szabolcs, P., Moore, M.A. (1995) Identification of dendritic cell colony-forming units among normal human CD34+ bone marrow progenitors that are expanded by c-kit-ligand and yield pure dendritic cell colonies in the presence of granulocyte/macrophage colony-stimulating factor and tumor necrosis factor alpha. *J. Exp. Med.* **182,** 1111–1119.
11. Szabolcs, P., Avigan, D., Gezelter, S., Ciocon, D. H., Moore, M. A., Steinman, R. M., and Young, J. W. (1996) Dendritic cells and macrophages can mature independently from a human bone marrow-derived, post-colony-forming unit intermediate. *Blood* **87,** 4520–4530.
12. Reid, C.D., Stackpoole, A., and Tikerpae, J. (1993) TNF and GM-CSF dependent growth of an early progenitor of dendritic Langerhans cells in human bone marrow. *Adv. Exp. Med. Biol.* **329,** 257–262.
13. Ardavin, C., Wu, L., Li, C. L., and Shortman, K. (1993) Thymic dendritic cells and T cells develop simultaneously in the thymus from a common precursor population. *Nature* **362,** 761–763.
14. Galy, A., Travis, M., Cen, D., and Chen, B. (1995) Human T, B, natural killer, and dendritic cells arise from a common bone marrow progenitor cell subset. *Immunity* **3,** 459–473.
15. Salomon, B., Cohen, J. L., Masurier, C., and Klatzmann, D. (1998) Three populations of mouse lymph node dendritic cells with different origins and dynamics. *J. Immunol.* **160,** 708–717.
16. Leenen, P. J., Radosevic, K., Voerman, J. S., Salomon, B., van Rooijen, N., Klatzmann, D., and van Ewijk, W. (1998) Heterogeneity of mouse spleen dendritic cells: in vivo phagocytic activity, expression of macrophage markers, and subpopulation turnover. *J. Immunol.* **160,** 2166–2173.

17. McWilliam, A. S., Napoli, S., Marsh, A. M., Pemper, F. L., Nelson, D. J., Pimm, C. L., Stumbles, P. A., Wells, T. N., and Holt, P. G. (1996) Dendritic cells are recruited into the airway epithelium during the inflammatory response to a broad spectrum of stimuli. *J. Exp. Med.* **184,** 2429–2432.
18. Schuler, G. and Steinman, R. M. (1985) Murine epidermal Langerhans cells mature into potent immunostimulatory dendritic cells in vitro. *J. Exp. Med.* **161,** 526–546.
19. Austyn, J. M., Hankins, D. F., Larsen, C. P., Morris, P. J., Rao, A. S., and Roake, J. A. (1994) Isolation and characterization of dendritic cells from mouse heart and kidney. *J. Immunol.* **152,** 2401–2410.
20. Matzinger, P. (1994) Tolerance, danger, and the extended family. *Annu. Rev. Immunol.* **12,** 991–1045.
21. Ludewig, B., Ehl, S., Karrer, U., Odermatt, B., Hengartner, H., and Zinkernagel, R. M. (1998) Dendritic cells efficiently induce protective antiviral immunity. *J. Virol.* **72,** 3812–3818.
22. Sallusto, F. and Lanzavecchia, A. (1994) Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor alpha. *J. Exp. Med.* **179,** 1109–1118.
23. Sallusto, F., Cella, M., Danieli, C., and Lanzavecchia, A. (1995) Dendritic cells use macropinocytosis and the mannose receptor to concentrate macromolecules in the major histocompatibility complex class II compartment: downregulation by cytokines and bacterial products. *J. Exp. Med.* **182,** 389–400.
24. Mason, D. W. (1987) Subpopulations of T lymphocytes. *Immunol. Lett.* **14,** 269–270.

19

Derivation of Murine Dendritic Cells from Thymic Precursors

Karen Lucas, Dolores Saunders, and Li Wu

1. Introduction

The earliest lymphoid precursor in the adult mouse thymus, the "low CD4 precursor," was found to be able to produce T cells, B cells, NK cells, and dendritic cells (DC) upon adoptive transfer ***(1–3)***. This precursor population represents only 0.03%–0.05% of total thymocytes and expresses low levels of CD4 and Thy-1, and positive for c-kit and CD44. The principle for isolating this minute precursor population is to enrich maximally for the population prior to fluorescence activated cell separation in order to reduce the cost and to maximize purity. A combination of density centrifugation and immunomagnetic bead depletion successfully removes mature and immature thymocytes and non-T-lineage cells. Note, it is important to deplete non-T-lineage cells, including erythrocytes, macrophages, and DC, which may otherwise contaminate the precursor preparation.

DC are generally considered to be of myeloid origin and can be grown in culture principally with granulocyte-macrophage colony-stimulating factor (GM-CSF) ***(4–6)***. The low CD4 precursor population, isolated from the adult mouse thymus, is lymphoid restricted and is unable to form detectable myeloid or erythroid colonies ***(1)***. However, in culture with a mix of seven cytokines, these thymic precursors proliferate and differentiate to DC ***(7)***. The in vitro production of DC from these thymic precursors does not require GM-CSF. At least 70% of the individual precursors have the capacity to form DC in culture. These DC express high levels of MHC Class II, CD11c, and DEC205, markers characteristic of mature DC in general ***(7)***. Therefore, it is believed that DC development via this particular lineage is of lymphoid origin.

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In the following sections two similar methods are described for growing murine DC from thymic precursors. The first, involves a "low-density culture." Low numbers of precursors, as low as 1 cell/well, can be incubated in wells of a Terasaki plate. In this system the expansion and differentiation of individual precursors can be closely monitored. A "high-density culture system" can be used when a high yield of DC is required. This second method is not as carefully controlled as the "low-density cultures," since cytokines and other factors may be produced by the cells in the high-density culture. These additional factors may affect the conditions of the culture, although we have not noted any differences in the properties of the DC produced by either method.

2. Materials

2.1. Mice

C57BL/6J mice at 5–6-wk-of-age are used for low CD4 precursor cell isolation.

2.2. Isolation of Thymic Precursors

2.2.1. Media

1. Balanced salt solution (BSS): a HEPES-buffered balanced salt solution at pH 7.2 and supplemented with 3% fetal calf serum (FCS) is used for single-cell suspension and immunofluorescent staining.
2. RPMI-1640-HEPES-FCS: RPMI-1640 culture medium buffered with HEPES at pH 6.8–7.0 and supplemented with 10% FCS is used for depletion of macrophages by adhesion to plastic Petri dish (*see* **Note 1**).
3. FCS: fetal calf serum is filtered through a 0.22 μm membrane and heat-inactivated at 56°C for 30 min.
4. Density separation medium: Nycodenz (Nycomed Pharma AS, Oslo, Norway) is purchased as an analytical-grade powder, 500 g bottles, MW 821. A stock of 0.372 *M* (30.55 g per 100 mL final), density approx 1.16 g/cm^3 at 4°C, is prepared with ddH_2O. The stock is diluted with BSS to give a density of 1.086 g/cm^3 at 4°C (*see* **Note 2**). A weighing bottle is used to obtain precise density. To calculate the dilution of stock, use the formula:

   $$100 \times 1.16 \text{ (stock density)} + 1.0 \times a = (100+a) \times 1.086 \text{ (required density)}$$

   where a is = additional volume of BSS to be added to 100 mL stock. Store Nycodenz frozen and protected from light. Prior to use, thaw and mix thoroughly.
5. EDTA: 0.1 *M* EDTA is prepared by dissolving the powder in ddH_2O and neutralizing with NaOH to pH 7.2. One-tenth volume of 0.1 *M* EDTA is added to BSS to prepare BSS-EDTA.

2.2.2. Monoclonal Antibodies (mAb) for Depletion

The mAb cocktail for depletion is prepared and stored in small aliquots at –20°C. The concentration of each mAb is determined by pretitration using

immunofluorescent staining with anti-Ig second stage. Each mAb is used at near saturating concentration in the final mix. The cocktail of Mab is used at 10 μL per 10^6 cells. The mAb cocktail contains: anti-CD3, KT3-1.1; anti-CD8, 53-6.7; anti-CD2, RM2-1; anti-CD25, PC61; anti-B220, RA3-6B2; anti-Mac-1, M1/70; anti-Gr-1, RB6-8C5; anti-erythrocyte antigen, TER-119; anti-MHC class-II, M5/114.

2.2.3. Immunomagnetic Beads for Depletion

1. Paesel and Lorei beads: Goat anti-rat IgG-coated magnetic beads are purchased from Paesel and Lorei (GMBH & Co., Frankfurt, Germany). These beads are used at a bead:cell ratio of 3:1. To remove preservative prior to use, the beads are washed three times in BSS-FCS on a Dynal magnet.
2. Dynabeads: Sheep anti-rat Ig-coated M450 Dynabeads (Dynal, Oslo, Norway) are used for the second-round magnetic-bead depletion. The Dynabeads are used at a bead:cell ratio of 5:1. To remove preservative, the beads are washed in BSS-FCS three times on a Dynal magnet before use.
3. Equipment: For immunobead depletion, a spiral mixer with a series of horizontal rollers is set up in the cold room. A "collar" (cut from thick rubber tubing) is fitted around the lid of 5 mL round-bottom Falcon tubes, so they will rotate slowly at an angle of approx 30° when placed on the roller.

2.2.4. Antibodies for Immunofluorescent Staining

The fluorescent conjugated antibodies for immunofluorescent staining are either purchased from Pharmingen (San Diego, CA.) or made in our laboratory. Anti-c-Kit, clone ACK-2, is used as an FITC conjugate and anti-Thy1.2, clone 30H-12, used as a PE- or Cy3- conjugate. The latter can be used interchangeably in the same fluorescent channel.

2.3. Culture of Thymic Precursors

2.3.1. Medium for Culture of Thymic Precursors

Mouse tonicity RPMI-1640 culture medium is buffered with HEPES at pH 7.2, and supplemented with 10% FCS, 10^{-4} *M* 2-ME, sodium pyruvate, penicillin G 500 U/mL, and streptomycin 25 mg/mL (Complete Medium; *see* **Note 1**).

2.3.2. Cytokines for Development of Dendritic Cells

1. Recombinant cytokines (Immunex Corp., Seattle, WA) are used at the following concentrations: interluekin-1 beta (IL-1β) (human), 200 U or 0.2 ng/mL; IL-3 (murine), 200 U or 400 ng/mL; IL-7 (human), 200 U or 10 ng/mL; stem cell factor (SCF) (murine), 10 ng/mL; tumor necrosis factor alpha (TNFα) (murine), 1 ng/mL.
2. Flt3 ligand is used at 100 ng/mL and is made by Dr. N. Nicola (The Walter and Eliza Hall Institute, Melbourne).

3. The mAb against CD40, FGK45.5, is made in our laboratory and is used at 1 μg/mL.
4. All cytokines are diluted in Complete Medium (*see* **Subheading 2.3.1.**), filtered, and stored at –20°C for no more than 1 mo prior to further dilution in culture.

2.3.3. Culture Plates

For "low density cultures" (250 cells/well) V-bottom Terasaki plates (NUNCLON, Denmark) are used. When a higher number of DC is required, for phenotyping, a 96-well flat-bottom microtiter tray (Falcon, Becton Dickinson, France) is used.

2.4. Analysis of Cultured Cells

2.4.1. Counting of Cells

Cell counts are carried out in a hemocytometer using a phase contrast microscope (Zeiss, West Germany).

2.4.2. Phenotypic Analysis of Cultured Cells

Phenotypic analysis of the cultured cells is performed using three-color fluorescent staining and propidium iodide to exclude dead cells. The fluorescent-conjugated mAb used are: anti-MHC Class II, Cy5-conjugated N22; anti-CD11c, Texas Red-conjugated N418; anti-DEC205, biotin-conjugated NLDC145. PE-conjugated streptavidin is used as the second stage.

3. Methods

3.1. Thymus Removal and Cell Dissociation

1. Remove thymuses from 16 mice under sterile conditions and place in RPMI-1640-3% FCS.
2. Prepare a thymocyte suspension by gently forcing thymus lobes through a stainless steel sieve in BSS-3% FCS.
3. Transfer the cell suspension into four 10 mL conical tubes (4 thymuses/tube), underlay with 1 mL FCS, and centrifuge at 580*g* for 7 min at 4°C in a benchtop centrifuge.

3.2. Purification of Thymic Precursors

3.2.1. Density Separation by Centrifugation

Twenty to thirty percent of thymocytes, including low CD4 precursors, have a density < 1.086 g/cm^3. It is this property that is used to separate the low CD4 precursors from the mature single-positive and double-positive thymocytes as well as other high-density cells.

1. Aliquot 5 mL of well-mixed Nycodenz medium at 4°C into four 14 mL round bottom polypropylene Falcon tubes (Becton Dickinson and Company, NJ).

2. Resuspend the thymocyte cell pellet of each conical tube in an additional 5 mL Nycodenz medium and overlay this cell/Nycodenz suspension onto the 5 mL Nycodenz medium in the Falcon tube.
3. Overlay with 2 mL FCS and slightly mix the interface bands with a Pasteur pipet.
4. Centrifuge the tubes in a swing-out rotor, at 4°C, for 10 min at 1800*g*.
5. Collect the cells at the interface and the FCS layer, using a Pasteur pipet. Dilute the collected fraction with BSS to a volume of 30–40 mL and mix well.
6. Take a small sample at this stage to count cell yield.
7. Centrifuge the cells to a pellet.

3.2.2. Adhesion Depletion of Macrophages

This step is to remove macrophages by adhesion to a plastic surface.

1. Resuspend the cell pellet in 20 mL RPMI-1640-10% FCS and transfer to two plastic Petri dishes (8 cm diameter). Ensure the suspension is distributed evenly over the entire area of the dish.
2. Incubate the plates in a 37°C CO_2-in-air incubator for 60 min.
3. Collect the nonadherent cells, after incubation, by gently washing the surface of the dish twice with 20 mL prewarmed (37°C) RPMI-1640-10% FCS.
4. Take a small sample for a cell count and then pellet the cells by centrifugation.

3.2.3. Immunomagnetic Bead Depletion

This procedure removes most cells bearing markers of mature thymocytes, non-T-lineage cells, and more mature precursor cells. The removal of antibody-coated cells is facilitated by using two rounds of a regime with anti-Ig coated beads.

1. Resuspend the cell pellet in the depletion Mab cocktail at 10 mL/10^6 cells (*see* **Materials**) and incubate on ice for 30–40 min.
2. Dilute cells in 9 mL BSS-3%FCS, underlay with 1 mL FCS, then centrifuge cells through the FCS layer (*see* **Note 3**).
3. Remove the supernatant carefully from the pelleted cells.
4. The amount of anti-rat IgG coated Paesel and Lorei (P&L) beads required is calculated at a ratio of 3:1 (beads:cells) (*see* **Note 4**).
5. The P&L beads are prewashed three times, in a 5 mL round bottom polypropylene Falcon tube, with BSS-3% FCS to remove preservative. Recover the beads with a Dynal magnet. Resuspend the antibody-coated cells in 300–500 mL BSS-3% FCS, then transfer the suspension to the Falcon tube containing the washed beads.
6. Mix the slurry of cells and beads, avoiding bubbles.
7. Seal the tube with the cap and place a "collar" around the cap. Then place the tube on a spiral mixer and rotate continuously for 20–30 min at 4°C, at a 30° angle.
8. To recover the undepleted cells, dilute the bead-cell mix in 4 mL BSS-3% FCS, then remove beads and attached cells with Dynal magnet (*see* **Note 5**). Use a Pasteur pipette to collect the bead-free cell suspension.
9. Take a small sample to count and recover the cells by centrifugation.

10. Use anti-rat Ig-coated Dynabeads at a ratio of 5:1 for the second-round depletion to remove any residual antibody-coated cells, in order to obtain the maximum enrichment. The appropriate amount of beads are washed three times prior to use.
11. Resuspend the cell pellet in 300–500 mL BSS-3% FCS and add to beads.
12. Mix the bead and cell slurry for 20–30 min on a spiral mixer.
13. Recover the bead-free cell suspension with a Pasteur pipette, on a Dynal magnet.
14. Centrifuge to pellet the cells. At this stage, the precursor population is enriched about 500-fold and makes up about 10–20% of the cells.

3.2.4. Immunofluorescent Staining and Sorting by Flow Cytometry

The depleted preparation is stained in two flourescent colors with FITC-anti-c-kit and PE-anti-Thy-1.2.

1. Resuspend the depleted cell preparation in BSS-FCS containing the staining antibodies and incubate on ice for 20–30 min.
2. Remove excess staining antibody by washing cells in BSS-3% FCS through a FCS layer (*see* **Note 3**).
3. Add propidium iodide (PI) to this final wash at 0.5–1.0 μg/mL.
4. Analyze stained cells using a FACStar-Plus.
5. Take a file of 10,000 cells to set gates for sorting: The low-CD4-precursor population is represented by the Thy-1^{lo}c-kit^{+} subpopulation, which is 10–20% of the total stained cells (*see* **Fig. 1**). Despite the name, CD4 is generally not used for staining; a combination of Thy-1 and c-kit gives the best separation of this population.
6. Sort the precursors by setting up live gates for Thy-1^{lo}c-kit^{+} cells and excluding dead cells using positivity for PI and very low forward scatter.
7. Determine the purity of the sorted population by reanalysis. It is usually > 98%.
8. The number of low CD4 precursors recovered is usually between $1.5–3 \times 10^5$ per experiment (or 16 thymus).

3.3. Culture of Thymic Precursors

A "low-density-culture" system is used to determine accurately the expansion of precursors in culture.

1. Count the sorted low-CD4-precursor cells after sorting and prior to dilution in Complete Medium (*see* **Materials**) containing cytokines. A seven cytokine cocktail, IL-1β, IL-3, IL-7, TNFα, SCF, Flt3L, and anti-CD40, yields an expansion of cells with the majority having the morphological appearance of DC by day 4.
2. Disperse the precursor cells in the cytokine medium, at a concentration of 2.5×10^4 cells/mL.
3. A 10 μL volume is carefully placed in each of 20 wells of a Terasaki tray (*see* **Note 6**).
4. To achieve even distribution of cells in the bottom of each well, the Terasaki tray is inverted on incubation at 37.5°C in a humidified 10% CO_2-in-air incubator.

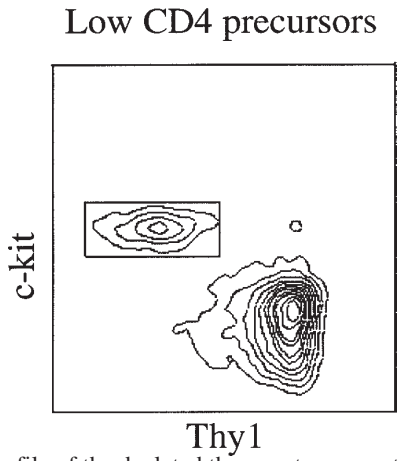

Fig. 1. A typical profile of the depleted thymocyte preparation by flow cytometric analysis. The cells are stained with anti-Thy1-PE and anti-c-kit-FITC. The low-CD4-precursor population is represented by the boxed Thy-1^{lo}c-kit^{+} population.

5. After overnight incubation, the tray is carefully turned over.
6. For surface phenotype analysis, a larger cell yield is required. In this case precursor cells are mixed with culture medium, at $1–2 \times 10^5$ cells/mL, and 100 μL-aliquots placed in flat-bottom wells of a 96-well culture tray.

3.4. Analysis of Cultured Cells

3.4.1. Cell Counts and Visualization of Dendritic Morphology

To recover cells after culture, one-tenth volume of 0.1 *M* EDTA pH 7.2, is first added and mixed well by repeated passage through a pipette tip. This procedure is necessary to break up the DC clusters, shown in **Fig. 2**, into a single-cell suspension (*see* **Note 7**). For low-density culture, the volume from each well is separately loaded into a hemocytometer channel, without the addition of eosin. DC numbers and total cell numbers are counted using a phase-contrast microscope. Over 95% of the individual cells recovered from cultures at day 4 have the morphological appearance of DC (**Fig. 2**).

3.4.2. Phenotypic Analysis of Cultured Cells

1. For phenotypic analysis, combine the cells from numerous wells in a 10 mL conical tube and centrifuge to a pellet.
2. Resuspend the cells in BSS-0.01 *M* EDTA-3% FCS and stain for DC markers: Cy5-anti-MHC Class II, Texas Red-anti-CD11c, and biotin-anti-DEC205, followed by PE-avidin as second stage (*see* **Note 8**).
3. The staining should be at 4°C and cells washed in BSS-EDTA-3%FCS through a layer of FCS between first and second stage (*see* **Note 8**).

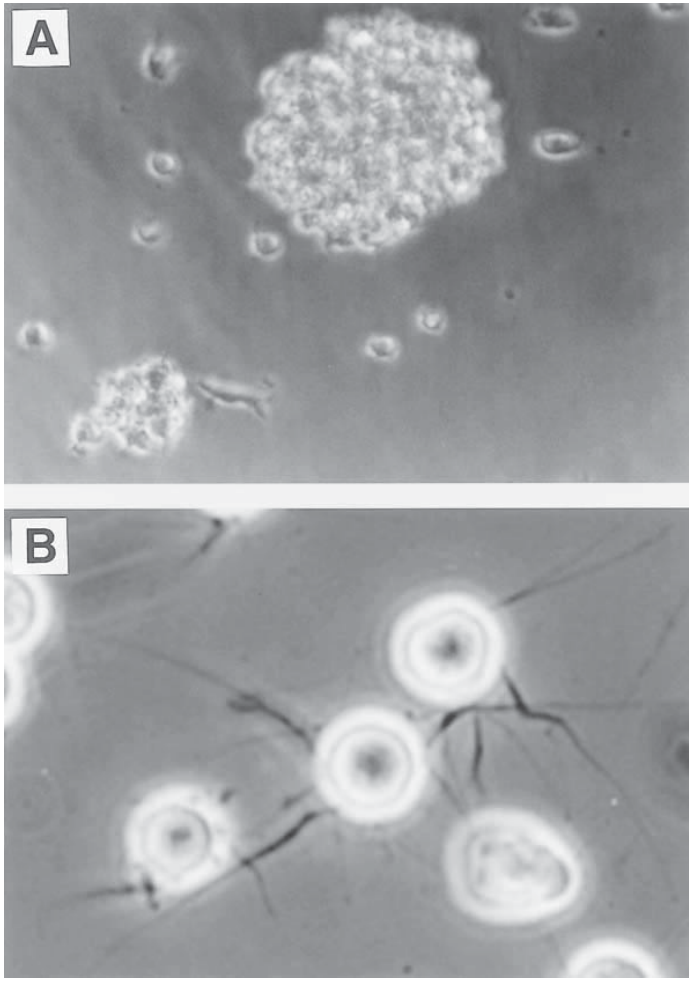

Fig. 2. The appearance of typical clusters and individual DC, which have developed in culture from the low CD4 precursors after 4 d. The clusters **(A)** were photographed directly in the well of a Terasaki tray. Magnification 280-fold. To photograph individual DC **(B)** the cells were first dissociated with 0.1 *M* EDTA, replaced in Culture Medium in a slide chamber and incubate at 37°C for 2 h. This allows for visualization of cytoplasmic extensions. Magnification 650-fold.

4. Include PI in the last wash to exclude dead cells on flowcytometric analysis (*see* **Note 9**).

4. Notes

4.1. Media

1. All media is of mouse tonicity, 308 milli-osmolar or equivalent to 0.168 *M* NaCl.
2. It is important that nycodenz be at 4°C for all density determinations and for use in experiment.

4.2. Immunomagnetic Bead Depletion

3. An FCS underlayer is used for all cell washes involving antibodies. This step allows for removal of residual antibody with only one washing step.
4. A large amount of beads are required for the first-round magnetic-bead depletion. For economic reasons P&L beads are used for this round of depletion.
5. Paesel and Lorei beads migrate very slowly to the magnet due to their small size. Therefore, it is important after the antibody/cell binding step that the tube be left on the magnet at least 3–5 min to successfully remove unwanted cells that are attached to the beads.

4.3. Culture and Analysis of Cells

6. To avoid evaporation from Terasaki wells during incubation, the edge wells of the tray are filled with 10 μL Complete Medium, without cytokines.
7. After culture of low CD4 precursors, EDTA is added to each well and mixed well by repeated passage through a pipet tip to break up cell clusters. Even after the addition of EDTA, many clusters of DC are still visible. In general, a cluster is counted as 20 cells, although it may contain up to 200 or more cells.
8. It is important to include EDTA in all medium after culture for phenotypic analysis. DC tend to form clumps which can cause nozzle blockages in the flowcytometer.
9. For phenotypic analysis the FITC channel is avoided due to high autofluorescence of the cells.

References

1. Wu, L., Antica, M., Johnson, G. R., Scollay, R., and Shortman, K. (1991) Developmental potential of the earliest precursor cells from the adult mouse thymus. *J. Exp. Med.* **174,** 1617–1627.
2. Matsuzaki, Y., Gyotoku, J., Ogawa, M., Nishikawa, S., Katsura, Y., Gachelin, G., and Nakauchi, H. (1993) Characterization of c-kit positive intrathymic stem cells that are restricted to lymphoid differentiation. *J. Exp. Med.* **178,** 1283–1291.
3. Ardavin, C., Wu, L., Li, C., and Shortman, K. (1993) Thymic dendritic cells and T cells develop simultaneously within the thymus from a common precursor population. *Nature* **362,** 761–763.
4. Steinman, R. M. (1991) The dendritic cell system and its role in immunogenicity. *Ann. Rev. Immunol.* **9,** 271–296.
5. Inaba, K., Inaba, M., Romani, N., Aya, H., Deguchi, M., Ikehara, S., Muramatsu, S., and Steinman, R. M. (1992) Generation of large numbers of dendritic cells

from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. *J. Exp. Med.* **176,** 1693–1702.

6. Caux, C. and Banchereau, J. (1996) In vitro regulation of dendritic cell development and function, in Blood cell biochemistry, *Vol. 7, Hemopoietic growth factors and their receptors* (Whetton, A. and Gordons, J., eds.), Plenum Press, London, pp. 263–301.
7. Saunders, D., Lucas, K., Ismaili, J., Wu, L., Maraskovsky, E., Dunn, A., and Shortman, K. (1996) Dendritic cell development in culture from thymic precursor cells in the absence of granulocyte/macrophage colony-stimulating factor. *J. Exp. Med.* **184,** 2185–2196.

IV

Generation of Murine Dendritic Cell Lines

20

Generation of Mouse Dendritic Cell Lines

Stefania Citterio, Maria Rescigno, Maria Foti, Francesca Granucci, Malgosia Matyszak, Giampiero Girolomoni, and Paola Ricciardi-Castagnoli

1. Introduction

Dendritic cells (DC) are now recognized as major players in the control of immune responses ***(1)***, since they direct both the quality and the extent of the adaptative response. Thus, DC represent a very appropriate means for the manipulation of harmful or protective immunity ***(2–4)***.

As DC are present in both lymphoid and nonlymphoid tissues, but in relatively low numbers, it is difficult to obtain large numbers of these cells with a high degree of purity. For many types of studies it would be beneficial to have reliable methods to generate and, therefore, to grow large numbers of homogeneous DC. In particular, the molecular basis for the unique immunostimulatory properties of DC, the precise mechanism of antigen handling, and the biochemical pathways of signal transduction have all been only marginally investigated. Furthermore, a complete characterization of DC physiology and the identification of DC-specific genes are a necessary prerequisite for an optimal use of DC in immunotherapy and for selective targeting of DC functions.

It has been shown that larger amounts of DC can be obtained by culturing precursors from bone marrow cells in granulocyte-macrophage colony stimulator factor (GM-CSF) ***(5,6)***. However, these cultures are very heterogeneous and will contain many other cell types. Moreover, up to now, this approach has not allowed the cloning of DC. A different approach has been to generate DC-like cells from peripheral blood monocytes ***(7,8)***.

In the mouse system, we succeeded in generating homogeneous, growth-factor-dependent DC lines from various tissues ***(9)*** using a conditioned

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medium, containing several cytokines, including mGM-CSF. These DC lines have an immature phenotype, and can continuously grow unless deprived of the conditioned medium. Also, in the last few years, immortalization of DC from mouse tissues, using v-myc^{MH2} oncogene ***(10)*** has successfully been achieved ***(11,12)***. The immortalized DC lines are homogeneous and easily grown, and have been extensively used for functional and biochemical DC characterization.

In the following sections, we describe the generation of two types of DC lines:

1. v-myc^{MH2} immortalized DC lines, which do not require growth factors for their propagation.
2. Growth-factor-dependent DC lines.

1.1. Immortalized DC Lines

Several immortalized DC lines have been generated, mainly by introducing the immortalizing v-myc^{MH2} gene into DC cultures ***(11,12)***. Retroviral vectors are among the most efficient means of transferring genes into cells. A major advance in their use has been the construction of packaging cell lines containing a helper-free retroviral vector defective in the RNA packaging signal sequence but with intact structural genes. When these cells are infected with a replication-defective retrovirus bearing the gene(s) of interest and with an intact sequence, they produce pure stocks of the engineered viruses without the recipient cell becoming a retrovirus producer ***(13)***. Recombinant retroviruses with the v-myc^{MH2} oncogene (VN11) inserted in the mouse AKRv viral genome can specifically immortalize hematopoietic cells with a myeloid/dendritic phenotype including macrophages, microglial cells, and dendritic cells ***(14–16)***.

Using the helper-free retroviral vector MIB-ψ2-N11, immortalized DC lines were generated from the spleen ***(11,15)*** and the fetal skin ***(12)***. DC lines did not require additional exogenous factors for their growth. The cells exhibited phenotypic (**Fig. 1**) and functional features of immature DC, including the ability to present exogenous antigens on class I and class II molecules and the capacity to induce primary T-cell response in vitro and in vivo ***(17–19)***. However, the inability to induce growth arrest in these immortal lines hampered the complete maturation of DC upon activation.

In contrast, growth-factor-dependent DC lines could be induced to full maturation in vitro, mimicking the whole natural process of DC differentiation that occurs after their activation with pathogens.

1.2. Growth-Factor-Dependent DC Lines

Several DC lines have been obtained from various mouse tissues including the spleen, bone marrow, and Peyer's patches. The spleen DC line (D1) was obtained by culturing cell suspensions in medium supplemented with a conditioned media (DC-GM) from fibroblast supernatant containing GM-CSF ***(9)***.

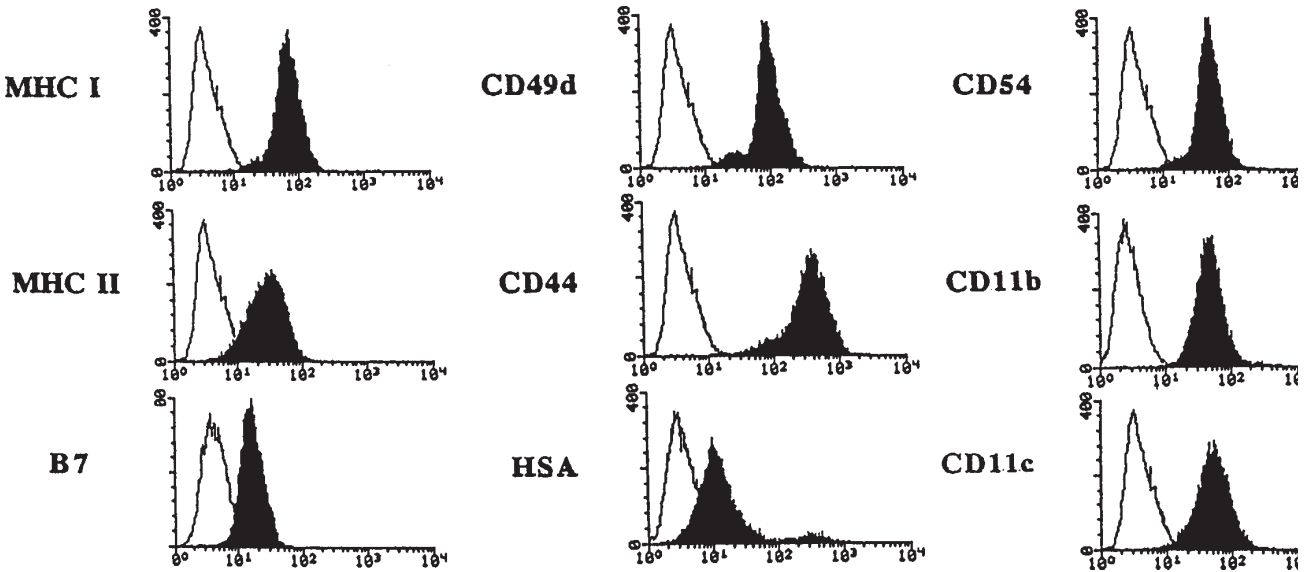

Fig. 1. Surface markers of immortalized DC lines by FACS analysis. Filled histograms show binding of specific antibodies, whereas isotype control antibodies are represented by open histograms.

Studies on these growth-factor-dependent long-term DC cell lines provided very important information about the maturation stages of DC and their interaction with T cells. Two different maturation stages of DC have been identified: an immature stage, also called "processing phenotype," in which DC are highly phagocytic and have low-intermediate levels of MHC and costimulatory molecules on the cell surface (**Fig. 2**), and, a mature stage, called "presenting phenotype," in which DC have lost the capacity to up-take particles and acquired high T-cell costimulatory activity and high levels of MHC and costimulatory molecules ***(9)***.

This phenotypic maturation can be followed in bone marrow culture after 15 d of culture (**Fig. 3**). In the presence of DC-GM medium, two distinct DC populations are generated: the mature DC population, characterized by high expression of MHC II molecules, and the immature DC population, with low-intermediate expression of MHC II molecules.

Long-term cultures (up to 3 mo) of the DC population may lead to the generation of growth-factor-dependent DC lines, which retain the immature phenotype. Morphologically, the immature DC are loosely adherent (**Fig. 4A**). After activation the cells acquire the mature phenotype (**Fig. 4B**) and will grow as clusters, mostly in suspension.

In conclusion, the possibility of having large amounts of long-term DC in the two distinct stages of maturation can be particularly useful for the development of powerful tools for the manipulation of the immune response ***(20,21)***.

2. Materials

2.1. Culture Media

All growth-factor-dependent DC lines are grown in conditioned medium, which is 70% complete Iscove's modified Dulbecco's medium (IMDM) with 10% FBS and 30% DC-GM medium.

All the immortalized cell lines are grown in complete IMDM with 10% FBS.

1. Complete IMDM: Iscove's modified Dulbecco's medium (IMDM, Sigma, St. Louis, MO), containing L-glutamine (2 m*M*, Sigma), 100 IU/mL penicillin, 100 µg/mL streptomycin (Sigma), 2β-mercaptoethanol (0.05 m*M*, Sigma), and 10% heat inactivated fetal bovine serum (FBS)–Australian origin (Life Technologies, Montgomery Country, MD) (*see* **Note 1**).
2. DC-GM medium: The DC-GM medium is a conditioned medium available upon request from P. Ricciardi-Castagnoli.

2.2. Cells and Reagents

1. Freshly removed organs from mice ≤ 6-wk-old.
2. 35 × 10 and 60 × 15 mm^2 tissue culture dishes (Corning Glass Works, Corning, NY).
3. 100 × 20 mm suspension culture dishes (Corning).

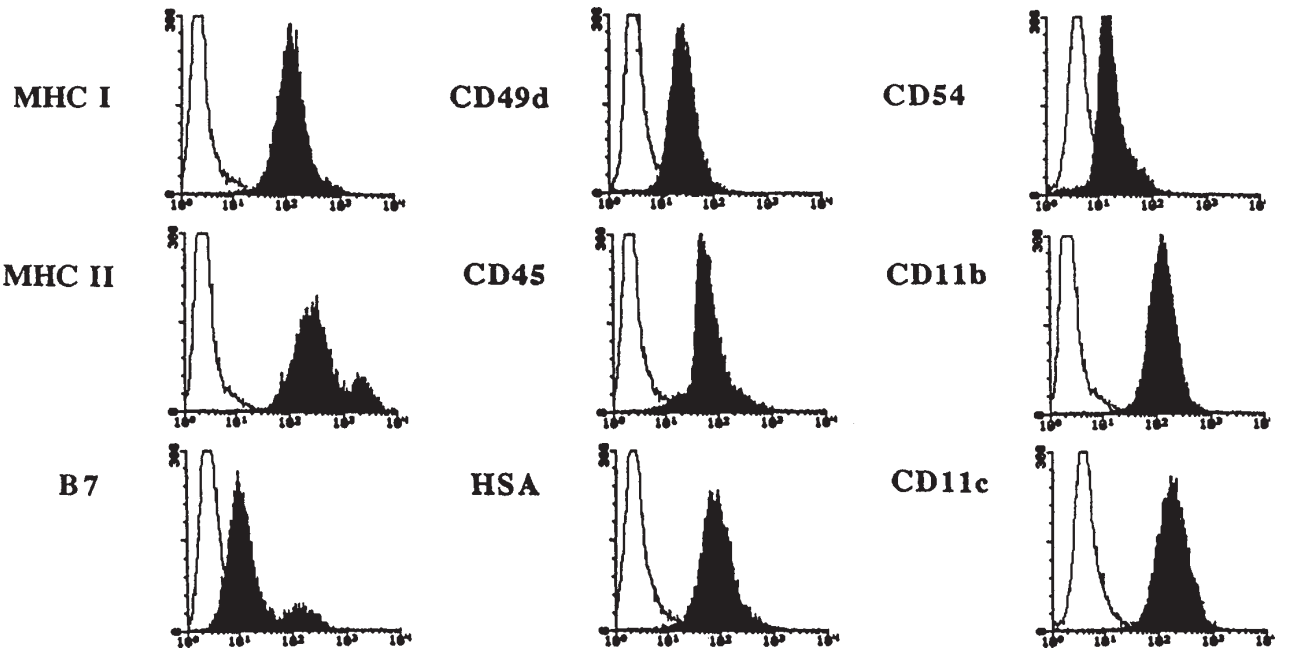

Fig. 2. Surface markers of growth factor dependent DC lines by FACS analysis. Filled histograms show binding of specific antibodies, whereas isotype control antibodies are represented by open histograms.

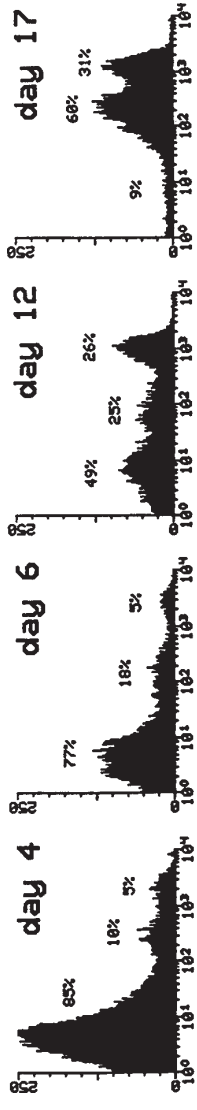

Fig. 3. Surface expression of MHC II molecules of bone marrow derived DC by FACS analysis. Analysis is performed at d 4, 6, 12, 17 of culture with DC-GM medium.

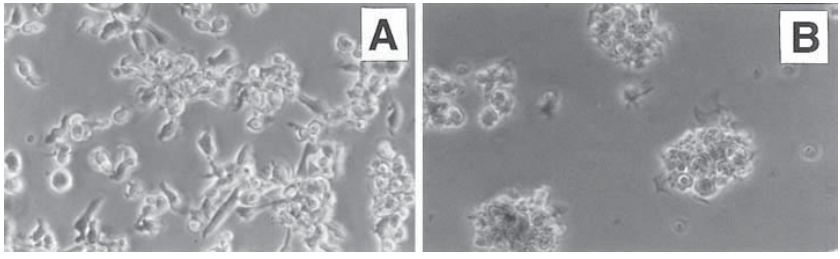

Fig. 4. Morphological appearance by phase contrast microscopy of the spleen DC line D1: **(A)** immature D1 cells; **(B)** Mature D1 cells.

4. Complete IMDM.
5. DC-GM medium.
6. Phosphate-buffered saline (PBS) and PBS-EDTA 2 m*M*.
7. ACK lysing buffer: 0.15 *M* NH_4Cl, 1.0 m*M* $KHCO_3$, 0.1 m*M* Na_2EDTA. pH 7.2–7.4.
8. 0.5% dispase (Neutral protease, Boehringer Mannheim, Mannheim, Germany).
9. Trypsin type XI (Sigma).
10. 200 μm nylon screen mesh.
11. Mouse recombinant GM-CSF (Pharmingen).
12. Viral producer cell line: MIB-ψ2-N11 ***(14)*** is available upon request from P. Ricciardi-Castagnoli.

3. Methods

3.1. Preparation of Cells Suspensions from Lymphoid Tissues

1. This section covers isolation from tissues such as spleen, thymus, lymph node, and Peyer's patches.
2. Place freshly removed organs in a 60 × 15 mm^2 tissue culture dish containing 3 mL PBS. With scissors, cut the organ into several pieces.
3. Using a circular motion, press the pieces against bottom of the dish with the plunger of a 6-mL syringe until mostly fibrous tissue remains.
4. Further disperse clumps in the suspension by drawing up and expelling the suspension several times through a 6-mL syringe equipped with a 19-G needle.
5. Filter cell suspension into a centrifuge tube through a 200-μm mesh nylon screen. Wash the tissue culture dish with 4 mL Complete Medium. Repeat if necessary, and add the wash to the tube.
6. Add 5 mL of complete IMDM, centrifuge for 10 min at 200*g* and discard supernatant (*see* **Subheading 3.4.** if removal of red blood cells is desired). Resuspend the pellet in 20 mL complete IMDM, centrifuge again, and resuspend in a volume suitable for counting.

3.2. Preparation of Cells Suspensions from Fetal Skin

1. Sacrifice mouse fetuses of 17–19 d gestational age.
2. Remove trunk skin and put it in a 60 × 15 mm^2 tissue culture dish with 3 mL of PBS.
3. Scrape subcutaneous surfaces with forceps, and then cut them into small pieces (less than 1 cm^2).
4. Incubate the pieces with 0.5% dispase (Boehringer) for 1 h at 37°C, to separate epidermis from dermis.
5. Separate epidermal and dermal sheets using forceps.
6. Disaggregate epidermal sheets using 0.25% trypsin (Type XI, Sigma) for 20 min at 37°C.
7. Add 10 mL of complete IMDM to inactive trypsin and then filter the cell suspension through a 200 μm mesh nylon screen, centrifuge, and resuspend pellet in complete IMDM.

3.3. Preparation of Cells Suspensions from Bone Marrow

1. Place the two femurs in 60 × 15 mm^2 Tissue Culture dish containing 3 mL PBS.
2. Remove excess muscle by holding one end of bone with forceps, and, using scissors, push muscle downward away from the forceps. Slice the head of both epiphyses (*see* **Note 2**).
3. Attach 1 mL syringe to 26-G needle and fill with PBS.
4. Insert the needle into the bone marrow cavity of the femural epiphysis. Flush the bone cavity with 5 mL of PBS, or, at least, until the cavity appears white. Allow wash medium to collect in the sterile 50 mL conical centrifuge tube on ice.
5. Centrifuge cells for 10 min at 500*g*.
6. Discard supernatant. Resuspend cell pellet in complete IMDM for counting the cells (*see* **Note 3**).

3.4. Removal of Red Blood Cells from Spleen Cell Suspension

Before counting the total number of spleen cell suspension, it is better to remove the red blood cells. From the thymus, lymph node, bone marrow, and Peyer's patches, this will not be necessary.

1. Resuspend pellet of spleen cells in ACK lysing buffer, using 5 mL per spleen, in a 15-mL conical centrifuge tube.
2. Incubate for 5 min at room temperature.
3. Add complete medium to fill the tube, spin for 10 min at 200*g*, and discard the supernatant. Wash the pellet again, and resuspend it in complete medium for counting.

3.5. Infecting Cells with Retroviral Supernatant

1. Collect the supernatant from a 24-h subconfluent MIB-ψ2-VN11 cell culture and centrifuge at 400*g* for 5 min to pellet cell debris. Filtering through 0.2-μm filter also removes the cells. Supernatants should be used immediately.

2. DC cultures should be growing in log phase at the time of infection. At 18–24 h prior to infection, plate cells at 5×10^5 cells/mL in a 35×10 mm^2 tissue culture dish containing 2 mL of medium.
3. Remove 1 mL media from each DC plate.
4. Add 1 mL of fresh viral supernatant containing 20 μg/mL Polibrene (Sigma) to each DC plate.
5. Place each DC plate at 37°C in 5% CO_2 with gentle manual shaking every 15 min for 2 h.
6. 24 h after the infection change medium in each DC plate (*see* **Note 4**).

During the first 2 wk after the infection, supplement the medium with rmGM-CSF (50–100 ng/mL). After that the cells can be grown in complete IMDM. One month after the infection, the proliferating cells can be replated. After about 10 further passages in vitro, they are considered established as continuous cell lines. Once established in vitro, the cells are cloned by limiting dilution and grown in complete IMDM, without any additional growth factors.

3.6. Generation of Long-Term Growth Factor-Dependent DC Cultures

1. Obtain single-cell suspensions from lymphoid or nonlymphoid tissues as previously described.
2. Plate remaining unfractionated cell populations at a density of $3–5 \times 10^5$ cells/mL in suspension culture dishes (Corning) (*see* **Note 5**) in conditioned medium (DC-GM) at 37°C with 5% CO_2.
3. Feed cultures with fresh DC-GM medium every 3–4 d (*see* **Note 6**).
4. Perform first passages of DC-enriched cultures around d 7 (for bone-marrow-derived DC) or 12–15 (for all the others) (*see* **Note 7**).
5. Then, once a week, collect both suspended and weakly adherent cells using PBS, centrifuged at 200*g*, and seed at a density of $2–3 \times 10^5$ cells/mL. Discard the remaining strongly adherent cells (*see* **Note 8**).
6. After 3 mo of continuous culture, cells can be passaged every 3–4 d using PBS-EDTA 2 m*M* and are considered an established line.

To define the DC line as growth-factor-dependent, the culture should be tested for its lack of survival in the absence of the DC-GM medium. In this condition DC should die within 3 d of culture.

As these DC lines are not clonally selected, it is necessary to prepare frozen stocks of the cells that can be kept as a backup storage.

3.7. Cryopreservation of DC Lines

3.7.1. Cell Freezing

1. Make a fresh solution of 10% DMSO in FBS and put on ice.
2. Collect cells in log phase of growth using PBS-EDTA 2 m*M* and centrifuge for 5 min at 200*g*.

3. Resuspend the pellet in complete IMDM (1–2 × 10^6 cells/tube). Centrifuge for 5 min at 200*g*.
4. Add dropwise 1 mL of DMSO solution to each tube.
5. Transfer the cells to the freezing vials and freeze using a temperature-controlled freezing apparatus.

3.7.2. Cell Thawing

1. Thaw vial quickly in 37°C water bath.
2. Add dropwise 1 mL of complete IMDM, while gently shaking.
3. Transfer the cell suspension to a 15 mL tube, add 5 mL of complete medium and centrifuge for 5 min at 200*g*.
4. Remove supernatant and wash the pellet once with complete IMDM.
5. Plate cells in DC-GM medium.

4. Notes

4.1. Culture Medium and Reagents

1. To optimize DC growth conditions, different batches of sera should be tested prior to use. In the presence of debris, it is better to filter serum, in order to avoid phagocytosis.

4.2. Generation of DC Lines

2. The number of cells obtained per mouse will vary, depending on the efficiency of flushing of the bone cavity. Cut carefully only the very end of both epiphysis, and then flush the cavity by inserting the needle once in one epiphysis and then in the other one.
3. If immediate processing is not possible, viability of cell suspensions is best maintained by keeping the pelleted cell suspension in a container with crushed ice. This will also reduce loss of cells that have a tendency to stick to the plastic. It is better to use only polypropylene tubes.
4. The infection can be repeated two or three times. Only freshly prepared viral supernatant must be used. After an overnight incubation, infected cells are washed once and fresh medium is added to each plate.
5. DC lines are more easily obtained and cultured using suspension-culture- than tissue-culture-treated dishes.
6. Half of the medium is collected and centrifuged, then the cell pellet is resuspended in fresh medium and replated.
7. Proliferating cell clusters are monitored daily and split, when necessary, at a concentration between 2 and 3 × 10^5 cells/mL.
8. It is often necessary to wash the cell pellet with PBS, in order to discard debris and dead cells.

References

1. Banchereau, J. and Steinman, R.M. (1998) Dendritic cells and the control of immunity. *Nature* **392,** 245–251.
2. Schuler, G., Thurner, B., and Romani, N. (1997) Dendritic cells: from ignored cells to major players in T-cell-mediated immunity. *Int. Arch. Allergy Immunol.* **112,** 317–333.
3. Girolomoni, G. and Ricciardi-Castagnoli, P. (1997) Dendritic cells hold promise for immunotherapy. *Immunology Today* **18,** 102–104.
4. Schuler, G. and Steinmann, R. M. (1997) Dendritic cells as adjuvants for immune-mediated resistance to tumors. *J. Exp. Med.* **186,** 1183–1187.
5. Inaba, K., Inaba, M., Romani, N., Aya, H., Deguchi, M., Ikehara, S., Muramatsu, S., and Steinman, R. M. (1992) Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with GM-CSF. *J. Exp. Med.* **176,** 1693–1702.
6. Szabolcs, P., Moore, M. A. S., and Young, J. W. (1995) Expansion of immunostimulatory dendritic cells among the myeloid progeny of human $CD34^+$ bone marrow precursors cultured with *c-kit* ligand, GM-CSF and TNF-α. *J. Immunol.* **154,** 5851–5861.
7. Romani, N., Gruner, S., Brang, D., Kampgen, E., Lenz, A., Trockenbacher, B., Konwalinka, G., Fritsch, P. O., Steinman, R. M., and Schuler, G. (1994) Proliferating dendritic cells progenitors in human blood. *J. Exp. Med.* **180,** 83–93.
8. Romani, N., Reider, D., Heuer, M., Ebner, S., Kampgen, E., Eibl, B., Neiderweiser, D., and Schuler, G. (1996) Generation of mature dendritic cells from human blood: an improved method with special regards to clinical applicability. *J. Immunol. Methods* **196,** 137–151.
9. Winzler, C., Rovere, P., Rescigno, M., Granucci, F., Penna, G., Adorini, L., Zimmermann, V.S., Davoust, J., and Ricciardi-Castagnoli, P. (1997) Maturation stages of mouse dendritic cells in growth factor-dependent long-term cultures. *J. Exp. Med.* **185,** 317–328.
10. Sassano, M., Granucci, F., Seveso, M., Marconi, G., Foti, M., and Ricciardi-Castagnoli, P. (1994) Molecular cloning of a recombinant retrovirus carrying a mutated env-AKR myc-MH2 fusion gene immortalizing cells of the monocytic-macrophage lineage. *Oncogene* **9,** 1473–1477.
11. Paglia, P., Girolomoni, G., Robbiati, F., Granucci, F., and Ricciardi-Castagnoli, P. (1993) Immortalized dendritic cell line fully competent in antigen presentation initiates primary T cell responses in vivo. *J. Exp. Med.* **178,** 1893–1901.
12. Girolomoni, G., Lutz, M. B., Pastore, S., Assmann, C. U., Cavani, A., and Ricciardi-Castagnoli, P. (1995) Establishment of a cell line with features of early dendritic cell precursors from mouse fetal skin. *Eur. J. Immunol.* **25,** 2163–2169.
13. Mann, R., Mulligan, R. C., and Baltimore, D. (1983) Construction of a retrovirus packaging mutant and its use to produce helper-free defective retrovirus. *Cell* **33,** 153–159.

14. Pirami, L., Stockinger, B., Betz-Corradin, S., Sironi, M., Sassano, M., Valsasnini, P., Righi, M., and Ricciardi-Castagnoli P. (1991) Mouse macrophage clones immortalized by retroviruses are functionally heterogenous. *Proc. Natl. Acad. Sci. USA* **88,** 7543–7547.
15. Lutz, M. B., Granucci, F., Winzler, C., Marconi, G., Paglia, P., Foti, M., Assmann, C.U., Cairns, L., Rescigno, M., and Ricciardi-Castagnoli, P.(1994) Retroviral immortalization of pahgocytic and dendritic cells clones as a tool to investigate functional heterogeneity *J. Immunol. Methods* **174,** 269–279.
16. Granucci, F., Girolomoni, G., Lutz, M. B., Gnocchi, P., Foti, M., Marconi, G., Nolli, L., and Ricciardi-Castagnoli, P. (1994) Modulation of cytokine expression in mouse dendritic cell clones. *Eur. J. Immunol.* **24,** 2522–2526.
17. Bachmann, M. F., Lutz, M. B., Layton, G. T., Harris, S. J., Fehr, T., Rescigno, M., and Ricciardi-Castagnoli, P. (1996) Dendritic cells process exogenous viral proteins and virus-like particles for class I presentation to CD8 cytotoxic T lymphocytes. *Eur. J. Immunol.* **26,** 2595–2600.
18. Lutz, M. B, Assmann, C. U., Girolomoni, G., and Ricciardi-Castagnoli, P. (1996) Different cytokines regulate antigen uptake and presentation of precursor dendritic cell line. *Eur. J. Immunol.* **26,** 586–594.
19. Lutz, M. B, Rovere, P., Kleijmeer, M. J., Rescigno, M., Assmann, C. U., Oorschot, V. M., Geuze, H. J., Trucy, J., Demandolx, D., Davoust, J., and Ricciardi-Castagnoli, P. (1997) Intracellular routes and selective retention of antigens in mildly acidic cathepsin D/lysosome-associated membrane protein-1/MHC class II-positive vesicles in immature dendritic cells. *J. Immunol.* **159,** 3707–3716.
20. Rescigno, M., Citterio, S., Thèry, C., Rittig, M., Medaglini, D., Pozzi, G., Amigorena, S., and Ricciardi-Castagnoli, P. (1998) Bacterial-induced neo-biosynthesis, stabilization and surface expression of functional class I molecules in mouse dendritic cells. *Proc. Natl. Acad. Sci. USA* **95,** 5229–5234.
21. Zitvogel, L., Regnault, A.,Lozier, A., Wolfers, J., Flament, C., Tenza, D., Ricciardi-Castagnoli, P., Raposo, G., and Amigorena, S. (1998) Eradication of established murine tumors using a novel cell-free vaccine: dendritic cell-derived exosomes. *Nature Medicine* **4,** 594–600.

21

Generation of Langerhans Cell-Like Dendritic Cells from Murine Fetal Skin

Thilo Jakob and Mark C. Udey

1. Introduction

The dendritic cell (DC) lineage is comprised of bone marrow-derived cells that are present in small numbers in nonlymphoid as well as lymphoid tissues *(1)*. Nonlymphoid DC, such as epidermal Langerhans cells (LC), display an immature phenotype, i.e., they are capable of acquiring and processing antigen, but express only low levels of MHC antigen and costimulatory molecules and do not effectively initiate primary immune responses. In contrast, interdigitating DC in lymphoid tissue display a mature phenotype, i.e., they have limited phagocytic and antigen-processing capacity, but express high levels of MHC antigen and costimulatory molecules and are very effective stimulators of naive T cells *(2)*. The conceptual link between nonlymphoid and lymphoid organ DC was provided by Schuler et al. several years ago when they demonstrated that epidermal LC cultured in GM-CSF–containing media for several days matured into cells with phenotypic and functional characteristics of interdigitating DC *(3)*. The transformation of immature epidermal LC into mature DC in vitro mimics what occurs in vivo during the migration of epidermal LC to regional lymph nodes. This model has been widely used for studies of LC/DC function.

LC represent only 1–3% of all cells present in epidermal cell preparations. Techniques used to enrich or isolate LC from epidermal cell suspensions are cumbersome, often lead to variable LC recovery, and result in preparations that are invariably contaminated with keratinocytes. In addition, freshly isolated LC spontaneously and rapidly acquire a mature phenotype in culture, and remain viable only in the presence of DC-activating cytokines. These limitations and others have led investigators to search for alternative methods to obtain immature DC from nonlymphoid organs like the skin. The availability

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of large numbers of DC of high purity will facilitate various kinds of studies of DC biology and function (e.g., studies of signal transduction pathways in DC).

Several years ago, Inaba et al. ***(4,5)*** demonstrated that DC could be expanded from murine blood and bone marrow in GM-CSF–supplemented media. Subsequently, other investigators generated DC from various tissues including liver ***(6)***, spleen ***(7,8)***, and skin ***(9–11)***. Although the availability of DC from these various sources has allowed studies of certain aspects of DC biology, the degree to which these cells recapitulate the biology of DC present in individual nonlymphoid and lymphoid tissues in vivo is uncertain. This may be especially true of DC lines that have been immortalized by oncogene-containing retroviruses ***(11)*** or that have been maintained in culture for long periods of time in the presence of growth factors ***(9,10)***.

Thus, we set out to establish a short term culture system in which cells that more closely resembled LC could be propagated. Based on the work of Elbe ***(9)*** and Girolomoni ***(11)*** and coworkers, we used murine fetal skin as a source of LC progenitors. Immature LC have been identified in the skin of fetal mice ***(12,13)***, and DC lines have been established from this tissue ***(9,11)***. The experience of Inaba ***(4,5)***, Witmer-Pack ***(14)***, and Takashima ***(15)*** and their coworkers led us to focus on GM-CSF and CSF-1 (M-CSF) as important growth factors for LC (or LC-like cells).

Herein we detail the conditions used to generate fetal-skin-derived DC (FSDDC) in primary culture. Extensive characterization revealed that FSDDC obtained after 14 d of primary culture display the phenotype of immature DC (FSDDC-I) and closely resemble LC with regard to surface antigen expression, cytokine profile, and functional activity ***(16)***. Similar to LC, FSDDC-I spontaneously mature into cells almost indistinguishable from mature interdigitating DC (FSDDC-M) when subcultured for 3–5 d ***(16)***. Because FSDDC-I maturation is slow (i.e., the phenotype of FSDDC-I is relatively stable), we have been able to begin to characterize mechanisms involved in DC maturation ***(17,18)***. To date, we have established over 200 primary cultures and reproducibly generated large numbers of FSDDC from fetal skin of different mouse strains (e.g., C57BL/6 or BALB/c). Since FSDDC generated in primary cultures closely mimic several aspects of LC biology, we anticipate that FSDDC will prove to be a useful tool to address aspects of LC biology that were previously unapproachable.

2. Materials

Quantities of reagents and solutions described are sufficient to prepare a fetal-skin single-cell suspension from fetuses of four pregnant mice, initiate cultures with 10^8 cells in twenty T-75 tissue culture flasks, and harvest FSDDC-I after 14 d of culture. For larger or smaller preparations, quantities have to be adjusted accordingly.

2.1. Preparation of Fetal-Skin-Cell Suspension

All instruments should be sterile and all reagents and solutions should be sterilized by filtration through a 0.22 μm Millipore filter.

1. Pair of fine, curved scissors.
2. Curved watchmaker forceps.
3. Nylon mesh (100 μm pore size; Tetko Inc., Briarcliff Manor, NY)
4. 50 mL conical polypropylene tubes.
5. Polystyrene Petri dishes.
6. Ethanol (70%).
7. Hank's balanced salt solution (HBSS; Biofluids, Rockville, MD).
8. Fetal bovine serum (FBS; Biofluids).
9. Trypsin (bovine pancreas, crystalline powder, 2.7 USP U/mg; USB, Amersham Life Science, Arlington Heights, IL).
10. Deoxyribonuclease I (DNase, bovine pancreas, lyophylized powder; Sigma, St. Louis, MO).
11. 500 mL of HBSS/FBS(5%) as washing solution.
12. 20 mL of HBSS/trypsin(0.25%, w/v).
13. 20 mL of HBSS/FBS(30%)/DNase(0.05%, w/v).

2.2. Culture and Harvest of FSDDC

1. Tissue culture flasks (T-75, ventilated; Costar, Cambridge, MA).
2. DC media: RPMI 1640 (Biofluids) supplemented with 5% FBS (Intergen, Purchase, NY), 1 μg/mL indomethacin, 50 μ*M* 2-mercaptoethanol (Sigma), 20 m*M* glutamine, 0.1 m*M* nonessential amino acids, 1 m*M* sodium pyruvate, 1% antibiotic-antimycotic (Gibco-BRL, Grand Island, NY), 10 m*M* HEPES (Biofluids).
3. Complete DC media: DC media supplemented with recombinant murine GM-CSF (10 ng/mL; Peprotech, Rocky Hill, NJ) and recombinant murine CSF-1 (10 ng/mL; R&D Systems, Minneapolis, NM). Prepare: 2000 mL complete DC media to initiate and feed 20 T-75 flasks over 14 d. Store complete DC media at 4°C.
4. HBSS/trypsin (0.01%; w/v) and HBSS/FBS (30%)/DNase (0,05%; w/v) (10 mL/T-75 flasks) for the harvest of FSDDC.
5. RPMI-1640/FBS (50%) and let equilibrate at RT prior to use (16 mL/T-75 flask) for gradient sedimentation of FSDDC.
6. Trypsin-EDTA (Ca^{2+}- and Mg^{2+}-free HBSS/trypsin (0.05%, w/v)/EDTA (0.53 m*M*); (Gibco-BRL) for cell counting.

2.3. Subculture of FSDDC—Generation of Mature FSDDC

1. 24-well plates or T-25 tissue culture flasks.
2. Complete DC media.
3. Recombinant murine IL-1α, IL-1β, TNFα (10 ng/mL; Genzyme, Cambridge MA) or LPS (Sigma).

3. Methods

3.1. Preparation of Fetal-Skin-Cell Suspension

1. Breed male and female mice (C57BL/6 or BALB/c) by housing 5 male and 15 female (at least 8-wk-old) in large breeding cage for 18 h. Verify pregnancies 12 d postcoitum by palpation of the uterus in the lower abdomen. Anticipate that 10% of the mated females will be impregnated (*see* **Note 1**).
2. Euthanize four, d 16 pregnant mice (C57BL/6 or BALB/c) by CO_2 inhalation and immerse in 70% ethanol for 5 min at room temperature.
3. In a laminar flow hood, place mice into 15 cm sterile Petri dish, lift abdominal skin with forceps, and incise the lower abdomen transversely taking care not to enter viscera. Extend excision on both sides laterally and in cephalad direction. This will allow you to fold back the abdominal wall onto the thorax and provide free access to remove the uterus.
4. Mobilize uterus with watchmaker forceps (grab it preferably between two fetuses) and pull it out of the peritoneal cavity. Cut attached ligaments and blood vessels close to the uterus and place uterus in Petri dish with 25 mL HBSS/5% FCS.
5. Incise uteri with scissors, carefully open each amnion sac, remove amnion from fetus and cut umbilical cord using watchmaker forceps. Place fetuses in new Petri dish with HBSS/5% FCS. Expect approx 7–8 fetuses/litter (range 2–12) (*see* **Note 2**). After recovering all fetuses, wash them three times in HBSS/5% FCS followed by three washes in HBSS by transferring them individually from one Petri dish to another.
6. Hold fetus with watchmaker forceps and remove extremities with a pair of scissors. Cut the skin along the side of the fetus from groin to axilla (on one side only) and completely around the shoulder girdle. Remove trunk skin with forceps and transfer to new Petri dish containing HBSS. Pool skins from all fetuses and wash three times in HBSS.
7. Place skins in empty Petri dish (*see* **Note 3**) and mince with curved scissors until skin fragments are smaller than 1 mm^2, add 10 mL of HBSS/0.25% trypsin, triturate, and add an additional 5 mL HBSS/0.25% trypsin.
8. Incubate for 30 min at 37°C and terminate trypsinization by adding 15 mL of HBSS/30% FBS/0.05% DNase. Obtain a single-cell suspension by vigorously aspirating the skin suspension into a 60 mL syringe and expelling it several times.
9. Filter cell suspension through sterile nylon mesh to remove residual clumps and debris.
10. Wash cell suspension twice in HBSS/5% FBS (sediment at 320*g* for 5 min at 4°C), resuspend in 50 mL complete DC media and count viable cells (trypan blue exclusion test) in a hemocytometer.

3.2. Cell Culture and Harvest of FSDDC

1. Adjust fetal-skin single-cell suspension to a final concentration of 2×10^5 cells/mL in complete DC media (*see* **Note 4**). Routinely, we obtain > 10^8 cells from the fetuses of four pregnant mice, which allows us to seed at least 20 T-75 tissue culture flasks each with 5×10^6 cells (25 mL).

2. Incubate at 37°C in a humidified incubator, 5% CO_2, for 12–14 d.
3. Feed at d 5, 7, 10, and 12 by replacing 60% of the media with fresh complete DC media.
4. Beginning around d 10 expect to observe small, tight aggregates of round cells forming on a background of fibroblastoid stromal cells (**Fig. 1A**). Over the next 2–4 d aggregates increase in number and size. Cultures are generally ready to be harvested on d 14 (**Fig. 1B**) (*see* **Note 5**).
5. Decant media (*see* **Note 6**) and liberate cells by adding 10 mL HBSS/trypsin (0.01%) to each T-75 flask (*see* **Note 7**). Incubate for 30 min at 37°C, in a 5% CO_2 incubator.
6. During the incubation, aliquot 20 mL gradient [RPMI 1640/FBS(50%)] into 50 mL conical polypropylene tubes. For harvesting 20 T-75 flasks, prepare two sets of eight gradients and let equilibrate at room temperature.
7. Terminate trypsinization by adding 10 mL HBSS/FBS(30%)/DNase(0.05%, w/v) to each T-75 flask and dissociate cells by vigorous pipeting. Pool cell suspensions from 20 T-75 flasks in eight 50 mL conical tubes and wash twice with HBSS (spin at 320*g* for 5 min at 4°C).
8. Resuspend cell pellet of each tube thoroughly in 10 mL HBSS and carefully layer over DC gradients in 50 mL tubes (*see* **step 6**) and let stand for 20 min at room temperature, while aggregates preferentially accumulate at the bottom of the tubes (1*g* sedimentation) (*see* **Note 8**).
9. Remove the upper phase, interface, and the upper two-thirds of the lower phase of the gradient so that only the bottom 5 mL remain. Be careful not to aspirate already sedimented cell aggregates at the bottom of the tubes.
10. Wash sedimented cell aggregates in HBSS.
11. Repeat **steps 8–10** and pool aggregates in one tube.
12. Pellet at 320*g* for 5 min at 4°C and resuspend in 20–40 mL of complete DC media (*see* **Note 9**).
13. Plate cell aggregate suspension into T-75 flasks in 10 mL aliquots and incubate at 37°C, in a 5% CO_2 incubator for 1 h. This step removes remaining stromal cells that preferentially and rapidly adhere to tissue culture flasks.
14. Dislodge loosely or nonadherent FSDDC aggregates from flask by tituration and pool in one 50 mL tube.
15. Pellet FSDDC aggregates at 320*g* for 5 min at 4°C and resuspend in 10 mL of complete DC media.
16. To assess the cell number, obtain a single-cell suspension from an aliquot of aggregate suspension. Transfer 250 µL of aggregate suspension into 1.5 mL microcentrifuge tube and pellet at 320*g* for 5 min. Discharge supernatant, resuspend in equal volume of trypsin-EDTA (Ca^{2+}- and Mg^{2+}-free HBSS/trypsin [0.05%, w/v]/EDTA [0.53 m*M*]) and incubate for 15 min in waterbath at 37°C. Dissociate aggregates by vigorous pipeting and count single cells in hemocytometer.
17. On average, 1–3 × 10^7 FSDDC with > 95% purity (*see* **Note 10**) are recovered from a starting population of 10^8 d 16 fetal skin cells after 14 d of culture in complete DC media.

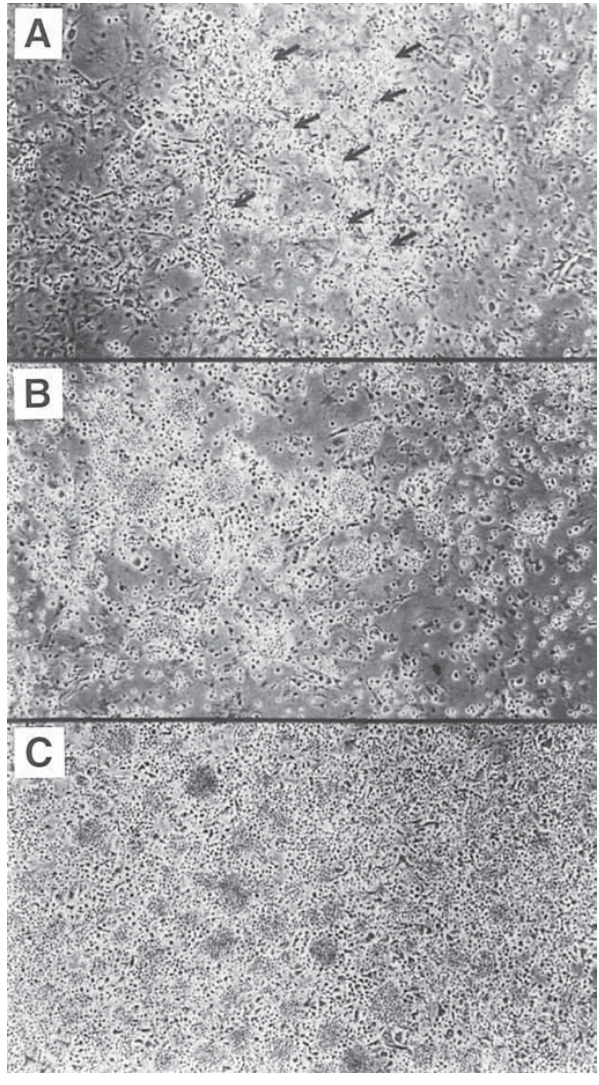

Fig. 1. FSDDC are generated from single-cell suspensions of d 16 fetal murine skin in GM-CSF and M-CSF supplemented primary culture. Starting at day 10 of culture, small clusters of tightly aggregated FSDDC (arrows) become visible **(A)**. Over the next 2–4 d, FSDDC aggregates grow in size and number and are generally ready to be harvested on day 14 **(B)**. Prolongation of the culture period is accompanied by the expansion of single round cells that overgrow FSDDC aggregates **(C)**.

3.3. Subculture of FSDDC—Generation of Mature FSDDC

1. Cells in freshly harvested FSDDC aggregates display the phenotype and function of immature DC ***(16)***. For subculture, FSDDC aggregates are adjusted to 2–5 × 10^5 cells/mL in complete DC media and seeded at a density of 1–2 × 10^5 cells/cm^2 into 24-well plates or T-25 flasks (*see* **Note 11**).
2. During the subsequent 3–5 d, FSDDC aggregates continuously release nonadherent FSDDC with pronounced dendritic morphology (**Fig. 2**). These nonadherent cells can be harvested by decanting and display function and phenotype of mature dendritic cells (FSDDC-M).
3. Alternatively, maturation of FSDDC characterized by loss of homotypic adhesion and up-regulation of MHC class II and costimulator molecules can be induced during subculture within 12 to 18 h by adding proinflammatory cytokines (e.g., IL-1, TNFα) or bacterial products like LPS or immunostimulatory DNA (**Figs. 2** and **3**) ***(17,18)***.

4. Notes

1. Because mice have a 3.5–4 d oestrous cycle, only 1:4 or 1:5 females will conceive during the 18 h mating period. For the generation of four timed pregnant mice, we therefore set up 40 females for mating.
2. The yield from four pregnant mice (28–32 fetuses) is sufficient to obtain 10^8 cells to set up 20 culture flasks as described in **Subheading 3.2.** Because the initial seeding density is crucial (compare **Note 4**), the size of the primary culture has to be reduced accordingly when the litter size is small.
3. We prefer to pool fetal skins in an empty Petri dish. The retained HBSS from previous washes is enough to prevent drying out of samples and helps to hold fetal skins in one place to allow for efficient mincing. When too much medium is present, tissue pieces start floating and mincing becomes difficult.
4. The initial seeding density is important for successful generation of FSDDC. In particular, higher cell densities seem to inhibit the outgrowth of FSDDC aggregates. We obtained optimal results with a seeding density of 2 × 10^5/mL and recommend a range of 1.5–2.5 × 10^5/mL.
5. The timing of cell harvesting is important for the outcome of the FSDDC culture. After 10–12 d, we generally observe three cell types: fibroblastoid stromal cells, clusters of tightly aggregated round cells (DC), and additional round cells that are larger and are usually found as single cells or as cells that loosely adhere to each other. This latter population of cells are leukocytes that display a vacuole-rich cytoplasm, are highly phagocytic, lack typical DC characteristics, and most likely represent macrophage-like cells. The two cell types display different growth characteristics in culture. Thus, cell cultures that were maintained for more than 17 d generally are overgrown by macrophage-like cells (**Fig. 1C**). Under these circumstances, the separation of DC aggregates from single cells yields unsatisfactory results (low numbers, low enrichment of DC). In contrast, when cultures are harvested before the optimal time (14 d), the purity of the preparation is usually high, but the yield rather low because the FSDDC have not

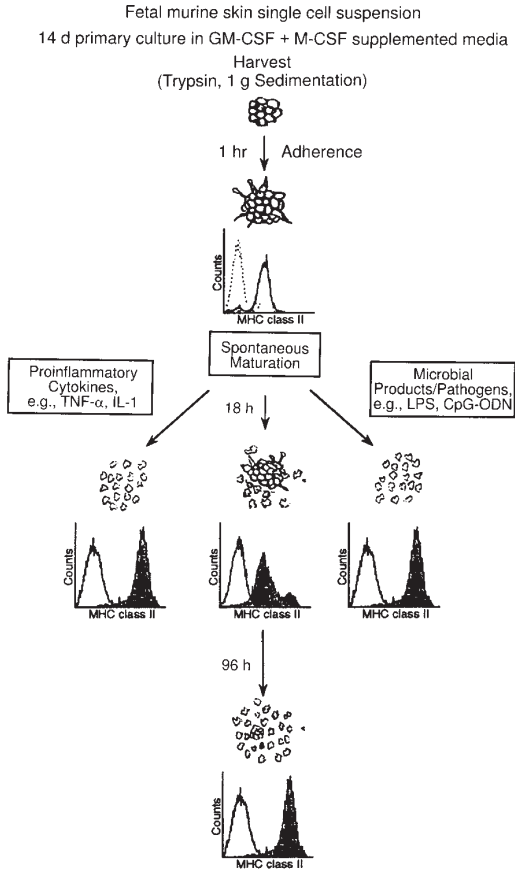

Fig. 2. FSDDC—a model for the study of DC activation and maturation. FSDDC are generated from single-cell suspensions of d 16 fetal murine skin. After 14 d of expansion in GM-CSF and M-CSF supplemented media, FSDDC can be harvested as tightly aggregated cell clusters. At this stage, FSDDC are immature DC with intermediate MHC class II surface staining and strong E-cadherin-mediated adhesion (hence the cell aggregates). After 18 h of subculture, the majority of FSDDC are clustered in aggregates and still display an immature phenotype (intermediate MHC class II). Only after subculture for prolonged periods FSDDC spontaneously acquire a mature phenotype with increased MHC class II expression and loss of E-cadherin-mediated adhesion leading to dissociation of cell aggregates into single cells. Similarly, stimulation of FSDDC with proinflammatory cytokines or microbial products [LPS, CpG-containing immunostimulatory oligodeoxynecleotides (CpG-ODN)] induces maturation, however with an accelerated time course.

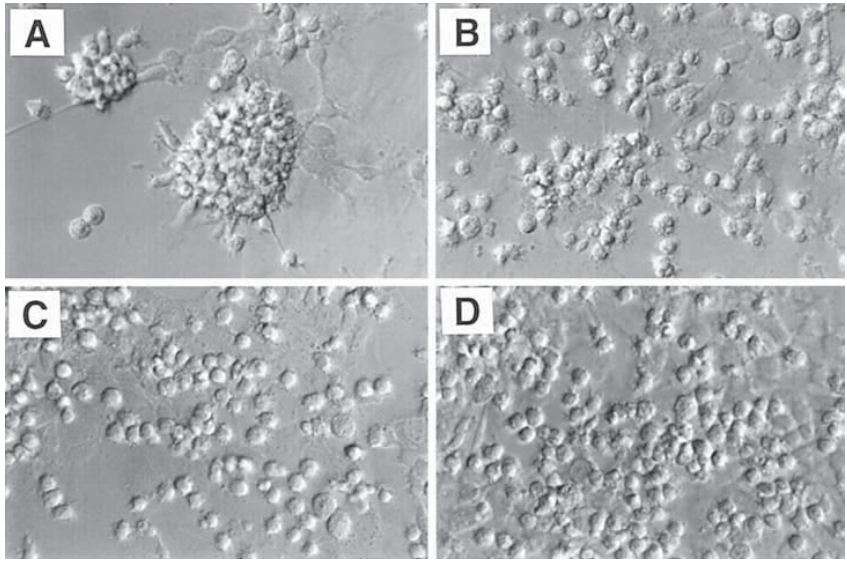

Fig. 3. Phase contrast microscopy of FSDDC after 18 h of subculture. In the absence of DC activation, FSDDC-I remain clustered in tight aggregates **(A)** in which adhesion is E-cadherin-mediated ***(16)***. Stimulation with proinflammatory mediators [e.g., IL-1 **(B)**, TNFα **(C)** or LPS **(D)**] induces activation of FSDDC-I as documented by loss of E-cadherin-mediated adhesion and dissociation into single cells.

expanded sufficiently. As a guideline, we recommend that the cultures be terminated when the following characteristics are apparent: a) multiple aggregates of tightly clustered round cells (FSDDC) are visible on a background of stromal cells, b) the approximate ratio of FSDDC to larger single round cells (marcophage-like cells) is 1:1, and c) stromal cells are still clearly visible and covered less then 50% by round cells or FSDDC aggregates.

6. It is important to drain thoroughly the media before adding the trypsin solution, because remaining culture media containing FBS also contains trypsin inhibitors and provides substrate for trypsin and thus reduces its activity.
7. We have previously demonstrated that FSDDC display homotypic adhesion (i.e., formation of tightly packed cell aggregates) mediated by the homophilic adhesion molecule E-cadherin ***(16)***. Limited trypsinization in the presence of divalent cations (Ca^{2+}, Mg^{2+}) disrupts most adhesive structures but renders cadherin function intact ***(19)***. This treatment therefore dissociates stromal cells and loosely adherent single round cells, but does not dissociate FSDDC aggregates. FSDDC aggregates have a high density/volume and thus a low buoyancy. The relatively large and single stromal cells and macrophage-like cells show the opposite charac-

teristics (low density/volume, high buoyancy). We use this difference to separate FSDDC from the remaining cells using a RPMI-1640/50% FBS gradient at 1*g*.

8. Depending on the yield, FSDDC aggregates may be faintly visible at the bottom of the tubes, whereas most of the cells remain at the interface.
9. Depending on the yield after sedimentation, i.e., size of the pellet, we resuspend FSDDC aggregates in 20, 30 or 40 mL of DC media and plate out 10 mL aliquots for the subsequent 1 h adherence step. The aim is to seed the cell suspension at a density low enough to allow all cells to contact the surface of the culture flask and high enough to avoid unnecessary loss of FSDDC during this purification step (approx 5–10 × 106 cells/10 mL media/T-75).
10. The purity of the FSDDC preparations is consistently high (> 95%) as determined by staining of DC markers such as DEC-205 or gp40 ***(20)***. Contaminating cells display fibroblastoid features in culture (actin stress fibers, fibroblastiod cell shape), are nonleukocytes (i.e., are CD45 negative) and have been designated stromal cells.
11. The seeding density is of particular relevance for the spontaneous maturation of FSDDC. The higher the cell density, the faster FSDDC acquire a mature phenotype. This may reflect autocrine production of cytokines (e.g., TNFα), which are known to induce DC maturation ***(17)***.

References

1. Steinman, R. M. (1991) The dendritic cell system and its role in immunogenicity. *Ann. Rev. Immunol.* **9,** 271–296.
2. Schuler, G. (1991) Epidermal Langerhans cells. CRC Press, Boca Raton, FL.
3. Schuler, G. and Steinman, R. M. (1985) Murine epidermal Langerhans cells mature into potent immunostimulatory dendritic cells in vitro. *J. Exp. Med.* **161,** 526–546.
4. Inaba, K., Steinman, R. M., Witmer-Pack, M., Aya, K., Inaba, M., Sudo, T., Wolpe, S., and Schuler, G. (1992) Identification of proliferating dendritic cell precursors in mouse blood. *J. Exp. Med.* **175,** 1157–1167.
5. Inaba, K., Inaba, M., Romani, N., Aya, H., Deguchi, M., Ikehara, S., Muramatsu, S., and Steinman, R. M. (1992) Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony stimulating factor. *J. Exp. Med.* **176,** 1693–1702.
6. Lu, L., Woo, J., Rao, A. S., Li, Y., Watkins, S. C., Qian, S., Starzl, T. E., Demetris, A. J., and Thompson, A. W. (1994) Propagation of dendritic cell progenitors from normal mouse liver using granulocyte/macrophage colony-stimulating factory and their maturational development in the presence of type-1 collagen. *J. Exp. Med.* **179,** 1823–1834.
7. Lu, L., Hsieh, M., Oriss, T. B., Morel, P. A., Starzl, T. E., Rao, A. S., and Thomson, A. W. (1995) Generation of DC from mouse spleen cell cultures in response to GM-CSF: Immunophenotypic and functional analyses. *Immunology* **84,** 127–134.
8. Lutz, M. B., Granucci, F., Winzler, C., Marconi, G., Paglia, P., Foti, M., Assmann, C. U., Cairns, L., Rescigno, M., and Ricciardi-Castagnoli, P. (1994) Retroviral

immortalization of phagocytic and dendritic cell clones as a tool to investigate functional heterogeneity. *J. Immunol. Methods.* **174,** 267–279.
9. Elbe, A., Schleischitz, S., Strunk, D., and Stingl, G. (1994) Fetal skin-derived MHC class I+, MHC class II- dendritic cells stimulate MHC class I-restricted responses of unprimed CD8+ T cells. *J. Immunol.* **153,** 2878–2889.
10. Xu, S., Ariizumi, K., Caceres-Dittmar, G., Edelbaum, D., Hashimoto, K., Bergstresser, P. R., and Takashima, A. (1995) Successive generation of antigen-presenting dendritic cell lines from murine epidermis. *J. Immunol.* **154,** 2697–2705.
11. Girolomoni, G., Lutz, M. B., Pastore, S., Assmann, C. U., Cavani, A., and Ricciardi-Castagnoli, P. (1995) Establishment of a cell line with features of early dendritic cell precursors from fetal mouse skin. *Eur. J. Immunol.* **25,** 2163–2169.
12. Romani, N., Schuler, G., and Fritsch, P. (1986) Ontogeny of Ia-positive and Thy-1-positive leukocytes of murine epidermis. *J. Invest. Dermatol.* **86,** 129–133.
13. Elbe, A., Tschachler, E., Steiner, G., Binder, A., Wolff, K., and Stingl, G. (1989) Maturational steps of bone marrow-derived dendritic murine epidermal cells. Phenotypic and functional studies on Langerhans cells and Thy-1+ dendritic epidermal cells in the perinatal period. *J. Immunol.* **143,** 2431–2438.
14. Witmer-Pack, M. D., Hughes, D. A., Schuler, G., Lawson, L., McWilliam, A., Inaba, K., Steinman, R. M., and Gordon, S. (1993) Identification of macrophages and dendritic cells in the osteopetrotic (op/op) mouse. *J. Cell Sci.* **104,** 1021–1029.
15. Takashima, A., Edelbaum, D., Kitajima, T., Shadduck, R. K., Gilmore, G. L., Xu, S., Taylor, R. S., Bergstresser, P. R., and Ariizumi, K. (1995) Colony stimulating factor-1 secreted by fibroblasts promotes the growth of dendritic cell lines (XS series) derived from murine epidermis. *J. Immunol.* **154,** 5128–5135.
16. Jakob, T., Saitoh, A., and Udey, M. C. (1997) E-cadherin-mediated adhesion involving Langerhans cell-like dendritic cells expanded from murine fetal skin. *J. Immunol.* **159,** 2693–2701.
17. Jakob, T. and Udey, M. C. (1998) Regulation of E-cadherin-mediated adhesion in Langerhans cell-like dendritic cells by inflammatory mediators that mobilize Langerhans cells in vivo. *J. Immunol.* **160,** 4067–4073.
18. Jakob, T., Walker, P. S., Krieg, A. M., Udey, M. C., and Vogel, J. C. (1998) Activation of cutaneous dendritic cells by CpG-containing DNA oligonucleotides: A role for dendritic cells in the augmentation of Th1 responses by bacterial DNA. *J. Immunol*. **161,** 3042–3049.
19. Takeichi, M. (1990) Cadherins: a molecular family important in selective cell-cell adhesion. *Ann. Rev. Biochem.* **59,** 237–252.
20. Borkowski, T. A., Nelson, A. J., Farr, A. G., and Udey M. C. (1996). Expression of gp40, the murine homologue of human epithelial cell adhesion molecule (Ep-CAM), by murine dendritic cells. *Eur. J. Immunol.* **26,** 110–114.

22

Derivation of Dendritic Cell Lines from Mouse Skin

Akira Takashima and Paul R. Bergstresser

1. Introduction

Dendritic cells (DC) are professional antigen presenting cells characterized morphologically by the extension of numerous dendrites, phenotypically by the expression of relatively large amounts of MHC class II molecules and costimulatory molecules, and functionally by their potent capacity to activate immunologically naive T cells. Members of this family reside not only in lymphoid tissues (e.g., spleen, lymph node, and thymus), but also in epithelial tissues at the environmental interface (e.g., skin). Therefore, external antigens that penetrate into bodies can be readily presented by DC to the immune system.

Langerhans cells (LC) are a skin-specific member of the DC family, and they possess all functional properties required for effective presentation of antigens to $CD4^+$ T cells. These properties include:

1. internalization and processing of antigens,
2. assembly of antigenic peptides with MHC class II molecules,
3. surface expression of peptide-bearing MHC class II molecules, as well as costimulatory molecules, such as CD40, CD54, CD80, and CD86,
4. secretion of T-cell activating cytokines, and
5. migration to regional lymph nodes, where antigen presentation takes place ***(1,2)***.

Importantly, resident LC in epidermis do not exhibit these features; rather, they acquire them only with "maturation." LC freshly isolated from skin express on their surfaces relatively small amounts of MHC class II molecules and costimulatory molecules. They also exhibit only a modest capacity to activate immunologically naive T cells. During subsequent short-term (2–3 d) culture, however, LC increase the expression of these molecules and elevate their capacity to activate naive T cells. These changes represent the transition of LC from "immature" DC specialized for incorporating and processing antigens

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into the "mature" DC that are specialized for delivering T-cell activation signals. Importantly, similar changes also occur in vivo following skin painting with contact sensitizers (i.e., haptens), a treatment that causes LC to move from the epidermis into draining lymphatics ***(3–5)***. Thus, LC at the environmental interface (epidermis) can up-take and process complex antigens, and when they migrate to the lymph nodes (where antigen presentation takes place), they acquire the capacities to stimulate naive T cells.

A major technical limitation in studying the biology of LC has been the unavailability of stable cell lines. Investigators have to sacrifice many experimental animals to purify reasonable numbers of LC, and the resulting LC preparations contain unavoidable contamination of other epidermal cells. Moreover, isolated LC undergo rapid and spontaneous maturation in culture, thus, losing their original features. To overcome this limitation, we have established a protocol to generate stable LC lines from mouse epidermis, the lines that maintain immature features of resident LC ***(6)***. The methods for generating these DC lines and their phenotypic and functional characteristics are described in this chapter.

2. Materials

1. Culture medium: All cell cultures are grown in "complete RPMI," which is RPMI 1640 medium supplemented with heat-inactivated fetal calf serum (FCS), HEPES, nonessential amino acids, L-glutamine, penicillin/streptomycin, sodium pyruvate. The recipe is as follows: 500 mL RPMI 1640 (Gibco-BRL or Sigma), 60 mL of heat-inactivated FCS (56°C, 60 min), 5 mL 1 *M* Hepes (Gibco-BRL), 5 mL nonessential amino acid mixture (100x, Gibco), 5 mL L-glutamine (100X, Gibco-BRL), 5 mL penicillin/streptomycin (100X, Gibco-BRL), and 5 mL sodium pyruvate (100X, Gibco-BRL). *Also see* **Note 1**.
2. Mice: All mice to be used for the generation of LC lines must be maintained in a specific pathogen-free environment. We routinely use newborn BALB/c or A/J mice as a source of LC lines. It is crucial to start from epidermal cells isolated from newborn mice; we have not been able to generate long-term LC lines from adult mice.
3. Epidermal cells isolated from mouse skin (*see* **Subheading 3.1.**).
4. Phosphate-buffered saline (PBS): 4 L distilled water containing 32 g NaCl, 0.8 g KH_2PO_4, 8.7 g $Na_2HPO_4{\cdot}7H_2O$, and 0.8 g KCl. Adjust the pH to 7.4. *See* **Note 1**.
5. 0.5% dispase in PBS: 100 mL PBS containing 500 mg dispase II (cat. no. 165859, Boehringer-Mannheim, Indianapolis, IN).
6. 0.3% trypsin in PBS: 100 mL PBS containing 300 mg trypsin (T1005, Sigma).
7. EDTA in PBS: 100 mL PBS containing 1 g EDTA (tetra sodium salt).
8. 0.3% trypsin/25 m*M* EDTA in PBS: 97.5 mL 0.3% trypsin in PBS plus 2.5 mL 1% EDTA in PBS.
9. Buffer A: 100 mL MEM (Gibco-BRL), 10 mL bovine calf serum (Gibco-BRL), 1 mL penicillin/streptomycin (100X, Gibco-BRL), 0.1 mL 0.1% DNAse in PBS (ICN Biochemicals, Cleveland, OH).

10. Histopaque 1.083 (Sigma, St. Louis, MO).
11. Mouse recombinant GM-CSF (R & D Systems, Minneapolis, MN).
12. Separa filters (Sera-Separa, 208-3084-020, Evergreen Scientific, Los Angeles, CA).
13. NS fibroblasts (*see* **Subheading 3.3.**).
14. 0.45 μm filter unit (Corning Costar Corp., Cambridge, MA).
15. LPS (Difco Laboratories, Detroit, MI).

See **Note 2** for advice on storage of buffers.

3. Methods

3.1. Isolation of Epidermal Langerhans Cells

To isolate epidermal cell suspensions, the epidermis is first separated from the dermis by dispase treatment and then dissociated into suspension after trypsin treatment. The resulting epidermal cell suspensions contain about 1% LC. We routinely use gradient centrifugation to enrich for LC up to 10–15%.

1. Sacrifice newborn BALB/c mice (within 3 d after birth) by methoxyflurene inhalation.
2. Rinse the entire body surface with 70% ethanol (*see* **Note 3**).
3. Harvest trunk skin surgically.
4. Rinse the skin specimen in sterile PBS.
5. Place and spread the skin sample over the lid of a 100 mm tissue culture dish with the epidermal side down and remove the subcutaneous tissue by scraping the dermal side using two pairs of eye forceps (*see* **Note 3**). Cut the skin into strips of about 0.5 cm width using a surgical scalpel.
6. Incubate the skin sample in 0.5% dispase in PBS in a 37°C water bath for 45 min. The incubation time required for epidermal separation may vary depending on the completeness of the subcutaneous tissue removal (*see* **Note 4**).
7. Place the sample on the lid of a 100 mm tissue culture dish with the epidermal side up and scrape the epidermis mechanically using two pairs of forceps.
8. Incubate the epidermal specimens in 4 mL of 0.3% trypsin in PBS in a 37°C water bath for 10 min (*see* **Note 4**).
9. Add 4 mL of ice-cold buffer A to the cell suspension (*see* **Note 5**).
10. Vortex the sample briefly.
11. Filter the cell suspension through Separa filter.
12. Collect cells by centrifugation at 250 *g* for 10 min at 4°C.
13. Resuspend the pellet in buffer A.
14. Place the cell suspension over 4 mL of Histopaque 1.083 gently.
15. Centrifuge at 600 *g* for 20 min at room temperature.
16. Harvest the cells in the medium/Histopaque interface and dilute them with complete RPMI (*see* **Note 6**).
17. Collect cells by centrifugation and resuspend the pellet in complete growth medium (*see* **Note 7**).

3.2. Establishment of Langerhans Cell Lines

Even after gradient centrifugation, the majority (85–90%) of cells in the epidermal-cell preparation are keratinocytes. Therefore, when placed in culture, keratinocytes will first form confluent monolayers in 5–7 d. Small colonies of round cells will then be observed on the surface of keratinocyte monolayers in 1–2 wk. These colonies, which can be harvested from keratinocyte monolayers by pipeting, will be plated onto new culture plates. Importantly, the resulting cultures will be a mixture of LC and fibroblasts. The contaminating fibroblasts must be removed by differential trypsin treatments (*see* **Note 8**). Cultures of growing LC must then be fed with the supernatant collected from independently established fibroblast cultures. (*See* **Fig. 1** for schematic illustration of the entire procedure.)

1. Culture epidermal cells on 24 well plates ($3–10 \times 10^5$ cells/2 mL/well) in complete RPMI with 5 ng/mL mouse granulocyte-macrophage colony stimulating factor (GM-CSF).
2. Place the culture in a humidified, 37°C, 5% CO_2 incubator.
3. Change medium initially on d 7 and every 3–4 d thereafter by gently removing culture media and adding fresh growth medium (*see* **Note 9**).
4. After 7–14 d, colonies of round cells will become detectable on the top of keratinocyte monolayers. Harvest these colonies by pipeting and plate them onto new 24-well plates (*see* **Note 10**).
5. LC will become firmly adherent, extending several long dendrites or numerous pseudopodia. Virtually all the wells containing growing LC colonies will be "contaminated" by fibroblastic cell populations.
6. Harvest the fibroblasts by 5 min treatment with 0.15% trypsin and 1 m*M* EDTA in PBS at room temperature. LC are relatively resistant to trypsin/EDTA treatment. Repeat this treatment until no fibroblasts are detectable in LC cultures.
7. Plate the harvested fibroblasts in complete RPMI (without added growth factors) to establish "NS" fibroblast lines.
8. Harvest supernatants from confluent NS fibroblast cultures (NS supernatant) for subsequent feeding of LC lines (*see* **Subheading 3.3.**).
9. Expand LC cultures (XS series) by feeding with fresh complete RPMI supplemented with 5 ng/mL GM-CSF and 10% v/v NS supernatant.
10. Harvest only the LC that are released into culture media spontaneously from confluent cultures and passage them onto new culture plates at relatively high densities ($3–10 \times 10^5$ cells/mL).
11. To obtain LC clones, harvest growing LC and plate onto flat bottom 96-well microculture plates at 0.5–5 cells/well.
12. Examine the resulting LC lines and clones for phenotypic and functional features (*see* **Subheading 3.4.**).
13. LC lines and clones may be frozen for long-term storage (*see* **Subheading 3.5.**).

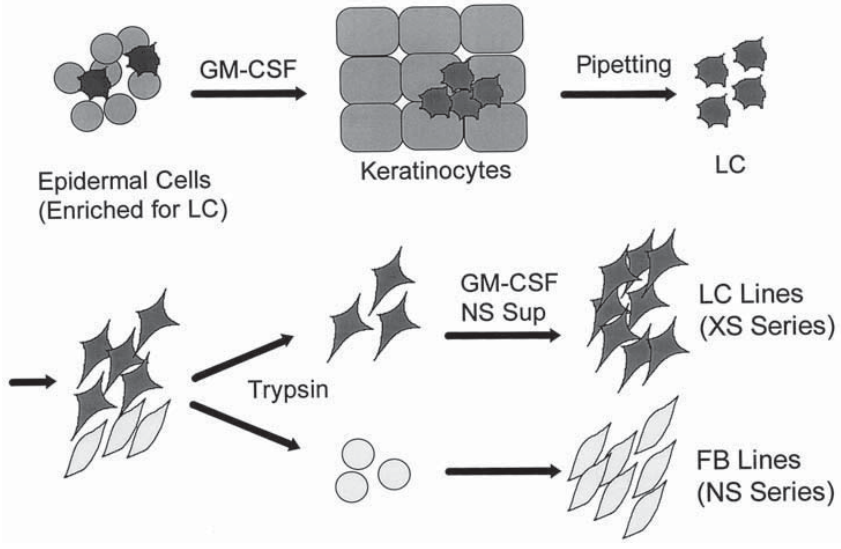

Fig. 1. Generation of long-term LC lines (XS series) from the epidermis of newborn mouse skin. Isolated epidermal cells from newborn mouse skin and enrich them for LC using gradient centrifugation (**Subheading 3.1.**). Culture these cells on 24-well plates in the presence of GM-CSF (**Subheading 3.2.**). Small colon!ies of round cells will become detectable on the surface of keratinocyte monolayers. These colonies can be harvested by pipeting, leaving the keratinocyte monolayers relatively intact. Plate the harvested colonies onto new plates. Resulting cultures will contain firmly adherent LC and rapidly growing fibroblasts. Separate contaminating fibroblasts from LC colonies by differential trypsin treatment, and establish long-term LC lines (XS series) and fibroblast lines (NS series) independently. Collect the supernatant from the NS fibroblast cultures (**Subheading 3.3.**). Feed the XS LC cultures with GM-CSF plus NS supernatant.

3.3. Preparation of NS Supernatant

We have found that, upon removal of contaminating fibroblasts from cultures, LC stop growing even in the presence of added GM-CSF. This suggests that one or more LC growth factors are secreted by these fibroblasts ***(6)***. In fact, LC growth can be restored by addition of fibroblast conditioned media, i.e., NS supernatants ***(6,7)***. We have identified that CSF-1 is responsible for the ability of NS supernatant to promote short-term proliferation of LC lines ***(8)***. Our subsequent attempt to maintain LC cultures in complete RPMI supplemented with GM-CSF and CSF-1 for a long term has not been successful, sug-

gesting that NS fibroblasts secrete additional factors that are required for continuous proliferation and/or survival. Thus, it is recommended to use the NS supernatant (in addition to GM-CSF) to expand LC lines.

1. Plate "NS" fibroblasts (2–5 × 10^4/mL in complete RPMI) onto either 25 cm^2 flasks (5 mL/flask) or 75 cm^2 flasks (15 mL/flask).
2. Change media twice a week.
3. Culture them until they become completely confluent as determined under phase-contrast microscopy.
4. Remove media and add minimal amounts of fresh media (2.5 mL/25 cm^2 flask or 7.5 mL/75 cm^2 flask).
5. Culture them for an additional 3–4 d.
6. Collect culture media and harvest cells for further passages by trypsin/EDTA treatment (*see* **Note 11**).
7. Centrifuge culture media at 1000 *g* for 20 min at 4°C and collect the supernatants (NS supernatant) (*see* **Note 12**).
8. Pass the supernatants through 0.45 μm filters. Because NS fibroblasts secrete relatively large amounts of type I collagen ***(7)***, the NS supernatant, when prepared correctly, should be relatively resistant to filter sterilization.
9. Aliquot and freeze the supernatant at –20°C. The supernatant can be stored for up to 6 mo without a significant loss of biological activities (*see* **Note 13**).

3.4. Phenotypic and Functional Characterization of LC Lines

3.4.1. Surface Phenotype

All the independent LC lines and LC clones, termed "XS" series, established by using the above protocol are virtually indistinguishable from LC freshly isolated from mouse skin in their surface phenotype ***(6,9)***. Briefly, they exhibit the phenotype of $CD45^+$, MHC class I^+,MHC class II^{low}, $CD11a^+$, $CD11b^+$, $CD11c^{low}$, $CD18^+$, $CD54^+$, $CD80^{low}$, $CD86^{low}$, $CD40^-$, $CD16^+$, CD23, $CD14^+$, $CD44^+$, $CD25^{low}$, $CD115^+$, $CD116^+$, $CD122^{low}$, and E-$cadherin^+$.

3.4.2. Antigen Presentation

All XS lines and clones showed extremely potent capacities to present protein and chemical antigens to relevant T-cell lines ***(6,10)***. On the other hand, all LC lines exhibited relatively poor capacities to activate immunologically naive T cells in primary allo-mixed lymphocyte reactions (MLR) ***(6)***. Finally, a single exposure of XS LC lines to relatively low fluences of ultraviolet B (UVB) radiation abrogated almost completely their antigen presenting capacity ***(10)***. Importantly, the same features have been reported for skin resident LC.

3.4.3. Cytokine Production

All XS LC lines secreted relatively large amounts of IL-1β, IL-6, TNFα, and IL-12 p40 in response to LPS stimulation. In addition to these cytokines,

XS lines also expresses mRNA for IL-1α, IL-7, IL-10, CSF-1, MIP-1α, MIP-1γ, and interferon-α ***(11,12)***.

3.5. Cryopreservation of LC lines (XS Series) and Fibroblast Lines (NS Series)

3.5.1. Cell Freezing

1. Harvest LC lines or fibroblast lines in their exponential growth phases.
2. Centrifuge cells and suspend the pellet in ice-cold 90% FCS/10% DMSO at concentrations of 3–10 × 10^6 cells/mL.
3. Transfer the cells to the freezing vials (1 mL/vial) and freeze them using a temperature-controlled freezing apparatus.
4. The frozen cells can be kept for >3 yr in liquid nitrogen (*see* **Note 14**).

3.5.2. Cell Thawing

1. Add 10 mL ice-cold complete RPMI in a 15 mL tube and keep it on ice.
2. Thaw vial quickly in a 37°C water bath.
3. Transfer cells to the 15 mL tube even before the vial content is completely thawed.
4. Rinse the vial with 1 mL ice-cold complete RPMI.
5. Centrifuge the tube at 250 *g* for 10 min at 4°C.
6. Suspend the pellet in 12 mL fresh complete RPMI.
7. Wash the cells once more. The cell viability should be above 70–80% as measured by trypan blue exclusion.
8. Culture the cells at relatively high densities.
9. Change media on the next day.

3.6. Establishment of LC Lines with Mature Features

As described in **Subheading 1.**, LC in the epidermis are "immature" both phenotypically and functionally. The long-term LC lines established in the above protocols also exhibit immature features of resident epidermal LC, even after prolonged culture of more than > 4 yr. Although exact mechanisms by which our LC lines maintain their immature features remain unclear, we have observed that NS supernatant contains one or more factors that prevent the maturation of the LC lines ***(11)***. Moreover, the fact that all our LC lines were generated from newborn mouse skin may be an important factor. Nevertheless, the immaturity of the LC lines has a clear advantage in that they more likely represent epidermal LC than do "conventional" cultured LC exhibiting mature features.

We and others have established protocols to induce the maturation of the LC lines of XS series. These protocols may be useful to study LC-induced immune responses in animals. Briefly, XS cells begin to exhibit the "mature" features of cultured LC when:

1. maintained in the absence of NS supernatant ***(11)***,
2. co-cultured with $CD4^+$ T cells in the presence of relevant antigen ***(13–17)***,
3. activated with bacterial lipopolysaccharide (LPS) ***(13–17)***, which is known to promote DC maturation ***(18,19)***, or
4. stimulated with a cocktail of cytokines ***(20)***.

Here we will describe an additional protocol that we have developed recently to generate "mature" LC lines directly from mouse skin.

3.6.1. Protocol for Generating Mature LC Lines from Mouse Skin

In our original protocol, all the XS lines were generated from highly adherent cell populations. Considering that these XS cells lose their adhesive potential upon maturation, we sought to modify our protocol in such a manner that mature LC lines will generate preferentially (see below). Briefly, nonadherent cells are selected in the initial phase of culture and subsequently expanded in the same culture media containing both GM-CSF and NS supernatant. Long-term LC lines generated with this modified protocol, in fact, exhibit features of fully mature LC, including the capacity to initiate cellular immune responses in mice ***(21)***. This protocol can be used to generate mature LC lines.

1. Isolate LC-enriched epidermal cells from the newborn A/J mice (*see* **Subheading 3.1.**)
2. Culture epidermal cells on 24-well plates ($3–10 \times 10^5$ cells/2 mL/well) in complete RPMI with 5 ng/mL GM-CSF and 10% NS supernatant.
3. Place the culture in a humidified, 37°C, 5% CO_2 incubator.
4. Leave the cultures without medium changes and harvest colonies of round cells from keratinocyte monolayers on d 7–10 by pipeting.
5. Plate the harvested colonies onto new 24-well plates.
6. The cultures will contain two adherent cell populations, i.e., immature LC and contaminating fibroblasts. Small numbers of nonadherent cells will also be detectable under phase contrast microscopy. Harvest only those nonadherent cells by pipeting and plate them into new culture plates.
7. Repeat this treatment until no adherent cells become detectable in cultures.
8. Expand "mature" LC cultures by feeding with fresh complete RPMI supplemented with 5 ng/mL GM-CSF and 10% v/v NS supernatant.
9. Harvest nonadherent cells from confluent cultures and passage them onto new culture plates at relatively high densities ($3–10 \times 10^5$ cells/mL).
10. During prolonged (> 6 mo) culture periods, some cultures may become loosely adherent to culture plates (*see* **Note 15**).
11. Examine the resulting LC lines and clones for phenotypic and functional features (*see* **Subheading 3.4.**). Select only the lines that express relatively large amounts of MHC class II molecules, CD80 and CD86, on their surfaces.
12. "Mature" LC lines may be frozen for long-term storage (*see* **Subheading 3.5.**).

4. Notes

1. Use endotoxin-free, tissue-culture-grade distilled water to prepare all culture medium, buffers, and reagents. Small amounts of endotoxin may affect the maturational states of resulting LC lines. It is also suggested to monitor routinely the tissue-culture reagents for endotoxin contamination by using E-Toxate kit (Gibco-BRL).
2. Complete RPMI, PBS, EDTA solution, and buffer A may be kept for up to 3 mo at 4°C after filter sterilization. On the other hand, the dispase and trypsin solutions will lose the enzymatic activities within a few weeks when stored at 4°C. Therefore, it is recommended to make small aliquots of freshly prepared enzyme solutions and keep them at –20°C for up to 12 mo.
3. To avoid bacterial or fungal contaminations, clean the skin well with 70% ethanol before harvesting the skin specimen. Check all the culture media and reagents, including PBS, dispase, trypsin, collagenase, and complete growth medium. Sterilize surgical blades and forceps with 70% ethanol. At the same time, avoid a typical problem of the fixation of tissue or cells with 70% ethanol being used.
4. The enzymatic digestion process is the most critical. If the cell viability of resulting epidermal cell suspensions is below 80% by trypan blue exclusion, this indicates overdigestion. When performed appropriately, the plated epidermal cells, mostly keratinocytes, should attach firmLy to culture plates within 24 h and begin to spread in 2–3 d. The incubation time required for epidermal separation may vary significantly depending on the batch of dispase, the degree of active hair growth in the skin specimen, and the completeness of removal of the subcutaneous tissue. Likewise, the incubation time required for epidermal cell dissociation may vary depending on the batch of trypsin and the completeness of epidermal separation.
5. Aggregates of epidermal cells frequently contain many viable cells that have been "trapped" by DNA released by dead cells. Those viable cells can be "released" by the DNAse contained in buffer A. Incubate the cells in buffer A at 37°C for 3–5 min to further dissociate the aggregates.
6. Try not to harvest too many cells from the medium/Histopaque interface; it will reduce the fraction of LC. All the gradient centrifugation procedures must be performed at room temperature. It is recommended to examine periodically the percentage of LC by FACS after immunofluorescence staining with anti-Ia monoclonal antibody. In our laboratory, the purity of Ia^+ epidermal cells (i.e., LC) is routinely above 10% after Histopaque separation.
7. Plate the LC preparations as soon as possible. When kept on ice for a prolonged period (> 60 min), the cell viability declines significantly.
8. Overgrowth of "contaminating" fibroblasts is a common technical problem. Because the immature LC are highly adhesive and resistant to trypsin/EDTA treatment, remove the fibroblasts by exposure to 0.15% trypsin and 1 m*M* EDTA in PBS (5 min at room temperature). This procedure may be repeated several times to eliminate the fibroblasts completely from LC cultures.
9. If LC do not grow rapidly, check the GM-CSF and the NS fibroblast supernatant.

Unlike other cytokines, human recombinant GM-CSF does not bind to murine GM-CSF receptor; always use murine recombinant GM-CSF. The concentration of GM-CSF to be added to culture may be increased up to 30 ng/mL, depending on the sources and batches.

10. The LC lines grow more rapidly when maintained at relatively high densities. Therefore, it is recommended always to keep them confluent; this will allow the investigators to harvest sufficient numbers of floating LC from the culture medium. Do not attempt to harvest the adherent cells with trypsin or EDTA; those adherent cells are usually less differentiated cells.
11. Care should be taken in harvesting NS fibroblasts from culture plates. After removal of culture media, we routinely wash culture plates briefly with PBS and then add minimal amounts of 0.3% trypsin/25 m*M* EDTA. These plates can be incubated at room temperature under a microscope; as soon as the cells become rounded (before being released spontaneously from plates), we stop the enzymatic reaction by the addition of ice-cold growth medium containing 10% FCS and harvest cells by tapping or gentle pipeting. These cells need to be centrifuged immediately to remove trypsin and EDTA. The cell viability should be above 90% by trypan blue exclusion.
12. The NS supernatant must be harvested from "completely" confluent cultures. When prepared appropriately, the NS supernatant should be relatively resistant to filter sterilization, owing to the presence of large amounts of type I collagen. The concentration of NS supernatant to be added to culture medium may be increased up to 20% (v/v), depending on the batches.
13. Do not store the NS supernatant for a prolonged period (> 4 wk) at 4°C. It is recommended to make small aliquots and keep them at –20°C.
14. Both LC and fibroblast lines can be frozen safely in 10% DMSO/90% FCS or 10% DMSO in complete DMEM and stored for > 3 yr in liquid nitrogen. Thus, it is recommended to freeze several aliquots in a relatively early phase in culture (e.g., after third passage). Because they may alter the original features during extended culture periods, it is not recommended to culture them continuously without experimental usage. If they suddenly stop dividing or the growth rate accelerates, such cultures need to be replaced. We routinely discard the original cultures after the sixth passage and start new cultures from a frozen stock.
15. The "mature" LC established using the protocol described in **Subheading 3.6.1.** may become slightly adherent during long-term culture periods (> 3 mo). Such adherent cells can be released into culture media by tapping or pipeting.

References

1. Steinman, R. M. (1991) The dendritic cell system and its role in immunogenicity. *Ann. Rev. Immunol.* **9,** 271–296.
2. Stingl, G., Hauser, C., and Wolff, K. (1993) *The Epidermis: An Immunologic Microenvironment.* Fitzpatrick, T. B. (ed.), McGraw Hill and Co., New York, pp. 172–197.
3. Aiba, S. and Katz, S. I. (1990) Phenotypic and functional characteristics of in vivo-activated Langerhans cells. *J. Immunol.* **145,** 2791–2796.

4. Aiba, S., Nakagawa, S., Ozawa, H., Miyake, K., and Tagami, H. (1993) Up-regulation of alpha 4 integrin on activated Langerhans cells: analysis of adhesion molecules on Langerhans cells relating to their migration from skin to draining lymph nodes. *J. Invest. Dermatol.* **100,** 143–147.
5. Enk, A. H. and Katz, S. I. (1992) Early molecular events in the induction phase of contact sensitivity. *Proc. Natl. Acad. Sci. USA* **89,** 1398–1402.
6. Xu, S., Ariizumi, K., Caceres-Dittmar, G., Edelbaum, D., Hashimoto, K., Bergstresser, P. R., and Takashima, A. (1995) Successive generation of antigen-presenting, dendritic cell lines from murine epidermis. *J. Immunol.* **154,** 2697–2705.
7. Schuhmachers, G., Xu, S., Bergstresser, P. R., and Takashima, A. (1995) Identity and functional properties of novel skin-derived fibroblast lines (NS series) that support the growth of epidermal-derived dendritic cell lines. *J. Invest. Dermatol.* **105,** 225–230.
8. Takashima, A., Edelbaum, D., Kitajima, T., Shadduck, R. K., Gilmore, G. L., Xu, S., Taylor, R. S., Bergstresser, P. R., and Ariizumi, K. (1995) Colony-stimulating factor-1 secreted by fibroblasts promotes the growth of dendritic cell lines (XS series) derived from murine epidermis. *J. Immunol.* **154,** 5128–5135.
9. Xu, S., Bergstresser, P. R., and Takashima, A. (1995) Phenotypic and functioinal heterogeneity among murine epidermal-derived dendritic cell clones. *J. Invest. Dermatol.* **105,** 831–836.
10. Caceres-Dittmar, G., Ariizumi, K., Xu, S., Tapia, F. J., Bergstresser, P. R., and Takashima, A. (1995) Hydrogen peroxide mediates UV-induced impairment of antigen-presentation in a murine epidermal-derived dendritic cell line. *Photochem. & Photobiol.* **62,** 176–183.
11. Xu, S., Ariizumi, K., Edelbaum, D., Bergstresser, P. R., and Takashima, A. (1995) Cytokine-dependent regulation of growth and maturation in murine epidermal dendritic cell lines. *Eur. J. Immunol.* **25,** 1018–1024.
12. Mohamadzadeh, M., Poltorak, A. N., Bergstresser, P. R., Beutler, B., and Takashima, A. (1996) Dendritic cells produce macrophage inflammatory protein-1 gamma, a new member of the CC chemokine family. *J. Immunol.* **156,** 3102–3106.
13. Kitajima, T., Ariizumi, K., Bergstresser, P. R., and Takashima, A. (1995) T cell-dependent loss of proliferative responsiveness to colony-stimulating factor-1 by a murine epidermal-derived dendritic cell line, XS52. *J. Immunol.* **155,** 5190–5197.
14. Kitajima, T., Ariizumi, K., Bergstresser, P. R., and Takashima, A. (1996) A novel mechanism of glucocorticoid-induced immune suppression: The inhibition of T cell-mediated terminal maturation of a murine dendritic cell line. *J. Clin. Invest.* **98,** 142–147.
15. Kitajima, T., Ariizumi, K., Bergstresser, P. R., and Takashima, A. (1996) UVB radiation sensitizes a murine epidermal dendritic cell line (XS52) to undergo apoptosis upon antigen presentation to T cells. *J. Immunol.* **157,** 3312–3316.
16. Kitajima, T., Ariizumi, K., Mohamadzadeh, M., Edelbaum, D., Bergstresser, P. R., and Takashima, A. (1995) T cell-dependent secretion of IL-1β by a dendritic cell line (XS52) derived from murine epidermis. *J. Immunol.* **155,** 3794–3800.

17. Kitajima, T., Caceres-Dittmar, G., Tapia, F. J., Jester, J., Bergstresser, P. R., and Takashima, A. (1996) T cell-mediated terminal maturation of dendritic cells: Loss of adhesive and phagocytotic capacities. *J. Immunol.* **157,** 2340–2347.
18. Sallusto, F., Cella, M., Danieli, C., and Lanzavecchia, A. (1995) Dendritic cells use macropinocytosis and the mannose receptor to concentrate macromolecules in the major histocompatibility complex class II compartment: Downregulation by cytokines and bacterial products. *J. Exp. Med.* **182,** 389–400.
19. Roake, J. A., Rao, A. S., Morris, P. J., Larsen, C. P., Hankins, D. F., and Austyn, J. M. (1995) Dendritic cell loss from nonlymphoid tissues after systemic administration of lipopolysaccharide, tumor necrosis factor, and interleukin 1. *J. Exp. Med.* **181,** 2237–2247.
20. Yamada, N. and Katz, S. I. (1998) Generation of fully mature dendritic cells from the CD14+ cell line XS52 using cytokines and agonistic anti-CD40 monoclonal antibody. *J. Leukocyte. Biol.* **supp 2,** 22–22.
21. Timares, L., Takashima, A., and Johnston, S. A. (1998) Functional roles of directly transfected dendritic cells in gene immunization. *Proc. Natl. Acad. Sci. USA* **95**, 13,147–13,152.

V

Generation of Human Dendritic Cells from Progenitor Cells

23

Propagation of Human Dendritic Cells In Vitro

Christophe Caux and Bertrand Dubois

1. Introduction

Considerable progress in the generation of dendritic cells (DC) from mouse and human precursors has recently been accomplished. Consequently, culture systems are now available for the in vitro generation of large numbers of DC.

1.1. Generation of DC from Human Peripheral Blood Monocytes

A decade ago, Knight et al. ***(1)*** described monocytes that have acquired a veiled and dendritic appearance after separation. More recently, monocytes were induced to express CD1a after treatment with granulocyte-macrophage colony stimulating factor (GM-CSF)+IL-4 ***(2)*** or GM-CSF alone ***(3)***. It is now well established that monocytes can be induced, without any proliferation, to differentiate into $CD1a^+$ DC, upon culture with GM-CSF and IL-4 ***(4–7)*** or IL-13 ***(8)***. These monocyte-derived DC possess the phenotype of immature DC characterized by no expression of CD83, low expression of CD80, CD86, CD58, expression of MHC class II within intracytoplasmic compartments, and expression of the monocyte markers (CD11b, CD36, CD68, cfms). The cells display efficient antigen up-take by macropinocytosis or by receptor-mediated endocytosis using the mannose receptor but a weak capacity to prime naive T cells. These DC can still be converted into macrophages upon culture with M-CSF, unless previously induced to mature ***(9)***. These monocyte derived DC undergo maturation when stimulated by inflammatory stimuli such as lipopolysaccharide (LPS), TNFα, or IL-1 (signals also inducing DC migration) or by T-cell signals such as CD40L ***(5,10)***. Following these stimuli, the DC develop a mature phenotype including a typical morphology with extended dendrites, loss of monocyte markers, a loss of antigen up-take capacity, up-regulation of

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accessory molecules (CD80, CD86, CD58), translocation of MHC class II onto the cell surface, and a strong capacity to prime naive T cells.

1.2. Generation of DC from Human CD34+ Hematopoietic Progenitor Cells

Tumor necrosis factors (α or β) strongly potentiate the proliferation induced by either IL-3 or GM-CSF of $CD34^+$ hematopoietic progenitor cells (HPC) that may be isolated from cord blood or bone marrow mononuclear cells ***(11–14)***. Under these culture conditions the cooperation between TNFα and GM-CSF/IL-3 is critical for the development of DC from $CD34^+$ HPC ***(14–19)***. Within 8 d in liquid cultures of $CD34^+$ HPC, addition of TNFα to GM-CSF leads to a 6–8-fold increase in cell number ***(11,15)***. At d 12, 50–80% of cells express CD1a thus yielding $10–30 \times 10^6$ $CD1a^+$ cells from 10^6 $CD34^+$ HPC. Moreover, SCF or FLT3-L increase by 3–4-fold the yield of $CD1a^+$ cells ***(20–23)***. These $CD1a^+$ cells are dendritic cells according to

1. a typical morphology;
2. the DC phenotype (expression of high MHC class II, CD4, CD40, CD54, CD58, CD80, CD86, CD83, and lack of CD64/FcγRI and CD35/CR1);
3. the presence of Birbeck granules (characteristic of LC) in 20% of cells;
4. a high capacity to stimulate proliferation of naive T cells and to present soluble antigen to $CD4^+$ T-cell clones ***(15,24,25)***.

$CD1a^+$ cells are $CD45RO^+$ and express the myeloid markers CD13 and CD33. In this culture system, the effect of TNFα on the development of DC seems to be mediated through the TNF-R1 ***(26)***. Using this culture system, DC have also been generated from $CD34^+$ HPC isolated after mobilization from blood ***(21,27)***.

Using semisolid cultures, DC were shown to arise within single colonies together with monocytes/macrophages, suggesting the existence of a common precursor cell ***(14,16,20)***.

1.3. Identification of Two Pathways of DC Development

Although most DC are $CD1a^+$ $CD14^-$ after 12 d of culture, at early time points (d 5–7) of the culture, two subsets of DC precursors, identified by the exclusive expression of CD1a and CD14, emerge independently ***(28)*** (**Fig. 1**). Both precursor subsets mature at d 12–14 into DC with typical morphology, phenotype (CD80, CD83, CD86, CD58, high HLA class II), and function. $CD1a^+$ precursors give rise to cells with Langerhans-cell (LC) characteristics (Birbeck granules, Lag antigen, and E-cadherin). In contrast, the $CD14^+$ precursors mature into $CD1a^+$ DC lacking Birbeck granules, E-cadherin, and Lag antigen, but expressing CD2, CD9, CD68, and the coagulation factor XIIIa

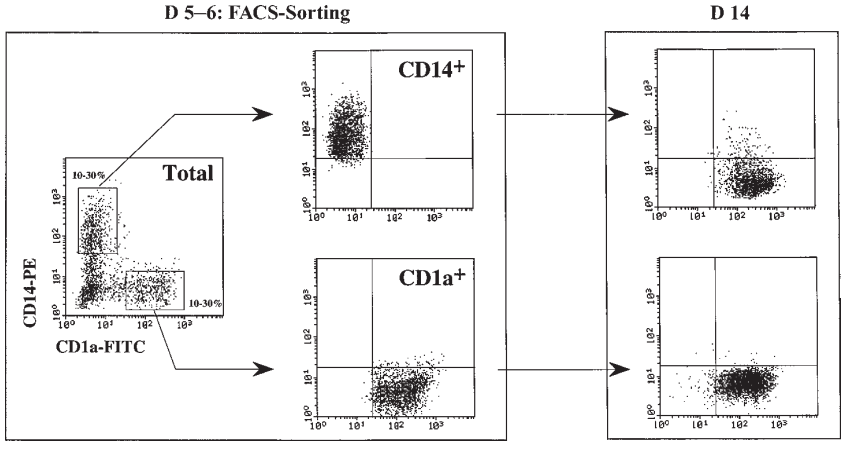

Fig. 1. Cord blood CD34+ HPC were cultured for 5–6 d in the presence of SCF+GM-CSF+TNFα. To allow clear definition of the populations, culture medium was supplemented with 2.5% AB+ pooled human serum at the initiation of the cultures. The cells were processed for double staining using anti-CD14-PE and anti-CD1a-FITC and FACS sorted into $CD1a^+CD14^-$ and $CD14^+CD1a^-$ cells (left panel). Each population represents between 10% and 30% of the total population. Sorted cells were then cultured in the presence of GM-CSF+TNFα ($1–2 \times 10^5$ cells/mL) for six or seven additional days with a last medium change at d 10. At d 12 the cells were reanalyzed for CD1a and CD14 expression by two-color flow cytometry (**right panel**).

described in dermal DC. Interestingly, the $CD14^+$ precursors, but not the $CD1a^+$ precursors, represent bipotent cells that can be induced to differentiate, in response to M-CSF, into macrophage-like cells, lacking accessory function for T cells. Furthermore, CD14-derived but not CD1a-derived DC express IL-10 mRNA and protein ***(29)***.

These two pathways of development have been documented and further characterized by others ***(30)***. In particular, the commitment into either pathway has already occurred at the level of $CD34^+$ cells ***(31)***. Peripheral-blood $CD34^+$, which expresses CLA (cutaneous-lymphocyte-associated antigen), differentiate in response to GM-CSF+TNFα into $CD1a^+$, Birbeck granule+, Lag^+ LC, whereas CLA^- progenitors differentiate into $CD1a^+$ Birbeck granule−, Lag^- interstitial DC (**Fig. 2**).

Although these two populations of DC are equally potent in stimulating naive CD45RA cord blood T cells, each also displays specific activities ***(32)***. In particular, CD14-derived DC demonstrate a potent and long lasting (from d 8 to d 13 of culture) antigen up-take activity (FITC-dextran or peroxidase)

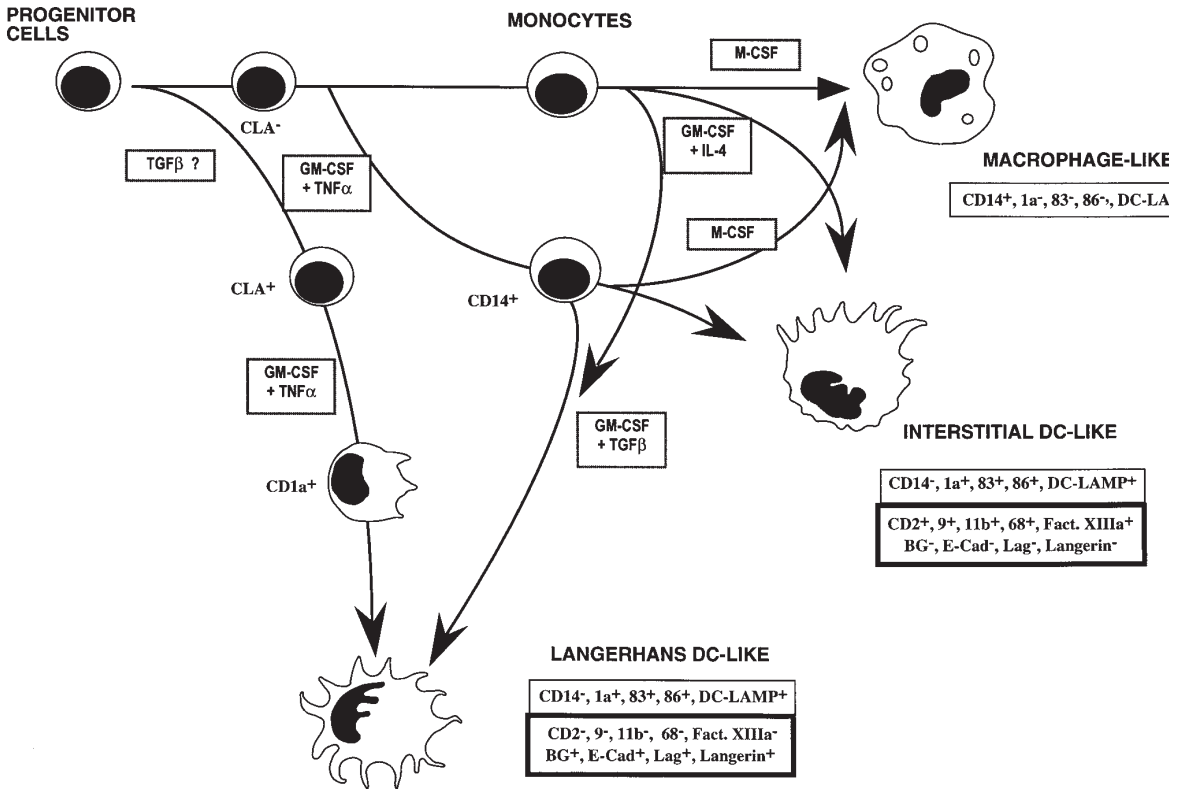

Fig. 2. Different pathways of myeloid DC development. In vitro studies in humans suggests that DC subsets may develop f different progenitors. DC-specific progenitors ($CD34^+CLA^+$) differentiate into $CD1a^+$ precursors leading to LC-type DC. In the prese of GM-CSF $CD34^+CLA^-$ progenitors differentiate into $CD14^+$ precursors leading to interstitial-type DC that are related to monoc derived DC. The same $CD14^+$ precursor, like blood monocytes, may differentiate into macrophage-like cells in the presence of M-CSF . However, under specific conditions requiring the addition of TGFβ, the $CD14^+$ precursors can differentiate into DC with LC features.

that is about 10-fold higher than that of $CD1a^+$ derived cells. In this model, the antigen capture is exclusively mediated by receptors for mannose polymers. The high efficiency of antigen capture of CD14-derived cells is coregulated with the expression of nonspecific esterase activity, a tracer of the lysosomal compartment. In contrast, the $CD1a^+$ population never expresses nonspecific esterase activity. A striking difference between the two populations is the unique capacity of CD14-derived DC to induce naive B cells to differentiate into IgM secreting cells, in response to CD40 triggering and IL-2. Importantly, CD14 derived DC are similar to germinal-center DC (GCDC) ***(33)*** in their capacity to strongly stimulate germinal-center B-cell expansion and to induce IL-10 independent isotype switching toward IgG1 ***(69)***. Thus, whereas T-cell priming is borne by both DC populations, initiation of humoral responses might be preferentially regulated by the CD14-derived DC.

1.4. Cytokines and the Regulation of Myeloid DC Development

Although GM-CSF appears to be a key factor required for DC development in vitro, other molecules are involved in this process. In particular, administration of FLT3-L to mice induces a large increase in the number of DC in the circulation and all organs ***(34–36)***. In vitro, TNFα is mandatory for the recruitment of human $CD34^+$ progenitors by GM-CSF ***(14–19)***. SCF and FLT3-L act in synergy with GM-CSF and TNFα to increase the yield of DC ***(20–23)***. Furthermore, CD40L induces a GM-CSF independent development of DC from CD34+ HPC ***(37)***.

TGFβ is a critical component of bovine serum required for LC development ***(22,38–40)***. Addition of exogenous TGFβ favors the development of DC with the characteristics of LC, e.g., E-cadherin expression, Birbeck granules, and the Lag molecule ***(70)*** (**Fig. 2**). Furthermore, TGFβ induces the expression of the molecule langerin recognized by the antibody Mab-4, which stains LC specifically in vivo (IDC and GCDC from lymphoid organs are negative) ***(71,72)***. Furthermore, TGFβ has been shown to induce the differentiation of monocytes into Langerhans-like cells ***(41)***.

Other molecules interfere with DC development. IL-10 prevents the differentiation of monocytes into DC and drives them into macrophage-like cells ***(42–44)***. These effects of IL-10 are also observed during CD34 progenitor differentiation (unpublished observations). M-CSF can also interfere with GM-CSF by skewing the differentiation of $CD34^+$ cells toward the monocyte/macrophage lineage. Similarly, and in synergy with M-CSF, IL-6 blocks DC differentiation and favors macrophage development. These last two molecules appear to be involved in the suppression of DC development by tumors ***(45)***. Vascular endothelial growth factor (VEGF) has also been proposed to be involved in this phenomenon ***(46)***.

Regarding DC maturation, IL-4, which is required for the differentiation of monocytes into DC, appears to block DC maturation when added to CD34 progenitor cultures. IL-4 blocks up-regulation of accessory (CD58, 80, 83, 86) and MHC class II molecules ***(30,47,48)***. TNFα, in addition to its involvement in progenitor recruitment, induces DC maturation ***(5,10,49)***. The TNFα effect is particularly evident on monocyte-derived DC, where maturation is also induced by LPS or CD40L. The effects of IL-4 on maturation seem to dominate over those of TNFα. In addition, IL-4 blocks the effects of TGFβ on LC development ***(70)***.

1.5. Different Pathways of Dendritic Cell Development

In vitro and in vivo studies in mice and humans indicate that several dendritic cell subsets may originate from different progenitors (Fig. 2). A progenitor cell common for granulocytes, monocytes, and dendritic cells (G-M-DC), identified in semisolid medium, further differentiates into several lineage-specific precursors ***(50–52)***. As discussed previously $CD1a^+$ precursors give rise to LC characterized by the expression of Birbeck granules, the Lag antigen, and E-cadherin. In contrast, the $CD14^+$ precursors mature into $CD1a^+$ DC lacking LC antigens but expressing CD11b, CD9, CD68, and the coagulation factor XIIIa described in dermal DC. Interestingly, the $CD14^+$ precursors, but not the $CD1a^+$ precursors, represent bipotent cells that can be induced to differentiate, in response to M-CSF, into macrophage-like cells (**Fig. 2**).

Following maturation signals, monocyte-derived DC display a full DC phenotype and may be closely related to dermal DC or $CD14^+$ precursor-derived DC as none of these cells possess Birbeck granules or express CD11b and CD68. Indeed, it is tempting to speculate that dermal DC may originate from monocytes that have entered tissues and encountered IL-4 or IL-13 released by tissue mast cells. Like $CD14^+$ precursors, monocytes can differentiate into macrophages in the presence of M-CSF ***(53)***. The LC type might be mainly involved in cellular immune responses, whereas the monocyte-derived DC could be involved in humoral immune responses ***(32)***.

However, the observed dichotomy between LC and monocyte-derived DC is not completely clarified (**Fig. 2**). In particular, TGFβ has been shown to induce the differentiation of monocytes into Langerhans-like cells ***(41)***. It is possible that depending on the physiological conditions different pathways leading to Langerhans cells might be utilized in vivo. In particular, the constitutive renewal of LC might involve early precursors already committed in the bone marrow ($CD34^+CLA^+$), whereas immune-mediated renewal might require rapid mobilization and differentiation of late precursors including monocytes. It has to be noted that differentiation of monocytes into DC could be achieved within 48 h in a model of reverse migration ***(54)***.

In mice, a thorough analysis of cellular populations within the mouse thymus has permitted the identification of progenitors that can differentiate into either T cells or DC when injected into thymic lobes or into B cells following homing to the spleen after iv injection ***(55–57)***. These progenitors have not been shown to differentiate into any other myeloid lineage. Such T-B-DC progenitors might have been identified in humans ***(58,59)*** and the CD11c- DC precursors identified in blood and tonsils ***(60,61)*** have been proposed to be of lymphoid origin ***(73)***. Human CD11c- DC precursors produce high levels of IFNα upon contact with viruses ***(74)*** and might play an important role between innate and acquired anti-viral immune responses. The function of this cell population remains undetermined. Finally, neutrophil granulocyte-committed cells have been shown to acquire DC characteristics upon culture in presence of GM-CSF, IL-4, and TNFα ***(62)***.

Recent experiments with mice, whose *Rel-B*, *TGFβ1*, or *Ikaros* genes have been disrupted, further illustrate the different origin/relationship of dendritic cell subsets. *Rel-B–/–* animals display epidermal LC but no DC in their thymus and spleen ***(63,64)***. In contrast, *TGFβ–/–* mice lack LC but display $CD11c^+$ DC in lymph nodes ***(65)***. *Ikaros–/–* mice lacking T, B, and NK cells display a deficiency in $CD11c^+$ splenic DC. In contrast epidermal LC as well as myeloid lineages (granulocytes, monocytes) are not affected ***(66)***. All of these studies support the hypothesis that distinct developmental pathways of DC exist.

Herein we describe the in vitro culture conditions that allow the generation of human myeloid DC from hematopoietic progenitors or from circulating blood monocytes.

2. Materials

2.1. Cell Purification

1. CPD (Sigma).
2. Ficoll-Hypaque.
3. Human Serum (HS). Pools of HS are used to block Fc-receptors during staining.
4. Monoclonal antibodies for cell depletion: anti-CD3 (OKT3, ascites), anti-CD16 (ION16, Immunotech, Marseille, France), anti-CD19 (4G7, ascites), anti-CD56 (NKH1, Ortho Diagnostic System, Raritan, NJ), anti-glycophorin A (Immunotech).
5. Beads for depletion. Goat anti-mouse immunoglobulin (Ig) beads (Dynabeads; Dynal, Oslo, Norway).
6. Magnetic Particle Concentrator (Dynal, Oslo, Norway).
7. Anti-CD34 for $CD34^+$ HPC purification (Immu-133.3, 10 μg/mL, Immunotech).
8. Mini/Midi-MACS anti-mouse-IgG-coated microbeads (Miltenyi Biotec GmBH, Bergish Gladbach, Germany).
9. Minimacs or Midimacs separation columns and appropriate magnet (Miltenyi Biotec).

2.2. DC Culture

1. Medium. Cultures are established in endotoxin-free medium consisting of RPMI 1640 (Gibco-BRL, Grand Island, NY) supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS) (Flow Laboratories, Irvine, UK), 10 m*M* HEPES, 2 m*M* L-glutamine, 5×10^{-5} M β2-mercaptoethanol, gentamicin (100 μg/mL) (referred to as complete medium).
2. FCS. Several batches of FCS are tested for their ability to supported the development of DC (*see* **Note 1**).
3. AB+ Human Serum. Pools of AB+ human serum are used for the first 5 d of culture of $CD34^+$ HPC (*see* **Note 2**).
4. Cytokines: rhGM-CSF (specific activity: 2.10^6 U/mg, Schering-Plough Research Institute, Kenilworth, NJ), rhTNFα (specific activity: 2×10^7 U/mg, Genzyme, Boston, MA), rhSCF (specific activity 4×10^5 U/mg, R&D Abington, UK), rhTGFβ1 (specific activity: 5×10^7 U/mg, R&D), rhIL-4 (specific activity: 10^7 U/mg, Schering-Plough Research Institute), and rhIL-10 (specific activity: 2×10^7 U/mg, Schering-Plough Research Institute).
5. Plastic vessels for DC cultures. 24-well culture plates (Linbro, Flow Laboratories, Mc Lean, VA) and 25–150 cm^2 culture vessels (Corning, Costar Corporation, Cambridge, MA).
6. Monoclonal antibodies for immunophenotypic studies. Anti-CD1a (Pharmingen, San Diego, CA or T6 Coulter, Hialeah, FL), anti-CD14 (Leu-M3, Becton-Dickinson, Mountain View, CA), anti-E-cadherin (Hecd-1, Takara: Shiga, Japan), anti-Lag, Langerin ***(71)***, anti-CD68 (EMB11, Dako, Glostrup, Denmark), anti-S100β (S2532, Sigma), anti-HLA-DR (L243, Becton Dikinson), anti-CD80 (Mab104, Immunotech or L307.4, Becton Dikinson), anti-CD86 (IT2.2, Pharmingen, San Diego, CA), anti-CD83 (HB15a, Immunotech), anti-CD40 (Mab89, Immunotech), anti-DC-LAMP, (Immunotech), and anti-CD11b (Leu-15, Becton Dickinson).
7. FACScan (Becton-Dickinson, Sunnyvale, CA) for immunophenotypic characterization.
8. FACSstar (Becton-Dickinson) for FACS sorting.

3. Methods

3.1. Derivation of DC from Human Peripheral Blood Monocytes

1. Collect 450 mL of blood into CPD (Sigma), and dilute by 50% with PBS. Layer the cell suspension on Ficoll-Hypaque and centrifuge at 600*g* for 30 min. Recover the mononuclear cells from the interface and wash in PBS.
2. Layer the mononuclear cells over 50% Percoll and centrifuge at 300*g* for 30 min. Harvest the interface cells and wash in PBS. Store the cells overnight at 4°C if necessary.
3. Resuspend cells at 2×10^7 cells/mL in PBS containing 2% HS and 0.5 m*M* EDTA and label with anti-CD3, -CD16, -CD19, -CD56, -glycophorin A. Incubate for 20–30 min at 4°C with gentle shaking, then wash three times in PBS containing 2% FCS and 0.5 m*M* EDTA.

4. Resuspend cells at 5×10^7 cells/mL and add goat anti-mouse Ig beads (Dynabeads; Dynal, Oslo, Norway) to the cell suspension (five beads per cell, original bead density = 4×10^8/mL). Incubate for 20–30 min at 4°C with gentle shaking.
5. Place the cell suspension in the Magnetic Particle Concentrator for 10 min. Aspirate the supernatant containing monocyte enriched unbound cells.
6. Mix supernatant fractions and centrifuge at 500*g* for 10 min. Discard the supernatant and resuspend the cells at 5×10^7 cells/mL. Perform another round of bead depletion by repeating **steps 4–6** to obtain a highly purified population of monocytes (*see* **Note 3**).
7. Seed purified monocytes at 2.5×10^5 cells/mL (25 mL in 75 cm^2 or 50 mL in 150 cm^2 culture vessels) in complete medium containing 100 ng/mL GM-CSF and 50 U/mL IL-4.
8. At d 4–5 feed the cultures by adding an equal volume of complete medium with fresh cytokines.
9. Harvest nonadherent and loosely adherent cells at d 8–10 of culture (*see* **Note 4**).

3.2. Derivation of DC from Human CD34+ Hematopoietic Progenitor Cells

1. Dilute umbilical cord blood samples by adding twice the volume of PBS. Carefully layer the cell suspension over Ficoll-Hypaque. Centrifuge at 600*g* for 30 min.
2. Harvest the interface cells and wash in PBS.
3. Resuspend the mononuclear cells at 2×10^7 cells/mL in PBS/HS/EDTA and add anti-CD34 (Immu-133.3, 10 μg/mL, Immunotech). Incubate for 20–30 min at 4°C with gentle shaking.
4. Wash the cells three times in PBS/FCS/EDTA and then resuspend the cells at 1×10^8cells/mL. Add goat anti-mouse Ig microbeads (0.5 μL bead suspension per 10^6 cells). Incubate for 20–30 min at 4°C with gentle shaking.
5. Wash the cells twice in PBS/FCS/EDTA.
6. Isolate $CD34^+$ progenitor cells using Minimacs separation columns as described below. Resuspend the cells at a maximum concentration of 5×10^7 cells/mL.
7. Fix the Minimacs column in the appropriate magnet according to the manufacturer's instructions. Sequentially layer four 500 μL volumes of the cell suspension on the top of the Minimacs column. The maximum cell number applied to each column is 200×10^6 cells.
8. Wash the column with 500 μL of PBS/FCS/EDTA four times.
9. Remove the column from the magnet and flush twice with 2 mL complete medium to obtain $CD34^+$ cells (*see* **Note 5**).
10. Cryopreserve the purified $CD34^+$ cells in 10% DMSO (*see* **Note 6**).
11. Establish cultures of $CD34^+$ cells in complete medium supplemented with 100 ng/mL rhGM-CSF, 2.5 ng/mL rhTNFα, and 25 ng/mL rhSCF ***(56,111)*** as described below.

12. Thaw $CD34^+$ cells and resuspend in complete medium supplemented with cytokines at 2×10^4 cells/mL. Place cells in 25–75 cm^2 culture vessels and culture at 37°C, 5% CO_2.
13. Split cultures at d 4 with complete medium containing fresh GM-CSF and TNFα to maintain a cell concentration of $1–3 \times 10^5$ cells/mL (*see* **Note 7**).
14. Harvest cells between d 11 and d 14 of culture (*see* **Note 8**).

3.3. Isolation of $CD1a^+$ and $CD14^+$ DC Precursors by FACS-Sorting

After 5–6 d of culture in the presence of SCF, GM-CSF, TNFα, and 2.5% AB+ human serum (*see* **Note 2**) distinct populations of $CD1a^+$ and $CD14^+$ DC precursors may be identified and purified from the cultures.

1. Harvest the cells from the cultures at d 5–6. Centrifuge the cells at 500*g* and resuspend in PBS containing 0.5 m*M* EDTA (*see* **Note 9**).
2. Label the cells with FITC-conjugated CD1a and PE-conjugated Leu-M3 (CD14) for 30 min at 4^OC. Wash the cells once in PBS supplemented with 0.5 m*M* EDTA.
3. Separate cells into $CD14^+CD1a^-$, $CD14^-CD1a^+$ fractions using a FACStarplus (**Fig. 1**) (*see* **Notes 10** and **11**).
4. Seed sorted cells in complete medium with supplemental GM-CSF±TNFα at $1–2 \times 10^5$ cells/mL. Culture for six or seven additional days at 37°C, 5% CO_2.
5. Replace the culture medium at d 10 with fresh medium and supplemental cytokines.
6. Harvest cells between d 11 and d 14 (*see* **Notes 10** and **12, 13–15**). Adherent cells may be recovered using a 0.5 m*M* EDTA solution.

4. Notes

1. Several batches of FCS are tested for their ability to support the development of DC. The FCS is essentially tested for its capacity to support differentiation of $CD14^+$ precursors into $CD1a^+$ DC. The yield of cells is of secondary importance. The absence of endotoxins has to be controlled.
2. AB+ human serum blocks the differentiation of $CD14^+$ into $CD1a^+$ DC. If $CD34^+$ HPC are cultured from d 0 to d 12 in the presence of AB+ human serum, essentially monocyte/macrophages will be generated, as a consequence of blocking $CD14^+$ precursor differentiation into $CD1a^+$ DC. When FACS-sorting of $CD1a^+$ and $CD14^+$ precursor subsets at d 5–6 is required, $CD34^+$ HPC should be cultured in the presence of 2.5% AB+ human serum in order to create clear subset identification (the early expression of CD1a on the CD14 subset is prevented by the human serum). After 5–6 d of culture, the human serum has to be washed away in order to optimize differentiation of $CD14^+$ precursors into $CD1a^+$ DC.
3. After two rounds of bead depletion the resulting suspension contains 90–95% monocytes as judged by anti-CD14 staining; 450 mL blood yield $30–150 \times 10^6$ monocytes.

4. At the end of the culture the number of DC represents 40–60% of the number of seeded monocytes ***(5)***. When monocytes are isolated by bead depletion, the DC that are subsequently generated are always immature at d 7. In contrast, if monocyte purification is performed by adherence, the activation status of the DC is less controlled, probably reflecting either monocyte activation during purification or the presence of contaminating cell populations (e.g., T cells).
5. The isolated cells are 80–90% $CD34^+$ as judged by staining with anti-CD34 MoAb.
6. $CD34^+$ HPC are routinely frozen after purification. This permits better control over experimental conditions (seeding and expansion of the cells may be performed the same day of the week from sample to sample). In addition for reasons that are unclear, DC development is usually more efficient from thawed $CD34^+$ HPC.
7. The generation of $CD1a^+$ DC from $CD34^+$ HPC is very sensitive to endogenous factors that can prevent the differentiation of $CD14^+$ precursors into $CD1a^+$ DC. Thus, in order to generate reproducible differentiation patterns, the cell density should never exceed 3×10^5 cells/mL.
8. In terms of yield, starting from 10^6 $CD34^+$ HPC (1 cord blood generates 50–80 mL of blood which is equivalent to $0.5–3 \times 10^6$ $CD34^+$ cells), $20–50 \times 10^6$ cells are generated by d 6, 10–30% of which are either $CD1a^+$ or $CD14^+$. At d 12, $40–100 \times 10^6$ are recovered, and 50–80% of the cells are $CD1a^+CD14^-$ DC. DC can be further enriched at d 10–12 by FACS-sorting according to CD1a expression.
9. All procedures for labeling and sorting cells are performed in the presence of 0.5 m*M* EDTA in order to avoid cell aggregation.
10. The proportions of each subset of DC precursor at d 5–6 is extremely variable and a wide range has been observed (from 5% to 30% for each population). However, the $CD1a^+$ subset is usually less frequent at d 6. In addition, from d 6 to d 12, $CD14^+$ cells still develop from $CD1a^-CD14^-$ proliferating progenitors, while $CD1a^+$ precursors do not. Finally, $CD1a^+$ are more fragile than $CD14^+$ cells. Altogether the $CD1a^+$ DC originating from $CD1a^+$ precursors can represent a very minor population in bulk culture at d 12.
11. Reanalysis of the sorted populations shows a purity higher than 98%. Contaminating cells are immature myeloid cells. (T cells have never been detected even using PCR amplification of T-cell receptor components.)
12. At equivalent time points, CD1a-derived cells are always more advanced in their maturation stage than CD14-derived cells.
13. The normal differentiation of $CD14^+$ precursor cells is characterized on d 12 by loss of CD14 expression and acquisition of CD1a expression. However, even in optimal conditions, some cells remain $CD14^+$ with or without expression of CD1a. This phenomenon is likely to be due to endogenous factors that favor monocyte/macrophage development. However, if IL-4 is added to the culture medium, this monocyte/macrophage development is never observed.
14. Although the different stages occurring during DC development/maturation (e.g., phenotype and functions) are always observed in the same sequence, the kinetics of the development/maturation can vary from sample to sample.

15. Different stages of development/maturation can be defined in this culture system. From d 0 to d 6 most of the cells are progenitors with high proliferative potential. From d 4 to 6 DC precursors lacking proliferative potential may be identified based on CD14 or CD1a expression. From d 8 to 10 most of the cells are immature DC. From d 12 to d 17 most of the cells are mature DC. Maturation can be further induced by the addition of agents such as LPS, TNFα, or CD40L (see below). To assess the effects of cytokines (e.g., TGFβ, IL-10) on DC development, these factors may be added at d 0 through to d 6. To assess the effects on DC differentiation, factors (e.g., IL-4, TGFβ) may be added at d 6. To assess the effects on DC maturation, factors (e.g., IL-4, TNFα) may be added at d 10. In order to prevent spontaneous maturation, TNFα may be removed from the cultures at d 6. TGFβ1, IL-4 and TNFα are studied for their effects on DC development and maturation using optimal concentrations of 1 ng/mL, 50 U/mL, and 2.5 ng/mL, respectively. The key markers to follow DC development in this culture system are: CD1a, CD14, E-cadherin, Lag ***(67)***, Langerine ***(71,72)***, CD68, S100b, and to follow DC maturation: HLA-DR, CD80, CD86, CD83, CD40, CD68, DC-LAMP ***(68)***, and CD11b.

References

1. Knight, S. C., Farrant, J., and Bryan, A. (1986) Non-adherent, low density cells from human peripheral blood contain dendritic cells and monocytes, both with veiled morphology. *Immunology* **57,** 595–603.
2. Porcelli, S., Morita, C. T., and Brenner, M. B. (1992) CD1b restricts the response of human CD4–8–T lymphocytes to a microbial antigen. *Nature* **360,** 593–597.
3. Kasinrerk, W., Baumruker, T., Majdic, O., Knapp, W., and Stockinger, H. (1993) CD1 molecule expression on human monocytes induced by granulocyte-macrophage colony-stimulating factor. *J. Immunol.* **150,** 579–584.
4. Romani, N., Gruner, S., Brang, D., et al. (1994) Proliferating dendritic cell progenitors in human blood. *J. Exp. Med.* **180,** 83–93.
5. Sallusto, F. and Lanzavecchia, A. (1994) Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor alpha. *J. Exp. Med.* **179,** 1109–1118.
6. Zhou, L.-J. and Tedder, T. F. (1996) $CD14^+$ blood monocytes can differentiate into functionally mature $CD83^+$ dendritic cells. *Proc. Natl. Acad. Sci. USA* **93**, 2588–2592.
7. Pickl, W. F., Majdic, O., Kohl, P., et al. (1996) Molecular and functional characteristics of dendritic cells generated from highly purified $CD14^+$ peripheral blood monocytes. *J. Immunol.* **157,** 3850–3859.
8. Piemonti, L., Bernasconi, S., Luini, W., et al. (1995) IL-13 supports differentiation of dendritic cells from circulating precursors in concert with GM-CSF. *Eur. Cytokine Netw.* **6,** 245–252.
9. Palucka, K. A., Taquet, N., Sanchez-Chapuis, F., and Gluckman, J. C. (1998) Dendritic cells as the terminal stage of monocyte differentiation. *J. Immunol.* **160,** 4587–4595.

10. Sallusto, F., Cella, M., Danieli, C., and Lanzavecchia, A. (1995) Dendritic cells use macropinocytosis and the mannose receptor to concentrate macromolecules in the major histocompatibility complex class II compartment: down-regulation by cytokines and bacterial products. *J. Exp. Med.* **182,** 389–400.
11. Caux, C., Saeland, S., Favre, C., Duvert, V., Mannoni, P., and Banchereau, J. (1990) Tumor necrosis factor-alpha strongly potentiates interleukin-3 and granulocyte-macrophage colony-stimulating factor-induced proliferation of human $CD34^+$ hematopoietic progenitor cells. *Blood* **75,** 2292–2298.
12. Backx, B., Broeders, L., Bot, F. J., and Lowenberg, B. (1991) Positive and negative effects of Tumor Necrosis Factor on colony growth from highly purified normal marrow progenitors. *Leukemia* **5,** 66–70.
13. Jacobsen, S. E. W., Ruscetti, F. W., Dubois, C. M., and Keller, J. R. (1992) Tumor necrosis factor α directly and indirectly regulates hematopoietic progenitor cell proliferation: role of colony-stimulating factor receptor modulation. *J. Exp. Med.* **175,** 1759–1772.
14. Reid, C. D. L., Stackpoole, A., Meager, A., and Tikerpae, J. (1992) Interactions of tumor necrosis factor with granulocyte-macrophage colony-stimulating factor and other cytokines in the regulation of dendritic cell growth in vitro from early bipotent $CD34^+$ progenitors in human bone marrow. *J. Immunol.* **149,** 2681–2688.
15. Caux, C., Dezutter-Dambuyant, C., Schmitt, D., and Banchereau, J. (1992) GM-CSF and TNF-α cooperate in the generation of dendritic Langerhans cells. *Nature* **360,** 258–261.
16. Santiago-Schwarz, F., Belilos, E., Diamond, B., and Carsons, S. E. (1992) TNF in combination with GM-CSF enhances the differentiation of neonatal cord blood stem cells into dendritic cells and macrophages. *J. Leukocyte Biol.* **52,** 274–281.
17. Szabolcs, P., Moore, M. A. S., and Young, J. W. (1995) Expansion of immunostimulatory dendritic cells among the myeloid progeny of human $CD34^+$ bone marrow precursors cultured with c-kit-ligand, GM-CSF, and TNFα. *J. Immunol.* **154,** 5851–5861.
18. Strunk, D., Rappersberger, K., Egger, C., et al. (1996) Generation of human dendritic cells/Langerhans cells from circulating $CD34^+$ hematopoietic progenitor cells. *Blood* **87,** 1292–1302.
19. Rosenzwajg, M., Canque, B., and Gluckman, J. C. (1996) Human dendritic cell differentiation pathway from CD34+ hematopoietic precursor cells. *Blood* **87**, 535–544.
20. Young, J. W., Szabolcs, P., and Moore, M. A. S. (1995) Identification of dendritic cell colony-forming units among normal human CD34+ bone marrow progenitors that are expanded by c-kit-ligand and yield pure dendritic cell colonies in the presence of granulocyte/macrophage colony-stimulating factor and tumor necrosis factor α. *J. Exp. Med.* **182,** 1111–1120.
21. Siena, S., Di Nicola, M., Bregni, M., et al. (1995) Massive ex vivo generation of functional dendritic cells from mobilized $CD34^+$ blood progenitors for anticancer therapy. *Exp. Hematol.* **23,** 1463–1471.

22. Strobl, H., Bello-Fernandez, C., Riedl, E., et al. (1997) Flt3 ligand in cooperation with transforming growth factor-beta1 potentiates in vitro development of Langerhans-type dendritic cells and allows single-cell dendritic cell cluster formation under serum-free conditions. *Blood* **90,** 1425–1434.
23. Saraya, K. and Reid, C. D. (1996) Stem cell factor and the regulation of dendritic cell production from $CD34^+$ progenitors in bone marrow and cord blood. *Br. J. Haematol.* **93,** 258–264.
24. Caux, C., Vanbervliet, B., Massacrier, C., Durand, I., and Banchereau, J. (1996) Interleukin-3 cooperates with tumor necrosis factor a for the development of human dendritic/Langerhans cells from cord blood $CD34^+$ hematopoietic progenitor cells. *Blood* **87,** 2376–2385.
25. Caux, C., Massacrier, C., Dezutter-Dambuyant, C., et al. (1995) Human dendritic Langerhans cells generated in vitro from CD34+ progenitors can prime naive $CD4^+$ T cells and process soluble antigen. *J. Immunol.* **155,** 5427–5435.
26. Lardon, F., Snoeck, H. W., Berneman, Z. N., et al. (1997) Generation of dendritic cells from bone marrow progenitors using GM-CSF, TNF-alpha, and additional cytokines: antagonistic effects of IL-4 and IFN-gamma and selective involvement of TNF-alpha receptor-1. *Immunology* **91,** 553–559.
27. Ratta, M., Rondelli, D., Fortuna, A., et al. (1998) Generation and functional characterization of human dendritic cells derived from CD34 cells mobilized into peripheral blood: comparison with bone marrow CD34+ cells. *Br. J. Haematol.* **101,** 756–765.
28. Caux, C., Vanbervliet, B., Massacrier, C., et al. (1996) $CD34^+$ hematopoietic progenitors from human cord blood differentiate along two independent dendritic cell pathways in response to GM-CSF+TNFα. *J. Exp. Med.* **184,** 695–706.
29. de Saint-Vis, B., Fugier-Vivier, I., Massacrier, C., et al. (1998) The cytokine profile expressed by human dendritic cells is dependent on cell subtype and mode of activation. *J. Immunol.* **160,** 1666–1676.
30. Canque, B., Camus, S., Yagello, M., and Gluckman, J. C. (1998) IL-4 and CD40 ligation affect differently the differentiation, maturation, and function of human $CD34^+$ cell-derived $CD1a^+CD14^-$ and $CD1a^-CD14^+$ dendritic cell precursors in vitro. *J. Leukoc. Biol.* **64,** 235–244.
31. Strunk, D., Egger, C., Leitner, G., Hanau, D., and Stingl, G. (1997) A skin homing molecule defines the Langerhans cell progenitor in human peripheral blood. *J. Exp. Med.* **185,** 1131–1136.
32. Caux, C., Massacrier, C., Vanbervliet, B., et al. (1997) $CD34^+$ hematopoietic progenitors from human cord blood differentiate along two independent dendritic cell pathways in response to GM-CSF+TNFa: II functional analysis. *Blood* **90,** 1458–1470.
33. Grouard, G., Durand, I., Filgueira, L., Banchereau, J., and Liu, Y. J. (1996) Dendritic cells capable of stimulating T cells in germinal centers. *Nature* **384,** 364–367.
34. Maraskovsky, E., Brasel, K., Teepe, M., et al. (1996) Dramatic increase in the numbers of functionally mature dendritic cells in Flt3 ligand-treated mice: multiple dendritic cell subpopulations identified. *J. Exp. Med.* **184,** 1953–1962.

35. Pulendran, B., Lingappa, J., Kennedy, M. K., et al. (1997) Developmental pathways of dendritic cells in vivo: distinct function, phenotype, and localization of dendritic cell subsets in Flt3 ligand-treated mice. *J. Immunol.* **159,** 2222–2231.
36. Shurin, M. R., Pandharipande, P. P., Zorina, T. D., et al. (1997) Flt3 ligand induces the generation of functionally active dendritic cells in mice. *Cell. Immunol.* **179,** 174–184.
37. Flores-Romo, L., Bjšrck, P., Duvert, V., van Kooten, C., Saeland, S., and Banchereau, J. (1997) CD40 ligation on human $CD34^+$ hematopoietic progenitors induces their proliferation and differentation into functional dendritic cells. *J. Exp. Med.* **185,** 341–349.
38. Strobl, H., Riedl, E., Scheinecker, C., et al. (1996) TGF-B1 promotes in vitro development of dendritic cells from $CD34^+$ hemopoietic progenitors. *J. Immunol.* **157,** 1499–1507.
39. Riedl, E., Strobl, H., Majdic, O., and Knapp, W. (1997) TGF-beta 1 promotes in vitro generation of dendritic cells by protecting progenitor cells from apoptosis. *J. Immunol.* **158,** 1591–1597.
40. Strobl, H., Riedl, E., Bello-Fernandez, C., and Knapp, W. (1998) Epidermal Langerhans cell development and differentiation. *Immunobiology* **198,** 588–605.
41. Geissmann, F., Prost, C., Monnet, J. P., Dy, M., Brousse, N., and Hermine, O. (1998) Transforming growth factor beta1, in the presence of granulocyte/macrophage colony-stimulating factor and interleukin 4, induces differentiation of human peripheral blood monocytes into dendritic Langerhans cells. *J. Exp. Med.* **187,** 961–966.
42. De Smedt, T., van Mechelen, M., De Becker, G., Urbain, J., Leo, O., and Moser, M. (1997) Effect of interleukin-10 on dendritic cell maturation and function. *Eur. J. Immunol.* **27,** 1229–1235.
43. Buelens, C., Verhasselt, V., de Groote, D., Thielemans, K., Goldman, M., and Willems, F. (1997) Interleukin-10 prevents the generation of dendritic cells from human peripheral blood mononuclear cells cultured with interleukin-4 and granulocyte/macrophage-colony-stimulating factor. *Eur. J. Immunol.* **27,** 756–762.
44. Buelens, C., Verhasselt, V., De Groote, D., Thielemans, K., Goldman, M., and Willems, F. (1997) Human dendritic cell responses to lipopolysaccharide and CD40 ligation are differentially regulated by interleukin-10. *Eur. J. Immunol.* **27,** 1848–1852.
45. Menetrier-Caux, C., Montmain, G., Dieu, M. C., et al. (1998) Inhibition of the differentiation of dendritic cells from CD34+ progenitors by tumour cells: role of Interleukin-6 and macrophage colony-stimulating factor. *Blood* **92,** 1–15.
46. Gabrilovich, D. I., Chen, H. L., Girgis, K. R., et al. (1996) Production of vascular endothelial growth factor by human tumors inhibits the functional maturation of dendritic cells. *Nat. Med.* **2,** 1096–1103.
47. Rougier, N., Schmitt, D., and Vincent, C. (1998) IL-4 addition during differentiation of CD34 progenitors delays maturation of dendritic cells while promoting their survival. *Eur. J. Cell. Biol.* **75,** 287–293.

48. Caux, C., Massacrier, C., Vanbervliet, B., et al. (1997) In $CD34^+$ hematopoietic progenitors from human cord blood differentiate along two independent dendritic cell pathways in response to GM-CSF+TNFα, Ricciardi-Castagnoli, P. (ed.) Plenum Press, New York, pp. 21–25.
49. Chen, B., Shi, Y., Smith, J. D., Choi, D., Geiger, J. D., and Mule, J. J. (1998) The role of tumour necrosis factor alpha in modulating the quantity of peripheral blood-derived, cytokine-driven human dendritic cells and its role in enhancing the quality of dendritic cell function in presenting soluble antigens to $CD4^+$ T cells in vitro. *Blood* **91,** 4652–4661.
50. Cella, M., Sallusto, F., and Lanzavecchia, A. (1997) Origin, maturation and antigen presenting function of dendritic cells. *Curr. Opin. Immunol.* **9,** 10–16.
51. Peters, J. H., Gieseler, R., Thiele, B., and Steinbach, F. (1996) Dendritic cells: from ontogenetic orphans to myelomonocytic descendants. *Immunol. Today* **17,** 273–278.
52. Young, J. W. and Steinman, R. M. (1996) The hematopoietic development of dendritic cells: a distinct pathway for myeloid differentiation. *Stem Cells* **14,** 376–387.
53. Chapuis, F., Rosenzwajg, M., Yagello, M., Ekman, M., Biberfield, P., and Gluckman, J. C. (1997) Differentiation of human dendritic cells from monocytes in vitro. *Eur. J. Immunol.* **27,** 431–441.
54. Randolph, G. J., Beaulieu, S., Lebecque, S., Steinman, R. M., and Muller, W. (1998) Differentiation of monocytes into dendritic cells in a model of transendothelial trafficking. *Science* **282,** 480–483.
55. Ardavin, C., Wu, L., Li, C. L., and Shortman, K. (1993) Thymic dendritic cells and T cells develop simultaneously in the thymus from a common precursor population. *Nature* **362,** 761–763.
56. Saunders, D., Lucas, K., Ismaili, J., et al. (1996) Dendritic cell development in culture from thymic precursor cells in the absence of granulocyte/macrophage colony-stimulating factor. *J. Exp. Med.* **184,** 2185–2196.
57. Wu, L., Li, C. L., and Shortman, K. (1996) Thymic dendritic cell precursors: relationship to the T-lymphocyte lineage and phenotype of the dendritic cell progeny. *J. Exp. Med.* **184,** 903–911.
58. Galy, A., Travis, M., Cen, D., and Chen, B. (1995) Human T, B, natural killer, and dendritic cells arise from a common bone marrow progenitor cell subset. *Immunity* **3,** 459–473.
59. Res, P., Martinez-Caceres, E., Jaleco, A. C., et al. (1996) $CD34^+$ $CD38^{dim}$ cells in the human thymus can differentiate into T, natural killer, and dendritic cells but are distinct from pluripotent stem cells. *Blood* **87,** 5196–5206.
60. Oehler, L., Majdic, O., Pickl, W. F., et al. (1998) Neutrophil granulocyte-committed cells can be driven to acquire dendritic cell characteristics. *J. Exp. Med.* **187,** 1019–1028.
61. Grouard, G., Rissoan, M. C., Filgueira, L., Durand, I., Banchereau, J., and Liu, Y. J. (1997) The enigmatic plasmacytoid T cells develop into dendritic cells with IL-3 and CD40–ligand. *J. Exp. Med.* **185,** 1101–1111.

62. O'Doherty, U., Peng, M., Gezelter, S., et al. (1994) Human blood contains two subsets of dendritic cells, one immunologically mature and the other immature. *Immunology* **82,** 487–493.
63. Burkly, L., Hession, C., Ogata, L., et al. (1995) Expression of relB is required for the development of thymic medulla and dendritic cells. *Nature* **373,** 531–536.
64. Weih, F., Carrasco, D., Durham, S. K., et al. (1995) Multiorgan inflammation and hematopoietic abnormalities in mice with a targeted disruption of RelB, a member of the NF-kB/Rel family. *Cell* **80,** 331–340.
65. Borkowski, T. A., Letterio, J. J., Farr, A. G., and Udey, M. C. (1996) A role for endogenous transforming growth factor B1 in Langerhans cell biology: the skin of transforming growth factor B1 null mice is devoid of epidermal Langerhans cells. *J. Exp. Med.* **184,** 2417–2422.
66. Wang, J., Taniuchi, I., Maekawa, Y., Howard, M., Cooper, M. D., and Watanabe, T. (1996) Expression and function of Fas antigen on activated murine B cells. *Eur. J. Immunol.* **26,** 92–96.
67. Kashihara, M., Ueda, M., Horiguchi, Y., Furukawa, F., Hanaoka, M., and Imamura, S. (1986) A monoclonal antibody specifically reactive to human Langerhans cells. *J. Invest. Dermatol.* **87,** 602–607.
68. de Saint-Vis, B., Vincent, J., Vandenabeele, S., et al. (1998) A novel lysosome associated membrane glycoprotein, DC-LAMP, induced upon DC maturation, is transiently expressed in MHC class II compartment. *Immunity* **9,** 325–336.
69. Dubois, B., Barthelemy, C., Durand, I., Lui, Y. J., Caux, C., and Briere, F. (1999) Toward a role of dendritic cells in the germinal center reaction: triggering of B cell proliferation and isotype switching. *J. Immunol.* **162,** 3428–3436.
70. Caux, C., Massacrier, C., Dubois, B., et al. (1999) Respective involvement of TGF-beta and IL-4 in the development of Langerhans cells and non-Langerhans dendritic cells from CD34+ progenitors. *J. Leukoc. Biol.* **66,** 781–791.
71. Valladeau, J., Duvert-Frances, V., Pin, J. J., et al. (1999) The monoclonal antibody DCGM4 recognizes Langerin, a protein specific of Langerhans cells, and is rapidly internalized from the cell surface. *Eur. J. Immunol.* **29,** 2695–2704.
72. Valladeau, J., Ravel, O., Dezutter-Dambuyant, C., et al. (2000). Langerin, a novel C-type lectin specific to Langerhans cells, is an endocytic receptor that induces the formation of Birbeck granules. *Immunity* **12,** 71–81.
73. Spits, H., Couwenberg, F., Bakker, A. Q., Weijer, K., and Uittenbogaart, C. H. (2000) Id2 and Id3 inhibit development of CD34(+) stem cells into predendritic cell (pre-DC)2 but not into pre-DC1. Evidence for a lymphoid origin of pre-DC2. *J. Exp. Med.* **192,** 1775–1784.
74. Kadowaki, N. Antonenko, S., Lau, J. Y., and Lui, Y. J. (2000) Natural interferon alpha/beta-producing cells link innate and adaptive immunity. *J. Exp. Med.* **192,** 219–226.

24

Generation of Dendritic Cells from Lymphoid Precursors

Anne Galy

1. Introduction

Dendritic cells (DC) are "professional" antigen presenting cells (APC) that are pivotal for the initiation of T-cell-dependent immune responses ***(1)***. DC are widely distributed cells in the body, but are scarce and thus difficult to purify. Methods have become available to grow DC in culture, facilitating the study of their biological and developmental properties. One such method is described in this chapter to generate DC in vitro from $CD34^+$ $Lineage^-$ (Lin^-) $CD10^+$ bone marrow (BM) progenitor cells that are easily committed to the lymphoid lineages. This source of DC precursors thus distinguishes itself from mature monocytes or from total $CD34^+$ cells, which have also been employed to generate DC in vitro ***(2–5)***. It is unclear at this point if DC produced from lymphoid progenitor cells or from monocytes or total $CD34^+$ cells are equivalent. Lymphoid progenitor cells displaying the cell-surface phenotype $CD34^+$ Lin^- $CD10^+$ rapidly lose CD34 cell-surface expression in culture and quickly differentiate into T-, B-, or natural-killer (NK) lymphocytes in the appropriate SCID-hu assay or culture systems ***(6,7***; Galy, unpublished observations). This population of $CD34^+$ BM progenitor cells contains cells expressing the IL-7Rα chain, TdT, RAG-1, the transcription factors Pax5 and Ikaros, and represent the earliest recognizable human B-cell precursor identified in human BM ***(7,8)***. BM cells of $CD34^+$ Lin^- $CD10^+$ phenotype can differentiate into antigen-presenting DC under the appropriate culture conditions, generating "lymphoid-related DC" ***(6)***. Limiting dilution analyses demonstrated that single clones of $CD34^+$ Lin^- $CD10^+$ progenitor cells can generate B cells, NK cells, and DC suggesting that these three lineages of cells share a common developmental stage in the BM and thus have close developmental relationships ***(6)***. In spite

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of being multipotent cells, $CD34^+$ Lin^- $CD10^+$ cells do not differentiate into myeloid or erythroid cells in the presence of cytokines permissive for the production of such lineages from $CD34^+$ cells ***(6,7)***. In contrast, cytokine combinations such as c-kit-ligand + GM-CSF + flt3-ligand + IL-1+ IL-7, which cause total $CD34^+$ cells to become myeloid cells, induce $CD34^+$ Lin^- $CD10^+$ to differentiate into DC. Such lymphoid-related DC display characteristic long membrane processes, have immunostimulatory properties, and express on the cell surface high levels of MHC class II molecules, CD1a, CD40 with little or no CD14, CD19, or CD15 antigens. Owing to their distinct hematopoietic origin, lymphoid-related DC may represent a separate population of DC. It is tempting to speculate that lymphoid-related DC may develop in lymphoid organs such as the thymus where progenitor cells with similar dual lymphoid/DC potential have been described ***(9)***. The method described here consists of preparing mononuclear cells (MNC) from BM that are depleted by negative selection of cells expressing lineage markers (CD2, CD3, CD19, CD20, CD14, CD15, CD32, glycophorin A), found on T cells, B cells, monocytes, and erythrocytes. Lineage-depleted cells are labeled with fluorochrome-conjugated antibodies and $CD34^+$ Lin^- $CD10^+$ cells are purified by flow cytometry. Sorted cells are placed in culture in the presence of Flt3-ligand, c-kit-ligand, GM-CSF, IL-1, and IL-7 for 2 wk to generate lymphoid-related DC.

2. Materials

All solutions and materials are sterile. Whenever possible, purchase reagents and solutions that have been tissue culture tested by the manufacturer (*see* **Note 1**).

1. Human adult bone marrow cells are obtained from the screen filters of used bone marrow transfusion pouches or, alternatively, from human ribs obtained as a surgical by-product in patients undergoing thoracic surgery (*see* **Note 2**).
2. Pipets (Sigma).
3. 50 mL polystyrene tubes (Sigma).
4. Bone-cutting scissors (Sigma).
5. Forceps (Sigma).
6. 100 mm Petri dishes (Sigma).
7. 1.5 mL Eppendorf tubes (Sigma).
8. 15 mL tubes (Sigma).
9. 5 mL tubes with caps (Sigma).
10. 100 mesh grids (Sigma).
11. 300 mesh copper electron microscope grids mounted on pipet tips as prefilters for sorter.
12. 24-well plates.
13. Phosphate-buffered saline (PBS) without calcium magnesium.
14. Ficoll (Pharmacia).
15. Wash buffer. PBS + 0.2% bovine serum albumin (BSA) (Sigma).

16. DNase I from bovine pancreas grade II (Boerhinger Mannheim). Prepare a stock at 1×10^5U/mL in RPMI. Prepare 200 µL aliquots in Eppendorf tubes and store at –20°C. Once thawed, keep at 4°C and discard at end of day. Working concentration is 100 U/mL in RPMI (or any other solution containing Mg^{2+}).
17. Heparin (Sigma). Prepare a stock at 1×10^4 U/mL in PBS and store at 4°C (*see* **Note 3**).
18. Propidium iodide (PI). Prepare a stock solution at 1 mg/mL in PBS. Store at 4°C for up to a year. Working solution is 5 µg/mL.
19. Culture medium. RPMI 1620 supplemented with 10% heat-inactivated fetal calf serum (FCS), penicillin, and streptomycin (respectively, 100 U/mL and 100 µg/mL), L-glutamine (2 m*M*), 2×10^{-5} *M* 2-mercaptoethanol (2-ME).
20. Antibodies: Mouse monoclonal antibodies against CD40 (G28.5), CD32 (IV.3), CD2 (OKT11), CD14 (3C10), and glycophorin A (10F7MN) are purified from hybridomas available at ATCC and used for debulking Lin^+ cells. Fluorochrome-conjugated antibodies: Fluorescein isothiocyanate (FITC) anti-CD3 (7D6, Caltag), FITC anti-CD15 (PR9, kind gift of Dr. B. Hill, SyStemix Inc, Palo Alto, CA), FITC anti-CD19 (HIB19, Pharmingen), FITC anti-CD45 (Becton Dickinson, used for positive control), FITC-irrelevant Ig control, phycoerythrin (PE) anti-CD10 (HI10a, Pharmingen), PE anti-CD38 (Becton Dickinson, used for positive control), PE-irrelevant Ig control, sulforhodamine (SR) anti-CD34 (PR3, kind gift of Dr. B. Hill, SyStemix Inc.), SR-irrelevant Ig control.
21. Magnetic sheep anti-mouse Ig beads M450 (Dynal), Dynal magnet for 15 mL tubes.
22. Vantage cell sorter (Becton Dickinson) utilizing an argon laser tuned to 488 nm, a dye pump laser tuned to 590–600 nm, a nozzle of 70 mm diameter vibrating at a frequency of approx 22 kHz, and sterile buffered saline. Cells are aspirated into the instrument through a 300 mesh prefilter to avoid clumping.
23. Cytokines: Human recombinant GM-CSF, flt3-ligand, c-kit ligand (final concentration 25 ng/mL each) (kind gift of Dr. B. Hill), and IL-1α and IL-7 (R&D Systems, final concentration 10 ng/mL each). Cytokine stocks are prepared at 100 mg/mL in wash buffer and small aliquots are stored frozen (–20°C). Working stocks are prepared at 10 µg/mL and stored for up to 2 wk at 4°C.

3. Methods

3.1. Preparation of Bone Marrow Mononuclear Cells

1. To obtain rib BM cells, place the tissue into a Petri dish with 5 mL PBS and cut the bone open longitudinally with bone-cutting scissors. Repeat the cut again to obtain long slivers of bone exposing the medullary cavity, which can be flushed extensively with a Pasteur pipet and cold PBS to extract the hematopoeitic cells. Extract BM cells from the filters by rinsing with PBS (*see* **Note 4**).
2. Prepare MNC $d < 1.077$ g/mL, by centrifuging BM cells through Ficoll at room temperature for 20 min at 700*g* (brakes off).
3. Load 20 mL of cell suspension over 10 mL of Ficoll in each 50 mL tube (*see* **Note 5**).
4. Recover MNC at the interface and wash twice in wash buffer.

5. Aliquot 5 × 10^7 MNC/mL/vial and cryopreserve with 10% DMSO, 50% FCS using standard procedures. Store vials in liquid nitrogen for long-term storage (*see* **Note 6**).
6. Before taking vials out of liquid nitrogen, prepare some cold wash buffer with 10 U/mL heparin + 100 U/mL DNase.
7. Thaw vials rapidly at 37°C and quickly dilute approx 4 × 10^8 MNC in 40 mL of cold wash buffer + heparin + DNase (*see* **Note 7**).
8. Layer the cells over Ficoll and centrifuge at 700*g* and room temperature for 20 min with no brake.
9. Collect the cells at the interface (avoiding large DNA clumps if they are present) in cold wash buffer + heparin + DNAse.
10. Spin at 600*g* at 4°C for 10 min, resuspend the cells and count cells (*see* **Note 8**).

3.2. Depletion of Lineage Positive (Lin+) Cells

1. Incubate MNC for 30 min with lineage antibodies against CD40, CD32, CD2, CD14, and glycophorin A recognizing respectively B cells, Fc binding cells, T-cells monocytes and erythroid cells, respectively. Dilute cells and antibodies in cold wash buffer + DNase to appropriate concentration (*see* **Note 9**). Incubate cells for 30 min on ice and shake cell suspension every few min.
2. After incubation, wash cells twice in cold wash buffer + DNase.
3. Incubate labeled cells with washed sheep anti-mouse Ig magnetic beads at the ratio of eight beads per cell.
4. Incubate beads and cells with gentle rotation for 20 min at 4°C in 15 mL tubes containing no more than 7 mL of a cell suspension at 1 × 10^7 cells/mL.
5. Deplete lineage positive cells in the by placing tube in Dynal magnet.
6. Aspirate the Lin^- cells that remain in suspension and transfer to another 15 mL tube.
7. Count cells.

3.3. Staining of Lineage-Depleted Cells for Flow Cytometric Sorting

1. Prepare controls to set each fluorescence channel on the FACS Vantage and to adjust compensation between FITC and PE detectors.
2. Transfer 1 × 10^5 cells into each of four 5 mL tubes for a negative control, FITC-positive control, PE-positive control and SR-positive control.
3. Spin cells at 600*g* at 4°C for 10 min, decant the supernatant and resuspend the cell pellet.
4. In each tube, add respectively, FITC-irrelevant Ig control plus PE-irrelevant Ig control plus SR-irrelevant Ig control (each at 1 μg/10^6 cells and 10 μg/mL); FITC anti-CD45 (10 μL); PE anti-CD38 (10 μL); SR anti-CD34 (1 μg/10^6 cells and 10 μg/mL).
5. Prepare the remaining cells for sorting. Spin the 15 mL tube at 600*g* at 4°C for 10 min, decant the supernatant and resuspend the pellet gently.
6. Add SR anti-CD34 (0.3 μg/10^6 cells), FITC anti-CD19, CD3, CD15 (1 μg/10^6 cells and 10 μg/mL each), PE-anti-CD10 (20 μL/10^6 cells). Dilute antibodies and

cells in cold wash buffer (*see* **Note 10**). Incubate cells at 4°C for 30 min and then wash twice in wash buffer.
7. Resuspend cells at approx 1×10^7 cells/mL in wash buffer + PI (5 μg/mL).
8. Filter cells through a stainless steel grid (100 mesh) into a 5 mL tube.
9. Rinse grid with 1 mL wash buffer to recover as many cells as possible. Do not fill tube with more than 4 mL of liquid.

3.4. Flow Cytometric Sorting of CD34$^+$ Lin$^-$ CD10$^+$ Cells

1. Use negative and positive controls to adjust the detectors, compensate the cytometer, and set the limits of the gates.
2. Sort cells that do not stain for PI or FITC-conjugated lineage antibodies, but express CD34 and CD10 (Fig. 1A) (*see* **Note 11**).

3.5. Culture of CD34$^+$ Lin$^-$ CD10$^+$ Cells

1. Place sorted cells into 1 mL of medium supplemented with the combination of cytokines Flt3-ligand, c-kit-ligand, GM-CSF, IL-1, and IL-7 in a well of a 24-well plate.
2. Incubate cells at 37°C, 5% CO_2 in humidified atmosphere for up to 2 wk.
3. Change medium twice weekly by demidepletion.
4. After 7 d, clusters of characteristic DC are visible (**Fig. 2**).

4. Notes

1. Although I herein specify which antibodies and cytokines were used to prepare lymphoid-related DC in my laboratory, I believe that equivalent preparations obtained elsewhere can be used as well.
2. The investigator should seek approval from an Ethics Committee or Institutional Review Board for the guidelines on the use of such material. Human tissues constitute biohazardous material and should be handled with precaution under Biosafety level 2 precautions. The investigator should wear gloves and lab coats at all times and discard waste in biohazard containers that will be autoclaved before disposal.
3. I routinely store this solution at 4°C for several months before renewing it.
4. It is important to keep cells cold throughout the processing, labeling. and sorting.
5. Do not load more than 1×10^8 leukocytes per 10 mL of Ficoll.
6. Although there is cell loss associated with freezing and thawing of BM, it is usually practical to freeze these cells due to time constraints. Furthermore, freezing usually eliminates granulocytes that may have remained after Ficoll without affecting CD34$^+$ cells.
7. Thaw MNC carefully, as it is a critical step that may cause clumping and cell loss.
8. Typically, one should expect to recover only 10% of the theoretical number of cells frozen but cells should be free of clumps, depleted of granulocytes, and enriched for CD34$^+$ cells (3–12% of MNC).
9. Although it is imperative to determine the optimal amount of antibody by titration, I suggest using on average 1 μg of antibody per 1×10^6 cells at concentra-

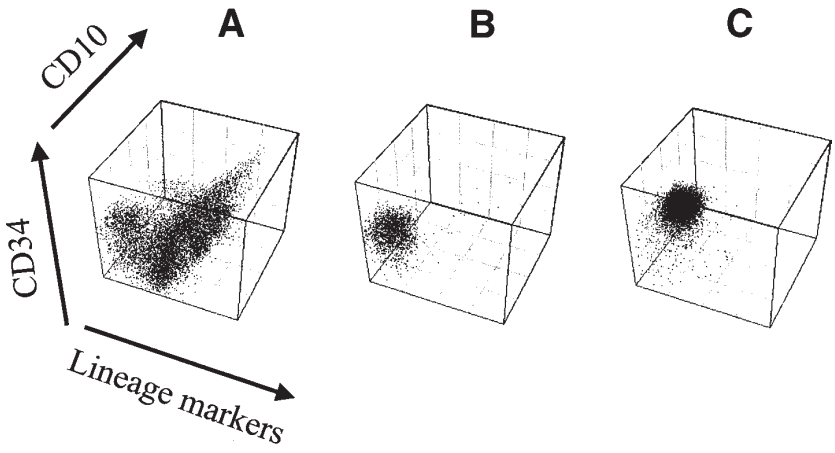

Fig. 1. Three-dimensional representation of a BM sort. Only live cells are represented. **(A)** cells after Lin depletion. **(B)** Sorted $CD34^+$ Lin^- $CD10^-$ cells. C: $CD34^+$ Lin^- $CD10^+$ cells.

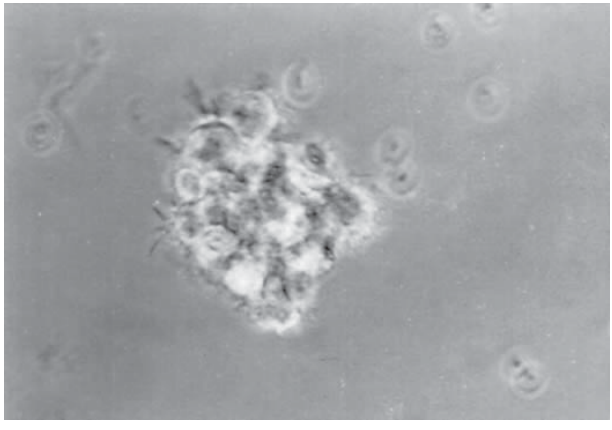

Fig. 2. Phase contrast photograph of a cluster of lymphoid-related DC grown in culture from $CD34^+$ Lin^- $CD10^+$ cells in the presence of Flt3-ligand + c-kit-ligand + GM-CSF + IL-1 + IL-7 for 11 d. Objective ×20.

tions of at least 10 μg/mL. Furthermore, keep cells and antibodies concentrated in a small volume (1–2 mL).

10. It is usually not necessary to include DNase here unless clumps and cell debris remain.

11. This target population represents approx 5% of adult BM $CD34^+$ Lin^- cells. Whereas sort purity of the $CD34^+$ Lin^- $CD10^-$ population usually exceeds 90% (**Fig. 1B**), sort purity is typically 50–85% for the more rare $CD34^+$ Lin^- $CD10^+$ cell subset (**Fig. 1C**). To obtain highly purified $CD34^+$ Lin^- $CD10^+$ cells, it is recommended that a second sort of the cells using the same gates be performed. This will cause cell loss but will provide a highly enriched population that does not differentiate into myeloid cells in culture with the cytokines used above. The cell separation that is described here may seem inefficient, but is practical and relatively economical. Antibodies and magnetic beads are used to enriched $CD34^+$ Lin^- cells before flow cytometry sorting. Negative depletion is the preferred method for enrichment. Although it is less effective than positive selection, it preserves cell yield yet facilitates sorting.

References

1. Steinman, R. M. (1991) The dendritic cell system and its role in immunogenicity. *Ann. Rev. Immunol.* **9,** 271–296.
2. Santiago-Schwarz, F., Belilos, E., Diamond, B., and Carsons, S.E., (1992) TNF in combination with GM-CSF enhances the differentiation of neonatal cord blood stem cells into dendritic cells and macrophages. *J. Leuk. Biol.* **52,** 274–281.
3. Caux, C., Dezutter-Dambuyant, C., Schrmitt, D., and Banchereau, J. (1992) GM-CSF and TNF-α cooperate in the generation of dendritic Langerhans cells. *Nature* **360,** 258–261.
4. Inaba, K., Inaba, M., Deguchi, M., et al. (1993) Granulocytes, macrophages, and dendritic cells arise from a common major histocompatibility complex class II-negative progenitor in mouse bone marrow. *Proc. Natl. Acad. Sci. USA* **90,** 3038–3042.
5. Sallusto, F. and Lanzavecchia, A. (1994) Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin-4 and down-regulated by tumor necrosis factor α. *J. Exp. Med.* **179,** 1108–1118.
6. Galy, A., Travis, M., Cen, D., and Chen, B. P. (1995) Human T, B, natural killer, and dendritic cells arise from a common bone marrow progenitor cell subset. *Immunity* **3,** 458–473.
7. Galy, A., Morel, F., Hill, B., and Chen, B. P. (1998) Hematopoietic progenitor cells of lymphocytes and dendritic cells. *J. Immunother.* **21,** 132–141.
8. Ryan, D. H., Nuccie, B. L., Ritterman, I., Liesveld, J., Abboud, C., and Insel, R. (1997) Expression of interleukin-7 receptor by lineage-negative human bone marrow progenitors with enhanced lymphoid proliferative potential and B-lineage differentiation capacity. *Blood* **89,** 928–940.
9. Ardavin, C., Wu, L., Li, C.-L., and Shortman, K. (1993) Thymic dendritic cells and T cells develop simultaneously in the thymus from a common precursor population. *Nature* **362,** 761–763.

25

Dendritic Cell Generation from Highly Purified CD14+ Monocytes

Winfried F. Pickl, Otto Majdic, and Walter Knapp

1. Introduction

Dendritic cells (DC) play a pivotal role in the function of the immune system, for they are the primary antigen-presenting cells (APC) in the activation of naive T-lymphocyte responses ***(1)***. Recent studies have uncovered complexity in the DC lineage with several subsets, functions, and maturational stages. Although it is generally accepted that human DC derive from hematopoietic progenitor cells ***(2–9)***, it is not clear at present whether DC cells and their precursors represent a separate hematopoietic lineage or whether DC should be seen as specialized macrophages with particular morphological, molecular, and functional features. Several lines of evidence point to DC and monocytes/macrophages being offspring of the same $CD34^+$ hematopoietic progenitor cell (***3–5***, ***12–14***, and reviewed in ***[10,11]***). DC committed precursor cells have also been identified in peripheral blood ***(15–18)***.

Immunostimulatory human dendritic cells can, however, also be generated from nonproliferating $CD14^+$ monocytes. Along that line it has been shown that at least some characteristic features of DC, such as CD1a expression and high HLA Class II expression, can be induced by culturing monocytes in medium with low paracrine stimulation ***(19)***. Furthermore, we could show that GM-CSF is required and responsible for the induction of CD1a expression on monocytes ***(20)***. Porcelli et al. ***(21)*** observed similar effects with a combination of GM-CSF plus IL-4.

Recently, we and others have demonstrated that highly purified monocytes or monocyte-enriched PBMC preparations cultured with GM-CSF plus IL-4 differentiate into $CD1a^+/CD14^-$ cells with the morphological and functional features of DC ***(22–28)***. These monocyte derived DC have an immature pheno-

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type, as determined by the lack of CD83 expression. Addition of TNF-α/IL-1β, or LPS, or CD40 ligation, or monocyte conditioned medium leads to terminal maturation ***(23,29,30)***.

In this chapter a detailed protocol for the generation of monocyte-derived DC, immature and mature, is described. Monocyte-derived DC populations can be identified after the 6–8 d of culture based on their morphology, their surface-marker profile, and their functional capabilities in APC/allostimulatory assays.

The reader should be aware that there are as many modifications to the theme (i.e., generation of DC from monocytes) as papers have been published (*see* **Table 1**), but all of these reports come finally to the conclusion that GM-CSF plus IL-4 differentiates monocytes toward DC.

2. Materials

2.1. Isolation of Peripheral Blood Mononuclear Cells (PBMC)

1. Buffy coats from healthy blood donors.
2. 70% (v/v) ethanol.
3. RPMI-1640 medium containing 500 U/mL heparin (Immuno AG, Vienna, Austria).
4. 50 mL centrifuge tubes (Sarstedt, Nürnbrecht, Germany).
5. 25 mL centrifuge tubes (Steriline, Bibby Steriline, UK).
6. Temperature-controlled centrifuge, Sorvall RC-3 or equivalent benchtop centrifuge.
7. Sterile pipets (10 mL).
8. Sterile Pasteur pipets.
9. Ficoll-Paque solution (Pharmacia Biotech; cat no. 17-0840-03). Store protected from light.
10. Hematocytometer plus inverted microscope or cell counter (Coulter, Luton, England).
11. PBS-HA-EDTA (separation buffer): PBS without Ca^{2+}/Mg^{2+} but supplemented with 0.5% (w/v) human albumin (Centeon Pharma GmbH, Vienna, Austria) and 0.5 m*M* EDTA. Filter through a 0.22 μm sterile filter.

2.2. Separation of PBMC Subpopulations

2.2.1. Labeling and Separation of Cells

1. Prechilled (4°C), temperature controlled centrifuge, Sorvall RC-3 or equivalent benchtop type centrifuge.
2. 25 mL Sterilin tubes.
3. PBMC single-cell suspension prepared as in **Subheading 3.1.**
4. Sterile Pasteur pipets.
5. Magnetic cell separator VarioMACS (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany).
6. Separation kit (i.e., columns, flow resistors, syringes) (Miltenyi; cat. no. 413-05).
7. Falcon cell strainer, 70 μm nylon (Becton Dickinson; cat no. 2350).

Table 1
Various Procedures for mdDC Generation

Author	year	Cytokines		Protein	%	Medium	Monocyte preparation
		rhGM-CSF	rhIL-4				
Sallusto et al.	1994	50 ng/mL (Hoffmann L)	1000 U/mL (Hoffmann L)	FCS	10	RPMI1640	lymphoprep>2h plastic adhesion alternative: percoll gradient>Dynal beads (CD19/CD2).
Romani et al.	1994	800 U/mL (Sandoz)	500 U/mL (Genzyme)	FCS	10	RPMI1640	Ficoll-Paque>2h plastic adherence
Zhou et al.	1996	800 U/mL (Genzyme)	500 U/mL (Genzyme)	FCS	10	RPMI1640	Ficoll-Paque>plastic adherence> 14.5% metrizamide gradient
		cytok. replenished every other day					
Pickl et al.	1996	50 ng/mL (Sandoz)	1000 U/mL (Sandoz)	FCS	10	RPMI1640	Ficoll-Paque>positive selection with two CD14 mAbs and MACS
Kiertscher et al.	1996	800 U/mL (Genetics Inst.)	500 U/mL (R&D Syst.)	FCS	10	RPMI1640	Ficoll-Paque>2h plastic adherence
Bender et al.	1996	1000 U/mL (private source)	1000 U/mL (Immunex)	human plasma & CM	1	RPMI1640	Ficoll-Paque>E rosetting
		cytok. replenished every other day					
Romani et al.	1996	800 U/mL (Sandoz)	1000 U/mL (Immunex)	human plasma & CM	1	RPMI1640 X-Vivo 15 AIM-V	lymphoprep>platelets removed> Dynal beads (CD19/CD2)
		cytok. replenished every other day					
Chapuis et al.	1997	800 U/mL (Genzyme)	1000 U/mL (Genzyme)	FCS	10	RPMI1640	Ficoll-Paque>2h plastic adherence
		cytok. replenished every other day					
Palucka et al.	1998	5 ng/mL (Schering-Plough)	250 U/mL (Genzyme)	FCS	10	RPMI1640	Ficoll-Paque>Dynal beads (CD19/CD2)
		cytok. replenished every other day					

8. Separation buffer: PBS-HA-EDTA (*see* **Subheading 2.1., item 11**). Use ice cold for the whole procedure.
9. Streptavidin microbeads (Miltenyi; cat no. 481-01).
10. Appropriate biotinylated monoclonal antibodies (Mabs). As an example we list below the names and concentrations of Mabs used in our studies. Other Mabs with similar specificity should also work; however, careful evaluation of their efficacy and their effective concentrations has to be performed. A protocol for the biotinylation of antibodies is also provided (**Subheading 2.2.** and **Subheading 3.2.**).
    a. Monocyte-positive selection pool: Biotinylated Mab directed against CD14 (VIM13: 15 μg/mL).
    b. T-cell negative selection pool: Biotinylated Mabs directed against:
        CD14 (e.g., VIM13: 15 μg/mL),
        CD19 (e.g., HD37: 15 μg/mL),
        CD16 (e.g., 3G8: 15 μg/mL),
        HLA-C II (e.g., VID1: 15 μg/mL),
        CD11b (e.g., LM2: 15 μg/mL),
        CD33 (e.g., CD33-4D3: 15 μg/mL).
    For preparation of $CD8^+$ T cells a CD4 mAb (e.g., VIT4: 15 μg/mL) is added.

2.2.2. Biotinylation of Monoclonal Antibodies

1. $NaHCO_3$/NaCl buffer: Prepare a 0.1 *M* $NaHCO_3$, 1 *M* NaCl pH 7.8 solution by dissolving 8.4 g $NaHCO_3$ and 58.4 g NaCl in 1 L of ddH_2O and adjusting pH to 7.8 with NaOH.
2. A dialysis system for small volumes (e.g., Slide-a-Lyzer from Pierce; cat. no. 66425 for volumes of 0.5–3 mL, or cat. no. 66415 for volumes of 0.1–0.5 mL).
3. Biotin-X-NHS (Calbiochem; cat. no. 203188).
4. Dimethylformamide (Sigma; cat. no. D-4254).
5. PBS.

2.3. Cell Freezing and Thawing

2.3.1. Freezing of Cells

1. Freezing solution: to 77 mL of RPMI-1640 (Gibco-BRL) supplemented with 10% fetal calf serum (FCS) add an additional 13 mL of pure FCS and 10 mL of dimethylsulfoxide (DMSO). Filter solution through a sterile 0.22 μm filter and store in a sterile glass bottle at 4°C and protected from light.
2. Cryotubes (Nunc).
3. Sterile Pasteur pipets.
4. Freezing container (Nalgene-Nunc; cat. no. 5100).
5. –80°C freezer.
6. Liquid-nitrogen storage system.

2.3.2. Thawing Frozen Cells

1. Water bath at 37°C.
2. Sterile Pasteur pipets.

3. 15 mL V-botttomed tubes (Falcon; cat. no. 2099).
4. Medium: RPMI-1640 (Gibco-BRL) supplemented with 10% (v/v) FCS (Gibco-BRL), 100 U/mL penicillin and 100 μg/mL streptomycin.

2.4. Generation of Monocyte-Derived DC (mdDC)

2.4.1. Culture of $CD14^+$ Monocytes

1. Culture medium. All cell cultures are grown in RPMI-1640 (Gibco-BRL) supplemented in the following order with:
   a. FCS (Gibco) at a final concentration of 10% (v/v).
   b. Penicillin (Gibco, concentration 10^4 U/mL) diluted 1:100 results in final concentration of 100 U/mL.
   c. Streptomycin (Gibco, concentration 10 mg/mL) diluted 1:100 results in final concentration of 100 μg/mL.
   d. Glutamin (Gibco, 200 m*M* solution), diluted 1:100 to give a final concentration of 2 m*M*.
   e. Recombinant human (rh)IL-4 (we used IL-4 from Novartis, or *see* **Table 1**). Add to give a final concentration of 1000 U/mL (*see* **Note 1**). Recombinant human (rh)GM-CSF (we used GM-CSF from Novartis, or *see* **Table 1**). Add to give a final concentration of 50 ng/mL.
2. 75 cm^2 tissue culture flasks (Costar).

2.4.2. Production of Monocyte Conditioned Medium

1. 100 mm bacterial plates (Falcon; cat. no. 1029).
2. Human immuoglobulin (human Ig for therapeutic purposes).
3. PBS.
4. Medium (**Subheading 2.4.1.**).
5. 0.22 μm filters.

2.5. Characterization of mdDC

2.5.1. Morphology

1. Medium (**Subheading 2.4.1.**).
2. Microscope slides.
3. Cytocentrifuge (Shandon Southern Products, Astmoor, UK) with buckets and punched filter paper sheets.
4. Glass drill.
5. Materials for May-Gruenwald Giemsa stain:
   a. May-Gruenwald solution (Merck cat. no. 101.424).
   b. Giemsa solution (Merck cat. no. 109.204).
   c. Buffer tabs (Merck cat. no. 9468).
   d. Cover medium "Entallan" (Merck cat. no. 7961).

2.5.2. Immunofluorescence

1. PBS containing 0.5% (w/v) bovine serum albumin (BSA) and 0.02% (w/v) NaN_3.
2. Vortex.

3. 4 mL tubes (Falcon; cat. no. 2054).
4. Monoclonal antibodies and corresponding fluorochrome labeled secondary antibodies.
5. Fluorescence microscope or flow cytometer.

2.5.3. Mixed Leukocyte Reaction (MLR)

1. 96-well cell plates, U-bottom (Costar, cat. no. 3799).
2. T cells or PBMC (as responder cells).
3. mdDC.
4. Irradiation device (^{137}Cs source).
5. Medium: RPMI-1640 containing 10% (v/v) human serum.
6. CO_2-incubator.
7. Methyl-[^{3}H]thymidine (Amersham, Buckinghamshire, UK).
8. Cell harvester, counter, scintillator (all depending on the available system).

3. Methods

3.1. Isolation of PBMC

Human mononuclear cells (PBMC) are isolated from heparinized buffy coat preparations of healthy blood donors by the use of density gradient centrifugation ***(31)***. All solutions and equipment coming into contact with cells must be sterile. The whole protocol is performed under sterile conditions!

1. Clean the buffy coat container with 70% ethanol to reduce bacterial contamination, and then let the ethanol dry off for 2 min.
2. Open container with sterile scissors and dilute blood with 3 vol of RPMI-1640 medium containing heparin.
3. Place 20 mL Ficoll-Paque solution into sterile centrifuge tubes (50 mL).
4. Carefully overlay the diluted buffy coat preparation onto the Ficoll-Paque cushion. It might be helpful to tilt the tube by 45° when applying the first milliliters of the cell suspension to prevent mixing of blood with Ficoll-Paque.
5. Separate cell populations by centrifugation of tubes at 400*g* at room temperature for 30 min (*see* **Notes 2** and **3**). Erythrocytes, granulocytes, and any dead cells will be found at the bottom of the tube. At the interface between the plasma and Ficoll, lymphocytes, monocytes, and some platelets will be visible as a distinct layer of cells.
6. Remove the cells from the interface (=PBMC) with a 10 mL pipet with a large orifice in a volume of about 10 mL and transfer them to a fresh 50 mL centrifuge tube. Discard the red cells and granulocytes that remained in the tube.
7. Fill tube(s) containing PBMC to top with ice-cold RPMI-1640 medium (containing heparin) and centrifuge for 8 min at 400*g*. This removes the residual Ficoll-Paque, which is toxic to cells.
8. Suck off supernatant and resuspend the cell pellet by gently flicking the tube, fill tube to top with ice-cold RPMI-1640 medium (containing heparin), and centrifuge for 8 min at 200*g* (approx 800–1000 rpm).

9. Carefully suck off and discard supernatant. This step removes the majority of the platelets in the preparation. Resuspend cell pellet in ice-cold separation (PBS-EDTA-HA) buffer and wash cells twice at 400*g* for 8 min and 4°C.
10. Resuspend cells in separation buffer with a Pasteur pipet, count cells and adjust cell concentration to 1×10^9 cells per 750 µL of separation buffer, and put on ice. A reasonably good buffy coat preparation should yield at least 1×10^9 PBMC (normal range $1.5 – 3.0 \times 10^9$). If much less than this amount is gained one should not start the large scale separation procedure described in **Subheading 3.2.**

3.2. Separation of PBMC Subpopulations

PBMC can be separated into different subpopulations (mainly monocytes and T cells) by magnetic cell sorting (MACS). The technique relies on the monoclonal-antibody-based separation of PBMC with colloidal microspheres using a magnet ***(32)***.

3.2.1. Labeling and Separation of PBMC

1. (Continued from **step # 10** of **Subheading 3.1.**) Resuspend 1×10^9 cells in 750 µL of separation buffer (PBS-HA-EDTA), add them to a 25-mL Sterilin tube, and put on ice. It is important to work with homogeneous cell suspension with no cell clumps whatsoever. If cell clumps already appear at this time point, pass cells through a cell strainer.
2. With a glass Pasteur pipet, add 250 µL of the appropriate Mab pool to the cells, mix well by pipeting up and down repeatedly, and incubate suspension for 10–15 min at 4–6°C in a refrigerator (not on ice!).
3. Fill tube to top with ice-cold separation buffer and centrifuge at 400*g* for 8 min in a prechilled centrifuge (4°C) and remove supernatant completely.
4. Resuspend cells and repeat washing step as above.
5. After removal of the washing buffer, resuspend cell pellet with a Pasteur pipet in 750 µL of separation buffer and incubate cells with 250 µL of streptavidin-coated magnetic beads for 10–15 min at 4°C in a refrigerator.
6. Pass cells through a nylon mesh (cell strainer, Falcon 70 µm) put onto a 50 mL tube to remove cell aggregates. For complete recovery of cells, wash mesh with 1 mL of separation buffer.
7. Prepare magnetic column:
   a. Place CS column attached to a three-way stop cock, a 10 mL syringe, and a 20-G needle onto VarioMACS separator. (For details refer to the operation instructions provided by Miltenyi.)
   b. Fill the column with ice-cold separation buffer via the attached syringe until the buffer reaches the top of the reservoir. Pump out any air bubbles.
   c. Remove buffer by turning the three-way stop cock until the buffer has reached the level of the sponge positioned in the column. Add more buffer from the side via the attached syringe until all bubbles are removed. Make sure that the column does not run dry.

d. Choose recommended needle for cell separation (monocyte-positive selection: for fraction 1 use 21-G; for fraction 2 use 20-G; for T-cell negative selection use 22-G) and attach to three-way stop cock.

8. Obtain monocytes by positive selection:
   a. Apply PBMC labeled with anti-CD14 Mabs and magnetic microspheres on top of the column. By turning the three-way stop cock, allow the cell suspension to penetrate into the matrix. Continuously add separation buffer from the top (total amount of 30 mL) and collect the effluent. This fraction contains the larger amount of CD14 negative cells (i.e., T cells, B cells, NK cells).
   b. Remove column from VarioMACS and flush cells with 10 mL of buffer and the syringe attached to the side of the column to the top of the column.
   c. Replace column in VarioMACS and allow labeled cells to readhere to the magnetized matrix by allowing the buffer to flow through the column.
   d. Repeat this step twice.
   e. Subsequently attach a fresh 20-G needle to the column.
   f. Flush the column with 50 mL of buffer from the top of the column. The effluent of **steps 2–6** should be mostly monocyte-free and still contains a small amount of T cells, B cell, and NK cells.
   g. Remove column from magnetic separator. Pull out monocyte-positive fraction with the syringe attached to the side of the column by adding a total of 50 mL of buffer to the top of the column. Be aware of the strong magnetic forces: the distance to the VarioMACS for this procedure should be at least 1 meter. *See* **Note 4** for expected results.
9. Obtain T cells by negative selection:
   a. Attach a 22-G needle to the three-way stop cock of the assembled column.
   b. Apply PBMC suspension or $CD14^-$ cells from previous monocyte separation step labeled with T-cell selection Mabs plus beads to the top of the column. Allow cells to penetrate into the matrix of the column and, when the cell suspension has reached the level of the sponge, start to add a total volume of 30 mL of buffer from the top of the column. Collect effluent containing purified T cells. *See* **Note 5** for expected results.

3.2.2. Biotinylation of Monoclonal Antibodies

1. Dilute Mab solution to 1 mg/mL with PBS and dialyze overnight against 1 L of $NaHCO_3$/NaCl buffer. The most convenient system for dialysis of small volumes is the Slide-a-Lyzer system from Pierce.
2. Next day, solubilize the biotin in dimethylformamide (DMF) at a concentration of 10 mg/mL.
3. Add 10 µL biotin/DMF solution per 1 mL of dialyzed Mab solution and incubate at 37°C under constant agitation for 3 h.
4. Afterward dialyze against PBS overnight. Change the dialysis buffer at least three times (3 × 1 L).
5. Store biotinylated Mab solution at 4°C and add NaN_3 to a final concentration of 0.05% (w/v).

3.3. Cell Freezing and Thawing

3.3.1. Freezing of Cells

1. Label a sufficient number of 1.8 mL cryotubes and place on a rack.
2. Resuspend monocytes or T cells at a concentration of 10–20 × 10^6/mL in ice-cold freezing medium with a Pasteur pipet.
3. Very quickly, apply aliquots of 1 mL cell suspension to cryotubes and place tubes into the freezing container that has been accommodated to room temperature.
4. Place freezing container with ampoules into –80°C freezer overnight.
5. Next day remove frozen ampules and place them into a liquid-nitrogen container for long-term storage.

3.3.2. Thawing of Cells

1. Remove ampule from the liquid-nitrogen container and put in a 37°C water bath.
2. Wear gloves and protection glasses. (I have seen bursting cryotubes!) Remove cells from the water bath when half of the cell suspension is thawed.
3. Shake vial in your hand until the last piece of ice in the vial disappears.
4. Immediately put cryotube on ice for 5 min.
5. With a Pasteur pipet transfer the cell suspension to a 15 mL tube with a V-formed bottom.
6. Add five drops of culture medium, mix by gently tipping the tube, and let sit for 1 min.
7. Repeat addition of medium in 1 min intervals three times and double the volume of added medium each time.
8. Fill tube with medium to top and collect cells by centrifugation at 400*g* for 8 min.
9. Suck off medium, resuspend cells with a Pasteur pipet, and repeat washing twice.

3.4. Generation of mdDC

3.4.1. Culture of $CD14^+$ Monocytes

Immature mdDC are obtained as follows:

1. Take an aliquot (10 × 10^6 cells) of purified $CD14^+$ cells, freshly prepared or thawed.
2. Resusupend at a cell density of 5 × 10^5 cells/mL in 20 mL of culture medium. Our culture medium contains GM-CSF (50 ng/mL, Novartis) and IL-4 (1000 or 100 U/mL, Novartis).
3. Add cell suspension to a standard 75cm^2 culture flask (Costar) and incubate at 37°C in a humidified CO_2-containing atmosphere.
4. Incubate cells for 6–8 d to obtain mdDC (*see* **Notes 6** and **7**). Mature ($CD83^+$) mdDC can be obtained by two methods:
   a. Follow the preceding protocol but in addition to GM-CSF and IL-4, also supplement medium with 100 U/mL of TNF-a at the beginning of the culture.
   b. Alternative protocol (according to ref. 30)
      Start culture as described above in **Subheadings 3.4.1.1.–4.** (e.g., in 20 mL).

On d 7 of culture remove cells from culture flask.
Seed cells into new flask in three-quaters of the original culture volume in original medium (i.e., 15 mL original medium).
Add one-quarter volume (i.e., 5 mL) of monocyte-conditioned medium (see Subheading 3.4.2.) and replenish with fresh GM-CSF and IL-4.
Culture cells for another 4 d and analyze.

3.4.2. Production of Monocyte Conditioned Medium (CM)

1. Incubate bacterial plates with 10 mL of a 10 mg/mL solution (in PBS) of human immunoglobulin for 1–2 h at room temperature.
2. Remove Ig-solution and wash plates by rinsing with PBS three times.
3. Add 5×10^7 PBMC in 8 mL of medium per plate and incubate for 1 h at 37°C.
4. Rinse off nonadherent cells, add 8 mL of fresh medium, and put cells for 24 h in the incubator.
5. Filter supernatant (medium) through 0.22 μm filter and store at –20°C until use.

Use the conditioned medium (CM) at 25 % (v/v) as described in **Subheading 3.4.1.**

3.5. Characterization of mdDC

3.5.1. Morphology

1. Resusupend cells at a density of 1×10^6 cells/mL in culture medium.
2. Mark standard microscope slides with a pencil, and assemble together with punched filter paper sheets in cytocentrifuge buckets.
3. Prewet the slides by addition of 50 μL of PBS and spin them at 1000 rpm for 1 min in a cytocentrifuge.
4. Subsequently, add 50 μL of well-resusupended cell suspension and centrifuge at 1000 rpm for 5 min.
5. After removal from the buckets, air-dry slides for 5 min and circle the area where the cells are sticking to the glass with a glass-drill.
6. Store samples at –70°C until use or proceed directly to May–Gruenwald Giemsa staining:
   a. Air-dry cytospin preparations on microscope slides.
   b. Add 150 mL of May–Gruenwald solution into standard staining cuvet and incubate microscope slides for 5 min in the solution.
   c. Prepare a cuvet filled with May–Gruenwald solution diluted 1:2 with buffer and incubate slides for another 5 min in this cuvet.
   d. Prepare a cuvet filled with 150 mL of Giemsa solution (8% in buffer) and incubate cytospins for 15 min.
   e. Rinse slides with tab water until no color goes off and air-dry subsequently.
   f. Analyze immediately or cover by putting one drop of Entallan on cover slide and sealing the stained cells.

After 8 d of culture you expect enlarged (double the size of monocytes) cells with long cytoplasmic projections (dendrites). For details read cited literature.

3.5.2. Immunofluorescence

1. Resusupend cells to be analyzed at a density of 1×10^7/mL in ice-cold PBS/BSA/NaN_3.
2. Add 50 µL of the cell suspension into a 4 mL tube and put on ice.
3. Add 20 µL of desired antibody solution (20 µg/mL), vortex for 2 s and incubate for 30 min on ice.
4. Fill tube to top with ice-cold PBS/BSA/NaN_3 solution and centrifuge at 400*g* for 5 min.
5. Discard supernatant, resusupend cells by gently flicking the bottom of the tube or by gently vortexing.
6. Add 50 µL of fluorochrome labeled secondary antibody (20 µg/mL), vortex for 2 s and incubate for 30 min on ice.
7. Fill tube to top with ice-cold PBS/BSA NaN_3 solution and centrifuge at 400*g* for 5 min. Resusupend cells as above and wash again.
8. Resuspend cells in a suitable vol (0.5–1.0 mL) of PBS and analyze by using a fluorescence microscope or a flow cytometer (*see* **Note 8**).

3.5.3. Mixed Leukocyte Reaction (MLR)

1. Irradiate stimulator cells (i.e., monocytes or mdDC) to be tested with 3000 rad (^{137}Cs source). This completely blocks DNA synthesis in contaminating T cells in the stimulator population.
2. Resusupend T cells or PBMC at a concentration of 1×10^6/mL in RMPI 1640 plus 10% human serum and apply 100 µL per well. Make sure that T cells/PBMC are histoincompatible with the monocyte-donor, otherwise no alloresponse would be detectable.
3. Prepare several dilutions of mdDC or native monocytes to be tested (e.g., 1×10^5/mL, 5×10^4/mL, 2.5×10^4/mL) and add 100 µL per well.
4. Incubate cell mixture in triplicate at 37°C for 5 d in a CO_2 incubator
5. Add 1 µCi of methyl-[^{3}H]thymidine per well (in a volume of 25–50 µL) and incubate for another 18 h to allow incorporation into newly synthesized DNA.
6. Harvest cells onto a glass filter using one of the common commercial harvesting systems (*see* **Note 9**).
7. Analyze samples and determine the stimulation index (SI) according to the formula (*see* **Note 10**):

$$SI = cpm(T\ cells + mdDC)/cpm\ (T\ cells\ alone)$$

4. Notes

1. 1:10 of the amount of IL-4 (i.e., 100 U/mL) also works fine in our hands.
2. Rpm can be calculated according to the formula: $rpm = 1000 \times \sqrt{(g/1.12\,r)}$, where r is the radius of the rotor in mm.
3. It is absolutely necessary to shut off brake-function for deceleration, otherwise the interphase will be disturbed. Be sure to operate the centrifuge at room temperature, separation does not work at 4°C.

4. Like the monocyte count of different individuals, the yield of monocytes is variable. It should lie somewhere between 50×10^6 and 250×10^6 monocytes per 1×10^9 PBMCs applied to the column. The purity can be as high as 99.9%, but can sometimes reach only 95%.
5. The starting populations for T-cell selection are in our hands either PBMC or monocyte-depleted PBMC fractions. Expect to recover $300–500 \times 10^6$ cells per 1×10^9 input cells. The purity can be as high as 99.5% and should be at least 95%. For convenience, we routinely store aliquots of cells in liquid nitrogen until use.
6. According to our experience, there is no need to change the culture medium during the 6–8 d of culture. The cell yield after the culture period should be at least 50–70% of input cells (when working with thawed cells), but can be as high as the number of input cells (freshly prepared cells).
7. The purification and the cultivation of monocytes have been performed in many different ways (*see* **Table 1**), and all the described methods seem to work well. Variations include
   a. resupplementation of cultures with fresh cytokines during the culture period,
   b. use of different protein sources and concentrations,
   c. use of different media,
   d. application of various monocyte enrichment procedures.
8. Compare fluorescence of labeled cells with that of unlabeled cells and of cells that have been incubated with a nonbinding isotype control Mab. As a minimum staining panel stain for CD1a, CD14, HLA-DR, HLA-DQ, CD40, CD80, CD83, CD86, plus nonbinding control (see literature for details).
9. Should immediate harvesting not be possible, freeze plates at –20°C.
10. Expect half maximal stimulation with a DC:T cell ratio of 1:100 to 1:33. Stimulation indices can be as high as 20-fold with DC:T cell ratios of 1:1000 ***(33)***.

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References

1. Steinman, R. M. (1991) The dendritic cell system and its role in immunogenicity. *Annu. Rev. Immunol.* **9**, 271–296.
2. Volc-Platzer, B., Stingl, G., Wolff, K., Hinterberger, W., and Schnedl, W. (1984) Cytogenetic identification of allogeneic epidermal Langerhans cells in a bone marrow-graft recipient. *N. Engl. J. Med.* **310**, 1123,1124.
3. Caux, C., Dezutter-Dambuyant, C., Schmitt, D., and Banchereau, J. (1992) GM-CSF and TNF-alpha cooperate in the generation of dendritic Langerhans cells. *Nature* **360**, 258–261.
4. Reid, C., Fryer, P., Clifford, C., Kirk, A., Tikerpae, J., and Knight, S. (1990) Identification of hematopoietic progenitors of macrophages and dendritic Langerhans cells (DL-CFU) in human marrow and peripheral blood. *Blood* **76**, 1139–1149.

5. Reid, C., Stackpoole, A., Meager, A., and Tikerpace, J. (1992) Interactions of tumor necrosis factor with granulocyte-macrophage colony-stimulating factor and other cytokines in the regulation of dendritic cell growth in vitro from early bipotent $CD34^+$ progenitors in human bone marrow. *J. Immunol.* **149**, 2681–2688.
6. Santiago-Schwarz, F., Divaris, N., Kay, C., and Carsons, S. (1993) Mechanisms of tumor necrosis factor and granulocyte-macrophage colony-stimulating factor-induced dendritic cell development. *Blood* **82**, 3019–3028.
7. Strunk, D., Rappersberger, K., Egger, C., et al. (1996) Generation of human dendritic cells/Langerhans cells from circulating CD34+ hematopoietic progenitor cells. *Blood* **87**, 435–445.
8. Young, J., Szabolcs, P., and Moore, M. (1995) Identification of dendritic cell colony-forming units among normal human $CD34^+$ bone marrow progenitors that are exprandend by c-kit-ligand and yield pure dendritic cell colonies in the presence of granulocyte/macrophage colony-stimulating factor and tumor necrosis factor alpha. *J. Exp. Med.* **182**, 1111–1120.
9. Galy, A., Travis, M., Cen, D., and Chen, B. (1995) Human T, B, natural killer, and dendritic cells arise from a common bone marrow progenitor cell subset. *Immunity* **3**, 459–473.
10. Peters, J. H., Gieseler, R., Thiele, B., and Steinbach, F. (1996) Dendritic cells: From ontogenetic orphans to myelomonocytic descendants. *Immunol. Tod* **17**, 273–278.
11. Reid, C. D. L. (1997) The dendritic cell lineage in hematopoiesis. *Br. J. Haematol.* **96**, 217–223.
12. Caux, C., Vanbervliet, B., Massacrier, C., et al. (1995) Characterization of human $CD34^+$ derived dendritic/Langerhans cells (D-Lc). In *Dendritic cells in fundamental and clinical immunology.* (Banchereau, J. and Schmitt, D., eds.) Plenum Press, New York, pp. 1–15.
13. Mackensen, A., Herbst, B., Köhler, G., et al. (1995) Delineation of the dendritic cell lineage by generating large numbers of Birbeck granule-positive Langerhans cells from human peripheral blood progenitor cells in vitro. *Blood* **86**, 2699–2707.
14. Szabolcs, P., Moore, M., and Young, J. (1995) Expansion of immunostimulatory dendirtic cells among the myeloid progeny of human $CD34^+$ bone marrow precursors cultured with c-kit ligand, granulocyte-macrophage colony-stimulating factor and TNF-α. *J. Immunol.* **154**, 5851–5861.
15. Thomas, R. L., Davis, L. S., and Lipsky, P. E. (1993) Isolation and characterization of human peripheral blood dendritic cells. *J. Immunol.* **150**, 821–834.
16. O'Doherty, U., Peng, M., Gezelter, S., et al. (1994) Human blood contains two subsets of dendritic cells, one immunologically mature and the other immature. *Immunology* 82, 487–493.
17. Thomas, R., and Lipsky, P. E. (1994) Human peripheral blood dendritic cell subsets—isolation and characterization of precursor and mature antigen-presenting cells. *J. Immunol.* **153**, 4016–4028.
18. Weissman, D., Li, Y., Ananworanich, J., et al. (1995) Three populations of cells with dendritic morphology exist in peripheral blood, only one of which is

infectible with human immunodeficiency virus type I. *Proc. Natl. Acad. Sci. USA* **92,** 826–830.
19. Rossi, G., Heveker, N., Thiele, B., Gelderblom, H., and Steinbach, F. (1992) Development of a Langerhans cell phenotype from peripheral blood monocytes. *Immunol. Lett.* **31**, 189–198.
20. Kasinrerk, W., Baumruker, T., Majdic, O., Knapp, W., and Stockinger, H. (1993) CD1 molecule expression on human monocytes induced by granulocyte-macrophage colony-stimulating factor. *J. Immunol.* **150**, 579–584.
21. Porcelli, S., Morita, C. T., and Brenner, M. B. (1992) CD1b restricts the response of human $CD4^{-}CD8^{-}$ T lymphocytes to a microbial antigen. *Nature* **360**, 593–597.
22. Romani, N., Gruner, S., Brang, D., et al. (1994) Proliferating dendritic cell progenitors in human blood. *J. Exp. Med.* **180**, 83–93.
23. Sallusto, F. and Lanzavecchia, A. (1994) Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulationg factor plus interleukin 4 and downregulated by tumor necorsis factor alpha. *J. Exp. Med.* **179**, 1109–1118.
24. Akagawa, K. S., Takasuka, N., Nozaki, Y., et al. (1996) Generation of $CD1^{+}$ $RelB^{+}$ dendritic cells and tartrate-resistant acid phosphatase positive osteoclast-like multinucleated giant cells from human monocytes. *Blood* **88**, 4029–4039.
25. Kiertscher, S., and Roth, M. (1996) Human $CD14^{+}$ leukocytes acquire the phenotype and function of antigen-presenting dendritic cells when cultured in GM-CSF and IL-4. *J. Leuk. Biol.* **59**, 208–218.
26. Pickl, W. F., Majdic, O., Kohl, P., et al. (1996) Molecular and functional characteristics of dendritic cells generated from highly purified $CD14^{+}$ peripheral blood monocytes. *J. Immunol.* **157**, 2001–2011.
27. Zhou, L.-J. and Tedder, T. (1996) $CD14^{+}$ blood monocytes can differentiate into functionally mature CD83+ dendritic cells. *Proc. Natl. Acad. Sci. USA* **93**, 2588–2592.
28. Chapuis, F., Ronsenzwajg, M., Yagello, M., Ekamn, M., Biberfeld, P., and Gluckman, J. C. (1997) Differentiation of human dendritic cells from nonproliferating progenitors in human blood. *Eur. J. Immunol.* **27**, 431.
29. Bender, A., Sapp, M., Schuler, G., Steinman, R. M., and Bhardwaj, N. (1996) Improved methods for the generation of dendritic cells from nonproliferating progenitors in human blood. *J. Immunol. Methods* **196**, 121–135.
30. Romani, N., Reider, D., Heuer, M., et al. (1996) Generation of mature dendritic cells from human blood: an improved method with special regard to clinical applicability. *J. Immunol. Methods* **196**, 137–151.
31. Boyum, A. (1968) Isolation of leucocytes from human blood-further observations. *Scand. J. Clin. Lab. Invest.* **21**, 31–50.
32. Miltenyi, S., Müller, W., Weichel, W., and Radbruch, A. (1990) High gradient magnetic cell separation with MACS. *Cytometry* **11**, 231–238.
33. Schuler, G. and Steinman, R. (1997) Dendritic cells as adjuvans for immune-mediated resistance to tumors. *J. Exp. Med.* **186**, 1183–1187.

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Generation of Blood-Derived Human Dendritic Cells for Antitumor Immunotherapy

Michel F. Gilliet and Frank O. Nestle

1. Introduction

Dendritic cells (DC) are a family of bone-marrow-derived professional antigen presenting cells (APC) with sparse, but wide, tissue distribution ***(1)***. They are classified primarily based on their localization: as interdigitating reticulum cells when present in lymphoid organs, as veiled cells when present in afferent lymph, as Langerhans cells when present in the epidermis, and as dermal dendritic cells when found in the dermis. Although DC are widely dispersed throughout the body, they exhibit many common features: an irregular shape with elongated dendritic processes, a distinctive cell-surface phenotype, low buoyant density, active motility, and the ability to stimulate vigorous proliferation of unprimed T cells. Like other professional APC such as macrophages and B cells, DC are able to ingest, process, and present antigen (Ag) in the context of major histocompatability (MHC) molecules. However, owing to their high expression of MHC class I and II, as well as costimulatory molecules and adhesion molecules, DC have the ability to induce primary T-cell-dependent immune responses in vivo and in vitro. This unique feature gives dendritic cells a central role in controlling immunity.

For years the low frequency of DC throughout the human body (e.g., $< 0.2\%$ of human mononuclear cells are mature blood DC) has been a major obstacle for the development of immunotherapeutic strategies based on the ex vivo antigen loading of DC. In recent years several laboratories have developed new culture systems for the generation of adequate numbers of human DC from blood, bone marrow, or $CD34^+$ stem cells. The first method in humans was described by Caux and Banchereau ***(2)*** in 1992. This approach, based on the isolation and culture of proliferating $CD34^+$ cells, is less practical for small

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samples of blood, where the frequency of $CD34^+$ cells is very low (0.1%). A major breakthrough came with the description of a simple method to generate large numbers of blood-derived DC from monocytes by culture with granulocyte-macrophage colony stimulating factor (GM-CSF) and IL-4 ***(3,4)***. This allowed the design of immunotherapeutic strategies using ex vivo–generated DC as adjuvants ***(5,6)***. Although reaching a good level of maturation at d 7, these culture conditions involved xenologous sera such as fetal calf serum (FCS), which may be suboptimal in clinical applications. FCS-free autologous conditions necessitate an additional maturation step for the generation of mature DC. In this two-step culture system peripheral blood mononuclear cells (PBMC) are first differentiated in autologous-plasma-supplemented medium with addition of GM-CSF and IL-4 for 7 d and then stimulated for three additional days with monocyte-conditioned medium (MCM) ***(7,8)***. This completely autologous culture system may have the disadvantage of an unpredictable DC quality due to the variation in the content of maturation-inducing cytokines in the conditioned medium. An improved method came upon with the identification of IL-1, IL-6, and TNF-α as substitutes for the monocyte-conditioned medium ***(9,10)***. PGE_2 was found to further enhance the yield and the quality of the DC generated ***(9)***. A pure population of mature DC can now be generated under these conditions in a 10-d autologous culture system. The in vitro maturation of blood-derived DC (BDC) has many similarities to the in vivo physiological maturation of Langerhans cells (LC) upon antigen up-take and migration to the T-cell areas of lymphoid organs. Like skin-resident LC, immature BDC have an efficient antigen up-take and processing machinery with high-level recirculation of MHC complexes ***(11)***. Mature BDC with their strong T-cell stimulatory capacity correspond qualitatively to LC after migration into the lymph node. The exact knowledge of their maturation pathway (**Fig. 1**) makes BDC optimal tools for immunotherapies based on DC as adjuvants: native protein antigens may be preferentially delivered at the immature stage when the processing machinery is highly activated. Further in vitro culture will generate mature DC, which are less able to capture new proteins but are better at stimulating resting CD4 and CD8 T cells, therefore representing optimal candidates for immunization. An additional maturation to "superactivated DC" takes place in vivo after DC-vaccination: interaction with CD40L on T cells will license DC to fully activate cytotoxic T cells without bystander T-cell help ***(12–14)***.

2. Materials

2.1. Culture Medium

1. Dendritic cell cultures are grown in X-VIVO 15 supplemented with 1% heat-inactivated autologous plasma (*see* **Note 1**), glutamine, and antibiotics. Heat-

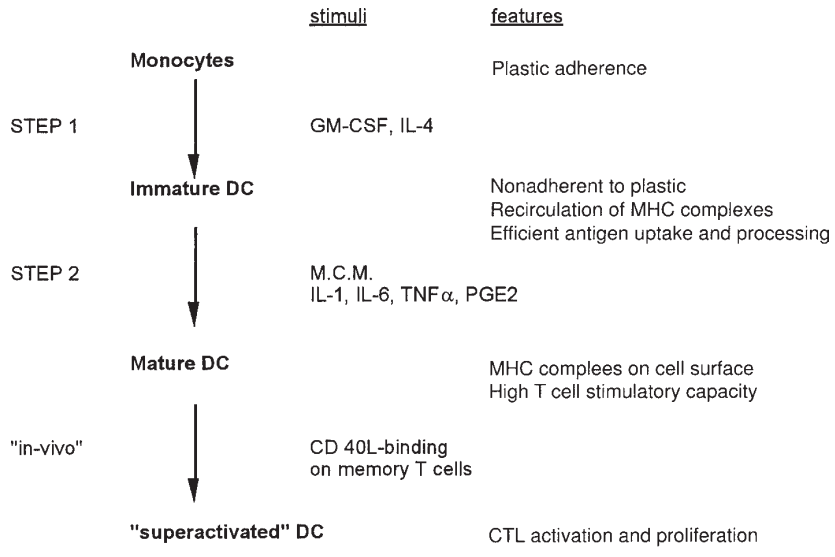

Fig. 1. A model of blood-derived dendritic cell differentiation from monocytes.

inactivated plasma is prepared as follows:

a. Centrifuge 10 mL heparinized autologous whole blood for 10 min at 1400*g*.
b. Collect the clear cell-free supernatant (plasma) and transfer into a 15 mL tube. Keep cellular components for PBMC isolation.
c. Heat-inactivate plasma in a 56°C water bath for 1 h (*see* **Note 2**).
d. Centrifuge and collect supernatant (heat-inactivated plasma).

2. Complete culture medium (CM) is prepared as follows: 96% X-VIVO 15 (BioWhittaker, Belgium; 03-418Q), 1% heat-inactivated autologous plasma (**Subheading 2.1., step 1**), 2% L-glutamin 200 m*M* (Seromed, Berlin, Germany; K0282), 1% penicillin/streptomycin 10000 IU/mL (Gibco, Basel, Switzerland; 15140-114). Filter through 0.2 mm filter, and store at 4°C.

2.2. Reagents

1. Phosphate-buffered saline (PBS).
2. Ficoll-Paque (Pharmacia Biotech, Dübendorf, Switzerland; 17-0840-02).
3. 75 cm^2 tissue culture flasks with 0.2 mm vented plug seal cap (Falcon, B&D, Meylan, France; 3111).
4. Recombinant human cytokines:
   a. GM-CSF (Leukomax, Sandoz, Bern, Switzerland).
   b. IL-4 (R&D, Wiesbaden-Nordenstadt, Germany; 204-IL-005).
   c. IL-1β (R&D; 201-LB-005).
   d. IL-6 (R&D; 206-IL-001).
   e. TNF-α (R&D; 210-TA-010).

5. FACS buffer: PBS, 1% bovine serum albumin (BSA) (Sigma; A-7906), 5 m*M* EDTA (Sigma, Buchs, Switzerland; E-5134), 0.01% NaN_3. Store at 4°C.
6. FACS fixative solution: FACS buffer with 0.5% formaldehyde solution (Fluka, Buchs, Switzerland; 47608). Solution is light sensitive. Store at 4°C and do not expose to light for prolonged periods.
7. Mouse anti-human Mab:
   a. CD83 FITC (Immunotech, Marseille, France; 4210).
   b. CD80 PE (B&D; 340294).
   c. CD86 FITC (Pharmingen, Hamburg, Germany; 33404).
   d. HLA-DR FITC (B&D; 7363).
8. Isotype controls:
   a. $IgG2_b$ FITC(Ancell, Bayport, MN; 284-040).
   b. IgG_1 PE (B&D; 9043).
   c. IgG_1 FITC (B&D; 9041).
   d. IgG_{2a} FITC (B&D 9010).

3. Methods

3.1. Generation of Immature Dendritic Cells

1. Collect 80 mL heparinized peripheral blood from patients or healthy donors.
2. Separate into four 50-mL tubes and dilute 1:1 in PBS (20 mL blood + 20 mL PBS).
3. Underlay carefully the 40 mL diluted blood sample with 10 mL Ficoll-Paque and centrifuge for 20 minutes at 600g.
4. Isolate PBMC by gently aspirating the interface between the plasma and the Ficoll-Paque with a 10-mL pipette, transfer to a clean centrifuge tube, wash twice with at least 3 volumes of PBS (*see* **Note 3**) and resuspend in 15mL CM.
5. Incubate for 2 hours at 37°C, 5% CO_2 in a 75cm^2 culture flask (adherence step) (see Note 4).
6. Discard non-adherent cells and wash flask surface twice with PBS (at room temperature) to eliminate residual lymphocytes and platelets.
7. Incubate adherent cells (monocytes) in 15 mL CM supplemented with 800U/mL GM-CSF and 1000U/mL IL-4 at 37°C, 5% CO_2 for 7 days (*see* **Note 5**).
8. At day 7 rinse off non-adherent cells (immature dendritic cells) and wash once with PBS.

3.2. Generation of Mature Dendritic Cells

1. Transfer the d 7 immature dendritic cells to a new 75 cm^2 culture flask with 15 mL CM supplemented with 800 U/mL GM-CSF and 1000 U/mL IL-4. Use either monocyte-conditioned medium (protocol 1) or proinflammatory cytokines (protocol 2) as maturation stimuli (*see* **Note 6**). Protocol 1 (modified from ***[7,8]***): Add 20% monocyte-conditioned medium, generated as described in **Subheading 3.3.** Protocol 2 (modified from ***[9]***): Add 10 ng/mL IL-1β, 1000U/mL IL-6, 10 ng/mL TNF-α. Add 1 mg/mL PGE_2 (*see* **Note 7**).

2. Incubate at 37°C, 5% CO_2 for 3 d (until d 10) and rinse off the nonadherent mature dendritic cells (*see* **Note 8**). Wash once with PBS.
3. Count cells, determine viability, and perform quality control (*see* **Note 9**).

3.3. Generation of Monocyte-Conditioned Medium

1. Apply 10 mL of 10 mg/mL human gamma globulin solution to a bacteriologic 10-mL dish and swirl the plate until the entire surface is coated. Leave for 1 min and then aspirate back the fluid.
2. Leave the coated plates at room temperature for 30 min.
3. Just before use, gently wash the plates with cold PBS twice: add PBS slowly to the side of the plate and let it run down onto the surface. Swirl the plate to cover and "rinse" the surface. Aspirate off between the washes and before addition of cells.
4. Draw 50 mL peripheral blood, isolate PBMC (approx 50×10^6) by Ficoll centrifugation as described in **Subheading 3.1.**, and resuspend in 10 mL CM. Add PBMC to coated plates and incubate at 37°C, 5% CO_2 for 30 min.
5. Vigorously rinse off the nonadherent cells.
6. Add 10 mL CM on adherent cells (monocytes adhering to IgG-coated plates via Fc receptor) and incubate at 37°C, 5% CO_2 for 24 h.
7. Isolate cell-free supernatant (monocyte-conditioned medium = MCM) and store at 4°C for maximally 1 wk or at –20°C for longer periods.

3.4. Quality Control

1. Phenotypic changes and DC purification are monitored by light microscopy and flow cytometric analysis of surface-marker expression: CD83 as DC maturation marker and CD80, CD86, and HLA-DR as functional relevant markers.
2. Put 10^5 cells into eight FACS tubes. Keep cells at 4°C on ice.
3. Wash twice with FACS buffer (keep solution at 4°C).
4. Add antibodies (anti-CD83,-CD80,-CD86,-HLA-DR, and respective iso-type controls) diluted in 100 µL FACS buffer and incubate for 30 min at 4°C, in the dark.
5. Wash twice with FACS buffer. Do not expose stained cells to light for prolonged periods to avoid bleaching of the fluorescent dye.
6. Fix cells by addition of 400 µL FACS fixative solution and keep at 4°C until flow cytometric analysis (should be done within 3 d for optimal results).

4. Notes

4.1. Materials

1. Culture conditions involving FCS-supplemented media produce fairly mature DC within 7 d but may be inappropriate for human applications owing to the presence of xenologous proteins. The use of autologous plasma requires an additional maturation step after the classical 7-d culture period in order to generate mature DC with potent T-cell stimulatory capacity.
2. Heat-inactivation of plasma is an essential procedure to prevent complement activation.

4.2. Generation of Immature DC

3. Addition of 5 m*M* EDTA to wash solution (PBS) may enhance the PBMC yield by avoiding clumping of cells.
4. Alternatively to plastic adherence, T and B cells may be eliminated by immunomagnetic depletion with CD19 and CD3 Dynabeads. This procedure aiming at the purity of the monocyte preparation may not be suitable for subsequent in vivo use of generated DC. We therefore prioritize a simple 2-h adherence step, which gives us routinely a recovered monocyte population of > 80%.
5. Adherent cells are cultured at approx 0.5×10^6 cells/mL, which allows an easy 7-d culture period without the need of medium and cytokine replacement. The convenience of the procedure and its safety regarding possible contamination steps by avoiding repetitive handling of cells make this protocol suitable for clinical applications. Increasing the cell density to 1×10^6 cells/mL by culturing the cells in 6-well plates (3×10^6 cells in 3 mL per well) slightly increases the yield of mature CD83-positive DC but necessitates a CM replacement every other day and bears the risk of contaminations.

4.3. Generation of Mature DC

6. Monocyte-conditioned medium was described as stimulus for the generation of mature DC in a FCS-free culture system ***(7,8)***. A major advantage of this cocktail is that it does not contain any foreign serum and can be obtained from autologous human blood. However, the considerable and unpredictable variations of the MCM quality represent a hazard for the generation of standardized mature DC. The identification of the growth factors mediating the MCM effect ***(9,10)*** allows the substitution of MCM with a well-defined cytokine cocktail (IL-1b, IL-6, TNF-α) for the generation of mature DC.
7. PGE_2 enhances the maturation, the homogeneity and the quality of DC when used as described ***(9)***. It is, however, important to keep in mind that the effects of PGE_2 on DC maturation are strictly dependent on culture conditions and the presence of additional activation signals such as IL-1 and TNF-α. Some authors have shown that in FCS-containing conditions the addition of PGE_2 in the absence of pro-inflammatory cytokines inhibits the maturation of DC precursors. Furthermore, addition of PGE_2 before d 5 of culture completely inhibits the maturation of DC and results in differentiation of macrophage-like cells ***(15)***.
8. Mature DC should be isolated between d 9 and d 10.
9. $4.0 (\pm 2.4) \times 10^6$ mature DC can be generated from 80 mL peripheral blood ($n = 29$). As a rule of thumb: 1×10^6 PBMC/mL blood are obtained after Ficoll centrifugation, 10% of PBMC are plastic adherent monocytes, half can be isolated as mature dendritic cells at d 10 of culture.
10. The use of cytokines described above does not fulfill GMP criteria.

References

1. Banchereau, J., and Steinman, R. M. (1998) Dendritic cells and the control of immunity. *Nature* **392,** 245–252.

2. Caux, C., Dezutter Dambuyant, C., Schmitt, D., and Banchereau, J. (1992) GM-CSF and TNF-alpha cooperate in the generation of dendritic Langerhans cells. *Nature* **360,** 258–261.
3. Sallusto, F. and Lanzavecchia, A. (1994) Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor alpha. *J. Exp. Med.* **179,** 1109–1118.
4. Romani, N., Gruner, S., Brang, D., Kampgen, E., Lenz, A., Trockenbacher, B., Konwalinka, G., Fritsch, P. O., Steinman, R. M., and Schuler, G. (1994) Proliferating dendritic cell progenitors in human blood. *J. Exp. Med* **180,** 83–93.
5. Hsu, F. J., Benike, C., Fagnoni, F., Liles, T. M., Czerwinski, D., Taidi, B., Engleman, E. G., and Levy, R. (1996) Vaccination of patients with B-cell lymphoma using autologous antigen-pulsed dendritic cells. *Nat. Med.* **2,** 52–58.
6. Nestle, F. O., Alijagic, S., Gilliet, M., Sun, Y., Grabbe, S., Dummer, R., Burg, G., and Schadendorf, D. (1998) Vaccination of melanoma patients with peptide- or tumor lysate-pulsed dendritic cells. *Nat.Med.* **4,** 328–332.
7. Bender, A., Sapp, M., Schuler, G., Steinman, R. M., and Bhardwaj, N. (1996) Improved methods for the generation of dendritic cells from nonproliferating progenitors in human blood. *J. Immunol. Methods* **196,** 121–135.
8. Romani, N., Reider, D., Heuer, M., Ebner, S., Kampgen, E., Eibl, B., Niederwieser, D., and Schuler, G. (1996) Generation of mature dendritic cells from human blood. An improved method with special regard to clinical applicability. *J. Immunol. Methods* **196,** 137–151.
9. Jonuleit, H., Kuhn, U., Muller, G., Steinbrink, K., Paragnik, L., Schmitt, E., Knop, J., and Enk, A. H. (1997) Pro-inflammatory cytokines and prostaglandins induce maturation of potent immunostimulatory dendritic cells under fetal calf serum-free conditions. *Eur. J. Immunol.* **27,** 3135–3142.
10. Reddy, A., Sapp, M., Feldman, M., Subklewe, M., and Bhardwaj, N. (1997) A monocyte conditioned medium is more effective than defined cytokines in mediating the terminal maturation of human dendritic cells. *Blood* **90,** 3640–3646.
11. Sallusto, F., Cella, M., Danieli, C., and Lanzavecchia, A. (1995) Dendritic cells use macropinocytosis and the mannose receptor to concentrate macromolecules in the major histocompatibility complex class II compartment: downregulation by cytokines and bacterial products. *J. Exp. Med.* **182,** 389–400.
12. Ridge, J. P., Di Rosa, F., and Matzinger, P. (1998) A conditioned dendritic cell can be a temporal bridge between a CD4+ T-helper and a T-killer cell. *Nature* **393,** 474–478.
13. Bennett, S. R., Carbone, F. R., Karamalis, F., Flavell, R. A., Miller, J. F., and Heath, W. R. (1998) Help for cytotoxic-T-cell responses is mediated by CD40 signalling. *Nature* **393,** 478–480.
14. Schoenberger, S. P., Toes, R. E., van der Voort, E. I., Offringa, R., and Melief, C. J. (1998) T-cell help for cytotoxic T lymphocytes is mediated by CD40–CD40L interactions. *Nature* **393,** 480–483.
15. Kalinski, P., Hilkens, C. M., Snijders, A., Snijdewint, F. G., and Kapsenberg, M. L. (1997) Dendritic cells, obtained from peripheral blood precursors in the presence of PGE2, promote Th2 responses. *Adv. Exp. Med. Biol.* **417,** 363–367.

VI

Aspects of Dendritic Cell Function

27

Chemotaxis of In Vitro Cultured Human Dendritic Cells

Walter Luini, Giovanna D'Amico, Sergio Bernasconi, Paola Allavena, Alberto Mantovani, and Silvano Sozzani

1. Introduction

Chemotaxis is defined as the directional locomotion of cells sensing a gradient of the stimulus. Some cell types, such as monocytes and neutrophils, can be considered as "professional migrants" and for many years the study of chemotaxis has been applied to these cells. However, other cell types including fibroblasts, melanoma cells, keratinocytes, and vascular endothelial cells exhibit directional locomotion in vitro.

Migration from the blood to nonlymphoid tissues and then to lymph nodes or spleen through afferent lymph or blood is pivotal for dendritic cells (DC) to accomplish the function of professional antigen-presenting cells ***(1,2)***. However, the study of chemotactic response of these in vivo professional migrants has been impaired for a long time due to difficulties in obtaining a large enough number of cells for in vitro studies.

Following the development of culture conditions that enable the in vitro generation of DC from blood precursors ***(3)***, we have shown that monocyte-derived DC (mono-DC) and $CD34^+$ cell-derived DC (CD34-DC) undergo directional migration. This migration occurs in response to formylated peptides (fMLP), lipids (platelet activating factor, PAF), products of the complement cascade (C5a), and a number of CC chemokines ***(4–6)***. Chemokines are a growing superfamily of low molecular weight chemotactic proteins that can be divided into four groups according to the position of the first cysteine pair (CXC or α and CC or β families), the lack of two of the four cysteines (C or γ), or the presence of three spacing amino acids in the first cysteine tandem (CX3C

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or δ) ***(7–9)***. With the exception of SDF-1 (CXC), only CC chemokines, such as MIP-1α, MIP-1β, MIP-5, and MCP-3, are active on in vitro generated-DC ***(4,5,10,11)***. Accordingly, DC express CXCR4, the SDF-1 receptor, CCR1, and CCR5 ***(5)***, and the PAF receptor ***(6)***. Only CD34-DC, but not mono-DC, express CCR6 and migrate to MIP-3α, its ligand ***(10)***.

Chemokines active in chemotaxis assays are also functional in inducing endothelial cell transmigration of DC ***(12)*** (*see* Chapter 29). Very recently, it was found that chemokine receptor expression in DC is modulated during DC maturation with down-regulation of CCR1 and CCR5, two receptors for inducible chemokines, and up-regulation of CCR7, the receptor for MIP-3β and SLC, two chemokines constitutively produced in lymphoid tissues ***(13)***. Altogether, these results indicate that chemoattractants are likely to contribute to localization and trafficking of DC and could provide tools to recruit these cells in the design of immunization strategies.

Two different methods can been used to evaluate DC migration in vitro: chemotaxis across polycarbonate filters using either microwell Boyden chambers or Transwells. Both approaches provide reliable results; the only difference is that the Boyden chambers allow work with smaller volumes of chemoattractants and a lower number of DC than the Transwell system.

2. Materials

2.1. Culture of Dendritic Cells from Monocytes with GM-CSF and IL-13

1. Buffy coat of blood donations or 50 mL of blood.
2. Ficoll from Biochrom KG (Berlin, Germany).
3. Percoll from Pharmacia Fine Chemical (Uppsala, Sweden).
4. RPMI 1640 medium from Biochrom KG screened for being endotoxin-free.
5. Fetal calf serum (FCS) from Hyclone (Logan, UT) screened for being endotoxin and *Mycoplasma* free.
6. Granulocyte macrophage-colony stimulating factor (GM-CSF) (Sandoz, Basel, CH) and IL-13, Sanofi (Labege, France).
7. Complete RPMI 1640 medium for DC culture: RPMI 1640 supplemented with 10% FCS, 50 ng/mL GM-CSF, and 10 ng/mL IL-13.

2.2. Chemotaxis Assay in 48-Well Boyden Chambers

1. Micro 48-well Boyden chamber (Neuroprobe, Pleasanton, CA).
2. Humidified 5% CO_2 37° C incubator.
3. 5 μm polycarbonate filters (Neuroprobe).
4. Glass slides.
5. RPMI 1640 medium containing 0.2% BSA or 1% FCS.
6. Monocyte-derived DC (or $CD34^+$ cell-derived DC).
7. Diff-Quik (Harleco).

8. Standard chemoattractants: Formyl-methionyl-leucyl-phenylalanine (fMLP) (Sigma, St. Louis, MO) and chemokines. Chemokines can be obtained from several companies, such as PeproTech (Rocky Hill, NJ) or R&D Systems Inc. (Minneapolis, MN).

2.3. Chemotaxis Assay in Tranwell Culture Inserts

1. Transwell culture inserts (5 μm pore size; Corning, Costar, Cambridge, MA).
2. Humidified 5% CO_2 37°C incubator.
3. RPMI 1640 medium containing 0.2% BSA, or 1% FCS.
4. Monocyte-derived DC (or $CD34^+$ cell-derived DC).
5. $Na_2{}^{51}CrO_4$ (37 MBq; 1 mCi) from Amersham (Buckinghamshire, UK).
6. Standard chemoattractants (*see* **Subheading 2.2., item 8**).

3. Methods

3.1. Culture of DC

1. Prepare highly enriched blood monocytes by Ficoll and Percoll gradients ***(12)*** or by using methods described elsewhere in this volume.
2. Culture monocytes for 7 d at 1×10^6/mL in six-well multiwell tissue-culture plates in complete RPMI 1640.
3. At d 5, replace half of the medium with fresh RPMI medium containing GM-CSF but no IL-13 (*see* **Note 1**).
4. At d 7, collect DC and resuspend at $1–1.5 \times 10^6$/mL in RPMI containing 1% FCS (*see* **Note 2**).

3.2. Chemotaxis Assay in 48-Well Boyden Chamber

1. Aliquot 25 μL of chemoattractant into each lower well (*see* **Note 3**). The 25 μL vol may have some variations (2–3 μL more or less), depending on the microchamber used. It is advisable to calibrate in advance the lower wells, so that having seeded the chemoattractant, the liquid in the lower well forms a small convex surface that guarantees a perfect adhesion of the filter avoiding air bubble formation.
2. Put the filter (25 × 80mm) on the lower compartment. To avoid confusion about the order of the experimental groups in the same filter, it is suggested that a small angle be cut off the filter to allow it to be orientated.
3. Mount the silicon gasket and cover piece. Press the cover piece tightly to avoid air bubbles.
4. Seed 50 μL cell suspension (1.5×10^6/mL cells) in the upper well by leaning the pipet tip on the border of the well and quickly ejecting the cell suspension.
5. Incubate the chamber at 37°C in 5% CO_2 for 1.5 h.
6. Unscrew and turn over the chamber. Hold the upper compartment tightly and remove the lower compartment, keeping the silicon trimming and the filter adhered to the upper compartment of the chamber. At this point the migrated cells are on the upper surface of the filter.

7. Lift the filter and hold it with a clamp on each end (the clamps are purchased from the manufacturer of the chamber: Neuroprobe).
8. Wash the opaque side of the filter, where the non migrated cells remain, by passing this side over PBS. Do not immerse entire filter in PBS or the migrated cells will be lost.
9. Hold the filter with one of the clamps and clean the opaque side by scraping the filter against a special rubber policeman (purchased from the manufacturer) to remove all nonmigrated cells.
10. Stain the filter with Diff-Quik.
11. Read the results with a microscope at X100 magnification. Usually the average number of cells present in 5 high power fields is used to express the number of migrated cells ***(4)*** *see* **Notes 4** and **5**).

3.3. DC Transmigration through Transwell Membrane Inserts

1. At d 7, collect DC and resuspend in RPMI containing 10% FCS at 2–4 × 10^7/mL and label with of $Na_2{}^{51}CrO_4$ (100 μCi for 2–4 × 10^7 cells), for 1 h at 37°C.
2. Add to the lower well of the Transwell insert RPMI 1640 medium containing 0.2% BSA, or 1% FCS and an optimal concentration of a chemotactic factor.
3. Layer 0.1 mL of radiolabeled DC (1 × 10^6/mL) onto the upper part of the Transwell insert and incubate at 37°C for 90 min.
4. After incubation, gently mix the medium on the top of the cell culture insert in order to resuspend the nonadherent DC. Collect medium and floating cells, and gently wash with 0.1 mL of medium.
5. Scrape the bottom of the filter and count together with the medium of the lower compartment: this represents the fraction of migrated DC.
6. Determine the total radioactivity in a 0.1 mL sample of radiolabeled DC and, as the cell concentration is known, chemotaxis can be expressed as the percentage of input cells that have migrated (*see* **Note 6**).

4. Notes

4.1. Dendritic Cells

1. Differentiation of DC from monocytes can be successfully achieved by using IL-13 instead of IL-4 ***(14)***.
2. The purity of monocyte-derived DC can be checked by expression of characteristic surface markers on most cells (CD1a, high levels of MHC II, CD80, and CD86, mannose receptor).

4.2. Chemotaxis Assay in 48-Well Boyden Chamber

3. It is very important to be sure that all reagents used are endotoxin-free. Endotoxin and other bacterial products are able to cause a very fast (15 min) inhibition (approx 80%) of chemotaxis to most of the chemokines. At longer timest (1–2 h) endotoxin also induces loss of membrane receptors and decreases mRNA for chemokine receptors ***(15)***.

4. The responsivity of DC to chemotactic factors is dependent on the stage of cell maturation. DC prepared as described in this chapter have an immature phenotype and migrate to a large panel of CC chemokines and classical chemotactic factors (fMLP and C5a). Following maturation DC lose their ability to respond to these agonists and strongly up-regulate the chemotactic response to MIP-3β ***(13)***.
5. Sometimes DC are somewhat sticky and they tend to strongly adhere to the upper part of the filter (in an agonist-dependent way) with only a few of them going through the filter.

4.3. DC Transmigration through Transwell Membrane Inserts

6. This assay works very well but requires the use of larger volumes of chemoattractants and the use of radiolabeled cells.

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References

1. Hart, D. N. J. (1997) Dendritic cells: unique leukocyte populations which control the primary immune response. *Blood* **90,** 3245–3286.
2. Banchereau, J. and Steinman, R. M. (1998) Dendritic cells and the control of immunity. *Nature* **392,** 245–252.
3. Sallusto, F. and Lanzavecchia, A. (1994) Efficient Presentation of Soluble Antigen by Cultured Human Dendritic Cells Is Maintained by Granulocyte/Macrophage Colony-Stimulating Factor Plus Interleukin-4 and Downregulated by Tumor Necrosis Factor-alpha. *J. Exp. Med.* **179,** 1109–1118.
4. Sozzani, S., Sallusto, F., Luini, W., Zhou, D., Piemonti, L., Allavena, P., Van Damme, J., Valitutti, S., Lanzavecchia, A., and Mantovani, A. (1995) Migration of dendritic cells in response to formyl peptides, C5a and a distinct set of chemokines. *J. Immunol.* **155,** 3292–3295.
5. Sozzani, S., Luini, W., Borsatti, A., Polentarutti, N., Zhou, D., Piemonti, L., D'Amico, G., Power, C. A., Wells, T. N., Gobbi, M., Allavena, P., and Mantovani, A. (1997) Receptor expression and responsiveness of human dendritic cells to a defined set of CC and CXC chemokines. *J. Immunol.* **159,** 1993–2000.
6. Sozzani, S., Longoni, D., Bonecchi, R., Luini, W., Bersani, L., D'Amico, G., Borsatti, A., Bussolino, F., Allavena, P., and Mantovani, A. (1997) Human monocyte-derived and CD34+ cell-derived dendritic cells express functional receptors for platelet activating factor. *FEBS Lett.* **418,** 98–100.
7. Rollins, B. J. (1997) Chemokines. *Blood* **9,** 909–928.
8. Baggiolini, M. (1998) Chemokines and leukocyte traffic. *Nature* **392,** 565–568.
9. Mantovani, A., Allavena, P., Vecchi, A., and Sozzani, S. (1998) Chemokines and chemokine receptors during activation and deactivation of monocytes and den-

dritic cells and in amplification of Th1 versus Th2 responses. *Int. J. Clin. Lab. Res.* **28,** 77–82.

10. Power, C. A., Church, D. J., Meyer, A., Alouani, S., Proudfoot, A. E. I., Clark-Lewis, I., Sozzani, S., Mantovani, A., and Wells, T. N. C. (1997) Cloning and characterization of a specific receptor for the novel CC chemokine MIP-3 alpha from lung dendritic cells. *J. Exp. Med.* **186,** 825–835.
11. Godiska, R., Chantry, D., Raport, C. J., Sozzani, S., Allavena, P., Leviten, D., Mantovani, A., and Gray, P. W. (1997) Human macrophage derived chemokine (MDC) a novel chemoattractant for monocytes, monocyte derived dendritic cells, and natural killer cells. *J. Exp. Med.* **185,** 1595–1604.
12. D'Amico, G., Bianchi, G., Bernasconi, S., Bersani, L., Piemonti, L., Sozzani, S., Mantovani, A., and Allavena, P. (1998) Adhesion, transendothelial migration and reverse transmigration of in vitro cultured dendritic cells. *Blood* **92,** 207–214.
13. Sozzani, S., Allavena, P., D'Amico, G., Luini, W., Bianchi, G., Kataura, M., Imai, T., Yoshie, O., Bonecchi, R., and Mantovani, A. (1998) Differential regulation of chemokine receptors during dendritic cell maturation: a model for their trafficking properties. *J. Immunol.* **161,** 1083–1086.
14. Piemonti, L., Bernasconi, S., Luini, W., Trobonjaca, Z., Minty, A., Allavena, P., and Mantovani, A. (1995) IL-13 supports differentiation of dendritic cells from circulating precursors in concert with GM-CSF. *Eur. Cytokine Netw.* **6,** 245–252.
15. Sica, A., Saccani, A., Borsatti, A., Power, C. A., Wells, T. N. C., Luini, W., Polentarutti, N., Sozzani, S., and Mantovani, A. (1997) Bacterial lipopolysaccharide rapidly inhibits expression of C-C chemokine receptors in human monocytes. *J. Exp. Med.* **185,** 969–974.

28

Adhesion of Dendritic Cells to Endothelia

K. Alun Brown

1. Introduction

Many of the interdigitating dendritic cells (DC) that reside in lymph nodes arise from the migration of tissue interstitial DC such as Langerhans cells in the skin *(1)*. Although this migration appears to be stimulated by cytokines *(2)*, relatively little is known of the mechanisms underlying the maintenance and expansion of DC in the skin. Langerhans cells are bone-marrow derived *(3)*, and their replacement in the epidermis following transportation of antigen to lymphoid tissue is likely to depend upon the tissue extravasation of circulating DC. Moreover, the continuous passage of DC across blood vessel walls could be responsible for the increase in DC numbers in tumors *(4)* and sites of chronic inflammation *(5,6)*. Thus, germane to both homeostasis and pathological disturbance would be the interaction of circulating DC with blood vessel walls, and their subsequent entry into the surrounding tissue. The first stage in leukocyte migration across blood vessel walls is binding to vascular endothelium, and for lymphocytes, monocytes and neutrophils this event is governed by adhesion molecules on their surface recognizing corresponding endothelial ligands commonly referred to as vascular adhesion molecules *(7)*. Despite the plethora of information concerning the molecular nature of the attachment of the major leukocyte subpopulations to endothelium, relatively few studies have been undertaken with DC. Understanding the controlling features of DC–endothelial cell interaction would be relevant to the clinical application of DC in immunodeficient disorders and malignancies *(8,9)* and to antagonizing their entry into sites of chronic inflammatory lesions.

Within the circulation, DC exist in two forms; one that is functional in that cells of this lineage present antigen to naive and memory T cells *(10)*, hereafter referred to as the mature DC cells, and the other is the $CD34^+$ progenitor

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(11,12). When the latter cells are cultured with cytokines such as granulocyte-macrophage colony-stimulating factor (GM-CSF) and TNFα, they differentiate into mature DC, which express CD11c, CD13, and CD33. Among the mature DC in peripheral blood, two subpopulations may be identified, both of which are devoid of the Langerhans cell marker CD1a and only one of which expresses CD11c, CD13, and CD33. To date, only the mature populations have been investigated for their ability to bind to endothelium *(15)*. Of the known leukocyte adhesion molecules the β2 integrin family (CD11a-c/CD18), CD44, and CD54 (ICAM-1) are expressed on the majority of mature DC, the β1 integrin family (CD49a-d/CD29) on less than half of the population, and CD62L (L-selectin) on a small proportion of the cells *(15)*. There is currently sparse information regarding the distribution and expression of leukocyte adhesion molecules on DC progenitors and their differentiated forms.

Further evidence of heterogeneic forms of circulating DC comes from the demonstration that blood monocytes differentiate into DC when cultured with the appropriate cytokines *(16,17)*. Differences in the adhesion molecule phenotype of circulating DC could be indicative of a distinct tissue migratory pathway. For example, some forms of blood DC may specifically "home" to certain areas of the body as illustrated by the Langerhans cell progenitor bearing the skin-homing molecule CLA, which binds to E-selectin and which is expressed on activated endothelium *(18)*. Examining the binding characteristics of DC of different lineages to endothelium derived from a variety of tissues could lead to the identification of molecules responsible for the organ-specific trafficking of DC, particularly when complemented by blocking studies with antibodies and synthetic peptides of known adhesion molecules.

The following method has successfully been applied to the measurement of the adherence properties of DC to cultured endothelial cells derived from human umbilical cord veins (*see* **Fig. 1**). It is likely that the system will also be suited to monitoring the adhesion of DC differentiated from cytokine-activated progenitors because of the large numbers of cells that will be available for experiment.

2. Materials

1. Culture medium: standard RPMI 1640 culture medium (with sodium bicarbonate) (Gibco-BRL, Paisley, UK) is supplemented with 4 m*M* L-glutamine, 2 m*M* sodium pyruvate (both Gibco-BRL), 200 U/mL penicillin (Britannia Pharmaceuticals, Redhill, UK) 100 U/mL streptomycin (Sigma Chemical Co., Poole, UK), and 100 U/mL gentamycin (Roussell, Uxbridge, UK). This medium is referred to as "RPMI culture medium." Heat-inactivated (56°C, 30 min) fetal calf serum (FCS, Gibco) is added to this basic formulation where indicated.
2. Preservative-free heparin (Leo Laboratories, UK).
3. Collagenase solution (Class II, Sigma).
4. Gelatin (Sigma).

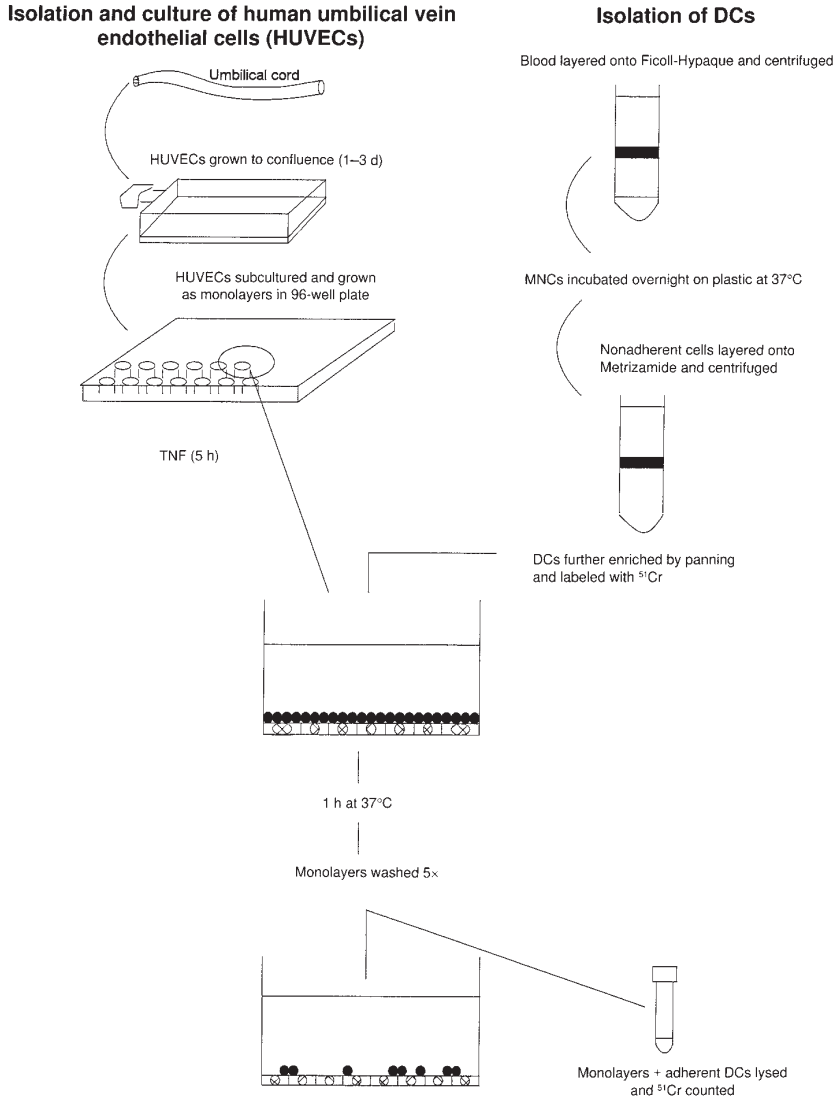

Fig. 1. Schematic representation of the adherence assay in which isolated DC bind to cultured endothelial cells.

5. Trypsin–EDTA solution for endothelial cell cultures (IX) (Sigma).
6. 25 cm^3 tissue culture flasks (Falcon Co., Cockeysville, MD).
7. Flat-bottomed 96-well microtiter plates (Costar, Cambridge, MA).
8. Multispot glass microscope slides (Hendley, UK).
9. Formaldehyde (BDH Chemicals Ltd., Poole, UK).
10. Saparin (Sigma).
11. Antifactor VII related antigen antibodies (Nordic Laboratories, London, UK).
12. Phosphate-buffered saline (PBS). A 10X normal strength stock solution is prepared by dissolving in 5 L of double-distilled water, 400 g NaC1, 10 g KC1, 10 g KH_2PO_4, and 57.5g Na_2HPO_4 (all BDH). This stock solution is diluted 1:10 with double-distilled water and the pH adjusted to 7.4 prior to use.
13. Ficoll-Paque (Pharmacia, Uppsala, Sweden).
14. Metrizamide (Nyegaard, Oslo, Norway).
15. Flasks coated with anti-mouse IgG (Laboratory Technology International, Sussex, UK).
16. $Na_2{}^{51}CrO_4$ (Amersham International PLC, Amersham, UK).

3. Methods

3.1. Human Umbilical Vein Endothelial Cells (HUVEC)

1. Collect umbilical cords into 0.9% saline.
2. Wipe the surface of each cord clean with 75% alcohol and discard approximately 2 cm of each end of the vein.
3. Cannulate each vein with a blunt 16-G needle that is secured with sutures.
4. Wash each vein with RPMI culture medium containing 10 U/mL preservative-free heparin to remove residual blood. Fill each vein with 200 U/mL collagenase in RPMI medium, and incubate for 10 min at 37°C.
5. Gently knead the umbilical cord to loosen the endothelium.
6. Aspirate the collagenase solution and then wash the vein out with RPMI containing 10% FCS.
7. Pool the collagenase solution and washings and centrifuge at 400*g* for 10 min.
8. Resuspend the endothelial cell pellet (*see* **Note 1**) in 5 mL of RPMI culture medium supplemented with 20% FCS. Transfer the cells to a 25 cm^2 culture flask, the floor of which has previously been treated with 1% (w/v) gelatin and incubate in a 10% CO_2 humidified atmosphere at 37°C.
9. When HUVEC have grown to form a confluent monolayer (**Fig. 2**) expel the medium and wash the monolayer with RPMI medium and then treat with 1 mL of a mixture of 0.05% trypsin and 0.025% EDTA.
10. After dislodging the endothelial cells arrest the enzyme digestion by adding 5 mL of RPMI with 20% FCS.
11. Resuspend the harvested endothelial cells, (usually $1–2 \times 10^6$), in 20 mL RPMI/20% FCS and add 20 µL to each well of a 1% gelatin-coated 96-flat bottomed well microtiter plate.
12. Culture at 37°C in a humidified atmosphere enriched with 10% CO_2. Confluent HUVEC monolayers are obtained after 3–5 d (*see* **Notes 2** and **3**).

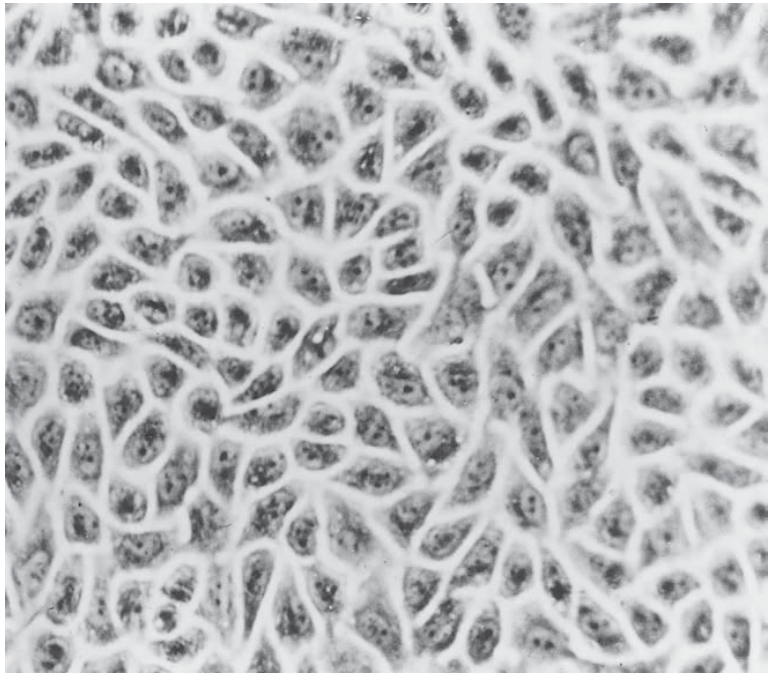

Fig. 2. Typical cobblestone morphology of endothelial cells when grown to confluence in culture. The cells form tight junctions that will not allow the passage of liquid through the monolayer.

3.2. Isolation of Blood Dendritic Cells

1. Defibrinate 70 mL of peripheral blood by shaking with glass beads.
2. Dilute the blood with an equal volume of RPMI and layer over Ficoll-Paque at a ratio of 3 vol Ficoll-Paque to 4 vol of diluted blood.
3. Centrifuge at 400*g* for 30 min and then harvest the mononuclear cells that equilibrate at the upper Ficoll-Paque/blood interface. Wash the cells twice in RPMI/10% FCS and culture overnight at a concentration of 5×10^6 cells/mL in 25 cm^3 tissue culture flasks in complete medium (RPMI 1640 with 100 U penicillin, 100 µg/mL streptomycin, and 10% FCS).
4. Harvest the nonadherent cells and layer over metrizamide (13.7% w/v). Centrifuge at 600*g* for 10 min.
5. Harvest the low-density cells (which are enriched for DC) from the interface and wash twice in medium.
6. Incubate the cells on human IgG-coated plates for 1 h at room temperature so that contaminating Fc receptor-positive monocytes bind to the plate. Aspirate the nonadherent cells.

7. The DC may be further enriched by removal of contaminating T cells, monocytes, B cells, and NK cells. Incubate the cells with antibodies directed against CD3 (T cells), CD14 (monocytes), CD19 (B cells), and CD56 (natural killer [NK] cells). Wash the cells twice in RPMI medium and then pan over flasks coated with anti-mouse IgG for 1 h. Harvest the nonadherent, DC enriched cells (*see* **Note 4**).

3.3. Radiolabeling of DC

1. Resuspend DC in 200 μL Dulbecco's modified Eagle's medium (DMEM) and incubate with 3 μCi (0.1 MBq) of $Na_2\ ^{51}Cr\ O_4/10^6$ for 1 h at 37°C.
2. Wash the cells three times by centrifugation for 10 min at 400*g* through 10 mL DMEM plus 10% FCS (culture media) to remove unincorporated radiolabel.
3. Resuspend the radiolabeled cells at 1×10^6 cells/mL in culture media.

3.4. Adherence Assay

1. Dispense 100 μL aliquots of the radiolabeled DC onto washed endothelial cell monolayers in quadruplicate wells.
2. Incubate each plate for 1 h at 37°C in 10% CO_2.
3. At the end of this period, carefully aspirate the DC remaining in suspension from each well by means of a water vacuum pump. Remove the nonadherent and loosely attached DC by gently washing the endothelial monolayers five times with warm tissue-culture medium (200 mL/well). The adherent DC will remain attached to the HUVEC (**Fig. 3**).
4. Osmotically disrupt the endothelial monolayers with bound DC by adding 200 μL of 0.1 *M* NaOH.
5. Measure the radioactivity of the lysate, (expressed as counts per minute [cpm]), with an automatic gamma counter set to detect emissions for ^{51}Cr.
6. Calculate the mean cpm of radiolysate collected from quadruplicate wells together with mean cpm from 4 × 100 μL aliquots of the radiolabeled DC suspension.
7. Express dendritic cell adhesion in terms of the percentage of DC originally dispensed onto endothelial monolayers by the following formula (*see* **Notes 5** and **6**):

$$\%\ \text{adhesion} = \frac{\text{mean cpm of monolayer with adherent DC} - \text{cpm background} \times 100}{\text{mean cpm of 100 μL radiolabeled DC} - \text{cpm background}}$$

3.5. Adherence of DC to Cytokine-Treated Endothelial Monolayers

Leukocyte entry into sites of infection/inflammation is dependent, in part, on receptors on the cell surface recognizing ligands (vascular adhesion molecules) on the endothelial surface whose expression is either up-regulated (e.g., ICAM-1) induced (e.g. VCAM-1) by inflammatory cytokines (e.g., IL-1, TNFα) generated by tissue insult. These conditions are artificially created in the laboratory by pre-treating endothelial cell monolayers with recombinant cytokines ***(19)***. It is likely that the entry of blood DC into inflammatory sites is also governed by the activity of inflammatory cytokines and, therefore, the

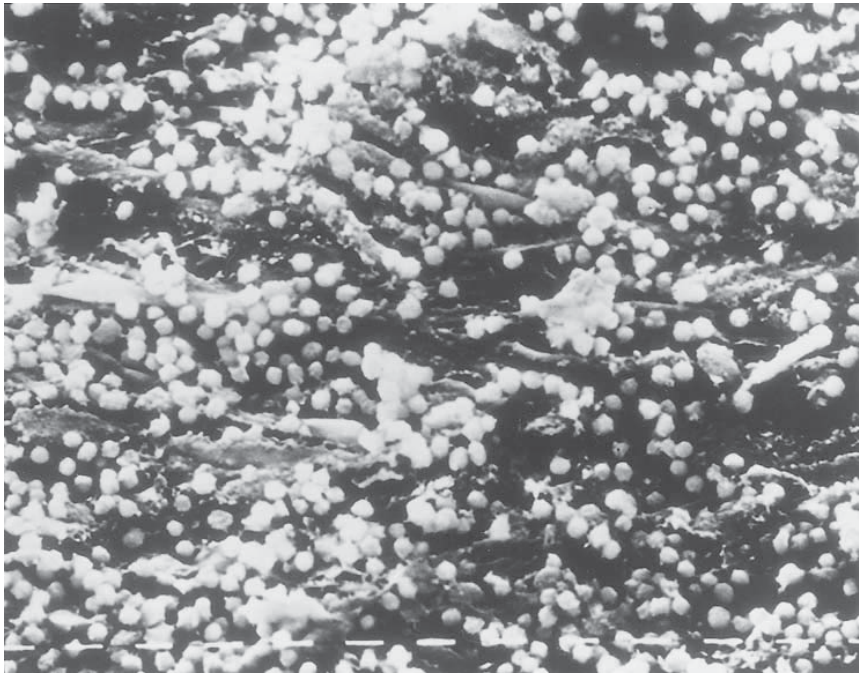

Fig. 3. Scanning electron microscopy of DC adhering to cultured endothelium. Occasionally, the DC bury themselves between neighboring endothelial cells and they become integrated into the monolayer.

following protocol is a useful model for identifying factors controlling the binding of DC to "inflamed" endothelium.

1. Pretreat confluent monolayers of endothelial cells grown in 96-well plates with recombinant cytokines (e.g., IL-1, TNFα) for 4–24 h over the concentration range 0.1–100 U/mL.
2. Dilute all cytokines in RPMI/10% FCS and control wells receive 200 μL of the diluent medium.
3. At the end of each incubation aspirate the media wash the monolayers once with 200 μL of RPMI plus 10% FCS.
4. Visually inspect all treated monolayers prior to the adherence assay to ensure that incubation with cytokine does not damage or disrupt the endothelial cells.
5. The enhanced attachment of DC to cytokine-treated endothelial cells is calculated as a percentage of the binding to untreated monolayers.

3.7. Contribution of Leukocyte Adhesion Molecules

The format outlined below is known to be applicable to assessing the role of CD11a and VLA-4, but for other potential adhesion molecules preliminary experiments need to be performed to ascertain the duration of incubation and concentration of antibody required to produce maximum inhibition of DC adhesion.

1. Suspend radiolabeled DC in culture media (1×10^6/mL) for 45 min at room temperature with anti-CD11a or anti-VLA-4 monoclonal antibodies (equivalent to 25 μg/10^6 cells).
2. Incubate control aliquots of DC with irrelevant antibodies whose concentration and subclass are the same as those of the test antibodies.
3. The cells are not washed prior to their introduction onto untreated or cytokine-treated endothelial monolayers.

4. Notes

1. Typically, 1.0 to 5.0×10^6 viable cells should be obtained from each umbilical cord.
2. Endothelial cells from other sources may be substituted for HUVEC in the adherence assay. Microvascular endothelial cells have been isolated and cultured successfully from a number of tissues, including skin ***(20)*** and hybrid endothelial cell lines are available for study ***(19)***.
3. Endothelial cells are identified by their morphology and staining by antibodies against factor VIII-related antigen. On attainment of confluence, HUVEC possess a characteristic cobblestone morphology (*see* **Fig. 2**). To confirm the purity of the HUVEC, the HUVEC are grown to confluence on gelatin-coated multispot glass microscope slides and fixed in 3.5% formaldehyde/PBS for 15 min at room temperature. The cells are washed, permeabilized with 0.2% saparin in PBS for 15 min, and incubated with monoclonal antibodies to factor VIII-related antigen for 30 min in a humid chamber at room temperature. Following washing with PBS, the monolayers are incubated with FITC-conjugated goat anti-mouse antibodies for 30 min. Washed slides are mounted with glycerol/PBS and viewed with a fluorescence microscope. The number of cells stained for factor VIII-related antigen is expressed as a percentage of the total number of cells. By the above criteria > 98% of cultured cells are deemed to be endothelial cells.
4. DC prepared in this way have a purity of 76%. Viability of enriched cells is > 99% as judged by trypan blue exclusion, and the cell yield will be approx 2×10^6 cells. Ultrastructural analysis reveals that they possess the characteristic morphology of DC, in that there are few cytoplasmic organelles, several cytoplasmic projections that range from a blunt to a veiled appearance, and the nucleus is irregularly shaped or oval. The enriched DC preparations stimulate an allogeneic mixed leukocyte reaction (MLR) and are more portent than monocytes in presenting recall antigens, purified protein derivative (PPD) influenza virus, and tetanus toxoid to lymphocytes.
5. The adherence assay described in the present report measures the leukocyte attachment to endothelial cells under static conditions, and this interaction is mainly

dependent on integrin recognition of endothelial ligands such as ICAM-1 and VCAM-1 ***(7)***. Selectins expressed on leukocytes and endothelial cells promote this initial contact of these cell types with one another, which involves the rolling of leukocytes along the endothelial surface ***(21)***. This primary adhesion event may be monitored by measuring leukocyte rolling along endothelial monolayers during flow rates and wall shear stress in physiological relevant ranges ***(22)***. Because relatively large numbers of leukocytes are required for this technique, it is not really amenable to the study of "mature" DC isolated from blood. Moreover, the problem is compounded by the need for highly purified preparations of DC, because cells that make contact with endothelium are identified by videomicroscopy. This difficulty could be obviated by the study of cytokine-differentiated CD34-positive progenitors, which would provide a plentiful supply of highly enriched DC. The inclusion of blocking studies with relevant antibodies and synthetic peptides of selectins and their receptors in this assay would provide much needed information concerning the mechanisms by which DC adhere to endothelial cells under flow conditions.

6. Monolayers of endothelial cells are not always necessary for studying the binding of isolated DC to endothelial cells. Stamper and Woodruff ***(23)*** established a technique that permitted the study of lymphocyte attachment to endothelial cells within blood vessel walls exposed in frozen sections of rat lymphoid tissue. Overlaid lymphocytes were shown to adhere preferentially to high endothelial venules, an observation that made a valuable contribution to unravelling the pathways by which circulating lymphocytes enter the extravascular compartment of peripheral lymph nodes. More recently, the "frozen section assay" has been applied to the study of lymphocyte binding to blood vessel walls in human skin ***(24)***, synovium ***(25)***, and brain ***(26)***. It is thought that the inclusion of DC in the frozen section assay will advance the understanding of factors governing the tissue localization of circulating DC. With the resurgent interest in the biology of DC and in their heterogeneous forms in blood, it is to be anticipated that the study of the binding of DC to endothelial cells will prove to be a rewarding and worthwhile pursuit.

References

1. Steinman, R., Pack, M., and Duaba, K. (1997) Dendritic cells in the T cell areas of lymphoid organs. *Immunological Reviews* **156**, 25–37.
2. Roake, J. A., Rao, A. S., Morris, P. J., Larsen, C. P., Hankins, D. F., Morris, P. J., and Austyn, J. J. (1995) Dendritic cell loss from nonlymphoid tissues after systemic administration of lipopolysaccharide, tumour necrosis factor and interleukin-1. *J. Exp. Med.* **181**, 2237–2248.
3. Katz, S. I., Tamaki, K., and Sachs, D. (1979) Epidermal Langerhans cells are derived from cells originating in bone marrow. *Nature* **282**, 324–327.
4. Chaux, P., Hammann, A., Martin, F. et al. (1993) Surface phenotype and functions of tumor-infiltrating dendritic cells: CD8 expression by a cell population. *Eur. J. Immunol.* **23**, 2517–2525.

5. Knight, S. C. (1988) Dendritic cells in inflammatory joint disease. In *Imunopathogenetic mechanisms of arthritis* (Goodacre, J. and Carson, D. W., eds.), MTP Press Ltd., Lancaster, p. 69.
6. Thomas, R., Davis, L. S., and Lipsky, P. E. (1994) Rheumatoid synovium is enriched in mature antigen-presenting dendritic cells. *J. Immunol.* **153**, 2613.
7. Bevilacqua, M. P. (1993) Endothelial-leukocyte adhesion molecules. *Ann. Rev. Immunol.* **11**, 767–804.
8. Girolomoni, G. and Ricciardi-Castagnole, P. R. (1997) Dendritic cells hold promise for immunotherapy. *Immunol. Today* **18**, 102–104.
9. Wong, J. (1997) Dendritic cells reach out to the clinic. *Nature Med.* **3**, 129.
10. Knight, S. C., Macatonia, S. E., Roberts, M. S., Harvey, J. J., and Patterson, S. (1993) Dendritic cells and antigen presentation. In *Viruses and the cellular immune response* (Thomas, D. B., ed.), Marcel Dekker, New York, pp. 49–74.
11. Caiux, C., Dezutter-Dambuyant, C., Schmitt, D. et al. (1992) GM-CSF and TNFα cooperate in the generation of dendritic Langerhans cells. *Nature* **360**, 258–261.
12. Reid, C., Stackpoole, A., Meager, A. et al. (1992) Interactions of tumor necrosis factor with granulocyte-macrophage colony-stimulating factor and other cytokines in the regulation fo dendritic cell growth *in vitro* and early bipotent CD34+ progenitors in human bone marrow. *J. Immunol.* **149**, 2681–2688.
13. O'Doherty, U., Peng, M., Gezelter, S. et al. (1994) Human blood contains two subsets of dendritic cells, one immunologically mature and the other immature. *Immunology* **82**, 487–493.
14. Thomas, R., and Lipsky, P. E. (1994) Human peripheral blood dendritic cell subsets. Isolation and characterisation of precursor and mature antigen-presenting cells. *J. Immunol.* **153**, 4016–4028.
15. Brown, K. A., Bedford, P., Macey, M., et al. (1997) Human blood dendritic cells: binding to vascular endothelium and expression of adhesion molecules. *Clin. Exp. Immunol.* **107**, 601–607.
16. Kasinrerk, W., Baumruker, T., Majdic, O., Knapp, W., and Stockinger, H. (1993) CD1 molecule expression on human monocytes induced by granulocyte-macrophage colony-stimulating factor. *J. Immunol.* **150**, 579–584.
17. Romani, N., Gruner, S., Brang, D., et al. (1994) Proliferating dendritic cell progenitors in human blood. *J. Exp. Med.* **180**, 83–93.
18. Strunk, D., Egger, C., Leitner, G., Hanau, D., and Stingl, G. (1997) A skin homing molecule defines the Langerhans cells progenitor in human peripheral blood. *J. Exp. Med.* **185**, 1131–1136.
19. Brown, K. A., Vora, A., Biggerstaff, J., et al. (1993) Application of an immortalized human endothelial cell line to the leukocyte-endothelial adherence assay. *J. Immunol. Methods* **6**, 13–22.
20. LeRoy, F., Brown, K. A., Vora, A. J., Greaves, M. W., Dowd, P. M., Slavin, B. M., and Dumonde, D. C. (1991) Blood mononuclear cells from patients with psoriasis exhibit an enhanced adherence to cultured vascular endothelium. *J. Invest. Dermatol.* **97,** 511–516.

21. Springer, T. A. (1994) Traffic signals for lymphocyte recirculation in leukocyte emigration: the multistep paradigm. *Cell* **76**, 301–314.
22. Cooke, B. M., Usami, S., Perry, I., and Nash, G. B. (1993) A simplified method for culture of endothelial cells and analysis of adhesion of blood cells under conditions of flow. *Microvas. Res.* **45**, 33–45.
23. Stamper, H. B. and Woodruff, J. J. (1977) An *in vitro* model of lymphocyte homing. 1. Characterization of the interaction between thoracic duct lymphocytes and specialized high-endothelial venules of lymph nodes. *J. Immunol.* **119**, 772–780.
24. Sackstein, R., Falanga,. V., Streilein, J. W., and Chin, Y-H. (1988) Lymphocyte adhesion to psoriatic dermal endothelium is mediated by a tissue-specific receptor/ligand interaction. *J. Invest. Dermatol.* **91**, 423–428.
25. Jalkanen, S., Steere, A. C., Fox, R. I., and Butcher, E. C. (1986) A distinct endothelial cell recognition system that controls lymphocyte traffic into inflamed synovium. *Science* **233,** 556–558.
26. Vora, A. J., Perry, M. E., Hobbs, C., Dumonde, D. C., and Brown, K. A. (1995) Selective binding of peripheral blood lymphocytes to the walls of cerebral vessels in frozen sections of human brain. *J. Immunol. Methods* **180**, 165–180.

29

Transendothelial Migration and Reverse Transmigration of In Vitro Cultured Human Dendritic Cells

Giancarlo Bianchi, Giovanna D'Amico, Silvano Sozzani, Alberto Mantovani, and Paola Allavena

1. Introduction

Dendritic cells (DC) are professional antigen-presenting cells (APC) that are believed to be indispensable to initiate a primary immune response ***(1,2)***. DC are migratory cells that exhibit complex trafficking properties in vivo, involving interaction with vascular and lymphatic endothelium and extracellular matrix (ECM). DC progenitors from the bone marrow enter the blood and seed nonlymphoid tissues. DC are spread all over the body, at epithelia (skin epidermis, airways, gastrointestinal, and genitourinary tracts) and at interstitial spaces of many solid organs (heart, liver, kidney), where they act as sentinels, waiting for an encounter with an antigen ***(1–3)***.

Some pathways of DC migration in vivo and recruitment of DC precursors from blood to tissues have been characterized. At epithelial lining surfaces, upon antigen capture, DC traffic to regional lymph nodes via lymphatics, and this process can be increased by locally produced inflammatory cytokines (e.g., IL-1 and TNF) and bacterial products (LPS). The same route is followed by DC present in the interstitium of organs such as heart and kidney; these cells, however, can also reach the spleen via the blood stream. A subset of DC present in the liver can capture circulating antigen and travel along the lymph/lymph nodes as well as the blood/spleen route ***(1,3)***.

Some molecular mechanisms of DC motility have recently been identified. DC migrate in vitro in response to chemotactic signals and to a unique spectrum of chemokines (*see* Chapter 27) including the CC molecules MCP-3, Rantes, MIP1α, MIP1β, MCP-4, MDC, and the C-X-C chemokine SDF-1 ***(4–7)***. The

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chemotactic response to chemokines suggests that these molecules may have a role in regulating the trafficking of DC in vivo.

The extravasation of leukocytes requires the adhesion and subsequent transmigration through the vascular endothelium. These processes are mediated by the interaction of different adhesion molecules on leukocytes with their respective endothelial ligands. In this chapter we will discuss the practical approaches for the in vitro study of DC transendothelial migration. The method reported here takes advantage of endothelial cell growth on porous polycarbonate membrane filters. Moreover, as DC have the ability to undergo the reverse process of migration from tissues into the lumen of blood (or lymphatic vessels), we established an assay to mimic the tissue-to-lymph/blood part of the natural history of DC (reverse transmigration). In this assay the upper filter (of a two-filter system) is coated with ECM, and the lower filter is coated by a monolayer of endothelial cells (EC), placed upside down.

2. Materials

2.1. Isolation and Culture of Endothelial Cells

1. Phosphate-buffered saline (PBS) with and without $Ca2^{+}$.
2. Endothelial Cell Growth Supplement (ECGS) from Collaborative Research (Lexington, MA). Dissolve ECGS in PBS (without $Ca2^{+}$) at 5 mg/mL.
3. Heparin sodium salt (grade I-A from porcine intestinal mucosa) from Sigma Chemical Co. (St. Louis, MO), dissolved in PBS (without $Ca2^{+}$) at 10 mg/mL.
4. Medium 199 (M199) and fetal bovine serum (FBS) from Gibco-Europe (Paisley, UK).
5. Complete M199 for endothelial cell culture onto membrane inserts: M199 supplemented with 20% FBS, ECGS, and heparin at the final concentrations of 0.05 and 0.1 mg/mL.
6. Fibronectin (from human plasma) from Sigma.
7. Tumor necrosis factor (TNF) Basf Knoll (Germany).
8. Transwell polycarbonate membrane inserts (6.5 mm diameter; 5.0 μm pore size) and PVP-free polycarbonate filters (13 mm diameter; 5.0 μm) from Costar (Cambridge, MA).
9. Cell incubator (37°C in a 5% CO_2 humidified atmosphere).
10. 6- and 24-well tissue-culture plates from Falcon (Becton Dickinson).
11. Modified Boyden chemotactic chambers (Neuroprobe).

2.2. Isolation and Culture of Dendritic Cells from Monocytes

1. Buffy coat of blood donations or 50 mL of blood.
2. Ficoll from Biochrom KG (Berlin, Germany).
3. Percoll from Pharmacia Fine Chemical (Uppsala, Sweden).
4. RPMI 1640 medium from Biochrom KG screened for being endotoxin-free.
5. FBS from Hyclone (Logan, UT) screened for being endotoxin and Mycoplasma free.

6. Granulocyte Macrophages-Colony Stimulating Factor (GM-CSF) (Novartis, Basel, CH) and IL-13, Sanofi (Labege, France).
7. Complete RPMI 1640 medium for DC: RPMI 1640 supplemented with 10% FBS together with GM-CSF and IL-13 respectively at the final concentrations of 50 and 10ng/mL.
8. $Na_2{}^{51}CrO_4$ (37 MBq; 1 mCi) from Amersham (Buckinghamshire, UK).

3. Methods

3.1. Culture of Endothelial Cells on Polycarbonate Membrane Inserts (Transwells) for Use in Transmigration Assay

1. Culture EC isolated from human umbilical vein as described elsewhere (*see* Chapter 28) ***(8)***. (*See* **Notes 1–3**.)
2. Coat Transwell culture inserts with 10 μg/mL of fibronectin (final vol 0.1) mL for 2 h at 37°C.
3. At the end of coating, wash membrane inserts with M199 (FBS-free).
4. Detach EC by brief exposure to trypsin (0.25%)-EDTA (0.022%) and then resuspend in complete M199 at a concentration of 4×10^5/mL.
5. Layer 0.1 mL EC suspension (4×10^5/mL) into each cell culture insert and place inserts into 24-well tissue culture plate filled with 0.6 mL of complete M199 in the lower chamber.
6. Culture until EC are confluent (usually after 3–4 d).

3.2. Culture of Endothelial Cells on PVP-Free Polycarbonate Filters for Use in Reverse Transmigration Assay

1. Coat PVP-free polycarbonate filters (5 μm pore) with gelatin (5 μg/mL) by boiling in distilled water for 1 h ***(9)***.
2. Wash filters with M199 (FBS- free) and place filters in 24-well plate.
3. Layer EC onto the filters at a concentration of 8–10 x 10^4/well, in a final vol of 1 mL. Two filters will be required per tube in the subsequent transmigration assay.
4. Culture until EC are confluent (usually after 3–4 d).

3.3. Culture of DC and Radiolabeling

1. Prepare highly enriched blood monocytes using Ficoll and Percoll gradients ***(8)*** or by following alternative protocols presented in this volume.
2. Culture monocytes for 7 d at 1×10^6/mL in six-well Multiwell tissue culture plates in complete RPMI 1640.
3. At d 5, replace half of the medium with fresh medium containing GM-CSF only (*see* **Notes 4** and **5**).
4. At d 7, collect DC and resuspend in RPMI containing 10% FBS at $2–4 \times 10^7$/mL. Label with $Na_2{}^{51}CrO_4$ (100 μCi for $2–4 \times 10^7$ cells), for 1 h at 37°C.
5. At the end of the incubation, wash the cells three times with 15 mL of PBS and then resuspend in RPMI + 10% FBS.

3.4. DC Transmigration through Transwell Membrane Inserts

1. Layer 0.1 mL of radiolabeled DC (7×10^5/mL) onto the EC monolayer and incubate for 60 min at 37°C (*see* **Notes 6** and **7**).
2. After incubation, gently mix the medium of the cell culture insert in order to resuspend the nonadherent DC. Collect medium and floating cells. Gently wash with 0.1 of medium. Both fractions are counted in a gamma counter and represent the amount of unattached DC.
3. Collect the intact endothelial cell monolayer together with bound DC using cotton buds: This is the fraction containing bound DC.
4. Scrape the bottom of the filter and count together with the medium of the lower compartment: This is the fraction of migrated DC.
5. Since adhesion to the EC monolayer is an early stage of transmigration, the total number of adherent DC is calculated by adding the fraction of bound DC to the fraction of transmigrated DC.
6. Determine the total radioactivity in a 0.1 mL sample of radiolabeled DC.

3.5. Reverse Transmigration in Modified Boyden Chamber

1. EC are subcultured to confluent monolayers on gelatin-precoated PVP-free polycarbonate filters (5 μm pore) in 24-well plates.
2. Treat half of the filters for 30 s with 20 m*M* NH_4O_4 containing 0.5% Triton X100, to strip away the EC monolayer and expose a natural ECM.
3. Mount the filters in the Boyden chambers: the lower compartment contains 0.2 mL of complete medium. Mount the filter coated with EC cell-side down. Add the second filter with the ECM facing up, and immediately cover with 0.15 mL of complete medium.
4. Seed 0.15 mL of radiolabeled DC (7×10^5/mL) in the upper compartment of the chamber and incubate at 37°C for 60 min.
5. After the incubation, gently resuspend the nonadherent DC. Collect the medium and floating cells. Gently wash with 0.3 mL of medium. Combine both fractions and count in a gamma counter. This represents the amount of unattached DC.
6. Collect the ECM-adherent DC and DC in the bottom of the EC-coated filter with cotton buds and count as the bound fraction.
7. Collect transmigrated cells in the lower compartment and count.
8. Calculate the proportion of migrating cells (*see* **Note 8**).

4. Notes

4.1. Endothelial Cells

1. The purity of EC culture may be checked by expression of von Willebrand factor. Positivity is usually > 99%.
2. Designated wells of EC cultures may be stimulated with 10–20 ng/mL of inflammatory cytokines (IL-1, TNF) in order to augment DC adhesion and therefore transmigration.
3. Expression of adhesion molecules on cytokine-activated EC depends on the time of exposure. Selectins are expressed within 2 h after stimulation and decline after

4 h. ICAM-1 and VCAM-1 are poorly expressed before 4 h and remain in plateau for 24–48 h.

4.2. Dendritic Cells

4. As IL-13 is inhibiting DC adhesion ***(8)***, at d 5 of differentiation IL-13 should be omitted from the culture medium, and only GM-CSF must be used.
5. DC can be further differentiated in vitro by exposure to inflammatory cytokines (IL-1, TNF) for 24–48 h. The stage of differentiation can be checked by expression of surface CD83, which increases with DC maturation.

4.3. Transmigration Assay

6. Before plating the assay, it is of major importance to check the integrity of the endothelial monolayer. Few spare filters (or membrane inserts) should be prepared and after 3–4 d are stained with Diff-Quick.
7. Spontaneous transmigration may be augmented by adding chemotactic factors in the lower compartment of the Transwell, at the time of leukocyte layering. fMLP, C5a, or chemokines may be used.

4.4. Reverse Transmigration Assay

8. As this double-filter system is much thicker than the Transwell insert, the response of DC to a chemotactic factor in the reverse transmigration assay is not as sensitive as in the direct transmigration.

Acknowledgments

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References

1. Hart, D. N. J. (1997) Dendritic cells: unique leukocyte populations which control the primary immune response. *Blood* **90,** 3245–3286.
2. Banchereau, J. and Steinman, R. M. (1998) Dendritic cells and the control of immunity. *Nature* **392,** 245–252.
3. Austyn, J. M. (1996) New insights into the mobilization and phagocytic activity of dendritic cells. *J. Exp. Med.* **183,** 1287–1292.
4. Sozzani, S., Sallusto, F., Luini, W., Zhou, D., Piemonti, L., Allavena, P., Van Damme, J., Valitutti, S., Lanzavecchia, A., and Mantovani, A. (1995) Migration of dendritic cells in response to formyl peptides, C5a and a distinct set of chemokines. *J. Immunol.* **155,** 3292–3295.
5. Xu, L. L., Warren, M. K., Rose, W. L., Gong, W. H., and Wang, J. M. (1996) Human recombinant monocyte chemotactic protein and other c- c chemokines bind and induce directional migration of dendritic cells in vitro. *J. Leukocyte Biol.* **60,** 365–371.

6. Sozzani, S., Luini, W., Borsatti, A., Polentarutti, N., Zhou, D., Piemonti, L., D'Amico, G., Power, C. A., Wells, T. N., Gobbi, M., Allavena, P., and Mantovani, A. (1997) Receptor expression and responsiveness of human dendritic cells to a defined set of CC and CXC chemokines. *J. Immunol.* **159,** 1993–2000.
7. Godiska, R., Chantry, D., Raport, C. J., Sozzani, S., Allavena, P., Leviten, D., Mantovani, A., and Gray, P. W. (1997) Human macrophage derived chemokine (MDC) a novel chemoattractant for monocytes, monocyte derived dendritic cells, and natural killer cells. *J. Exp. Med.* **185,** 1595–1604.
8. D'Amico, G., Bianchi, G., Bernasconi, S., Bersani, L., Piemonti, L., Sozzani, S., Mantovani, A., and Allavena, P. (1998) Adhesion, transendothelial migration and reverse transmigration of in vitro cultured dendritic cells. *Blood* **92,** 207–214.
9. Bianchi, G., Sironi, M., Ghibaudi, E., Selvaggini, C., Elices, M., Allavena, P., and Mantovani, A. (1993) Migration of natural killer cells across endothelial cell monolayers. *J. Immunol.* **151,** 5135–5144.
10. Sozzani, S., Allavena, P., D'Amico, G., Luini, W., Bianchi, G., Kataura, M., Imai, T., Yoshie, O., Bonecchi, R., and Mantovani, A. (1998) Differential regulation of chemokine receptors during dendritic cell maturation: a model for their trafficking properties. *J. Immunol.* **161,** 1083–1086.

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In Vivo Assays of Langerhans Cell Migration

Marie Cumberbatch, Rebecca J. Dearman, and Ian Kimber

1. Introduction

The skin is an immunologically active tissue; the integrated skin immune system comprises epidermal Langerhans cells (LC), dermal dendritic cells (DC), tissue macrophages, mast cells, and T lymphocytes in transit. Cutaneous immune function is orchestrated through the action of cytokines produced locally in the skin by LC, keratinocytes, and other cell types ***(1–3)***.

Key cellular vectors of cutaneous immune and allergic responses are LC. These are bone-marrow-derived DC of characteristic phenotype, which constitute a contiguous network in the epidermis where they serve as sentinels of the immune system forming a cellular trap for exogenous antigen. The contribution of LC to adaptive immune responses is dependent on their mobilization and the ability to adapt their functional activity to the needs of different tissue compartments (the skin and regional lymph nodes). In the epidermis, LC function primarily as antigen-processing cells, which are able to recognize, internalize, digest, and then display antigen. Following encounter with antigen, LC are stimulated to leave the epidermis and to travel to draining lymph nodes via the afferent lymphatics. By the time they reach the paracortical regions of the lymph nodes they have lost the capacity for antigen processing and have acquired instead immunostimulatory function and the ability to present antigen to responsive T lymphocytes ***(4–6)***. Much of what is known of the behavior of LC during the induction phase of cutaneous immune responses has come from investigations in mice of contact sensitization ***(7)***. Following topical exposure of mice to skin-sensitizing chemicals, a proportion of LC local to the site of allergen encounter (some of which bear high levels of antigen) is mobilized and stimulated to leave the epidermis. These migrating cells accumulate in

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draining lymph nodes as potent antigen presenting cells and play a pivotal role in the initiation of T-lymphocyte activation and contact sensitization ***(8–13)***.

The functional maturation of LC and their transition from antigen-processing to antigen-presenting cells are associated with a number of important changes in the expression of membrane determinants and are affected by epidermal cytokines. There is increased expression of those molecules that are required for effective interaction with, and presentation of antigen to, T lymphocytes including MHC Class II (Ia) antigens, intercellular adhesion molecule-1 (ICAM-1), and B7-1 and B7-2 costimulatory molecules ***(14–18)***. Conversely, there is decreased expression of E-cadherin, a molecule believed to retain LC within the epidermal tissue matrix ***(19–21)***. Such changes are mediated primarily by granulocyte/macrophage colony-stimulating factor (GM-CSF), tumor necrosis factor α (TNF-α), and interleukin 1β (IL-1β), each of which are constitutive or inducible products of epidermal cells ***(20,22–27)***.

In addition to mediating the functional maturation of LC, there is evidence that epidermal cytokines provide a stimulus for LC migration. Of particular importance is TNF-α, an inducible product of keratinocytes. Investigations in mice have shown that homologous recombinant TNF-α when administered intradermally via the ear pinnae induces a time- and dose-dependent loss of a proportion of LC from the local epidermis and an accumulation, some time later, of DC in draining lymph nodes ***(28,29)***. The response to TNF-α is rapid with a measurable reduction in epidermal LC frequency evident within 30 min and an increase in lymph node DC numbers detectable within 2 h. The inference drawn is that TNF-α, under certain inflammatory conditions, is able to provide a signal for the stimulation of LC migration. It is apparent, however, that migration of LC from the skin is dependent also on the availability of a second signal provided by IL-1β; a constitutive product in murine epidermis of LC exclusively ***(30–33)***. Homologous recombinant IL-1β, like TNF-α, when injected intradermally into ear pinnae of mice causes both a reduction in epidermal LC density and an increase in the number of DC found in draining nodes ***(34)***. The changes induced by IL-1β, however, exhibit somewhat slower kinetics. Following administration of IL-1β, a significant reduction in epidermal LC frequency is first detectable within 2 h and is accompanied by measurable increases in lymph node DC numbers by 4 h. The delayed kinetics of the response induced by IL-1β, coupled with the demonstration that intradermal administration of IL-1β is associated with a rapid increase in keratinocyte expression of mRNA for TNF-α, suggests that IL-1β may contribute to LC migration by stimulating the production by keratinocytes of TNF-α and that TNF-α then triggers LC migration ***(35)***. Certainly, LC migration stimulated by intradermal injection of IL-1β is prevented if mice are first treated with a neutralizing anti-TNF-α antibody ***(36)***. It is clear, however, that IL-1β contributes

to LC migration in other ways since LC migration induced by intradermal injection of TNF-α is itself inhibited by systemic pre-treatment of mice with an anti-IL-1β antibody ***(36)***. It is proposed therefore that LC require at least two signals to migrate, one of which is provided by TNF-α and the other by IL-1β. Intradermal administration of IL-1β is by itself effective at stimulating LC migration because it is able to induce local production of TNF-α by keratinocytes. TNF-α administered alone stimulates LC movement because there is sufficient constitutive IL-1β present to provide the second signal for migration. Taken together, it would appear that TNF-α and IL-1β are essential signals for the mobilization of LC and their directed movement through the skin to draining lymph nodes.

Various forms of cutaneous trauma, including contact sensitization, skin irritation, and exposure to ultraviolet B (UVB) light, are associated with the migration of LC from the skin and their accumulation as DC in draining lymph nodes ***(7,37,38)***. Exposure via the skin to these same signals is associated also with enhanced expression of epidermal cytokines ***(30,39,40)***. The assumption is that in each case LC migration is induced secondary to the stimulation of local cytokine production. In accordance with this, LC migration induced following contact sensitization, UVB irradiation, or exposure to a non-sensitizing skin irritant such as sodium lauryl sulfate is inhibited with a neutralizing anti-TNF-α antibody ***(35,36,41)***. Independent investigations using TNF receptor knockout mice also indicate that TNF-α is required for LC migration ***(42)***. The activity of TNF-α raises an important issue regarding the nature of cutaneous insults that cause LC migration. It may prove that all forms of dermal trauma of the type and severity necessary to stimulate the production by keratinocytes of TNF-α will result in the migration of LC from the epidermis and the accumulation of DC in draining lymph nodes irrespective of an overt antigen challenge. It is assumed that such changes occur in the absence of induced IL-1β expression and that sufficient constitutive IL-1β is available to provide the second signal, together with TNF-α, for migration. With respect to contact sensitization, in addition to local TNF-α production, the induction of IL-1β expression is required for optimal responses. Both LC migration and contact sensitization are compromised in IL-1β gene knockout mice or in mice exposed to a neutralizing anti-IL-1β antibody ***(35,36,43,44)***.

With respect to the regulation of LC migration in humans, there is only limited information available to suggest that similar stimuli are responsible. Preliminary results suggest that sensitization, via the skin, to diphenylcyclopropenone (a chemical contact allergen) is associated with a reduction in epidermal $CD1a^+$ LC frequency measured 17 h following exposure (**Fig. 1**; ***45***). In addition, a significant increase in the flow and cellular content of lymph following exposure of human volunteers to sodium lauryl sulfate has been demonstrated ***(46)***. Direct evidence

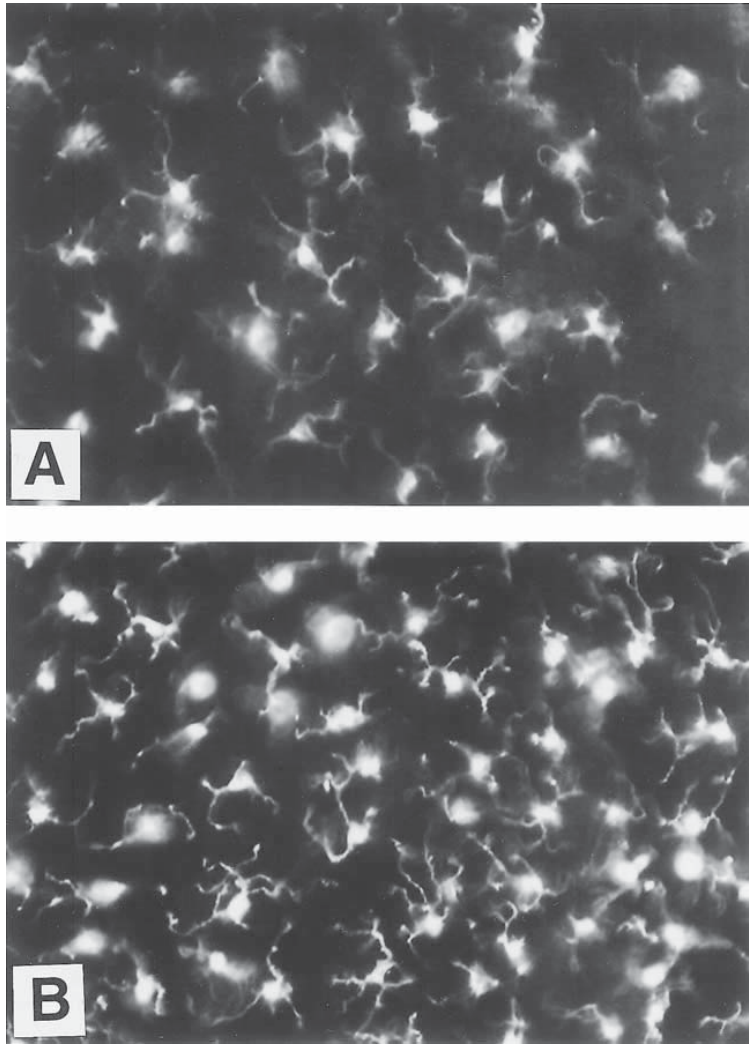

Fig. 1. Allergen-induced changes in human epidermal LC frequency and morphology. Sites identified on the hip or buttock were exposed to 50 μL of **(A)** 2% diphenylcyclopropenone in acetone, or an equivalent volume of **(B)** vehicle (acetone) alone. LC frequency and morphology were assessed following indirect immunofluorescence staining for CD1a expression of epidermal sheets prepared from skin biopsies taken 17 h later. Magnification ×50.

that TNF-α may provide a signal for LC migration in humans comes from investigations where individuals have been exposed by intradermal injection to homologous recombinant TNF-α. In two separate studies, treatment with TNF-α was associated with a reduction in the frequency of epidermal $CD1a^+$ LC ***(47,48)***.

In the following sections, methods are described for the assessment of LC migration as a function of either changes in epidermal LC frequency (for murine and human studies) or increases in draining lymph node DC number (murine studies only). Examples of conditions that result in LC migration are given along with an indication of the kinetics of these responses.

2. Materials

2.1. Stimulation of LC Migration

1. For murine studies: 4-Ethoxymethylene-2-phenyloxazol-5-one (oxazolone; Sigma, 0.5% in acetone:olive oil [4:1]) and fluorescein isothiocyanate (FITC; Sigma, 1% in acetone:dibutylphthalate [1:1]) are used commonly for the investigation of allergen-induced LC migration in mice. Dosing solutions should be prepared freshly, immediately prior to use. FITC requires mixing for 5 min on a magnetic stirrer and may form a fine suspension.
2. For human studies: Diphenylcyclopropenone (DPC; 2% in acetone) may be used for the study of allergen-induced LC migration in humans (**Fig. 1**; *see* **Note 1**).
3. Irritant, for example, sodium lauryl sulfate (SLS) ***(37)***.
4. Cytokines: For murine studies recombinant TNF-α and IL-1β ***(28,29,34)*** may be used. Cytokines should be prepared immediately prior to use and suspended in a carrier protein such as BSA (0.1% in PBS) to prevent loss of activity (*see* **Note 2**). For human studies cytokines should be of clinical grade suitable for injection into human volunteers.

2.2. Preparation and Analysis of Epidermal Sheets

1. Phosphate-buffered saline (PBS; pH 7.2).
2. Ethylenediamine tetraacetic acid, tetrasodium salt (EDTA; Sigma Chemical Co., St. Louis, MO). Prepare fresh as required.
3. Acetone (technical grade). Aliquots (in small glass bijou bottles) can be stored at –20°C ready for use.
4. Primary antibodies (*see* **Note 3**): For murine studies, rat anti-mouse $I\text{-}A^d/I\text{-}\varepsilon^d$ monoclonal antibody (clone 2G9; rat lgGzak) from Pharmingen (San diego, CA), or an irrelevant isotype control (rat lgGzak; Pharmingen), in place of the specific antibody, diluted equally in the concentration range of 1-10 μg/mL. For human studies, mouse anti-human HLA-DR (DAKO, Ely, Cambridge, UK), mouse anti-human CD1a (DAKO), and mouse IgG2a isotype control, each diluted to an equivalent extent in the concentration range of 1-10 μg/mL.

5. Bovine serum albumin (BSA; Sigma).
6. Tissue culture plates, e.g., 24-well Cell Culture Cluster (Costar, Cambridge, MA).
7. Fine tip sterile pastettes (Alpha Laboratories Ltd, Hampshire, UK).
8. Secondary antibodies (*see* **Note 4**): For murine studies, goat $F(ab')_2$ anti-rat IgG conjugated with FITC, horseradish peroxidase (HRP), or biotin (TCS Biologicals Ltd., Birmingham, UK). For human studies, goat $F(ab')_2$ anti-mouse IgG conjugated as above (DAKO).
9. Glycerol (AR). Mix 9 parts glycerol to 1 part PBS and store for up to 6 mo at room temperature.
10. Citifluor (Citifluor Ltd., London, UK).
11. Avidin (Sigma) and biotin (Sigma). Solutions of avidin and biotin, diluted appropriately in deionized water, may be stored in working aliquots at –20°C.
12. Diaminobenzidine tablets (DAB; Sigma). Diaminobenzidine is hazardous, the risk to health is reduced by handling the chemical in the tablet form.

2.3. Analysis of Lymph Node Dendritic Cells

1. 200-mesh stainless steel gauze (John Staniar & Co., Manchester, UK).
2. RPMI-1640 growth medium (Gibco, Paisley, UK) supplemented with 25 m*M* HEPES, 400 μg/mL streptomycin, 400 μg/mL ampicillin, and 10% heat-inactivated fetal calf serum.
3. Trypan blue (Flow Laboratories Ltd., Herts., UK).
4. Metrizamide (Sigma Chemical Co., *see* **Note 5**).
5. Long nose glass Pasteur pipets (John Poulton Ltd., Essex, UK).

3. Methods

3.1. Stimulation of Langerhans Cell Migration in the Mouse

3.1.1. Allergen/Irritant-Induced Migration

1. Mice receive 25 μL of the test chemical, or an equivalent volume of vehicle, on the dorsum of both ears (*see* **Subheading 2.1.**, **steps 1** and **2** and **Note 6**).
2. At various periods following treatment, ears and/or draining auricular lymph nodes are removed for analysis. Changes induced in epidermal LC frequency and lymph node DC number following topical exposure of mice to the contact allergen oxazolone are shown in **Fig. 2** (*see* **Note 7**).

3.1.2. Cytokine-Induced Migration

1. Dilute cytokine with sterile PBS containing 0.1% BSA as carrier protein.
2. Anesthetize mice, using, for example, a mixture of Halothane and oxygen.
3. Administer 30 μL vol of cytokine, or carrier protein alone, intradermally into both ear pinnae using 1-mL syringes with 30-gauge stainless-steel needles (*see* **Notes 6** and **8**).
4. Ears/draining lymph nodes may be removed at various periods thereafter for assessment of LC migration (*see* **Note 9**).

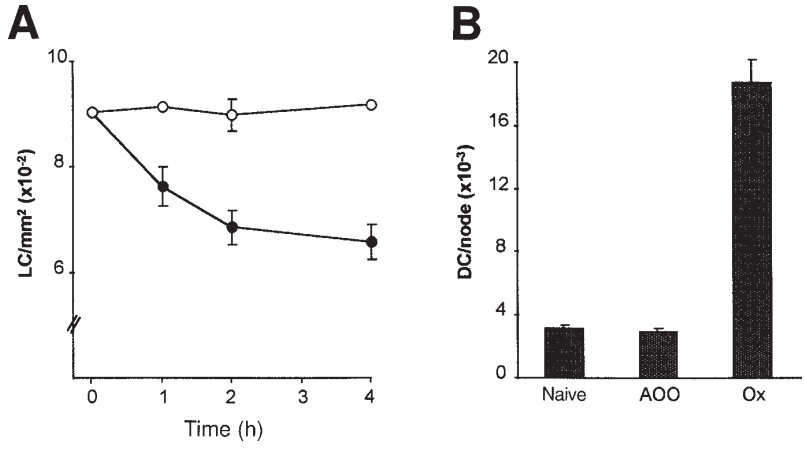

Fig. 2. Changes induced in **(A)** epidermal LC frequency and **(B)** draining lymph node DC number following topical exposure of mice to the contact allergen oxazolone. Groups of mice [**(A)** $n = 2$, **(B)** $n = 10$] received 25 μL of 0.5% oxazolone (Ox) in acetone:olive oil (AOO) vehicle, or an equal volume of vehicle alone, on the dorsum of both ears. Control mice were untreated. (**A**) Ears were removed from Ox- (●) or AOO-treated (○) animals at various times thereafter and the frequency of MHC class II^+ LC assessed following indirect immunofluorescence staining of epidermal sheets. Results are expressed as the mean number of cells/mm^2 (± SE) of counts derived from examination of 10 fields/ear for each of four samples. Standard errors of less than 10.0 are not shown. (**B**) Draining auricular lymph nodes were removed 18 h later and the number of DC/node measured. Mean ± SE of results from four independent experiments are shown.

3.2. Stimulation of LC Migration in Humans

3.2.1. Allergen-Induced Migration

1. Mark out 2 cm^2 (1.41 cm × 1.41 cm) areas at selected sites (*see* **Note 10**).
2. Apply 50 μL of allergen (e.g., diphenylcyclopropenone; 2% in acetone), or 50 μL of acetone alone, using a calibrated pipet.
3. Cover site loosely with gauze (i.e., do not occlude) (*see* **Note 11**).
4. At various intervals biopsy exposed sites and assess the number of LC remaining in the epidermis (**Fig. 1**).

3.2.2. Cytokine-Induced Migration

1. Dilute cytokine in sterile normal saline to appropriate concentration.
2. Inject volunteers with 50 μL of cytokine, or 50 μL of saline alone, intradermally into identified sites (*see* **Note 12**).
3. At various intervals, biopsy injected sites and assess epidermal LC frequency ***(47)*** (*see* **Subheading 3.3.**).

3.3. Preparation of Epidermal Sheets

3.3.1. Murine Epidermal Sheets

1. Remove ears and split with the aid of forceps into dorsal and ventral halves (*see* **Note 13**).
2. Incubate ear halves for 90 min at 37°C with 0.02 *M* EDTA dissolved in PBS (*see* **Notes 14** and **15**).
3. Separate the epidermis from the underlying dermis using forceps and wash epidermal sheets in PBS (*see* **Note 16**).

3.2.2. Human Epidermal Sheets

1. Take 6 mm punch biopsies under local anesthesia (*see* **Note 17**).
2. Incubate biopsies for 120 min at 37°C with 0.02 *M* EDTA dissolved in PBS (*see* **Note 18**).
3. Separate the epidermis from the underlying dermis using forceps and wash tissue in PBS.

3.4. Detection of Epidermal Langerhans Cells

3.4.1. Indirect Immunofluorescence

1. Fix epidermal sheets in prechilled acetone for 20 min at –20°C.
2. Wash tissue in three changes of PBS for a total of 15 min (*see* **Note 19**).
3. Dilute primary antibody, or appropriate isotype control antibody, in 0.1% BSA/PBS and add 500 μL vol to tissue culture plates.
4. Carefully blot epidermal sheets dry on paper tissue and add to tissue culture wells using forceps. Tease epidermal sheets using fine pipet tips until evenly distributed in antibody solution and incubate for 30–60 min at room temperature (*see* **Note 20**).
5. Remove antibody solution using a pastette and wash tissue in several changes of PBS (1-2 mL vol) for a total of 15 min.
6. Remove excess PBS using a pastette and add 500 μL of secondary (FITC or other fluorescent-conjugated) antibody, diluted appropriately in 0.1% BSA/PBS. Tease epidermal sheets using fine pipet tips until evenly distributed in antibody solution and incubate in the dark for a further 30–60 min at room temperature (*see* **Notes 20** and **21**).
7. Discard antibody solution using a pastette and wash tissue for a total of 15 min in several changes of PBS.
8. Carefully blot epidermal sheets dry on paper tissue and mount on microscope slides in glycerol/saline (9:1) or Citifluor (with antifade). Seal with nail varnish (*see* **Note 22**).

3.4.2. Indirect Immunoperoxidase Staining Procedure

1. Fix epidermal sheets in prechilled acetone for 20 min at –20°C.
2. Wash tissue in three changes of PBS for a total of 15 min.

3. Incubate sheets with 3% hydrogen peroxide (30% stock solution diluted 1:10 in PBS) for 5 min (*see* **Note 23**).
4. Wash in several changes of PBS for a total of 15 min.
5. Follow **steps 3–6** as described in **Subheading 3.4.1.** using peroxidase (HRP)-conjugated secondary antibody.
6. Wash tissue in several changes of PBS for a total of 5 min.
7. Visualize specific staining by incubation of epidermal sheets in a peroxidase substrate, such as DAB.
8. Wash sheets in several changes of water for a total of 10 min, blot dry on paper tissue, and mount on microscope slides in glycerol/saline (9:1). Seal with nail varnish.

3.4.3. Peroxidase-Conjugated Streptavidin Staining Procedure

1. Follow **steps 1-4** as described in **Subheading 3.4.2.**
2. Incubate epidermal sheets in 0.01% avidin for 20 min followed by 0.001% biotin for 20 min to block endogenous avidin-binding activity (*see* **Note 24**).
3. Wash tissue in several changes of PBS for a total of 15 min.
4. Follow **steps 3–6** as described in **Subheading 3.4.1.** using a biotinylated secondary antibody diluted in 0.1% BSA/PBS containing 10% normal mouse serum (*see* **Note 25**).
5. Wash tissue in several changes of PBS for a total of 15 min.
6. Incubate sheets with 500 μL vol of a streptavidin–peroxidase conjugate (e.g., ExtrAvidin–peroxidase from Sigma diluted 1:50 in PBS) for 45–60 min at room temperature.
7. Wash tissue in several changes of PBS for a total of 5 min.
8. Visualize specific staining by incubation of epidermal sheets in a peroxidase substrate, such as DAB (*see* **Note 26**).
9. Wash sheets in several changes of water for a total of 10 min, blot dry on paper tissue, and mount on microscope slides in glycerol/saline (9:1). Seal with nail varnish.

3.5. Analysis of Epidermal Sheets

3.5.1. Murine Epidermal Sheets

1. To determine the frequency of LC per mm^2 of epidermis the mean number of stained cells within at least 10 consecutive calibrated grid areas per tissue sample for at least four ears per treatment group is assessed microscopically (*see* **Note 27**).
2. Results may be expressed as the mean number of LC/mm^2 ± SEM (**Fig. 2**).
3. The statistical significance of differences between experimental groups may be calculated using a two-sided Student's *t*-test or other appropriate statistical methods.

3.5.2. Human Epidermal Sheets

1. The mean number of stained cells within at least 50 consecutive calibrated grid areas per biopsy is assessed microscopically.

2. Results may be expressed as the mean number of LC/mm^2 ± SD.
3. The statistical significance of differences between treatments may be calculated using a two-sided Student's *t*-test based on the error mean square in the analysis of variance or other appropriate statistical methods.

3.6. Isolation of Lymph Node DC

1. Excise and pool lymph nodes for each experimental group into 5 mL PBS (*see* **Note 28**).
2. Prepare a 4 cm^2 stainless-steel gauze, folded upward at the edges to prevent loss of lymph nodes, and place within a 6 cm plastic Petri dish.
3. Transfer pooled lymph nodes to gauze and add 1-2 mL of RPMI-FCS (*see* **Note 29**).
4. Prepare a single-cell suspension of lymph node cells (LNC) by gentle mechanical disaggregation using the plunger from a 5 mL plastic syringe.
5. Rinse the gauze with a further 1-2 mL of RPMI-FCS and suspend LNC in a total of 10 mL of medium (*see* **Note 29**).
6. Perform viable cell counts by exclusion of 0.5% trypan blue and adjust cell concentration to 5×10^6 cells/mL in RPMI-FCS, noting the total cell yield per experimental group.
7. Prepare a 14.5% solution of Metrizamide (w/v in RPMI-FCS) at room temperature using a magnetic stirrer (*see* **Notes 5**, **29**, and **30**).
8. Layer 2 mL Metrizamide under 8 mL LNC in a 10 mL conical-bottomed centrifuge tube using a long-nosed glass Pasteur pipet (*see* **Note 31**).
9. Centrifuge for 20 min (600*g*) at room temperature with centrifuge brake off.
10. Collect cells accumulating at the interface using a long-nose glass Pasteur pipet and wash once with RPMI-FCS.
11. Resuspend cells in a measured volume of medium (usually between 200–400 μL).

3.7. Assessment of Lymph Node DC Frequency

1. The number of DC in the volume of medium measured in **Subheading 3.6.**, **step 11** may be assessed by direct morphological examination using an improved Neubauer hematocytometer and phase contrast microscopy. DC are characterized by a "ruffled" appearance (*see* **Note 32**).
2. The total number of DC recovered (e.g., 44,850 in 230 μL) is divided by the number of LNC from which they were derived, i.e., the number of LNC/gradient: 8 mL at 5×10^6 (40×10^6).
3. This figure is then multiplied by the total yield of LNC (e.g., 45×10^6; *see* **Subheading 3.6.**, **step 6** for that experimental group to give the total DC yield for that group.
4. To compare the number of DC/node between groups, the total DC yield is divided by the number of nodes for that group (e.g., 20 if 10 mice/group are used).
5. An example calculation using the figures above:

   from **step 2**: $44{,}850 \div (40 \times 10^6) = 0.00112$.
   from **step 3**: $0.00112 \times (45 \times 10^6) = 50{,}400$.
   from **step 4**: $50{,}400 \div 20 =$ **2520 DC/node**.

4. Notes

4.1. Materials

1. Diphenylcyclopropenone (DPC) is a chemical contact allergen used routinely (at 2% in acetone) for the treatment of alopecia areata. A possible adverse effect observed in some individuals exposed to DPC is a delayed irritant response that may manifest itself up to 10 d later and may require conventional anti-inflammatory treatment.
2. Controls should include injection of carrier protein alone and no treatment to control for potential trauma caused by the injection procedure.
3. Epidermal LC may be detected by virtue of their expression of MHC class II (murine and human) and CD1a (human only). It is important to note that these membrane antigens are regulated differentially by human LC during their development into immunostimulatory DC; an up-regulation of MHC class II and a down-regulation of CD1a is reported ***(49,50)***.
4. Use of $F(ab')_2$ fragments will reduce nonspecific binding.
5. Metrizamide (14.5%) should be prepared immediately prior to use and should be dissolved in medium that has been allowed to reach room temperature. Metrizamide takes approx 5 min to dissolve at room temperature using a magnetic stirrer.

4.2. Stimulation of Langerhans Cell Migration

6. Groups of at least three mice are used for assessment of epidermal LC frequency; two mice (four ears) for specific antibody and one mouse (two ears) for isotype controls. Groups of at least 10 mice are used for measurement of DC accumulation in draining lymph nodes to ensure sufficient numbers of LNC for density gradient centrifugation.
7. The time point at which epidermal LC and lymph node DC measurements are made is critical. With respect to LC migration, the response to allergen/irritant is fairly rapid, and a significant reduction in epidermal LC frequency is detectable within 4 h of exposure (**Fig. 2**). At later time points (18–24 h), particularly following application of strong allergens such as oxazolone, LC numbers start to return to pre-exposure levels (unpublished observation). Assessment of lymph node DC accumulation is made most accurately before the onset of significant lymphocyte proliferative activity (*see* **Note 32**).
8. Intradermal injections should be given slowly and carefully to ensure even spread of the cytokine; a "white, flat bubble" should appear across the site. Attempting to deliver volumes greater than 30 μL into a mouse ear will result in LC migration itself as a result of the trauma induced.
9. Kinetics of migration induced by TNF-α and IL-1β are described in **Subheading 1.** At later time points (12 h in the skin and 18 h in lymph nodes), LC respond with migration to 0.1% BSA alone as a foreign protein ***(34)***.
10. LC activity is influenced by UVB light. It is advisable, therefore, to choose non-sun-exposed sites such as the hip or buttock.
11. Occlusion may cause sufficient trauma in itself to cause LC migration/activation.
12. Cytokines for human use should be of clinical grade and suitable for injection into human volunteers.

4.3. Preparation and Analysis of Epidermal Sheets

13. Following topical exposure to a test chemical, ears should be removed and rinsed quickly in 70% ethanol followed by PBS to remove excess chemical.
14. Incubation in EDTA may be performed in small (5 mL) plastic bijou bottles containing up to six samples per experimental group. Larger group sizes will require larger volumes of EDTA.
15. LC will start to respond immediately to the trauma of tissue isolation. Samples should be placed promptly into EDTA and epidermal sheets fixed immediately following separation.
16. Murine epidermal sheets are fragile. The aim is to prepare complete epidermal sheets, rather than pieces, as this enables subsequent quantitative measurements of LC frequency suitable for statistical analyses. LC may become activated if extreme care is not taken at this stage.
17. A local anaesthetic without adrenaline is advised. Adrenaline may influence the process of LC migration.
18. Incubation may be performed in a thermos flask if, for example, material requires transportation from hospital to laboratory (*see* also **Notes 14** and **15**).
19. Once fixed, samples may be stored overnight at 4°C in PBS prior to subsequent immunostaining if desired.
20. Uneven distribution of epidermal sheets in antibody solution results in uneven staining.
21. Fluorescence staining is preserved if samples are protected from light during antibody incubation and subsequent washes.
22. Mounted samples will keep for at least 1 mo if stored covered and at 4°C.
23. Best results are achieved using fresh hydrogen peroxide that has been open for no more than 2–3 mo.
24. Failure to block avidin-binding activity will result in strong nonspecific staining of hair follicles.
25. The addition of 10% normal mouse serum reduces nonspecific binding of the second antibody. Any nonspecific binding will be amplified by subsequent steps.
26. The signal may be amplified further by the addition of nickel chloride (dissolved in water) to the DAB solution at a final concentration of 3%. This converts the brown reaction product to a more intense blue/black product.
27. LC frequency varies across the surface of the ear, with higher LC density apparent toward the base of the ear and lower LC numbers toward the edges. Accurate counts are achieved only if similar areas per ear are examined. For analysis of cytokine-induced changes in LC frequency, it is important to count cells in the center of the ear (i.e., the injection site) and avoid cell counts toward the outer edges.

4.4. Isolation of Lymph Node DC

28. Auricular lymph nodes located in the neck region represent the draining lymph nodes for ears dosed either with allergen or cytokine.
29. RPMI-FCS should be maintained at room temperature throughout.

30. DC yields are compromised if medium/Metrizamide gradients are not at room temperature, particularly if the medium used is too cold.
31. This step must be performed gently to maintain a clean interface between medium and Metrizamide.
32. DC are routinely 100% viable and comprise 60–90% of the DC-enriched fraction prepared from naive lymph nodes and from lymph nodes taken before the onset of significant lymphocyte proliferative activity. Contaminating cells include macrophages (less than 1%) and T and B lymphocytes. Activated T lymphocytes (up to 15%) and macrophages will cofractionate with DC on Metrizamide gradients prepared from lymph nodes isolated after the onset of lymphocyte proliferation i.e., after 24 h of allergen exposure ***(12)***.

References

1. Streilein, J. W. (1978) Lymphocyte traffic, T-cell malignancies and the skin. *J. Invest. Dermatol.* **71,** 167–171.
2. Bos, J. D. and Kapsenberg, M. L. (1993) The skin immune system: Progress in cutaneous biology. *Immunol. Today* **14,** 75–78.
3. Kimber, I. (1996) The skin immune system. In *Dermatotoxicology* 5th ed. (Marzulli, F. N. and Maibach, H. I., eds.), Taylor and Francis, Washington, DC, pp. 131–141.
4. Schuler, G. and Steinman, R. M. (1985) Murine epidermal Langerhans cells mature into potent immunostimulatory dendritic cells in vitro. *J. Exp. Med.* **161,** 526–546.
5. Streilein, J. W. and Grammer, S. F. (1989) In vitro evidence that Langerhans cells can adopt two functionally distinct forms capable of antigen presentation to T lymphocytes. *J. Immunol.* **143,** 3925–3933.
6. Streilein, J. W., Grammer, S. F., Yoshikawa, T., Demidem, A., and Vermeer, M. (1990) Functional dichotomy between Langerhans cells that present antigen to naive and memory/effector T lymphocytes. *Immunol. Rev.* **117,** 159–183.
7. Kimber, I. and Cumberbatch, M. (1992) Dendritic cells and cutaneous immune responses to chemical allergens. *Toxicol. Appl. Pharmacol.* **117,** 137–146.
8. Knight, S. C., Krejci, J., Malkovsky, M., Colizzi, V., Gautam, A., and Asherson, G. L. (1985) The role of dendritic cells in the initiation of immune responses to contact sensitizers. I. In vivo exposure to antigen. *Cell. Immunol.* **94,** 427–434.
9. Macatonia, S. E., Edwards, A. J., and Knight, S. C. (1986) Dendritic cells and the initiation of contact sensitivity to fluorescein isothiocyanate. *Immunology* **59,** 509–514.
10. Macatonia, S. E., Knight, S. C., Edwards, A. J., Griffiths, S., and Fryer, P. (1987) Localization of antigen on lymph node dendritic cells after exposure to the contact sensitizer fluorescein isothiocyanate. Functional and morphological studies. *J. Exp. Med.* **166,** 1654–1667.
11. Kinnaird, A., Peters, S. W., Foster, J. R., and Kimber, I. (1989) Dendritic cell accumulation in draining lymph nodes during the induction phase of contact allergy in mice. *Int. Arch. Allergy Appl. Immunol.* **89,** 202–210.

12. Cumberbatch, M. and Kimber, I. (1990) Phenotypic characteristics of antigen-bearing cells in the draining lymph nodes of contact-sensitized mice. *Immunology* **71,** 404–410.
13. Kripke, M. L., Munn, C. G., Jeevan, A., Tang, J-M., and Bucana, C. (1990) Evidence that cutaneous antigen-presenting cells migrate to regional lymph nodes during contact sensitization. *J. Immunol.* **145,** 2833–2838.
14. Cumberbatch, M., Gould, S. J., Peters, S. W., and Kimber I. (1991) MHC Class II expression by Langerhans cells and lymph node dendritic cells: Possible evidence for the maturation of Langerhans cells following contact sensitization. *Immunology* **74,** 414–419.
15. Cumberbatch, M., Peters, S. W., Gould, S. J., and Kimber, I. (1992) Intercellular adhesion molecule-1 (ICAM-1) expression by lymph node dendritic cells: Comparison with epidermal Langerhans cells. *Immunol. Lett.* **32,** 105–110.
16. Larsen, C. P., Ritchie, S. C., Pearson, T. C., Linsley, P. S., and Lowry, R. P. (1992) Functional expression of the costimulatory molecule, B7/BB1, on murine dendritic cell populations. *J. Exp. Med.* **176,** 1215–1220.
17. Girolomoni, G., Zambruno, G., Manfredini, R., et al. (1994) Expression of B7 costimulatory molecule in cultured human epidermal Langerhans cells is regulated at the mRNA level. *J. Invest. Dermatol.* **103,** 54–59.
18. Inaba, K., Witmer-Pack, M., Inaba, M., et al. (1994) The tissue distribution of the B7–2 costimulator in mice: Abundant expression on dendritic cells in situ and during maturation in vivo. *J. Exp. Med.* **180,** 1849–1860.
19. Borkowski, T. A., Van Dyke, B. J., Schwarzenberger, K., McFarland, V. W., Farr, A. G., and Udey, M. C. (1994) Expression of E-cadherin by murine dendritic cells: E-cadherin as a dendritic cell differentiation antigen characteristic of epidermal Langerhans cells and related cells. *Eur. J. Immunol.* **24,** 2767–2774.
20. Schwarzenberger, K. and Udey, M. C. (1996) Contact allergens and epidermal proinflammatory cytokines modulate Langerhans cell E-cadherin expression in situ. *J. Invest. Dermatol.* **106,** 553–558.
21. Cumberbatch, M., Dearman, R. J., and Kimber, I. (1996) Adhesion molecule expression by epidermal Langerhans cells and lymph node dendritic cells: A comparison. *Arch. Dermatol. Res.* **288,** 739–744.
22. Witmer-Pack, M. D., Oliver, W., Valinsky, J., Schuler, G., and Steinman, R. M. (1987) Granulocyte/macrophage colony-stimulating factor is essential for the viability and function of cultured murine epidermal Langerhans cells. *J. Exp. Med.* **166,** 1484–1498.
23. Heufler, C., Koch, F., and Schuler, G. (1988) Granulocyte/macrophage colony-stimulating factor and interleukin 1 mediate the maturation of murine epidermal Langerhans cells into potent immunostimulatory dendritic cells. *J. Exp. Med.* **167,** 700–705.
24. Picut, C. A., Lee, C. S., Dougherty, E. P., Asherson, K. L., and Lewis, R. M. (1988) Immunostimulatory capabilities of highly enriched Langerhans cells in vitro. *J. Invest. Dermatol.* **90,** 201–206.

25. Chang, C.-H., Furue, M., and Tamaki, K. (1995) B7–1 expression of Langerhans cells is up-regulated by proinflammatory cytokines and is down-regulated by interferon-γ or by interleukin-10. *Eur. J. Immunol.* **25,** 394–398.
26. Furue, M., Chang, C.-H., and Tamaki, K. (1996) Interleukin-1 but not tumour necrosis factor α synergistically upregulates the granulocyte-macrophage colony-stimulating factor-induced B7–1 expression of murine Langerhans cells. *Br. J. Dermatol.* **135,** 194–198.
27. Ozawa, H., Nakagawa, S., Tagami, H., and Aiba, S. (1996) Interleukin-1β and granulocyte-macrophage colony-stimulating factor mediate Langerhans cell maturation differently. *J. Invest. Dermatol.* **106,** 441–445.
28. Cumberbatch, M. and Kimber I. (1992) Dermal tumour necrosis factor-α induces dendritic cell migration to draining lymph nodes, and possibly provides one stimulus for Langerhans cell migration. *Immunology* **75,** 257–263.
29. Cumberbatch, M., Fielding, I., and Kimber, I. (1994) Modulation of epidermal Langerhans cell frequency by tumour necrosis factor-α. *Immunology* **81,** 395–401.
30. Enk, A. H. and Katz, S. I. (1992) Early molecular events in the induction phase of contact sensitivity. *Proc. Natl. Acad. Sci. USA* **89,** 1398–1402.
31. Matsue, H., Cruz, P. D., Jr., Bergstresser, P. R., and Takashima, A. (1992) Langerhans cells are the major source of mRNA for IL-1β and MIP-1α among unstimulated mouse epidermal cells. *J. Invest. Dermatol.* **99,** 537–541.
32. Heufler, C., Topar, G., Koch, F., et al. (1992) Cytokine gene expression in murine epidermal cell suspensions: Interleukin 1β and macrophage inflammatory protein 1α are selectively expressed in Langerhans cells but are differentially regulated in culture. *J. Exp. Med.* **176,** 1221–1226.
33. Schreiber, S., Kilgus, O., Payer, E., et al. (1992) Cytokine pattern of Langerhans cells isolated from murine epidermal cell cultures. *J. Immunol.* **149,** 3525–3534.
34. Cumberbatch, M., Dearman, R. J., and Kimber, I. (1997) Interleukin 1β and the stimulation of Langerhans cell migration: comparisons with tumour necrosis factor α. *Arch. Dermatol. Res.* **289,** 277–284.
35. Enk, A. H., Angeloni, V. L., Udey, M. C., and Katz, S. I. (1993) An essential role for Langerhans cell-derived IL-1β in the initiation of primary immune responses in the skin. *J. Immunol.* **150,** 3698–3704.
36. Cumberbatch, M., Dearman, R. J., and Kimber, I. (1997) Langerhans cells require signals from both tumour necrosis factor-α and interleukin-1β for migration. *Immunology* **92,** 388–395.
37. Cumberbatch, M., Scott, R. C., Basketter, D. A., et al. (1993) Influence of sodium lauryl sulfate on 2,4–dinitrochlorobenzene-induced lymph node activation. *Toxicology* **77,** 181–191.
38. Moodycliffe, A. M., Kimber, I., and Norval, M. (1992) The effect of ultraviolet B irradiation and urocanic acid isomers on dendritic cell migration. *Immunology* **77,** 394–399.
39. Holliday, M. R., Corsini, E., Smith, S., Basketter, D. A., Dearman, R. J., and Kimber, I. (1997) Differential induction of TNF-α and IL-6 by topically applied chemicals. *Am. J. Contact Derm.* **8,** 158–164.

40. Kimber, I., Holliday, M. R., and Dearman, R. J. (1995) Cytokine regulation of chemical sensitization. *Toxicol. Lett.* **82/83,** 491–496.
41. Moodycliffe, A. M., Kimber, I., and Norval, M. (1994) Role of tumour necrosis factor α in ultraviolet B light-induced dendritic cell migration and suppression of contact hypersensitivity. *Immunology* **81,** 79–84.
42. Wang, B., Kondo, S., Shivh, G. M., Fujisawa, H., Mak, T. W., and Sauder, D. N. (1996) Tumour necrosis factor receptor II (p75) signalling is required for the migration of Langerhans cells. *Immunology* **88,** 284–288.
43. Cumberbatch, M. and Kimber, I. (1995) Tumour necrosis factor-α is required for accumulation of dendritic cells in draining lymph nodes and for optimal contact sensitization. *Immunology* **84,** 31–35.
44. Schornick, L. P., De Togni, P., Mariathasan, S., et al. (1996) Mice deficient in IL-1β manifest impaired contact sensitivity to trinitrochlorobenzene. *J. Exp. Med.* **183,** 1427–1436.
45. Griffiths, C. E. M., Cumberbatch, M., Tucker, S. C., Dearman, R. J., Andrew, S., Headon, D. R., and Kimber, I. (2001) Exogenous topical lactoferrin inhibits allergen-induced Langerhans cell migrations and cutaneous inflammation in humans *Br. J. Dermatol*. (accepted for publication).
46. Brand, C. U., Hunziker, T., and Braathen, L. R. (1992) Isolation of human skin-derived lymph: flow and output of cells following sodium lauryl sulfate-induced contact dermatitis. *Arch. Dermatol. Res.* **284,** 123–126.
47. Groves, R. W., Allen, M. H., Ross, E. L., Barker, J. N. W. N., and Macdonald, D. M. (1995) Tumour necrosis factor alpha is pro-inflammatory in normal human skin and modulates cutaneous adhesion molecule expression. *Br. J. Dermatol.* **132,** 345–352.
48. Cumberbatch, M., Griffiths, C. E. M., Tucker, S. C., Dearman, R. J., and Kimber, I. Tumour necrosis factor α induces Langerhans cell migration in humans. Br. J. *Dematol.* **141,** 192-200.
49. Aiba, S. and Katz, S. I. (1990) Phenotypic and functional characteristics of in vivo-activated Langerhans cells. *J. Immunol.* **145,** 2791–2796.
50. Romani, N., Lenz, A., Glassel, H., et al. (1989) Cultured human Langerhans cells resemble lymphoid dendritic cells in phenotype and function. *J. Invest. Dermatol.* **93,** 600–609.

31

In Vivo Migration of Rat Dendritic Cells

Kenjiro Matsuno and Taichi Ezaki

1. Introduction

Dendritic cells (DCs) comprise a dynamic cellular system with a continuous traffic throughout the body. DCs connect nonlymphoid and lymphoid tissues via the lymph and blood, and transport antigenic information from most parts of the body to the immune system ***(1)***. To understand the migratory behavior of DCs in vivo is very important, because in different immunological situations, this behavior could change easily and, in turn, greatly affect immune responses ***(2)***. Factors that determine the behavior of DCs, such as adhesion molecules, could potentially be the targets for manipulating and regulating immune responses. The study of DC migration, therefore, should provide useful information for understanding the role of DCs not only in local but also in systemic host-defense mechanisms ***(3)***. The aim of this chapter is to introduce several useful methods that help to examine DC migration in vivo.

First, a method for monitoring migrating DCs in vivo, especially for the collection and isolation of DCs in rat afferent lymph fluid, is described. A problem in using laboratory rodents for lymph collection is that most lymphatics except the thoracic duct are too small to manipulate. As a rule, most nonlymphoid cells in the afferent lymph are trapped in the regional lymph nodes (LN), and very few emerge in the central thoracic duct lymph ***(4)***. Pugh et al. established a method of lymphadenectomy of the whole LN draining the gastrointestinal tract in rats ***(5)***. The regenerating afferent lymphatics anastomose within 6 wk with the efferent vessels that connect the thoracic duct. Consequently, a direct influx of afferent lymph from the digestive tract into the thoracic duct occurs without intervening LN. Thus, one can collect nonlymphoid cells in afferent lymph from the whole digestive tract by cannulating the thoracic duct (**Fig. 1**). Rat DC can also be enriched by using Metrizamide ***(6)*** or Nycodenz

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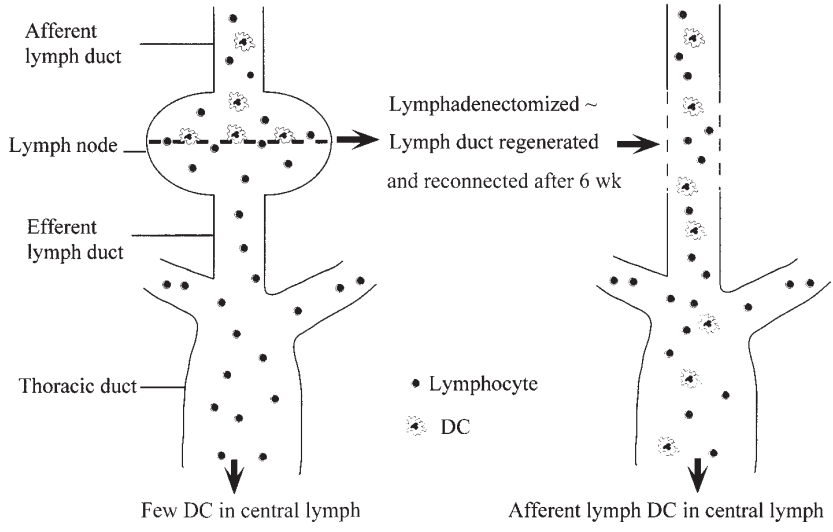

Fig. 1. The principle of lymphadenectomy to collect afferent lymph DCs from the central thoracic duct lymph.

(7–9) density gradient separation. We describe a method to collect and isolate rat DCs in hepatic or intestinal lymph by selective lymphadenectomy ***(10)*** and also to collect particle-laden DCs in the hepatic lymph that appear after intravenous injection of particulates ***(11,12)***. In addition, a method for the adoptive transfer assay of purified DCs into allogeneic or syngeneic rats and following their migratory behavior in the blood ***(12,13)*** is also described.

Second, immunocytochemical and histochemical methods for the identification of DCs and for examining *in situ* immune responses elicited by migrating DCs are presented. Specific staining of DC is not so easy, because few useful monoclonal antibodies (Mabs) that exclusively recognize DC are available ***(14)***. DCs constitutively express major histocompatibility class II antigen (MHC II) ***(1)***, which is, however, also expressed by B cells, some T cells, and macrophages. Thus, discrimination of DCs from other cell types is sometimes very difficult solely by MHC II immunostaining. If T cells, B cells, and macrophages are immunostained with lineage-specific marker prior to MHC II staining, cells that are singly positive for MHC II represent mostly the DC population. Therefore, cytosmears or cryosections are double immunostained by combining the indirect alkaline phosphatase (ALP) and peroxidase (HRP) methods ***(15)***. Immunostaining with Mab OX62, specific for a rat DC subpopulation ***(14)***, is especially useful for analyzing the spatial relationship

between DC and other cell types by double or triple immunostaining ***(11)***. For the immune response *in situ*, rosette formation of DCs with T cells and proliferative response of T cells to alloantigen presented by DCs can be studied by double immunostaining ***(12)***.

Third, an *in situ* cell binding assay for the analysis of selective migration of DCs is described. This method was reported previously as a tissue-binding reaction, which lead to the first discovery of an adhesion molecule, L-selectin, as a lymphocyte homing receptor ***(16)***. This should be applicable for examining the capability of DCs to bind via adhesion molecules to the vasculature or other tissue components.

2. Materials

2.1. Isolation of Migrating DCs in Afferent Lymph

1. Animals: Inbred male DA rats (MHC haplotype $RT1A^aB^a$) and Lewis rats ($RT1A^lB^l$) are used. Rats are reared under specific pathogen-free conditions to reduce background proliferating activities in the lymphoid tissues.
2. Density gradient medium: For preparing 15% (w/v) Metrizamide (M3383, Sigma, St Louis, MO) solution, dissolve 15 g of Metrizamide in phosphate buffered saline (PBS) supplemented with 0.1% bovine serum albumin (BSA, Sigma; A3311) to make 100 mL. Sterilize by filtration through 0.45 μm filter. The solution can be stored at 4°C for up to 6 mo.
3. Paramagnetic latex particles: Paramagnetic latex microspheres (0.8 μm diameter, 10% solid, L0898) are purchased from Sigma. First sonicate them lightly and resuspend in PBS supplemented with 1% fetal calf serum (FCS). Wash twice by magnetic attraction using a magnetic cell separator (Dynal, Oslo, Norway; MPC-1) and by removal of the supernatant and resuspension with the same buffer. For injection, adjust the concentration of latex to 0.25 mL of original suspension/100 g body weight.

2.2. Immunocytochemical and Histochemical Analysis of DCs In Situ

2.2.1. Specimen Preparation

1. For labeling proliferating cells with a thymidine analog 5-bromo-2'-deoxyuridine (BrdU), prepare BrdU solution in saline (6 mg/mL, Sigma; B5002) and store 1 mL aliquots frozen at –20°C for up to 6 mo. For use, thaw and mix well by vortexing.
2. Cryomolds (disposable vinyl specimen molds, Tissue-Tek, Miles Inc, Elkhart, IN; 4565, 4566 or 4557). OCT embedding compound (Tissue-Tek; 4583).
3. Poly-L-lysine-coated slides (Sigma; P0425) (*see* **Note 1**). Water-repellent pen (DAKO pen red, DAKO, Carpinteria, CA; S4002).
4. For formol calcium solution ***(17)***, dissolve 20 g paraformaldehyde (Merck, Darmstadt, Germany; 4005) in 400 mL distilled or deionized water (DW) with 200 μL of 1 *N* NaOH by heating and stirring (60–70°C). After cooling, add

50 mL of 10% $CaCl_2$ in DW and adjust pH to 7 by adding dropwise 1 *N* NaOH. Add DW to make final 500 mL solution. The solution is stored at 4°C and can be used for 1 mo.

5. For 1% glutaraldehyde solution, add 8 mL of 25% glutaraldehyde solution (Nacalai tesque, Kyoto, Japan; electron microscopic grade, or Sigma; G5882) to 192 mL PBS. The solution is stored at 4°C and can be used up to 6 mo.
6. A blocking solution (Dainippon Seiyaku, Tokyo, Japan; UK-B25, or Sigma; M7409).
7. An aqueous mounting medium (Aquatex, Merck; 1.08562, see Note 2).
8. Prepare PBS-Tween solution by mixing PBS with 0.05% Tween 20 (polyoxyethylene sorbitan monolaurate, Sigma; P1379).
9. For unmasking of BrdU epitope before immunostaining ***(15)***, dissolve pepsin (Sigma; P7012) in 0.01 *N* HCl solution at a final concentration of 0.001–0.01%.
10. For neutralization of specimens after HCl treatment, prepare borate buffer by dissolving 19.07 g of $Na_2B_4O_7 \cdot 10H_2O$ (Sigma; B9876) in 400 mL DW and adjust pH to 8.5 with 6 *N* HCl. Add DW to make up final 500 mL.

2.2.2. Antibodies

Mouse monoclonal antibodies (Mabs) and polyclonal antibodies specific for rat determinants ***(10–12,14,18–20)***, and enzyme-labeled secondary antibodies are listed in **Table 1** (*see* **Note 3**). Just before use, dilute antibody to optimal working concentration (usually 5–50 μg/mL) with PBS containing 0.2% BSA (Sigma; A7888) and 0.05% NaN_3 (Sigma; S2002) (*see* **Note 3**). For secondary antibodies, try to use those preadsorbed with rat serum proteins (serum proteins of the species used for target specimens) to minimize their cross-reactivity to rat immunoglobulin (Ig). Dilute secondary antibodies with 1% heat-inactivated normal rat serum and 0.2% BSA in PBS. Rat serum is necessary to adsorb cross-reactivity, which may be present even in the preadsorbed secondary antibodies. Heat normal rat serum for 30 min at 57^0C in water bath incubator to inactivate complement, which may cause a false-positive staining of antibodies.

2.2.3. Substrates for HRP Reaction

1. DAB (brown chromogen; ***15***):
   a. Dissolve 250 mg of DAB (3,3'-diaminobenzidine tetrahydrochloride, WAKO Pure Chemical Industries, Osaka, Japan; 349-00903) in 50 mL Tris-buffered saline, pH 7.5 (5 mg/mL). Store in small aliquots, 1 mL each in an Eppendorf tube, at –20°C (*see* **Note 4**).
   b. For use, thaw aliquot and mix with 40 mL PBS and 10 μL of 30% H_2O_2.
   c. Incubate sections in the above solution vertically in a Coplin jar (Sigma; S5516) for 10–15 min at room temperature (RT).
   d. Wash slides in tap water. The used substrate solution should be disposed of as toxic waste according to local government regulations.

Table 1
List of Primary and Secondary Antiodies Used

Categories	Clone or Catalog No.	Isotype	Antigen Recognized	Supplier[a]	Reference
Dendritic Cells	OX62	mouse IgG_1	Rat $\alpha_E\beta_7$ Integrin ?	1	***14***
Macrophages	ED1	mouse IgG_1	Rat CD68? (related to phagocytosis)	1	***18***
	ED2	mouse IgG_1	Rat $\alpha_D\beta_2$ Integrin ? (tissue macrophages)	1	***18***
	ED3	mouse IgG_{2a}	Rat sialoadhesin	1	***18***
	TRPM3	mouse IgG_{2a}	Rat pan-macrophages	2	***19***
MHC Antigens	OX6	mouse gG_1	Rat monomorphic MHC II	1	***10***
	MN4-91-6	mouse IgG_1	Rat polymorphic MHC class I ($RT1A^a$-specific)	1	***12***
	OX76	mouse IgG_{2a}	Rat polymorphic MHC class II ($RT1B^a$-specific)	1	
T-cells	R73	mouse IgG_1	Rat T cell receptor $\alpha\beta$	1	***12***
	OX34	mouse IgG_{2a}	Rat CD2	1	***12***
B-cells	HIS14	mouse IgG_1	Rat pan B-cells	3	***20***
	HIS 24	mouse IgG_{2b}	Rat recirculating and follicular B-cells	1	***20***
	MARM4	mouse IgG_1	Rat IgM (pan B-cells, plasma cells)	1	***12***
Proliferating Cells	85-2C8	mouse IG_1	5-bromo-2'-deoxyuridine (BrdU)	4	***12***
Tissue Framework	LB-1403	rabbit IgG	mouse type IV collagen (reacts also with rats)	5	***11***
HRP-labeled Antibodies	P0161	rabbit IgG	mouse IgG (whole molecule)	6	
	#55693	goat $F(ab')_2$	rabbit IgG (whole molecule)	7	
ALP-labeled Antibodies	A9316	goat IgG	mouse IgG (whole molecule)	8	
	A4812	sheep $F(ab')_2$	mouse IgG (whole molecule)	8	

a1: Serotec Ltd., Kidlington, England. 2: BMA Biomedicals AG, Augst, Switzerland. 3: Kindly donated from Dr. F. G. M. Kroese, Groningen Univ., The Netherlands. 4: Novocastra, Newcastle upon Type, England. 5: Cosmo Bio, Tokyo, Japan. 6: DAKO. 7: Cappel, Aurora, OH. 8: Sigma.

e. The DAB reaction product is alcohol insoluble and can be mounted in either nonaqueous or aqueous media.

2. 4-Chloro-1-Naphthol (dark violet chromogen; ***21***):
   a. Prepare small aliquots of 6 mg each of 4-chloro-1-naphthol (Sigma; C-8890) in Eppendorf tubes and store at –20°C.
   b. For use, thaw and dissolve aliquot in 100 µL of absolute ethanol.
   c. Add it to 50 mL of 0.1 *M* Tris-HCl, pH 7.4, and stir for 30 min at RT.
   d. Filter and add 10 µL of 30% H_2O_2.
   e. Incubate sections in the above solution vertically in Coplin jar for 15 min at RT.
   f. Wash in tap water. Used substrate solution should be disposed of as toxic waste according to local government regulations.
   g. Mount in an aqueous mounting medium such as Aquatex (Merck) because the reaction product is alcohol soluble (*see* **Note 5**).
3. Modified tetramethylbenzidine (black chromogen; ***11,22***):
   a. Because this substrate provides 10–50 times higher sensitivity than typical DAB preparations, the primary and secondary antibodies must be diluted accordingly, usually three to five times more dilute than those used for the DAB method.
   b. Incubate sections horizontally in a moist chamber for 5 min with a substrate kit (True Blue, KPL. Inc., Gaithersburg, MD).
   c. Wash in DW several times.
   d. Incubate sections with 5 mg DAB in 40 mL PBS with 0.02% $CoCl_2$ (Sigma; C2644) and 0.01% H_2O_2 vertically in a Coplin jar for 5 min. Do not develop too long, because this results in blackening of the whole sections.
   e. Wash in DW several times.
   f. Mount in aqueous media (*see* **Note 5**). Used substrate solutions should be disposed of as toxic waste according to local government regulations.
4. Amino-ethyl carbazole (red chromogen):
   a. Incubate sections horizontally in a moist chamber with a substrate kit (DAKO; K3464) for 5–15 min at RT.
   b. Wash in DW several times. Used substrate solution should be disposed of as toxic waste according to local government regulations.
5. Enhancing reagents for HRP substrate reaction:
   a. $CoCl_2 \cdot 6H_2O$ (Sigma; C2644): add 0.5 mL of 1% solution in the above DAB solution ***(23)***. Color becomes bluish black.
   b. $NiCl_2 \cdot 6H_2O$ (Sigma; N5756): add 0.5 mL of 1% solution in the above DAB solution ***(23)***. Color becomes purplish blue.
   c. Imidazole (Sigma; I2399): add 0.01 *M* in the above DAB solution ***(14)***. Color becomes dark brown.

2.2.4. Substrates for Alkaline Phosphatase (ALP) Reaction ***(17)***.

ALP can yield red, black, or blue color with ALP substrate kit I, II, or III (Vector Laboratories Inc., Burlingame, CA), respectively. Briefly, for kit I (Vector Red), incubate sections with substrate solution for 20–30 min at RT

horizontally in a moist chamber in the dark (black box). For kit III (Vector Blue), similarly incubate sections with the substrate solution for < 10 min (*see* **Note 6**). Wash the sections well in tap water for > 20 min to reduce nonspecific precipitates. Used substrate solution should be disposed of as toxic waste according to local government regulations.

2.3. In situ *Cell Binding Assay*

The medium for DC suspension is RPMI 1640 (Gibco-BRL, Grand Island, NY) containing 5% FCS and 5 m*M* HEPES (Sigma; H0887).

3. Methods

3.1. Isolation of Migrating DC in Afferent Lymph

3.1.1. Selective Lymphadenectomy and Thoracic Duct Cannulation of the Rat

(These procedures are also discussed in Chapter 4). In rats, celiac LN (**Fig. 2**) are the regional LN of the upper abdominal organs including the liver, stomach, and spleen (*see* **Note 7**). Celiac lymphadenectomy (HX) can be performed using blunt dissection of the nodes from 5- to 7-wk-old rats with a stereomicroscopic aid.

1. Under ether anesthesia, shave the abdominal skin with electric clippers and swab with 70% ethanol. Make a midline incision through the abdominal wall.
2. Excise the gastrosplenic ligament and pull the stomach upward.
3. Locate the celiac nodes as a group of four to six nodes on or beneath the trifurcation of the celiac artery where the common hepatic, gastric, and splenic arteries arise (**Fig. 2**).
4. Remove the nodes using fine forceps and a cotton swab (*see* **Note 8**). Press bleeding sites with cotton swab for several min, usually the bleeding is minimal and will stop soon.
5. Close the abdomen by suturing the muscle and skin with a 2/0 braided silk thread.

For collection of DCs in the intestinal lymph, perform a modified mesenteric lymphadenectomy (MX) as previously described ***(5)*** but leave the celiac nodes intact (**Fig. 2**).

For thoracic duct cannulation, the rats are allowed to recover for more than 6 wk, then thoracic duct leukocytes (TDL) are obtained by a routine thoracic duct cannulation (24). In principle, insert a polyethylene cannula (PE50, OD 1 mm, Becton Dickinson, Sparks, MD; 427411) into the abdominal part of the thoracic duct just before it penetrates the diaphragm and put the rat into position in a Bollman restraining cage ***(24)*** for collection of TDL.

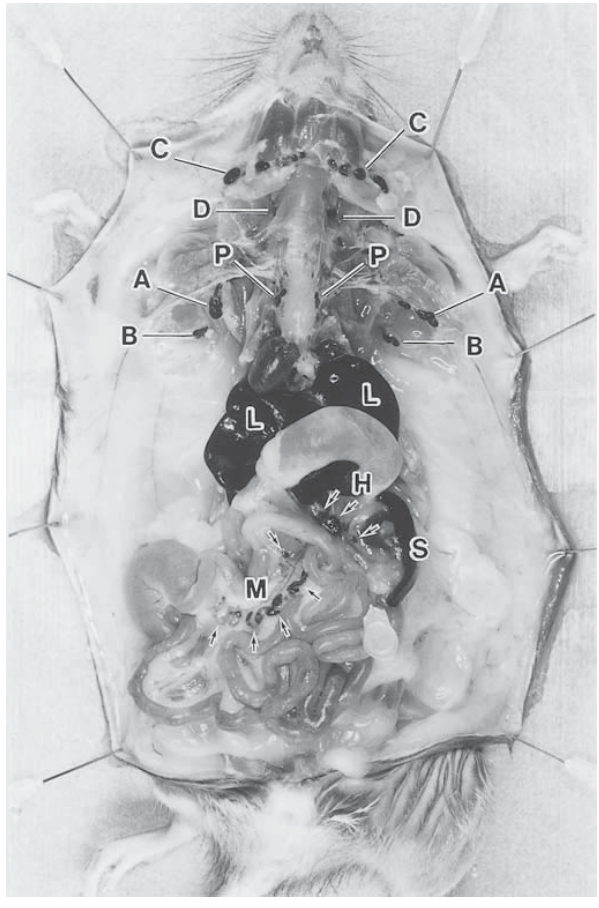

Fig. 2. Distribution of major LN in the dissected adult DA rat demonstrated by the injection of Rotring ink (*see* **Note 7**). A: axillary LN, B: brachial LN, C: superficial cervical LN, D: deep cervical LN, H (arrows): celiac LN, L: liver, M (arrows): mesenteric LN, P: parathymic LN, S: spleen.

3.1.2. Isolation of DC by Density Gradient Centrifugation

When adult male DA rats are used, the interface of HX-TDL and MX-TDL obtained by the first overnight collection (16 h) contains DC with 72% and 67% purity, and the absolute number of DC in the fractions are 4.8×10^5/rat and 7.2×10^5/rat, respectively (**Fig. 3**). Contaminating cells are mainly lymphocytes and pan B^- $CD5^-$ MHC II^- monocytic cells.

1. Collect TDL overnight at RT in 5 mL PBS containing 150 IU heparin (*see* **Notes 9** and **10**).
2. After spinning down (*see* **Note 11**), resuspend TDL in PBS with 0.1% BSA and overlay 1 to 2 × 10^8/2.5 mL onto 4 mL of 15% Metrizamide in a 15 mL conical centrifuge tube and centrifuge at 400*g* for 30 min ***(6)*** (*see* **Note 12**).
3. Collect the interface cells and wash twice in the same medium.

3.1.3. Isolation of Paramagnetic Latex-Ingesting DCs by Magnetic Attraction

Relatively immature DCs that have ingested paramagnetic latex are isolated from the hepatic lymph after intravenous administration of particulates ***(11,12)***. The purity of latex-laden DC is usually 80–90% with a viability of more than 95%. Contaminating cells are mainly polymorphonuclear leukocytes.

1. Collect HX-TDL of rats that received an intravenous injection of paramagnetic latex overnight at RT.
2. Clamp 15 mL test tube containing 1 to 3 × 10^8 HX-TDL/mL of PBS supplemented with 1% FCS in the separator and leave for 10 min with occasional gentle agitation.
3. After the supernatant is removed by pipeting, release test tubes from the separator and resuspend the remaining cells in the same medium.
4. Repeat this procedure once and pool the partially purified cells.
5. The pooled cells are subjected to a further round of sorting (*see* **Note 13**).

3.1.4. Adoptive Transfer Assay of Purified DCs

Intravenously transfer 1 × 10^4 to 1 × 10^6 of allogeneic DC or syngeneic DC or 1 × 10^5 to 1 × 10^8 allogeneic unseparated cells to hosts. Sacrifice host rats immediately, and at 1, 2, 3, 4, 5, and 7 d after cell transfer, excise the tissues and fresh-freeze them. Give host rats an intravenous injection via the tail vein of BrdU (2 mg/100 g body weight) 1 h prior to sacrifice. Detect transferred DC by immunostaining for donor type MHC class I or II antigen (*see* **Subheading 3.2.7.**) and by the existence of latex particles when paramagnetic latex-laden DCs are used. Host proliferative responses induced by transferred DC can be studied with respect to the organ specificity, dose dependence on donor cells, host cell types, time kinetics, and the phenotype of proliferating cells (*see* **Subheadings 3.2.8.** and **3.2.9.**).

3.2. Immunocytochemical and Histochemical Analysis of DCs In Situ

3.2.1. Specimen Preparation

For cytosmear preparation, adjust cell concentrations to 1 to 30 × 10^5/mL in PBS with 30% FCS and smear onto poly-L-lysine-coated microscopic slides by a cytocentrifuge.

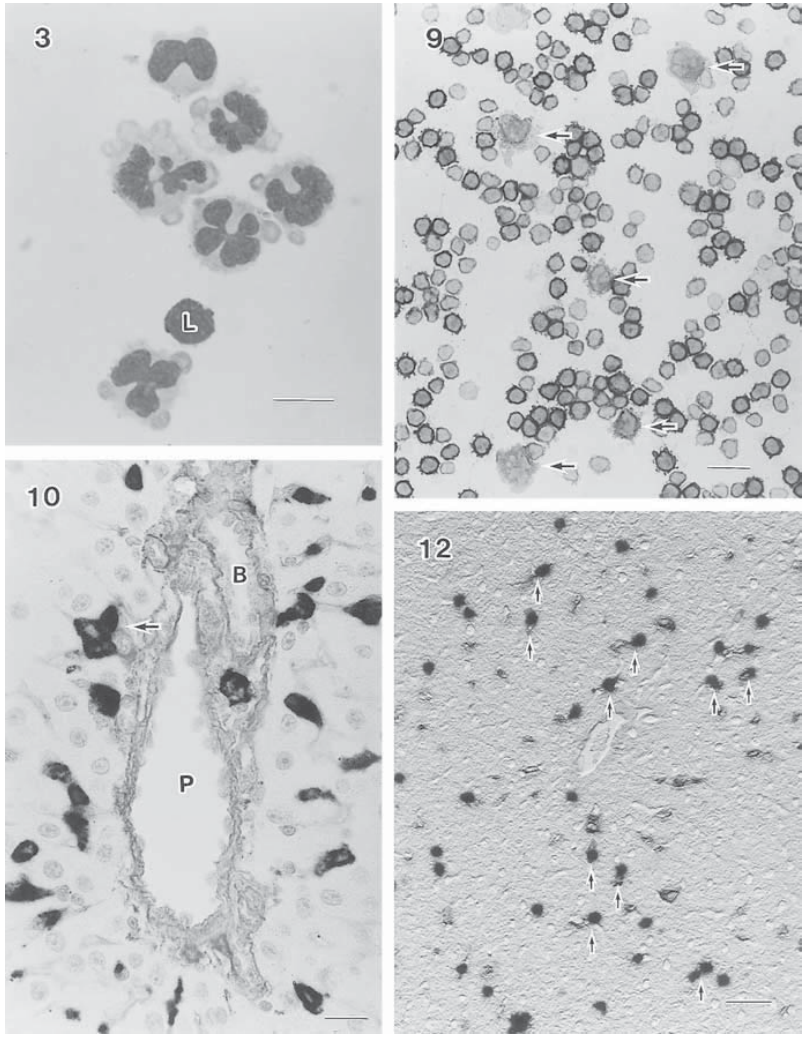

Fig. 3 Cytosmears of a Metrizamide fraction of HX-TDL ***(10)***. May-Grünwald Giemsa stain. Most cells have typical morphology of DC with a clover-leaf or flower-like nucleus with many cytoplasmic veils. L: contaminated lymphocyte. Bar = 10 μm.

Fig. 9. Double immunostaining of a cytosmear of HX-TDL with a cocktail of antipan B cells and anti-CD5 Mabs, then with anti-MHC II MAb ***(10)***. Typical DC can be readily determined as brown cells (arrows), whereas B and T cells are recognized as either blue–brown or blue cells. Bar = 20 μm.

For immunohistochemical study, a paraffin section does not work because of antigen masking or loss during preparation. Prepare and process fresh cryosections without prefixation as below:

1. Remove fat tissues as much as possible from target tissues since fat does not freeze at –18°C, which makes sectioning difficult. Never pinch the target tissues with forceps, which causes destruction of tissues and results in poor morphology.
2. Trim tissue blocks suitable for sectioning and place into cryomolds filled with OCT compound. Different tissues can be grouped in one mold so that several tissues can be examined at once (**Fig. 4**).
3. Place the cryomold onto the surface of liquid nitrogen in a glass beaker, but do not immerse the specimen completely to prevent boiling. Take out when about 80% of tissues in OCT compound is frozen. Then quickly widen the mold horizontally by pulling the edges with both hands several times by which cracking of frozen tissues can be avoided (**Fig. 5**).
4. Prepare cryostat sections at a thickness of 4–8 μm and put them onto poly-L-lysine-coated slides (*see* **Note 14**).
5. Both cytosmears and cryosections are air-dried for several hours or overnight at RT (*see* **Note 15**).
6. Fix slides in pure acetone for 10 min at RT in a Coplin jar and air-dry for 5 min.
7. Encircle smears and sections with a water-repellent pen (DAKO) and air-dry for 15 min. This is to prevent spread of antibody solution over the slide and make wiping off of liquids around the sections unnecessary (**Fig. 6**).
8. Rehydrate samples in Tris-buffered saline for 10 min and fix in formol calcium at RT for 1 min, then wash in PBS three times (*see* **Note 16**).
9. Rinse in PBS-Tween (*see* **Note 17**), then apply a blocking solution onto samples and decant after 10 min when samples are ready for the antibody reaction in the next session.
10. By using fresh cryosections as target specimens, endogenous HRP activity is considerably reduced and mostly found in eosinophilic leukocytes. In cases where specimens contain too many eosinophils to discriminate them from the specific staining, eliminate the endogenous HRP activity after Kelly et al. ***(25)***.

Fig. 10. (*opposite page) T*riple immunostaining of the normal liver with Kupffer cells ($ED2^+$), DC ($OX62^+$) and tissue framework (antitype IV collagen) ***(11)***. In the portal area, several $OX62^+$ cells are seen in the connective tissue surrounding the portal triads. Note a close association between DC and Kupffer cell (arrow). B: Bile ducts, P: portal vein. Bar = 20 μm.

Fig. 12.. *(opposite page) In situ* cell binding assay followed by double immunostaining with $RT1A^a$ and ED2 ***(12)***. Associations of bound DCs with Kupffer cells are frequently observed (arrows). Bar = 40 μm.

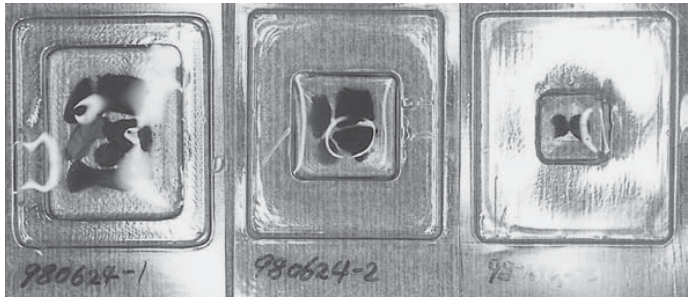

Fig. 4 A layout example of group of tissues in cryomolds filled with OCT compound.

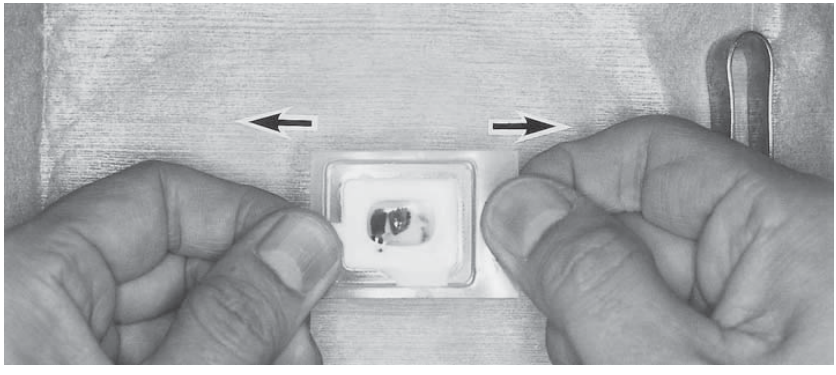

Fig. 5. A cryomold is frozen to about 80%, then widened with both hands (arrows).

3.2.2. Antibody Reactions for One to Three Color Immunostaining

1. After the blocking, incubate the samples with the first Mab or a cocktail of several Mabs for 1 h at RT in a moist chamber (**Fig. 7**, *see* **Note 18**) and wash three times in PBS (**Fig. 8**, *see* **Note 19**).
2. Rinse in PBS-Tween, then apply an ALP-labeled goat Ig to mouse Ig (1:120, A9316, Sigma) or HRP-labeled rabbit Ig to mouse Ig (1:120, P0161, DAKO) and incubate for 40–60 min at RT (*see* **Note 20**). Wash three times in PBS.
3. Fix in 1% glutaraldehyde for 30 s and wash in DW (*see* **Note 21**).
4. Develop ALP or HRP activity by using an ALP substrate kit or an HRP substrate solution, respectively. Wash in tap water for > 20 min, then in DW.
5. Rinse smears in PBS-Tween, then apply blocking solution, incubate for 10 min and decant.
6. Apply the second Mab and incubate for 1 h at RT. Wash three times in PBS.

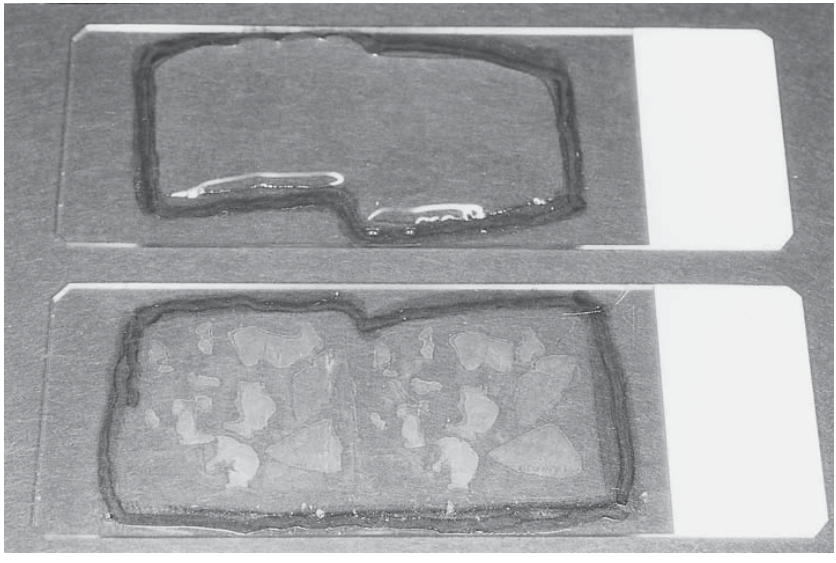

Fig. 6. Specimens encircled with a water-repellent pen (black line) by which reagents do not spread over the slide.

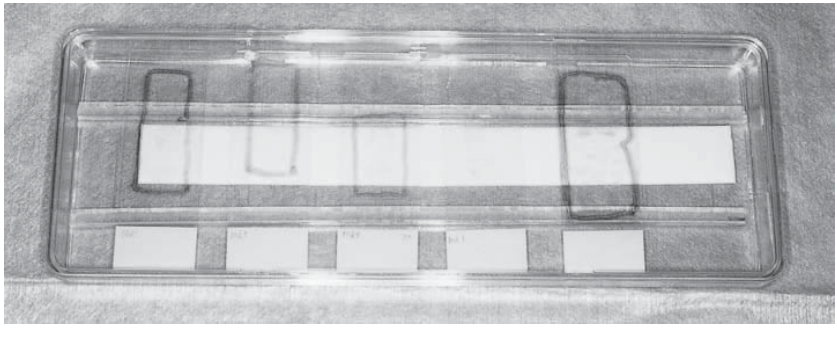

Fig. 7. An example of a moist chamber (see Note 18).

7. Rinse in PBS-Tween, then apply an HRP-labeled rabbit Ig to mouse Ig (1:120, P0161, DAKO) or an ALP-labeled goat Ig to mouse Ig (1:120, A9316, Sigma) and incubate for 40–60 min at RT. Wash three times in PBS.
8. Develop HRP or ALP activity by using an HRP substrate solution or an ALP substrate kit, respectively. Wash in tap water for 20–30 min, then in DW.

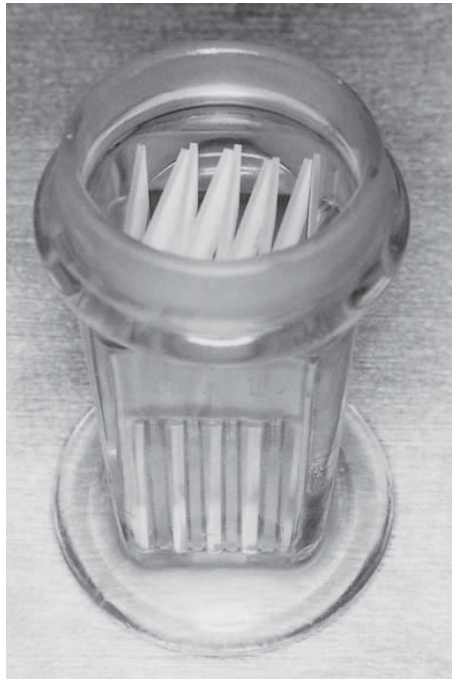

Fig. 8. A Coplin jar.

9. Rinse smears in PBS-Tween, then apply a rabbit polyclonal antibody to mouse type IV collagen (1:1000, Cosmo Bio) and incubate for 1 h at RT. Wash three times in PBS.
10. Rinse in PBS-Tween, then apply an HRP-labeled goat $F(ab')_2$ to rabbit Ig (1:120, Cappel) and incubate for 40–60 min at RT. Wash three times in PBS.
11. Develop HRP activity by using DAB substrate solution. Wash in tap water for 5 min, then in DW.
12. Fix again in 1% glutaraldehyde in PBS for 10 min and wash (*see* **Note 21**). Counterstain with Meyer's hematoxylin and wash in tap water for 5 min (*see* **Note 22**). Mount in Aquatex.

3.2.3. Antibody Reactions Including BrdU Immunostaining **(12,15)**

1. Give host rats an intravenous injection via tail vein of BrdU (2 mg/100 g body weight) 1 h prior to sacrifice.
2. Process target tissues as described in **Subheading 3.2.1.** and immunostain for the first Mab(s) as described in **Subheading 3.2.2.**

3. Fix in 1% glutaraldehyde for 10 min at RT and wash in DW. This is to prevent digestion of sections by pepsin.
4. Digest sections with pepsin, 0.001–0.01% in 0.01 *N* HCl for 10 min at 37°C. Wash in tap water for 5 min. The concentration of pepsin should be determined individually by comparing the intensity of BrdU staining and morphology.
5. Treat sections in 4 *N* HCl solution for 30 min at RT. Wash in tap water for 5 min, then in DW. Neutralize sections with borate buffer (0.1 *M*, pH 8.5) for 3 min.
6. Wash sections twice in PBS-Tween, then apply blocking solution. Decant after incubation for 10 min.
7. Apply a mouse MAb to BrdU (1: 200, Novocastra) and incubate for 1 h at RT. Wash three times in PBS.
8. Rinse in PBS-Tween, then apply an ALP-labeled second antibody to mouse Ig (sheep $F[ab']_2$, 1: 60, A-4812, Sigma) and incubate for 1 h at RT (*see* **Notes 20** and **23**). Wash three times in PBS.
9. Develop ALP activity by using ALP substrate kit I (Vector Red) for < 30 min. Wash in tap water for > 20 min, then in DW (*see* **Note 6**).
10. Lightly counterstain with Meyer's hematoxylin, if necessary (*see* **Note 22**) and mount in Aquatex.
11. The immunostaining techniques described in **Subheadings 3.2.1.**, **3.2.2.**, and **3.2.3.** are applied to various studies as below (*see* **Note 24**).

3.2.4. Identification of DC on Cytosmears by Double Immunostaining **(10)**

Immunostain first with a cocktail of Mabs, HIS14, and OX19 (ALP, blue) and second with OX6 (HRP, brown). HIS14 can be replaced by commercially available HIS24 (*see* **Table 1**). Consequently, typical DC can be easily determined as brown cells (pan B^- $CD5^-$ MHC II^{high+}) (**Fig. 9**), whereas B and T cells are either blue–brown or blue cells (pan B^+ MHC II^+, $CD5^+$ MHC II^+ or $CD5^+$ MHC II^-).

3.2.5. Identification of DC on Cryosections by Double Immunostaining

Immunostain first with a cocktail of Mabs, HIS24, OX19, and TRPM3 (ALP, blue) and second with OX6 (HRP, brown). Typical DC can be easily determined as brown cells (pan B^- $CD5^-$ $macrophage^-$ MHC II^{high+}), whereas B cells, T cells, and macrophages are either blue–brown or blue cells (MHC II^+ or MHC II^-).

3.2.6. Analysis for a Spatial Relationship Between DC and Other Cell Types by Triple Immunostaining **(11)**

Immunostain first with a cell-lineage specific MAb (HRP, black) and second with OX62 (ALP, red). Stain further with the third rabbit antibody to type IV collagen (HRP, brown) (**Fig. 10**).

3.2.7. Immunostaining for Transferred DCs and Host T Cells In Situ **(12)**

Immunostain first with donor type MHC antigens (ALP, blue), and second with a cocktail of mabs to CD2 and TcRαβ (HRP, brown).

3.2.8. Immunostaining for Proliferative Response (BrdU-Positive Cells) In Situ **(11,12,15)**

Immunostain BrdU (ALP, red) as described in **Subheading 3.2.3.**

3.2.9. Immunostaining for Phenotype of Proliferating (BrdU$^+$) Cells In Situ **(12,15)**

Immunostain first with lineage-specific Mabs, either T-cells, B-cells or macrophages (ALP, blue) and second with anti-BrdU (ALP, red, *see* **Subheading 3.2.3.**).

3.3. In Situ Cell-Binding Assay

1. Prepare fresh cryosections (6 μm) of liver and other tissues of either Lewis or DA rats, circle with DAKO pen, and air-dry for 1–4 h at RT. Sections can be preserved in air-tight box at –20°C up to several mo. Either latex-laden DC, mature DC, or unseparated cells from lymph of DA rats are resuspended at a concentration of 1×10^6/mL (*see* **Subheading 2.3.**).
2. Binding of DC to frozen sections is studied with a slight modification of previous reports ***(16,26)***. Rehydrate a target cryosection and overlay with 50 μL of cell suspension and incubate horizontally at either 4°C, 20°C, or 37°C in a moist chamber for 30 min with horizontal rotation at 80 rpm. DC binding to Kupffer cells is temperature independent ***(12)***. Aspirate carefully cell suspensions, wash very gently in PBS, and then fix samples in formol calcium solution for 3 min. After washing very gently in PBS, fix further with 1% glutaraldehyde for 1 min at RT and mount in Aquatex (*see* **Note 24**, **Fig. 11**). Sections can be directly examined under a differential interference light microscopy or can be further immunostained.
3. Sections can be double immunostained for RT1A^a ($MN_{4\text{-}91\text{-}6}$, ALP, blue black) and ED2 (HRP, brown) to detect allogeneic DC and Kupffer cells, respectively (*see* **Subheading 3.2.2.**, **Fig. 12**).

4. Notes

4.1. Reagents for Immunostaining

1. Microscopic slides should be coated with either poly-L-lysine or silane to prevent detachment. They are available from Sigma, DAKO, or other companies.
2. For an aqueous mounting medium, Aquatex is recommended because it hardens quickly after several h and a coverglass can be stabilized soon. Free-floating coverglasses result after mounting in ordinary aqueous media and can easily destroy the specimens.

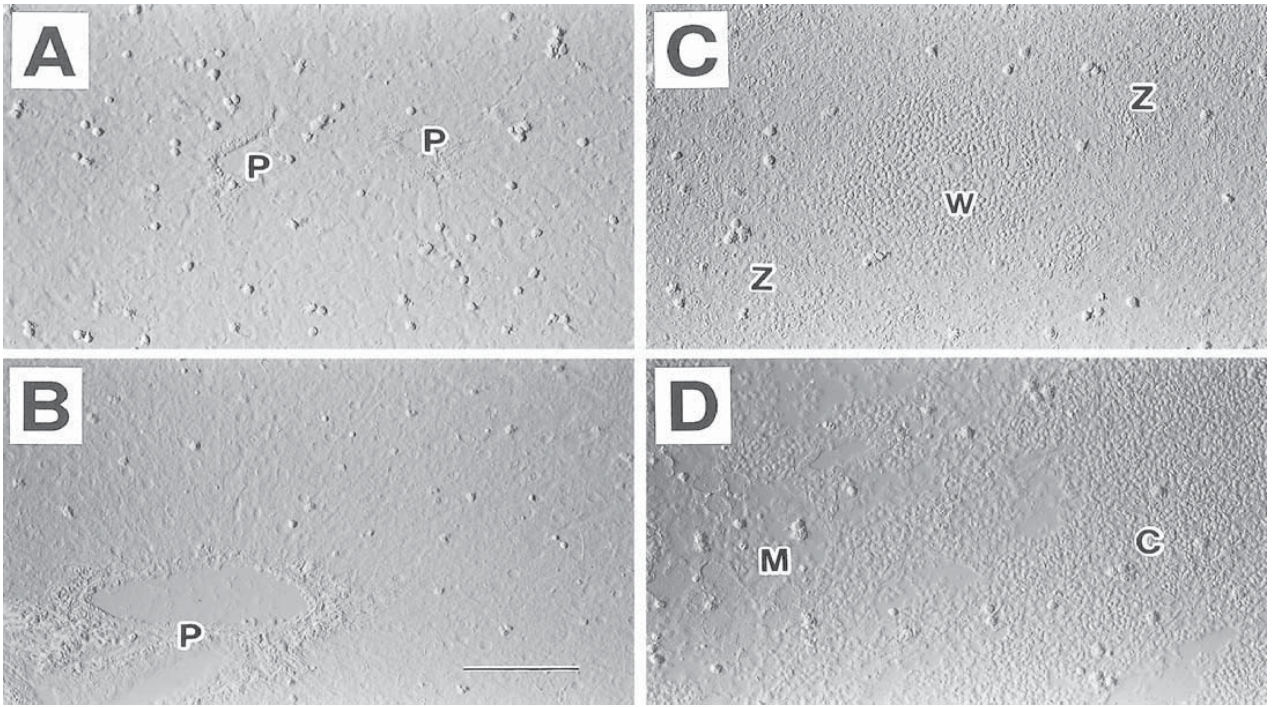

Fig. 11. *In situ* cell binding assay examined under differential interference light microscopy. DC show preferential binding to liver cryosections **(A)** compared with other tissues **(C,D)** ***(12)***. In spleen **(C)**, DC attach mainly to the marginal zone (Z) but not to the white pulp (W). In LN **(D)**, bound DC are not obviously localized to specific areas. The same concentration of unseparated cells show less binding to the liver cryosections than DCs **(B)**. P, portal area; M, medulla; C cortex. Bar = 100 μm.

3. Most Mabs in ascites fluid or purified Ig solution may be stored in small aliquots, 10–100 μL each as stock solutions at –80°C for up to several years. Mabs in culture supernatant can be stored at 4°C for usually up to 1–2 yr when 0.05% NaN_3 is added. For working (optimal) concentration, dilute each Mab to the lowest concentration that gives the intense specific staining comparable to other thicker solutions. This is important for reducing nonspecific and background staining.
4. A DAB reagent from WAKO is recommended because it is highly purified and the solution has no autocatalysis reaction, being colorless during substrate reaction.
5. Chloronaphthol and TMB reaction products are light sensitive and the colors fade in a few days. So photomicrographs should be taken within a few days after preparation.
6. For ALP reaction, adjust incubation time by examining the intensity of reaction product intermittently under light microscopy. If the intensity is not strong enough, wash specimens and repeat incubation in a newly prepared substrate solution. Do not incubate too long, < 10 min for Vector Blue and < 30 min for Vector Red, otherwise dirty precipitates appear. Wash specimens well in tap water for > 20 min (to overnight) to reduce undesirable precipitates.

4.2. Isolation of DCs in Afferent Lymph

7. Identification of LN *in situ* ***(27)*** is not easy for beginners. To become familiar with their locations *in situ*, LN can be readily demonstrated by the intravenous injection of Rotring ink (0.5 mL of 40% [v/v] suspension in saline/100 g body weight, Rotring, Germany; 5901017), 16 h before examination. Dialyze Rotring ink against saline overnight to remove toxic preservatives before use. These carbon particles preferentially deposit in the high endothelial venule of lymphoid tissues by which LN become apparent as a black dotted mass (**Fig. 2**).
8. For HX, all celiac nodes are removed, because hepatic peripheral lymphatics (hepatic ducts) tend to reconnect with remnant LN, if any, after the interruption of lymphatics ***(10)***.
9. For TDL collection, rats are subcutaneously and intraperitoneally injected with 10–15 mL saline and fed 4.5% NaCl solution in their drinking water to increase lymph flow. By this, thoracic duct lymph flow increases to around 1 mL/h. Injection of heparin into rats should be avoided because it causes blood contamination in TDL and facilitates clotting of lymph.
10. Intravenous injection of lipopolysaccharide (approx 50 μg/rat, Sigma; L2262) at the time of the cannulation of MX rat is reported to increase DC output 8–15 times without functional deterioration ***(28)***.
11. TDL suspension should be spun down intensely (1200 rpm for 15 min) because DCs are low density and easily lost in the supernatant during centrifugation.
12. Since Metrizamide itself may affect the maturation and differentiation of monocytes ***(29)***, Nycodenz as an alternative gradient material has been proposed ***(7)***. Nycodenz (Nycomed Pharma, Oslo, Norway, analytical grade) can be used in a similar fashion to Metrizamide. Briefly, after washing, TDL (approx 1×10^8/3 mL)

are overlaid onto 5 mL of 14.5% Nycodenz in a 15 mL conical centrifuge tube and centrifuged at 500*g* for 20 min at RT ***(7–9)***.

13. Isolation of DC from the other organs: Enzyme digestion with/without culture overnight then density gradient separation is performed ***(8,9)***. Briefly, mince and digest the lymphoid tissues in culture medium containing collagenase and DNAse (150 U/mL collagenase type III, C0255; 10 U/mL DNAse type I, D4527; both from Sigma) for 30–90 min at 37°C. Culture the collected cells overnight (5 to 8 × 10^6/mL in RPMI1640 supplemented with 10% FCS) and enrich for DCs using Nycodenz gradient ***(8)***.

4.3. Immunostaining

14. For sectioning, fresh frozen tissues are easier to cut with a cryostat than prefixed tissues, and the antigen preservation is also better. Keep temperature of cryostat chamber at –18°C, which is ideal for sectioning of lymphoid and many other tissues.
15. Cytosmears and cryosections should be air-dried enough, because it prevents sections from detaching during staining procedure, e.g., overnight at RT. Specimens can be preserved in air-tight boxes up to 1 year at –20°C or up to several years at –80°C.
16. Formol calcium fixation improves nuclear morphology a great deal. Avoid mixing formol calcium solution with PBS because calcium phosphate precipitation results.
17. Before applying antibodies or any reagents, rinse slides in PBS-Tween each time. This step decreases the surface tension and reagents can easily spread over the slide surface.
18. Perform every incubation in a moisturized chamber horizontally placed. Never allow specimens to dry out throughout the procedure, which will result in no staining. An example of a self-made moist chamber with a wet filter paper and two plastic bars to hold slides is shown in **Fig. 7**. This plastic chamber (Eiken Kizai, Tokyo, Japan; Sterile No.1 square dish) is originally used for bacterial cultures. Incubation time of antibodies is not so strict and can be prolonged up to several hours at RT or overnight to several days at 4°C. If incubation seemed too long, wash specimens well in PBS-Tween.
19. Pour PBS in a Coplin jar containing specimens, leave 2 min and then decant. Repeat this three times. Two minutes each is enough for washing antibodies or fixatives.
20. For negative controls, omit either the primary or secondary antibodies, which should result in loss of specific staining. Absorb small amount of the secondary antibodies to the filter paper every time after the secondary antibody reaction and drop the substrate solution onto it. Activities of both the secondary antibody and the substrate solution can be inspected by the appearance of color reaction products.
21. 1% glutaraldehyde fixation hardens the specimens, thus preventing destruction of specimens during the staining procedure, especially the DAB substrate reaction.

22. For counterstaining, dip slides in Meyer's hematoxylin for several seconds to a few minutes for desired intensity and wash in tap water for longer than 5 min. Hematoxylin fades easily by rinsing sections in 1 *N* HCl and washing in tap water if it is too strong.
23. For BrdU immunostaining, $F(ab')_2$ fragment of ALP-labeled second antibody seems superior to whole Ig possibly because the penetration of antibody molecule into sections may be more difficult with the latter reagent.
24. Select a color combination that produces good contrast. Because the first and second Mabs are of mouse origin in this protocol, the first color should be dark and intense enough to avoid a cross-reactive superimposition of the second color on the first staining. In other words, it is difficult to define double-positive cells using this protocol, although a relationship between two types of cells or phenotype of proliferating cells can be studied by the present technique. Avoid using the ALP systems twice for the double staining because superimposition of ALP substrate reaction is very intense. For the BrdU staining (*see* **Subheading 3.2.9.**), however, the ALP systems can be used twice, because the HCl treatment dissociates antibodies bound to the targets during the first immunostaining.

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References

1. Steinman, R. M. (1991) The dendritic cell system and its role in immunogenicity. *Annu. Rev. Immunol*. **9,** 271–296.
2. Banchereau, J. and Steinman, R. M. (1998) Dendritic cells and the control of immunity. *Nature* **392,** 245–252.
3. Austyn, J. M. (1996) New insights into the mobilization and phagocytic activity of dendritic cells. *J. Exp. Med.* ***183*****,** 1287–1292.
4. Smith, J. B., McIntosh, G. H., and Morris, B. (1970) The traffic of cells through tissues: a study of peripheral lymph in sheep. *J. Anat.* **107**, 87–100.
5. Pugh, C. W., MacPherson, G. G., and Steer, H. W. (1983) Characterization of non-lymphoid cells derived from rat peripheral lymph. *J. Exp. Med.* **157,** 1758–1779.
6. MacPherson, G. G. (1989) Properties of lymph-borne (veiled) dendritic cells in culture. 1. Modulation of phenotype, survival and function: partial dependence on GM-CSF. *Immunology* **68,** 102–107.
7. McLellan, A. D., Starling, G. C., and Hart, D. N. J. (1995) Isolation of human blood dendritic cells by discontinuous Nycodenz gradient centrifugation. *J. Immunol. Methods* **184,** 81–89.

8. Havenith, C. E. G., van Miert, P. P. M. C., Breedijk, A. J., Beelen, R. H. J., and Hoefsmit, E. C. M. (1993) Migration of dendritic cells into the draining lymph nodes of the lung after intratracheal instillation. *Am. J. Respir. Cell Mol. Biol.* **9,** 484–488.
9. Damoiseaux, J. G. M. C., Döpp, E. A., Neefjes, J. J., Beelen, R. H. J., and Dijkstra, C. D. (1989) Heterogeneity of macrophages in the rat evidenced by variability in determinants: two new anti-rat macrophage antibodies against a heterodimer of 160 and 95 kd (CD11/CD18). *J. Leuk. Biol.* **46,** 556–564.
10. Matsuno, K., Kudo, S, Ezaki, T., and Miyakawa, K. (1995) Isolation of dendritic cells in the rat liver lymph. *Transplantation (Baltimore)* **60,** 765–768.
11. Matsuno, K., Ezaki, T., Kudo, S., and Uehara, Y. (1996) A life stage of particle-laden rat dendritic cells in vivo: their terminal division, active phagocytosis, and translocation from the liver to the draining lymph. *J. Exp. Med.* **183,** 1865–1878.
12. Kudo, S., Matsuno, K., Ezaki, T., and Ogawa, M. (1997) A novel migration pathway for rat dendritic cells from the blood: hepatic sinusoids-lymph translocation. *J. Exp. Med.* **185,** 777–784.
13. Austyn, J. M. and Larsen, C. P. (1990) Migration patterns of dendritic leukocytes. *Transplantation (Baltimore)* **49,** 1–7.
14. Brenan, M. and Puklavec, M. (1992) The MRC OX-62 antigen: a useful marker in the purification of rat veiled cells with the biochemical properties of an integrin. *J. Exp. Med.* **175,** 1457–1465.
15. Matsuno, K., Ezaki, T., and Kotani, M. (1989) Splenic outer periarterial lymphoid sheath (PALS): an immunoproliferative microenvironment constituted by antigen-laden marginal metallophils and ED2–positive macrophages in the rat. *Cell Tissue Res.* **257,** 459–470.
16. Stamper, H. B. Jr. and Woodruff, J. J. (1976) Lymphocyte homing into lymph nodes: in vitro demonstration of the selective affinity of recirculating lymphocytes for high-endothelial venules. *J. Exp. Med.* **144,** 828–833.
17. Ezaki, T., Yao, L., and Matsuno, K. (1995) The identification of proliferating cell nuclear antigen (PCNA) on rat tissue cryosections and its application to double immunostaining with other markers. *Arch. Histol. Cytol.* **58,** 103–115.
18. Dijkstra, C. D., Döpp, E. A., Joling, P., and Kraal, G. (1985) The heterogeneity of mononuclear phagocytes in lymphoid organs: distinct macrophage subpopulations in the rat recognized by monoclonal antibodies ED1, ED2 and ED3. *Immunology* **54,** 589–599.
19. Takeya, M., Hsiao, L., and Takahashi, K. (1987) A new monoclonal antibody, TRPM-3, binds specifically to certain rat macrophage populations: immunohistochemical and immunoelectron microscopic analysis. *J. Leuk. Biol.* **41,** 187–195.
20. Kroese, F. G. M., Wubbena, A. S., Opstelten, D., et al. (1987) B lymphocyte differentiation in the rat: production and characterization of monoclonal antibodies to B lineage-associated antigens. *Eur. J. Immunol.* **17,** 921–928.
21. Nakane, P. K. (1968) Simultaneous localization of multiple tissue antigens using the peroxidase-labeled antibody method: a study on pituitary glands of the rat. *J. Histochem. Cytochem.* **16,** 557–560.

22. Rye, D. B., Saper, C. B., and Wainer. B. H. (1984) Stabilization of the tetramethylbenzidine (TMB) reaction product: application for retrograde and anterograde tracing, and combination with immunohistochemistry. *J. Histochem. Cytochem.* **32**, 1145–1153.
23. Hsu, S.-M. and Soban, E. (1982) Color modification of diaminobenzidine (DAB) precipitation by metallic ions and its application for double immunohistochemistry. *J. Histochem. Cytochem.* **30,** 1079–1082.
24. Ford, W. L. (1978) The preparation and labelling of lymphocytes. In *Handbook of experimental immunology,* 3rd ed., (Weir, D. M., ed.), Blackwell Scientific Publications, Oxford, pp. 23.1–23.22.
25. Kelly, J., Whelan, C. A., Weir, D. G., and Feighery, C. (1987) Removal of endogenous peroxidase activity from cryostat sections for immunoperoxidase visualisation of monoclonal antibodies. *J. Immunol. Methods* **96,** 127–132.
26. Austyn, J. M., Kupiec-Weglinski, J. W., Hankins, D. F., and Morris. P. J. (1988) Migration patterns of dendritic cells in the mouse: homing to T cell-dependent areas of spleen, and binding within marginal zone. *J. Exp. Med.* **167,** 646–651.
27. Tilney, N. L. (1971) Patterns of lymphatic drainage in the adult laboratory rat. *J. Anat*. **109,** 369–383.
28. MacPherson, G. G, Jenkins, C. D., Stein, M. J., and Edwards, C. (1995) Endotoxin-mediated dendritic cell release from the intestine. Characterization of released dendritic cells and TNF dependence. *J. Immunol.* **154,** 1317–1322.
29. Kabel, P. J., de Haan-Meulman, M., Voorbij, H. A. M., Kleingeld, M., Knol, E. F., and Drexhage, H. A. (1989) *Immunobiol.* **179**, 395–411.

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Antigen Uptake by Dendritic Cells

Christophe Caux and Bertrand Dubois

1. Introduction

Newly generated dendritic cells (DC) migrate from the bone marrow to the nonlymphoid tissues presumably through the blood stream (peripheral blood DC). During injury, tissue DC (such as Langerhans cells [LC] in the epidermis) capture antigen and then, under microenvironmental signals, leave the nonlymphoid tissues through the afferent lymph as veiled cells (*see* **Fig. 1**). During their migration, DC undergo maturation, including loss of antigen uptake and acquisition of costimulatory function. Then, DC enter into lymph nodes where they home to the T-cell rich area (inderdigitating cells) and induce an antigen specific primary T-cell response.

Although described as professional antigen-presenting cells (APC), DC were considered, until recently, as displaying poor endocytic and phagocytic capacities ***(1,2)***. Yet, LC were shown to perform all steps of receptor mediated endocytosis ***(3)*** and to phagocytose relatively large particles such as latex beads ***(4)***, apoptotic bodies ***(5)***, viruses ***(6)***, bacteria ***(7)***, and intracellular parasites such as *Leismania major* ***(8)***. DC efficiently concentrate extracellular solutes into vacuoles through macropinocytosis ***(9)***. Antigen uptake by afferent lymph DC can also occur in the form of immune complexes ***(10)***. Lastly, DC express surface receptors with multiple lectin domains such as the mannose receptor and DEC205 ***(9,11)***. Such molecules mediate through specific glycan recognition, efficient antigen uptake, and delivery to the MHC class II compartment allowing optimal antigen presentation to $CD4^+$ T-cell clones ***(12,13)***. These membrane lectins are likely to contribute to the uptake of antigens from bacteria that display glycosylation patterns different from mammals, and might therefore represent a first line of discrimination between self and non-self ***(14)***.

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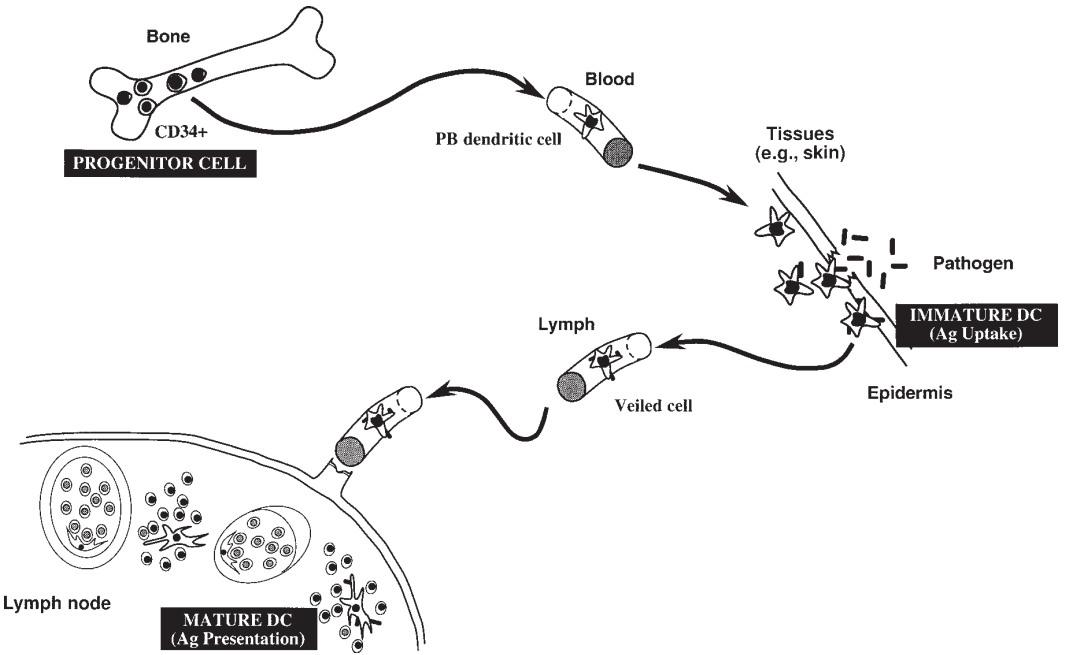

Fig. 1. Life cycle of dendritic cells. Newly generated dendritic cells migrate, from the bone marrow to the nonlymphoid tissues presumably through the blood stream (peripheral blood DC). During an injury, tissue dendritic cells (such as LC in the epidermis) capture the antigen and then, under microenvironmental signals (such as TNFα production in the dermis) leave the nonlymphoid tissues through the afferent lymph (veiled cells). During their migration, DC undergo maturation, including loss of antigen uptake and aquisition of costimulatory function. Then, DC enter into lymph nodes where they home to the T-cell rich area (interdigitating cells) and induce an antigen specific primary T-cell response. Few DC exit lymph nodes possibly because they may either be programmed to die after antigen presentation or destroyed by the afferent immune response.

The form of antigen appears to determine the efficiency with which DC may generate peptides for presentation in association with MHC molecules. In particular, uptake of exogenous antigens from apoptotic bodies allows efficient presentation of MHC class I determinants ***(15)***.

After Ag uptake, during their migration, DC undergo phenotypic and functional changes (*see* **Fig. 2**). Freshly isolated LC are immature DC able efficiently to take up native proteins and to present processed peptides to memory T cells. Conversely, cultured LC and IDC of lymphoid organs are relatively inefficient in Ag uptake, although they have a noticeable capacity to activate naive T cells ***(16–21)***. In this context, epidermal LC express lower levels of class II MHC Ag and accessory molecules than cultured LC and IDC ***(18,22–25)***. Maturation of LC as it occurs in vitro is considered as a physiological event occurring during the in vivo migration of LC from the skin to the draining lymph nodes ***(19,26)***.

Described below are two methods by which the uptake of antigen by DC may be quantified. As outlined above, the phagocytic activity of the DC being studied will depend upon their state of maturity and is therefore dependent on the prior manipulation of the cells. DC derived from any source may be studied with these methods.

2. Materials

1. Medium. RPMI 1640 (Gibco-BRL, Grand Island, NY) supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS) (Flow Laboratories, Irvine, UK), 10 m*M* HEPES, 2 m*M* L-glutamine, 5×10^{-5} M β2-mercaptoethanol, gentamicin (100 μg/mL) (referred to as complete medium).
2. HEPES 25 m*M*.
3. Phosphate-buffered saline (PBS) containing 1% FCS and 0.01% NaN_3.
4. 0.05% Triton X-100 in 10 m*M* Tris buffer, pH 7.4.
5. FITC-dextran: Lysine-fixable fluorescein isothiocyanate (FITC) dextran (Molecular Probes Inc., Eugene, OR).
6. Mannan: from *Saccharomyces cerevisiae* (Sigma).
7. Horseradish peroxidase (HRP, Sigma).
8. ABTS (2.2 azinobis-3-ethylhenthiazoline-6-sulfonic acid) 1 mg/mL in buffer (0.1 *M* citric acid, 0.2 *M* disodium hydrogenophosphate) supplemented with 0.1 μL/mL H_2O_2 (30%).

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where they home to the T-cell rich area (interdigitating cells) and induce an antigen specific primary T-cell response. Few DC exit lymph nodes possibly because they may either be programmed to die after antigen presentation or destroyed by the afferent immune response.

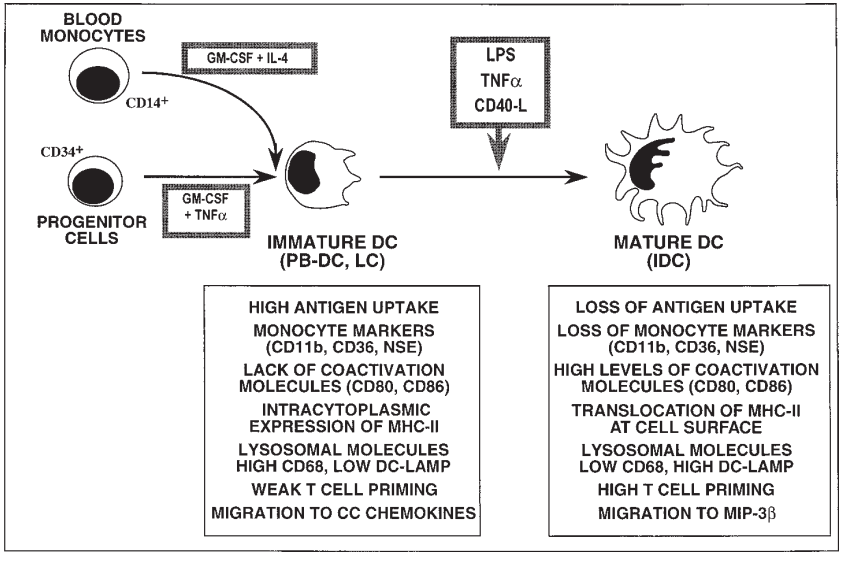

Fig. 2. Coordinated regulation of DC maturation and function. CD40 engagement on immature DC induces changes in morphology, phenotype, and function. CD40 triggering allows DC survival and induces loss of antigen uptake capacity, up-regulation of coactivation molecules, secretion of regulatory cytokines (IL-10, IL-12) and chemokines, changes in lysosomal protein expression (DC-LAMP >> CD68), translocation of MHC class II to the cell surface and up-regulation of the capacity to activate naive T cells. CD40 triggering also induces loss of responsiveness to various CC chemokines (MIP-1α, MIP-3α, RANTES) and induction of migration in response to MIP-3β and 6Ckine related to CCR7 up-regulation. TNFα and LPS induce similar changes in DC functions. The immature stage of DC corresponds to peripheral DC such as LC. The mature stage of DC corresponds to interdigitating cells of secondary lymphoid organs.

9. Monoclonal antibodies: anti-CD1a-PE (Coulter) and anti-CD14-PE (Becton Dickinson).
10. FACScan (Becton-Dickinson, Sunnyvale, CA) for phenotype characterization.
11. 5 mL polypropylene tubes (Falcon, Becton Dickinson).

3. Methods

3.1. Quantitation of Endocytosis Using FITC Dextran Capture and Flow Cytometry

1. Resuspend DC at 5×10^5 cells/mL in complete medium buffered with 25 m*M* HEPES at 37°C in a water bath.

2. Prepare aliquots of 1×10^5 cells in 5 mL polypropylene tubes.
3. Add FITC-dextran at a final concentration of 0.1 mg/mL and incubate separate aliquots in the waterbath for between 5 and 60 min.
4. Add mannan 1 mg/mL to some of the aliquots in order to block the mannose receptor (*see* **Note 1**).
5. Wash the cells four times with cold PBS containing 1% FBS and 0.01% NaN_3 (*see* **Note 2**).
6. If required label the cells with PE conjugated anti-CD1a or anti-CD14 at 4°C for 30 min (*see* **Note 3**).
7. After staining, analyze cells using an FACScan (*see* **Notes 4** and **5**).

3.2. Quantitation of Endocytosis by HRP Capture

1. Resuspend DC 5×10^5 cells/mL in complete medium buffered with 25 m*M* HEPES at 37°C in a water bath.
2. Prepare aliquots of 1×10^5 cells in 5 mL polypropylene tubes.
3. Add HRP, at a final concentration of between 0.1 µg/mL to 0.1 mg/mL for 15 min.
4. Wash the cells four times with cold PBS containing 1% FCS and 0.01% NaN_3.
5. Lyse the cells with 0.05% Triton X-100 in 10 m*M* Tris buffer, pH 7.4, for 30 min.
6. Centrifuge the lysate at 600*g* for 10 min.
7. Add ABTS (2.2 azinobis-3-ethylhenthiazoline-6-sulfonic acid) 1 mg/mL in buffer (0.1 *M* citric acid, 0.2 *M* disodium hydrogenophosphate) supplemented with 0.1 µL/mL H_2O_2 (30%) as substrate for the HRP.
8. Measure the enzymatic activity of the lysate as the OD read at 420 nm with reference to a standard curve.

4. Notes

1. It is not excluded that receptors other than the macrophage mannose receptor can internalize FITC-dextran and mannan and immature DC express many polylectin receptors.
2. To further characterize the endocytosing cell population, double staining may be done using anti-CD1a-PE (Coulter) or anti-CD14-PE (Becton Dickinson) or any other PE coupled MoAb.
3. To analyze the fate of endocytosed material, cells may be pulsed at 37°C; washed four times in cold medium, and recultured at 37°C for different times in marker-free medium.
4. When the results are expressed as MFI, the background (cells pulsed with FITC-dextran at 4°C) is subtracted.
5. Some DC populations may not express the mannose receptor but still have efficient up-take of other antigens through other receptors. Many DC functions (e.g., antigen uptake, T-cell activation, response to chemokines) are regulated during DC maturation. As the maturation stage of DC may vary from sample to sample, the kinetic studies of functions should be performed during DC maturation.

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References

1. Austyn, J. D. (1996) New insights into the mobilization and phagocytic activity of dendritic cells. *J. Exp. Med.* **183,** 1287–1292.
2. Steinman, R. M. and Swanson, J. (1995) The endocytic activity of dendritic cells. *J. Exp. Med.* **182,** 283–288.
3. Hanau, D., Fabre, M., Schmitt, D. A., Stampf, J.-L., Garaud, J.-C., Bieber, T., Grosshans, E., Benezra, C., and Cazenave, J.-P. (1987) Human epidermal Langerhans cells internalize by receptor-mediated endocytosis T6 (CD1 "NA1/34") surface antigen. Birbeck granules are involved in the intracellular traffic of the antigen. *J. Invest. Dermatol.* **89,** 172–177.
4. Matsuno, K., Ezaki, T., Kudo, S. and Uehara, Y. (1996) A life stage of particle-laden rat dendritic cells in vivo: their terminal division, active phagocytosis, and translocation from the liver to the draining lymph. *J. Exp. Med.* **183,** 1865–1878.
5. Rubartelli, A., Poggi, A. and Zocchi, M. R. (1997) The selective engulfment of apoptotic bodies by dendritic cells is mediated by the av§3 integrin and requires intracellular and extracellular calcium. *Eur. J. Immunol.* **27,** 1893–1900.
6. Barfoot, R., Denham, S., Gyure, L. A., Hall, J. G., Hobbs, S. M., Jackson, L. E., and Robertson, D. (1989) Some properties of dendritic macrophages from peripheral lymph. *Immunology* **68,** 233–239.
7. Reis e Sousa, C., Stahl, P. D., and Austyn, J. M. (1993) Phagocytosis of antigens by Langerhans cells in vitro. *J. Exp. Med.* **178,** 509–519.
8. Moll, H. (1993) Epidermal Langerhans cells are critical for immunoregulation of cutaneous leishmaniasis. *Immunol. Today* **14,** 383–387.
9. Sallusto, F., Cella, M., Danieli, C., and Lanzavecchia, A. (1995) Dendritic cells use macropinocytosis and the mannose receptor to concentrate macromolecules in the major histocompatibility complex class II compartment: down-regulation by cytokines and bacterial products. *J. Exp. Med.* **182,** 389–400.

10. Harkiss, G. D., Hopkins, J., and McConnell, I. (1990) Uptake of antigen by afferent lymph dendritic cells mediated by antibody. *Eur. J. Immunol.* **20,** 2367–2373.
11. Jiang, W., Swiggard, W. J., Heufler, C., et al. (1995) The receptor DC-205 expressed by dendritic cells and thymic epithelial cells is involved in antigen processing. *Nature* **375,** 151–155.
12. Tan, M. C., Mommaas, A. M., Drijfhout, J. W., et al. (1997) Mannose receptor mediated uptake of antigens strongly enhances HLA-class II restricted antigen presentation by cultured dendritic cells. *Adv. Exp. Med. Biol.* **417,** 171–174.
13. Engering, A. J., Cella, M., Fluitsma, D. M., Hoefsmit, E. C., Lanzavecchia, A., and Pieters, J. (1997) Mannose receptor mediated antigen uptake and presentation in human dendritic cells. *Adv. Exp. Med. Biol.* **417,** 183–187.
14. Stahl, P. D. and Ezekowitz, R. A. (1998) The mannose receptor is a pattern recognition receptor involved in host defense. *Curr. Opin. Immunol.* **10,** 50–55.
15. Albert, M. L., Sauter, B., and Bhardwaj, N. (1998) Dendritic cells acquire antigen from apoptotic cells and induce class I-restricted CTLs. *Nature* **392,** 86–89.
16. Streilein, J. W. and Grammer, S. F. (1989) In vitro evidence that Langerhans cells can adopt two functionally distinct forms capable of antigen presentation to T lymphocytes. *J. Immunol.* **143,** 3925–3933.
17. Inaba, K., Schuler, G., Witmer, M. D., Valinksy, J., Atassi, B., and Steinman, R. M. (1986) Immunologic properties of purified epidermal Langerhans cells. *J. Exp. Med.* **164,** 605–613.
18. Romani, N., Lenz, A., Glassl, H., et al. (1989) Cultured human Langerhans cells resemble lymphoid dendritic cells in phenotype and function. *J. Invest. Dermatol.* **93,** 600–609.
19. Larsen, C. P., Steinman, R. M., Witmer-Pack, M. D., Hankins, D. F., Morris, P. J., and Austyn, J. M. (1990) Migration and maturation of Langerhans cells in skin transplants and explants. *J. Exp. Med.* **172,** 1483–1494.
20. Schuler, G. and Steinman, R. M. (1985) Murine epidermal Langerhans cells mature into potent immunostimulatory dendritic cells in vitro. *J. Exp. Med.* **161,** 526–546.
21. Shimada, S., Caughman, S. W., Sharrow, S. O., Stephany, D., and Katz, S. I. (1987) Enhanced antigen-presenting capacity of cultured Langerhans cells is associated with markedly increased expression of Ia antigen. *J. Immunol.* **139,** 2551–2555.
22. Larsen, C. P., Ritchie, S. C., Pearson, T. C., Linsley, P. S., and Lowry, R. P. (1992) Functional expression of the costimulatory molecule, B7/BB1, on murine dendritic cell populations. *J. Exp. Med.* **176,** 1215–1220.
23. Teunissen, M. B. M., Wormeester, J., Krieg, S. R., Peters, P. J., Vogels, I. M. C., Kapsenberg, M. L., and Bos, J. D. (1990) Human epidermal Langerhans cells undergo profound morphological and phenotypical changes during in vitro culture. *J. Invest. Dermatol.* **94,** 166–173.
24. Inaba, K., Witmer-Pack, M., Inaba, M., et al. (1994) The tissue distribution of the B7–2 costimulator in mice: abundant expression on dendritic cells in situ and during maturation in vitro. *J. Exp. Med.* **180,** 1849–1860.

25. Larsen, C. P., Ritchie, S. C., Hendrix, R., et al. (1994) Regulation of immunostimulatory function and costimulatory molecule (B7–1 and B7–2) expression on murine dendritic cells. *J. Immunol.* **152,** 5208–5219.
26. Weinlich, G., Heine, M., Stossel, H., et al. (1998) Entry into afferent lymphatics and maturation in situ of migrating murine cutaneous dendritic cells. *J. Invest. Dermatol.* **110,** 441–448.

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Uptake of Antigen–Antibody Complexes by Human Dendritic Cells

Neil A. Fanger, Paul M. Guyre, and Robert F. Graziano

1. Introduction

Fc receptors specific for IgG (FcγR) potentiate the immune response by facilitating the interaction between myeloid cells and antibody-coated targets ***(1–3)***. Monocyte and neutrophil FcγR engagement can lead to the induction of lytic-type mechanisms associated with innate responses. FcγR triggering can also play a key role in adaptive immune responses. For example, FcγR-directed capture and uptake of antigens (Ag) by dendritic cells (DC) results in processing and presentation to naive Ag-specific T cells, leading to their expansion and maturation into effector T-cell populations. This chapter describes methodology currently in use to explore and manipulate antigen–antibody (Ag-Ab) uptake by FcγR expressed on DC.

1.1. Expression of Fc Receptors on the Surface of Human DC

Two subsets of DC with distinct morphologic and phenotypic features circulate in the peripheral blood ***(4–9)***. One subset possesses an eccentric nucleus and is defined phenotypically by the absence of lineage-committed antigens (e.g., CD3, CD14, CD19, and CD56); the presence of the myeloid-associated antigen, CD33; low levels of CD11c and CD4; and high levels of MHC class II (MHCII) ***(5,7,10)***. These DC ($CD33^+CD11c^+$) constitutively express the high-affinity (FcγRI/CD64) and low-affinity (FcγRII/CD32) IgG receptors ***(5,11)***, both of which are capable of mediating phagocytosis by the DC ***(4)***. DC with a similar morphology and phenotype are dispersed throughout the germinal center (GC) dark and light zones of human tonsils, spleen, and lymph node ***(11)***. These GCDC, similar to blood DC, have cell-membrane-associated Ig. Whether this cytophilic Ig represents monomeric IgG associated with the high-affinity

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FcγRI/CD64, or Ag–Ab complexes associated with the low-affinity FcγRII/CD32, remains to be determined. Nonetheless, expression of FcγRI/CD64 and FcγRII/CD32 by these $CD33^+CD11c^+$ blood DC and tissue-residing $CD33^+CD11c^+$ GCDC suggests that FcR are important for Ag–Ab complex uptake for subsequent Ag-specific T-cell activation, and, possibly, Ag–Ab complex docking for Ag-specific B-cell activation.

Another subset of blood DC ($CD33^-CD11c^-$) possess a plasmacytoid morphology and lack expression of all lineage-committed antigens as well as CD33 and CD11c. They express CD4, high surface levels of MHCII and IL-3Ra ***(12)***, low levels of FcγRII/CD32, but no detectable levels of FcγRI/CD64. They appear phenotypically and morphologically identical to the plasmacytoid DC that reside in the extra-follicular T-cell-rich regions of the tonsil ***(11,12)***. Their lack of myeloid-associated antigens, coupled with the fact that these blood DC and tissue-residing DC possess a plasmacytoid appearance, suggests that they may arise from lymphoid-committed progenitors ***(13)***. Alternatively, they may represent a more immature stage in the DC developmental pathway. Based on their tissue localization and unique expression of Ag surface markers, these $CD33^-CD11c^-$ DC may play a different role in immunoregulation than the $CD33^+CD11c^+$ DC. In fact, a precedent for functionally distinct DC subsets has been firmly established in the mouse system ***(14)***.

1.2. FcR-Mediated Antigen–Antibody Complex Uptake by Dendritic Cells

Early attempts to measure DC FcγR expression employed methods of cell isolation that led to an underestimation of DC FcγR importance ***(6,15,16)***. Therefore, a method was developed to enrich blood DC without modulating FcγR expression or removing DC that hold cytophilic IgG. This two-step procedure utilizes elutriation followed by magnetic bead depletion to rapidly (approx 4 h) enrich 20 to 50 million blood DC from a single donor ***(4)***. These blood DC comprise both $CD33^+CD11c^+$ and $CD33^-CD11c^-$ subsets. The ratio of these two subsets varies slightly among donors, with some demonstrating as high as 60% $CD33^+CD11c^+$ to 40% $CD33^-CD11c^-$ cells, and other donors demonstrating the opposite percentages. This method, which allows high numbers of blood DC to be isolated without modulating FcγR expression or removing DC that express FcγR, is ideal to critically defining DC FcγR function.

It has been shown using DC derived from monocytes cultured in granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-4 that internalization of Ag–Ab complexes through FcγRII/CD32 enhances Ag presentation at least 100-fold over noncomplexed Ag ***(17)***, demonstrating that FcγRII/CD32 is efficient at mediating uptake of Ag–Ab complexes. In contrast to FcγRII/CD32, which only binds immune-complexed IgG, FcγRI/CD64 is continuously

saturated with monomeric IgG1 and IgG3 in blood and during culture in the presence of serum ***(3)***. This high affinity IgG interaction makes it difficult to investigate DC Ag-Ab uptake by FcγRI/CD64. To circumvent this IgG–FcγRI/CD64 interaction, a monovalent fusion protein was constructed that binds specifically to FcγRI/CD64 outside the natural IgG binding domain. The fusion protein was constructed by altering the humanized sequence of the anti-FcγRI/CD64 Mab (H22) heavy chain ***(18)***. The DNA encoding CH2 and CH3 domains was removed, and the 3' end was modified to contain the *Xho*I and *Not*I cloning sites toward the end of the hinge region ***(19)***. This modification allowed coding sequences for either peptides or full-length proteins to be inserted immediately past the hinge region of the H22 heavy chain. Cotransfection of the expression vectors containing a modified heavy chain and unmodified light chain into non-Ig expressing hybridomas resulted in the production of an anti-FcγRI/CD64 F(ab') Mab containing the universal antigenic Th epitope of tetanus toxoid, TT830-844 ***(20)***. The enhancement of T-cell activation that resulted from FcγRI/CD64 targeting with this fusion protein ranged from 50- to 500-fold greater than with TT830 peptide alone, demonstrating that low levels of FcγRI/CD64 expressed on blood DC can efficiently capture and process Ag–Ab complexes for Ag presentation to T cells ***(5)***.

2. Materials

2.1. FcR-Specific Ag–Ab Complexes

1. The myelomonocytic U937 (ATCC; Manassas, VA) and the non-Ig synthesizing myeloma NSO (ECACC 85110503) cell lines are cultured in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% fetal bovine serum (FBS; Gibco-BRL, Gaithersburg, MD).
2. Cell transfections are performed in 0.4 cm cuvets (Bio-Rad, Hercules, CA) using a Gene Pulser electroporation apparatus (Bio-Rad).
3. Transfectants are selected with mycophenolic acid (Sigma, St. Louis, MO) and xanthine (Sigma).
4. Protein purification is performed using a Q-Sepharose FF column (Pharmacia, Alameda, CA), SP-Sepharose FF column (Pharmacia), and ABx column (JT Baker, Phillipsburg, NJ).

2.2. Human Blood Dendritic Cells

1. Leukophoresis products are obtained from a local blood donation center. Cell separation is performed on a JE-5 elutriation system (Beckman, Palo Alto, CA) in the presence of phosphate-buffered saline supplemented with 5% fetal bovine serum (PBS-FBS).
2. The Mab anti-CD3, anti-CD7, anti-CD8, anti-CD11b, anti-CD19, anti-CD34, anti-CD56, and anti-glycophorin A are available from Pharmingen (San Diego, CA); anti-CD14 and anti-CD16 are available from Medarex, Inc. (Annandale,

NJ); and PE-labeled anti-CD33, FITC-labeled anti-CD14, PE-labeled isotype matched control, and FITC-labeled isotype matched control are available from Caltag (San Francisco, CA).
3. Cell depletion is performed using goat anti-mouse Ig-coated magnetic beads (Dynal, Lake Success, NY).

2.3. T-Cell Proliferation Assays

1. Peripheral blood mononuclear cells (PBMC) are isolated using Ficoll-Hypaque (Sigma).
2. Short-term, antigen-specific T-cell lines are generated in AIM-V medium (Gibco-BRL). Aliquots of interleukin-2 (IL-2) (Genzyme, Boston, MA) are stored at –80°C until needed.
3. Antigen presentation assays are performed in flat-bottom 96-well tissue culture plates (Costar, Cambridge, MA). Cell proliferation is measured with ^{3}H-thymidine (Amersham, Arlington Heights, IL).

3. Methods

3.1. Genetically Engineered FcR-Specific Antigen-Antibody Complexes

3.1.1. Construction of the Anti-FcγRI/CD64 Fusion Protein

The following procedure describes the construction of an anti-FcγR-specific Ag–Ab complex that contains an antigenic peptide sequence. Full-length proteins and multiple antigenic peptide sequences may also be inserted (**Fig. 1**).

1. Generate sense and antisense DNA sequences that correspond to the peptide of interest. These DNA sequences should be designed with *Xho*I and *Not*I cloning sites on the ends (*see* **Note 1**).
2. Anneal and ligate the DNA into the pUC19/H22CH1(X+N) *Xho*I and *Not*I sites (*see* **Note 2**).
3. Sequence the inserted region to confirm the integrity of the DNA construct.
4. Transfer the pUC19 *Bam*HI fragment containing the CH1 region, the hinge region, and the corresponding DNA peptide sequence to the pSVgpt expression vector immediately downstream of the VH1 heavy chain region.

3.1.2. Expression and Purification of the Anti-CD64 Fusion Protein

1. Transfect the pSVgpt (H22 heavy chain–fusion expression construct) and pSVhyg (H22 light chain) vectors into the murine non-Ig synthesizing myeloma NS0 cell line at 200 V and 960 μF. Culture cells in IMDM containing 10% FBS in 24-well plates.
2. Two days after transfection, add mycophenolic acid (0.8 μg/mL final) and xanthine (2.5 μg/mL final) to the media to select for transfectants expressing guanine phosphoribosyltransferase (gpt).

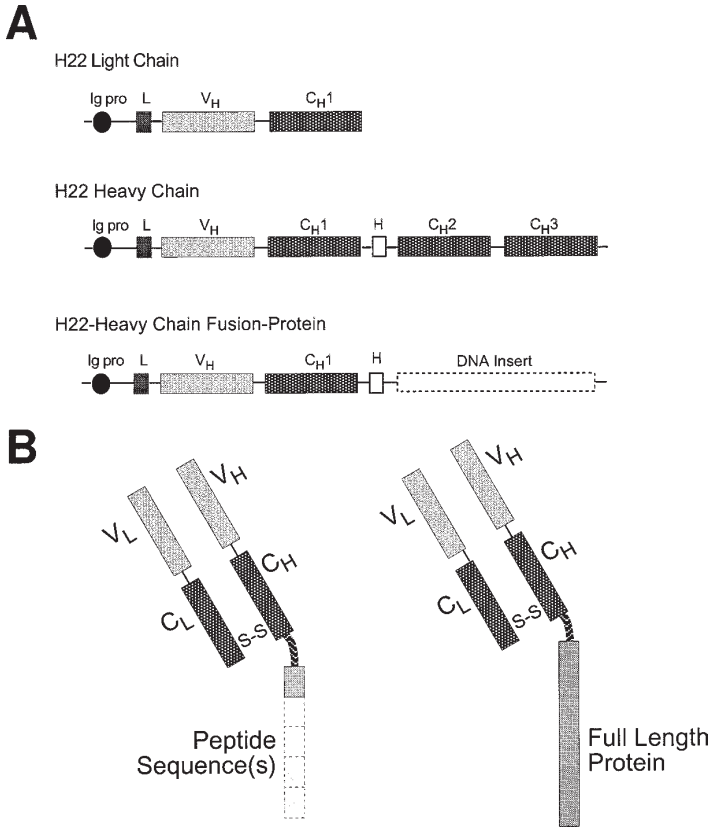

Fig. 1. Generation of the Antigen-antibody Complex. **(A)** The Ag–Ab fusion protein is constructed by inserting either the peptide coding sequence or full-length cDNA immediately past the hinge region of the H22 heavy chain. **(B)** Dual transfection of the vectors containing the genes for the H22 k chain and H22 heavy chain containing the antigen into NS0 hybridoma cells results in production of the Ag–Ab complex.

3. Approx 2 wk later, select colonies according to binding activity of the culture supernatants to FcγRI/CD64-expressing myelomonocytic U937 cells using a PE-labeled $F(ab')_2$ goat anti-human polyclonal Ab and FACS for detection.
4. Purify the fusion protein by ion-exchange chromatography using a Q-Sepharose FF column (Pharmacia), followed by an SP-Sepharose FF column (Pharmacia). The conditions for binding and eluting may vary depending on the protein structure. Use an ABx column (JT Baker) as a final purification step (*see* **Note 3**).

5. Monitor the protein purification process by SDS-PAGE on a 5–20% acrylamide gradient gel under nonreducing conditions. Final protein purity should be greater than 90%.
6. Calculate protein concentration on a spectrophotometer using an absorbance at 280 nm and the extinction coefficient of IgG F(ab') = 1.53 (*see* **Note 4**).

3.2. Isolation of Human Blood DC

3.2.1. Rapid Enrichment DC by Elutriation

1. Following autoclaving, wash the JE-5 elutriation system with 1 L hydrogen peroxide (6%), followed by 1 L ethanol (70%). Rinse away the ethanol with sterile water for injection (Baxter, Deerfield, IL) and equilibrate the elutriation system with PBS-FBS.
2. Load the leukophoresis product onto the elutriator, rotating at 870*g* with a flow rate of 15 mL/min. Collect five 50 mL fractions at each 10 mL/min flow rate increase from 15 to 45 mL/min (*see* **Note 5**).
3. Stain a small aliquot of cells from each fraction with PE-labeled anti-CD33 and FITC-labeled anti-CD14 in the presence of 30% human serum. Analyze by FACS to identify the fractions that contain the highest percentage of $CD33^+CD14^-$ cells. **Figure 2** represents an enriched fraction stained with PE-labeled anti-CD33 and FITC-labeled anti-CD14.
4. Incubate the cells with anti-CD3, anti-CD14, anti-CD16, anti-CD19, and anti-CD56 Mab (10 mg/mL final) in the presence of 30% human serum for 10 min at RT. After three washes, remove cells expressing CD3, CD14, CD16, and CD19 using goat anti-mouse Ig-coated magnetic beads at a 1:1 bead:cell ratio (*see* **Note 6**).
5. Incubate the remaining cells with the anti-CD7, anti-CD8, anti-CD11b, anti-CD34, and anti-glycophorin A. Remove the cells expressing CD7, CD8, CD11b, and CD34 using goat anti-mouse Ig-coated magnetic beads at a 3:1 bead:cell ratio.

3.2.2. Sorting of Blood DC Subsets

1. For cell sorting, stain the cells with PE-labeled anti-CD33 (or PE-labeled anti-CD11c) for 10 min at RT in the presence of 30% human serum.
2. Wash the cells three times to remove excess antibody, and resuspend cells at a concentration of 20×10^6/mL for cell sorting.
3. Sort $CD33^+$ ($CD11c^+$) and $CD33^-$ ($CD11c^-$) cells at approx 5000 cells/s. Separation of these two populations will yield greater than 90% pure populations of either $CD33^+CD11c^+$ or $CD33^-CD11c^-$ blood DC.

3.3. T-cell Proliferation Assays

3.3.1. Generation of Short-Term, Antigen-Specific T Cells

Generating antigen-specific T-cell lines against recall Ag, such as tetanus toxoid, can simplify the DC studies, particularly when donor histocompatibility Ag are unknown.

1. Remove 20–30 mL of cells from the leukophoresis pack prior to elutriation.

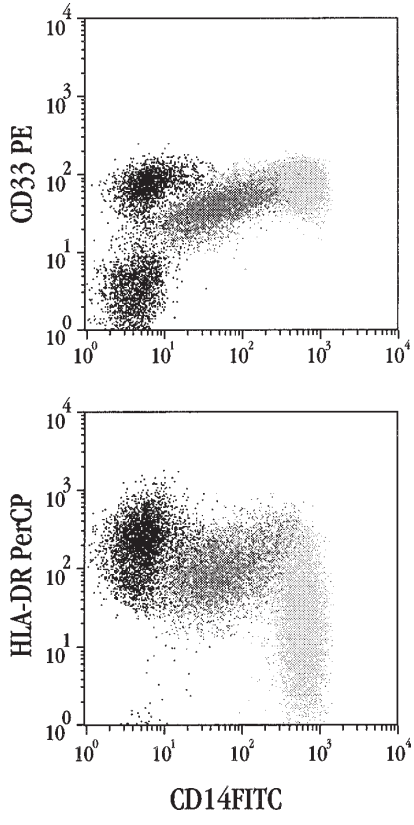

Fig. 2. Phenotype of Blood Dendritic Cell Subsets Following Elutriation. Countercurrent elutriated fractions containing a high percentage of circulating blood DC were stained with FITC-labeled anti-CD14, PE-labeled anti-CD33, and PerCP-labeled anti-HLA-DR, and biotin-labeled anti-CD16, followed by APC-labeled streptavidin. The $CD33^+CD11c^+$ and $CD33^-CD11c^-$ blood DC (black dots) are distinguishable from the $CD33^+CD14^+CD16^-$ monocytes (light dots) and $CD33^+CD14^+CD16^+$ monocytes (medium dots). Cells were analyzed using Paint-A-Gate software (Becton Dickinson).

2. Isolate PBMC by centrifuging cells over Ficoll-Hypaque at 600*g* for 30 min (*see* **Note 7**).
3. Incubate the PBMC (150×10^6) in the presence of 10 m*M* peptide or 10 µg/mL protein in 50 mL AIM-V medium.
4. Three days later, gently remove the nonadherent cells and add fresh AIM-V medium containing 20 U/mL rIL-2 and 2% PHS for further T-cell expansion.

5. After an additional 10–14 d, harvest the T cells and remove cell clumps by centrifuging cells over HISTOPAQUE® 1077. Cryopreserve until Ag proliferation assays.

3.3.2. Antigen Proliferation Assays

1. Irradiate the DC with 3000 rad to inhibit any low level of cell division.
2. Incubate the T cells (5×10^4) and DC (5×10^3) in AIM-V supplemented with 10% PHS with titrated amounts of soluble Ag or Ag–Ab complex. Assays should be completed in flat-bottomed 96-well tissue culture plates at a final volume of 200 µL/well (*see* **Note 8**).
3. After 72 h, add 10 µL (1 µCi/well) ^{3}H-thymidine for an additional 20 h.
4. Harvest and count plates in a liquid scintillation counter.

4. Notes

1. The DNA sequence of full-length proteins can be inserted by incorporating *Xho*I and *Not*I sites into the cDNA using PCR. Difficulties may arise if the original DNA sequence contains one or more internal *Xho*I and/or *Not*I sites. This may be circumvented by partial digestion of the PCR product.
2. The CH1 and hinge region containing the *Xho*I and *Not*I sites are in the pUC19 vector (pUC19/H22CH1[X+N]) for ease of ligation and sequence analysis. Once the integrity of the insert is confirmed by sequence analysis, the CH1 and hinge region containing the peptide sequence is transferred to the final pSVgpt expression vector.
3. High levels of unassociated light chain may be secreted by the NSO transfectants. In some instances, this light chain may complicate purification of the Ag–Ab complex. Fusion proteins can also be purified using Sepharose coupled with rabbit anti-human heavy chain-specific antibody. Alternatively, to avoid production of this light chain, a cDNA encoding single-chain Fv may be constructed. The resulting protein comprises the V_H and V_L of the FcγR-specific antibody joined by a flexible linker with the peptide or protein of interest following at the C-terminus.
4. The protein concentration for constructs containing a full-length protein can be determined by spectroscopy (OD 280 nm) using the extinction coefficient calculated according to the method of Gill and von Hippel ***(21)***.
5. The flow rate and *g* described here are for the standard chamber (5 mL) JE-5 elutriation chamber. If the large chamber (40 mL) is used, similar fractions may be obtained by increasing the flow rate 5 mL/min intervals from 65 to 85 mL/min at 750*g*.
6. Since approximately one billion cells need to be removed, the magnetic beads are maximized using a 1:1 bead:cell ratio in the first round, followed by a 3:1 bead:cell ratio in the second step. In addition, removing the majority of the T cells, B cells, monocytes, and granulocytes in the first step means that lower amounts of Mab are required in the second selection step.
7. Alternately, short-term antigen-specific T-cell lines can be generated using the lymphocyte fractions of the elutriated product. Extremely low numbers of blood

DC and monocytes will be present in these lymphocyte fractions, so monocytes may need to be added back to enhance the T-cell proliferation.

8. Several factors contribute to the observed DC stimulatory capacity in vitro. Monocytes cultured in the presence of GM-CSF and IL-4 for several days develop into DC-like cells that are extremely efficient at stimulating T cells in an Ag-specific manner ***(17)***. These monocyte-derived DC are several times larger in size and express higher levels of MHCII, CD80, and CD86 than freshly isolated blood DC. In contrast, freshly isolated blood DC lack CD80 surface expression, and express low levels of CD86. Thus, lower levels of T-cell proliferation may be measured with blood DC compared to DC generated using other methods.

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References

1. Daëron, M. (1997) Fc receptor biology. *Annu. Rev. Immunol.* **15,** 203–234.
2. Wallace, P. K., Howell, A. L., and Fanger, M. W. (1994) Role of Fc gamma receptors in cancer and infectious disease. *J. Leukoc. Biol.* **55,** 816–826.
3. Capel, P. J. A., van de Winkel, J. G. J., van den Herik-Oudijk, I. E., and Verbeek, J. S. (1994) Heterogeneity of human IgG Fc receptors. *Immunomethods* **4,** 25–34.
4. Fanger, N. A., Wardwell, K., Shen, L., Tedder, T. F., and Guyre, P. M. (1996) Type I (CD64) and type II (CD32) Fcγ receptor-mediated phagocytosis by human blood dendritic cells. *J. Immunol.* **157,** 541–548.
5. Fanger, N. A., Voigtlaender, D., Liu, C., et al. (1997) Characterization of expression, cytokine regulation, and effector function of the high affinity IgG receptor FcγRI (CD64) expressed on human blood dendritic cells. *J. Immunol.* **158,** 3090–3098.
6. O'Doherty, U., Steinman, R. M., Peng, M., et al. (1993) Dendritic cells freshly isolated from human blood express CD4 and mature into typical immunostimulatory dendritic cells after culture in monocyte-conditioned medium. *J. Exp. Med.* **178,** 1067–1076.
7. O'Doherty, U., Peng, M., Gezelter, S., et al. (1994) Human blood contains two subsets of dendritic cells, one immunologically mature and the other immature. *Immunology* **82,** 487–493.
8. Xu, H., Friedrichs, U., Gieseler, R. K., Ruppert, J., Ocklind, G., and Peters, J. H. (1992) Human blood dendritic cells exhibit a distinct T-cell-stimulating mechanism and differentiation pattern. *Scand. J. Immunol.* **36,** 689–696.
9. Zhou, L. J. and Tedder, T. F. (1995) Human blood dendritic cells selectively express CD83, a member of the immunoglobulin superfamily. *J. Immunol.* **154,** 3821–3835.

10. Nijman, H. W., Kleijmeer, M. J., Ossevoort, M. A., et al. (1995) Antigen capture and major histocompatibility class II compartments of freshly isolated and cultured human blood dendritic cells. *J. Exp. Med.* **182,** 163–174.
11. Grouard, G., Rissoan, M.-C., Filgueira, L., Durand, I., Banchereau, J., and Liu, Y.-J. (1997) The enigmatic plasmacytoid T cells develop into dendritic cells with interleukin (IL)-3 and CD40-ligand. *J. Exp. Med.* **185,** 1101–1111.
12. Olweus, J., BitMansour, A., Warnke, R., et al. (1997) Dendritic cell ontogeny: a human dendritic cell lineage of myeloid origin. *Proc. Natl. Acad. Sci. USA* **94,** 12,551–12,556.
13. Galy, A., Travis, M., Cen, D., and Chen, B. (1995) Human T, B, natural killer, and dendritic cells arise from a common bone marrow progenitor cell subset. *Immunity* **3,** 459–473.
14. Pulendran, B., Lingappa, J., Kennedy, M. K., et al. (1997) Developmental pathways of dendritic cells in vivo. Distinct function, phenotype, and localization of dendritic cell subsets in FLT3 ligand-treated mice. *J. Immunol.* **159,** 2222–2231.
15. Thomas, R. and Lipsky, P. E. (1994) Human peripheral blood dendritic cell subsets. Isolation and characterization of precursor and mature antigen-presenting cells. *J. Immunol.* **153,** 4016–4028.
16. Weissman, D., Li, Y., Ananworanich, J., Zhou, L.-J., et al. (1995) Three populations of cells with dendritic morphology exist in peripheral blood, only one of which is infectable with human immunodeficiency virus type 1. *Proc. Natl. Acad. Sci. USA* **92,** 826–830.
17. Sallusto, F. and Lanzavecchia, A. (1994) Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor α. *J. Exp. Med.* **179,** 1109–1118.
18. Graziano, R. F., Tempest, P. R., White, P., et al. (1995) Construction and characterization of a humanized anti-g-Ig receptor type I (FcγRI) monoclonal antibody. *J. Immunol.* **155,** 4996–5002.
19. Goldstein, J., Graziano, R. F., Sundarapandiyan, K., Somasundaram, C., and Deo, Y. M. (1997) Cytolytic and cytostatic properties of an anti-human Fc gammaRI (CD64) × epidermal growth factor bispecific fusion protein. *J. Immunol.* **158,** 872–879.
20. Liu, C., Goldstein, J., Graziano, R. F., et al. (1996) F(c)gammaRI-targeted fusion proteins result in efficient presentation by human monocytes of antigenic and antagonist T cell epitopes. *J. Clin. Invest.* **98,** 2001–2007.
21. Gill, S. C. and von Hippel, P. H. (1989) Calculation of protein extinction coefficients from amino acid sequence data. *Anal. Biochem.* **182,** 319–326.

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Immunoelectron Microscopy of Antigen Processing in Dendritic Cells

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1. Introduction

The cell biology of intracellular compartments and their interrelationships require detailed knowledge of the proteins that characterize the compartment and that are involved in the communication between them. To date, this can be best achieved by high resolution immunoelectron microscopy (IEM). Other methods, which make use of different embedding materials, such as EPON, Spurr's resin, LR white, or Lowicryls, also allow the detection of immuno–determinants. However, IEM is in many cases the optimum technique owing to better accessibility of the immunodeterminants to antibodies and the absence of denaturing solvents. In our laboratory for IEM we use immunogold labeling on cryosections. This technique combines optimal ultrastructure and good preservation of protein and/or lipid antigens. The ultrathin cryosections (50–100 nm) are prepared from small tissue blocks or cell pellets with a cryo-ultramicrotome. The sections are thawed, and labeled with antibodies, which are visualized with protein A-gold particles (PAG). We recommend the books by Larson ***(1)*** and Griffith ***(2)***, and chapters in *Handbook of Experimental Immunology* ***(3)*** and *Methods: a Companion to Methods in Enzymology* ***(4)***. The present chapter will describe the different aspects of IEM in detail, such as fixation procedures, the processing of samples, ultrathin cryosectioning, and immunogold labeling.

Although the technique of cutting ultrathin sections at low temperature was developed in 1952 ***(5)***, it has taken considerable time to develop further. The introduction of fixation, before freezing and sectioning ***(6)***, greatly improved the subcellular morphology, but the major step ahead was the use of sucrose as a cryoprotectant, which prevents ice crystal formation during freezing of the

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specimen *(7,8)*. Furthermore, cryo-ultramicrotomes have been improved greatly, which has made cryosectioning achievable for many researchers. More recently, an alternative picking-up solution for sections has been introduced *(9)*, which allows better visualization of membranes and storage of sections for several weeks to months *(10)*. Finally, monodispersed and uniformly sized gold particles can now easily be prepared, and enable quantative single- and double-labeling IEM *(3)*.

IEM can be used to address many cell biology questions, given that appropriate marker proteins for intracellular compartments are available. Depending on the species, markers for endoplasmic reticulum, intermediate compartment, Golgi complex, and endocytic organelles have been described for many cell types. The major advantage of IEM is the high resolution, which enables one to distinguish between small 50–100 nm vesicles, even when they are in close proximity to each other. This kind of resolution cannot be achieved by fluorescence/confocal microscopy, although for studying overall distribution of proteins or to reveal processes in living cells this is a very valuable technique. In combination with molecular biology, biochemistry, and antigen-presentation assays, IEM is a very powerful tool for studying the cell biology of many different processes. In this chapter, which deals with IEM on antigen processing in dendritic cells (DC), we will highlight several aspects that play an important role in such an IEM study. IEM allows the characterization of the different pathways of antigen uptake in these cells, such as macropinocytosis, phagocytosis, and receptor-mediated endocytosis. For each pathway we will discuss possible model antigens, and in **Subheading 3.1.** a pulse-chase experiment with an endocytic marker is described in detail. IEM can be performed on cells in suspension or adherent cells and on tissues such as skin, lymph node, spleen, or thymus, which, as shown in **Subheading 3.2.**, may each require a different fixation procedure. The number of cells available can be a limiting factor in IEM. Preparation of suitable blocks for sectioning requires at least 500,000 cells, but preferably more, which are not always available. Possible ways to solve this problem are described in **Subheading 4.2.** Importantly, when working with isolated DC, contamination by other cell types cannot be excluded, and proper identification of DC is absolutely necessary. The morphology of DC is a quick, though not foolproof way, to identify them. In general, mature DC exhibit a lobulated nucleus and much cytoplasm. This in contrast to B and T lymphocytes, which generally have a round nucleus and little cytoplasm. Furthermore, labeling with cell-specific markers can be of help, especially when studying DC precursors or immature DC, which do not exhibit typical DC morphological characteristics. In general, it is easiest to label contaminating cells with specific antibodies, because only a few DC-specific markers are available. In the case of mature DC the high expression of major histocompat-

ibility complex proteins can be very helpful in their identification. Finally, finding antibodies that work in IEM can be a time-consuming effort, and we will refer to several IEM studies that describe antibodies specific for DC studies.

2. Materials

2.1. Processing of Samples

1. Paraformaldehyde (PFA, methanal; $O=CH_2$, MW 30 Dalton). To prepare 8% PFA, dissolve 8 g of PFA powder (Polysciences, Inc.) in 90 mL of distilled water and heat it to 65°C. While stirring, add 1 *N* NaOH until the solution has become clear. Cool down on ice and add distilled water to a final volume of 100 mL. Finally, cool and filtrate the solution. PFA is stored in small aliquots at –20°C. PFA has one aldehyde group that can react with amino acids and cross-linking occurs in time due to the formation of methylene bridges. This cross-linking is reversible and makes PFA a relatively weak fixative. The formation of methylene bridges between molecules is enhanced by using higher PFA concentrations (>5%) and a higher pH (8–8.2). Owing to the size of PFA, the penetration into tissues and cells is very fast. It provides good morphology especially after prolonged fixation (8–72 h).
2. Glutaraldehyde (EM-grade) 8% or 70% in distilled water (Polysciences, Inc.). Glutaraldehyde [pentaandial, $O=CH–(CH_2)_3$-$CH=O$, MW 100 Dalton) possesses two reactive aldehyde moieties, which mainly react with lysine residues of proteins. Proteins can be crosslinked internally or to each other. The fixation is irreversible and its penetration rate is relatively slow. The morphology is very well preserved.
3. Acrolein, 98% pure solution (BDH Chemicals). Acrolein (propenal; $O=CH–CH=CH_2$, MW 56 Dalton) has one reactive aldehyde group that mainly reacts with lysine. Furthermore, the double bond between the carbon atoms may play a role in cross-linking. Its fixation is irreversible and its penetration into tissues and cells is fairly quick. Note that this fixative is extremely toxic and it must be handled with great care.
4. Phosphate buffer (PB) 0.2 *M*, pH 7.4 (stock solution). Prepare separately solutions of 0.2 *M* Na_2HPO_4 and 0.2 *M* NaH_2PO_4. Prior to use mix the two solutions in approx 4:1 ratio, respectively, while measuring the pH. The required fixatives are diluted to the appropriate concentration in 0.1 *M* PB (*see* **Note 2**).
5. Sucrose 2.3 *M*. 2.3 *M* of sucrose (Baker 0334) in 0.1 *M* PB. Stir until the sucrose is completely dissolved and aliquot it in 1 mL vials (*see* **Note 3**).
6. Gelatin 10%. 10 g of gelatin powder (Merck 4078) is put in 100 mL 0.1 *M* PB. Stir for 10 min at room temperature, and warm the solution to 60°C for 2 h. When all the gelatin has dissolved, cool the solution to 37°C, and add 200 µL of a 10% azide solution. The homogeneous 10% gelatin solution is poured into 5 mL vials and placed in the refrigerator until use.
7. Phosphate-buffered saline (PBS) supplemented with 50 m*M* glycine.
8. The following fixatives may then be prepared for use in IEM. PFA in 0.1 *M* PB, 2–96 h, 20°C. PFA + 0.1 to 0.5% glutaraldehyde in 0.1 *M* PB, 1–2 h, 20°C. 2–4%

(6–8%) PFA (10 min each step) 1–96 h, 20°C. PFA + 1% acrolein in 0.1 *M* PB, 1–48 h, 20°C . The PB can be replaced by 0.1 *M* PIPES of HEPES

2.2. Sectioning

1. Formvar-carbon coated grids. To prepare the Formvar solution dissolve 1.2 g Formvar powder (Formvar 1595 E Merck 12164) in a volumetric flask and add 100 mL chloroform while stirring. Formvar powder is kept under vacuum or at 60°C in the presence of silica gel. To prepare a clean solution all materials that are used should be rinsed with chloroform prior to use. For preparation of the film, clean microscope slides thoroughly. Introduce the slide upright in a glass column (rinsed with chloroform) filled with the Formvar solution. The glass column is funnel-shaped and specially designed to accommodate the slide (**Fig. 1A**). Set the draining time of the Formvar along the slide with a stopcock. This will leave a thin film of Formvar on the slide. The draining time determines the thickness of the film, and is usually 12 to 15 s. A shorter draining time will result in a thicker film. Remove the slide from the funnel and cut the edges with a razor blade. Put the slide on a water surface in an upright position. Push the slide gently into the water. This allows the film to be released from the slide and to float on the water surface (**Fig. 1B**). The thickness of the film can be judged by its color, which should be gray. Place the grids on the film while floating on the water surface (**Fig. 1C**). The grids are previously cleaned with acetone and dried in a stove at 37°C. After covering a film with grids, remove it from the water by putting a microscope slide covered with a sticker in an upright position on the edge of the film, and push it downwards into the water (**Fig. 1D**). The film will adhere to the sticker and has to be dried carefully before it can be supplemented with a carbon layer. A microscope slide covered with grids is shown in **Figure 1E**.
2. Knives. Cryosectioning can be performed on either a diamond (11–13) or a glass knife. Diamond knives and trimming tools are commercially available (Diatome, Biel, Switzerland; Drukker, Cuijk, The Netherlands) (*see* **Note 4**). Glass knives are prepared from glass rods (Leica, Vienna, Austria) using a Leica knife maker (*see* **Note 5**).
3. Cryo-ultramicrotome (Ultracut S/FCS, Leica, Vienna, Austria; RMC, Tucson, AZ).
4. Ionizer (Diatome, Biel, Switzerland).
5. Wooden stick with mounted eyelash on top for section guiding (**Fig. 2A**).
6. Stainless-steel loop (diameter 2 to 2.5 mm, wire diameter 0.3 mm) for section retrieval (**Fig. 2B**).
7. Gelatin plates. A 2% gelatin solution is made in the same way as the 10% solution. The solution is poured into small Petri dishes (diameter of 3 cm) and cooled in the refrigerator.
8. Picking-up solution. 2.3 *M* sucrose or 1:1 mixture of 2.3 *M* sucrose and 2% methyl cellulose in water. The preparation of methyl cellulose is described in **Subheading 2.3.**

2.3. Immunogold Labeling

1. Antibodies. The specific antibody can either be a polyclonal or a monoclonal antibody, a bridging antibody, or IgG conjugated to the gold probe. Bridging antibodies are commercially available (Nordic, Immunochemicals, Tilburg, The Netherlands; Dakopatt, Denmark). Antibodies for IEM on DCs have been described in several studies (reviewed in 4, ***14–19***).
2. Bovine serum albumin (BSA). 10 % BSA (Sigma A-9647) in distilled water (Milli Q). The solution must be stirred slowly to prevent foaming. The pH is set to 7.4 with 1 *N* NaOH and 200 µL of 10% azide is added. Finally the solution must be centrifuged for 1 h at 100,000*g*. The supernatant is stored in small aliquots in the refrigerator.
3. Gold conjugates (*see* **Note 6**) including protein A colloidal gold (PAG).
4. Uranyl acetate pH 4 and pH 7. A 4% uranyl acetate (UA) (Merck 8473) solution is made in distilled water. To prepare UA pH 7 mix the UA solution pH 4 with a 0.15 *M* solution of oxalic acid in a 1:1 ratio. The pH is set with 25% ammonium hydroxoid (NH_4OH). This must be added drop by drop while stirring, otherwise insoluble precipitates are formed. Uranyl acetate is sensitive to light and is stored in the dark at 4°C.
5. Methylcellulose. For a final volume of 200 mL, heat 196 mL of distilled water to a temperature of 90°C, and add 4 g of methylcellulose (Sigma, 25 centipoises) while stirring. Cool the solution rapidly on ice while stirring, until the solution has reached a temperature of 10°C. Slow stirring is continued overnight in the cold-room and then stopped to let the solution "ripen" for 3 d at 4°C.
6. Methylcellulose/uranyl acetate pH 4. For 200 mL, add to 180 mL of the methylcellulose solution to 20 mL of 4% uranyl acetate and mix gently. Centrifuge the solution for 95 min at 29,000 rpm (4°C). The supernatant is aliquoted and can be stored at 4°C in the dark for about 3 mo.
7. Picking-up loops.

3. Methods

3.1. Internalization of Endocytic Tracers

As an example, a pulse-chase experiment with BSA conjugated to 5 nm gold particles is described, but any endocytic tracer can be used.

1. Wash cells, which can be either adherent or in suspension, in serum free medium (*see* **Note 1**). When the amount of tracer is limited, one can first release adherent cells with either trypsin or EDTA, and perform short pulse-chase experiments in suspension. After releasing the cells, centrifuge and resuspend them in medium plus 0.5% serum (a higher concentration of serum will compete with the BSA-gold), and let them recover for 10 min at 37°C. For 10×10^6 cells, 1 mL of medium in a 50 mL tube is sufficient. With sufficient amount of tracer the experiment is best performed in the Petri dish.

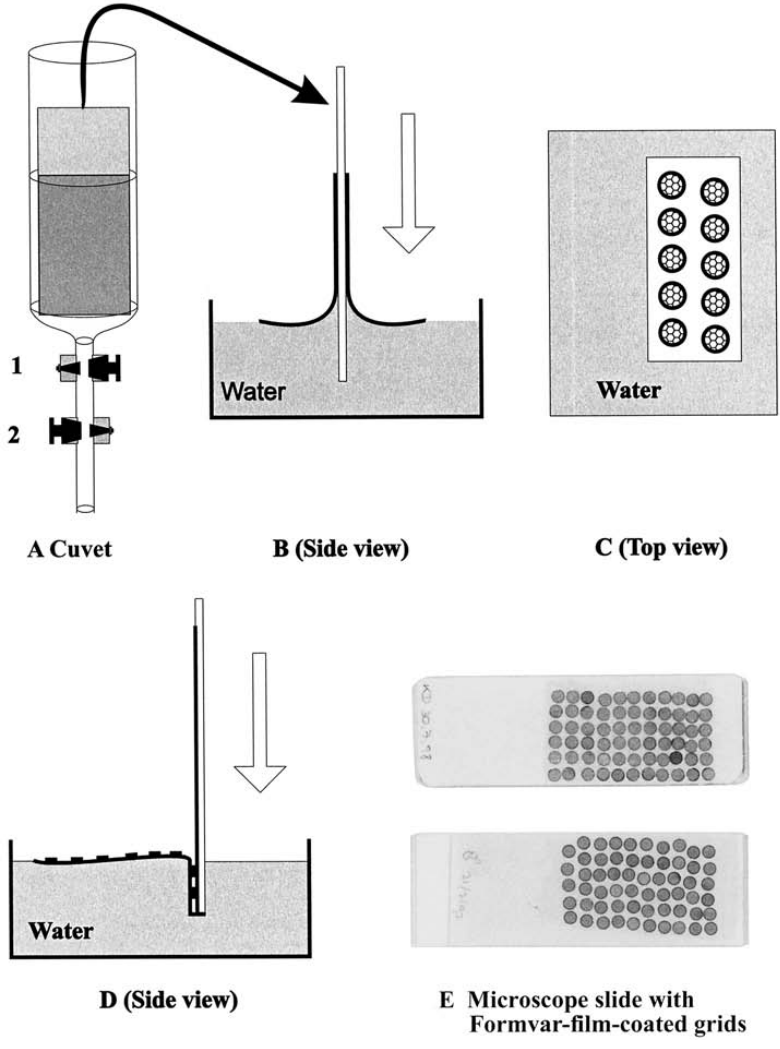

Fig. 1. This figure shows a schematic representation of the different steps in the preparation of Formvar coated grids. **(A)** The cuvet, which contains a microscope slide, and is filled with Formvar solution, is specially designed for making films. Numbers 1 and 2 are stopcocks, which are used to determine the draining time of the Formvar along the slide and to start the flow, respectively. **(B)** The slide with films is pushed into the

2. Add warm BSA-gold (final concentration OD 5) and pulse the cells for 5–10 min at 37°C.
3. Add an excess of cold serum-free medium and spin the cells gently. Repeat this two times.
4. Resuspend the cells in warm medium and immediately take one aliquot for fixation (*see* **Subheading 3.2.**).
5. Incubate the remaining cells for the desired time intervals (i.e., 20 and 50 min) at 37°C, and then fix them.

3.2. Fixation Procedure

The method of fixation is dependent on the source of cells. The commonly used fixatives in IEM are outlined in **Subheading 2.1.8** (23–25) (*see* **Note 7**).

3.2.1. Cell Suspensions

1. Pellet the cells at 200*g* (maximum) in culture medium.
2. Remove the supernatant and resuspend the pellet in the serum-free medium. When dealing with fragile cells, the fixation can be performed in medium supplemented with serum. In this case the serum proteins will compete with the cells for the fixative, and replacement with fresh fixative should be performed after 10 min.
3. While swirling add the fixative (equal volume, double concentration, molarity of the buffer should not be increased!).
4. After 10 min of fixation, pellet the cells at 200*g*, resuspend the pellet in 1 mL fixative, and transfer to an Eppendorf tube for further handling (in general, a minimum of 500,000 cells is required; *see* **Note 8**).

3.2.2. Cells Growing in Petri Dishes

1. Rinse the cells with serum-free medium.
2. Add an equal volume (as compared to the medium) of twice concentrated fixative.
3. Replace the medium/fixative mixture after 10 min with the standard fixative.
4. Discard after an appropriate fixation period most of the fixative (leave approx 1 mL in the dish) and remove the cells from the bottom of the dish using a standard cell scraper (*see* **Note 9**).
5. Transfer to an Eppendorf vial, and handle cells further as described for suspension cells.

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water, whereby the films, from both sides of the slide, are released and float on the water surface. **(C)** A film is covered with grids, while floating on the water surface. **(D)** To remove the film from the water surface, a microscope slide, covered with a sticker, is put in an upright position on the edge of the film. By pushing the slide downward, the film adheres to the sticker. **(E)** As an example two slides covered with grids are shown.

3.2.3. Tissue

1. Remove the tissue and rinse quickly in PBS.
2. Transfer to fixative and cut it into small pieces (1–2 mm^3) while submersed in fixative.
3. Put the pieces of tissue in fixative for prolonged fixation (*see* **Note 10**).

3.2.4. Storage of Fixed Specimens

After fixation with nonreversible fixatives, specimens can be stored in a buffer for weeks or even months at 4°C. After fixation with PFA, 1.0% PFA in a buffer is a suitable storage fluid.

When the specimens are used for cryosectioning after sucrose infiltration, the specimens can be stored in 2.3 *M* sucrose in a buffer at 4°C for a prolonged period of time, although this may affect the sectioning properties of the specimens as the tissue becomes more brittle. It is also possible to freeze an appropriate vial with 2.3 *M* sucrose and specimens in liquid nitrogen and store them indefinitely.

3.3. Support of Fixed Samples

Cells in suspension, loose tissues, or even solid tissues require support during the procedure ***(26)***. The specimens are more easy to handle and sectioning properties are improved. The most commonly used embedding medium is 10% gelatin in buffer. Owing to the size of the gelatin constituents, the gelatin does not enter the cells (*see* **Note 11**).

3.3.1. Gelatin Embedding of Cell Suspensions

1. After fixation, pellet the cells in an Eppendorf centrifuge at 200*g*.
2. Remove the fixative and rinse the cells twice with PBS/50 m*M* glycine (to quench free aldehyde groups).
3. Remove the supernatant and resuspend the pellet in 1 mL 10% gelatin in a buffer.
4. After 10 min at 37°C, pellet the cells and remove the excess of gelatin.
5. Solidify the gelatin on ice by placing the Eppendorf tube in an ice bucket. This results in a high density of cells in the final specimens (*see* **Note 12**).
6. After solidification cut off the bottom of the tube, including the pellet, with a sharp razor blade and put it in cold PBS or 2.3 *M* sucrose.
7. After 15–30 min remove the pellet from the tube and prepare small blocks.
8. Transfer the block to a small vial containing 2.3 *M* sucrose and let them rotate for at least 2 h at 4°C (*see* **Note 13**).

3.3.2. Gelatin Embedding of Tissue

Tissue with (large) intracellular spaces require support during the procedure. Solid tissue usually does not need extra gelatin support. However, when the tissue has large intercellular cavities or indentations, e.g., the villus lining of the gastrointestinal tract, a gelatin support is advisable.

1. After fixation cut small blocks (<1 mm^3) or thin sheets (thickness smaller than 1 mm) using a sharp razor blade.
2. Rinse the blocks or sheets in PBS/50 m*M* glycine for 10 min.
3. Infiltrate with 10% gelatin in a buffer at 37°C during 10 min while gently swirling the specimens.
4. Remove most of the gelatin and transfer the specimens and some gelatin to a microscope slide provided with a piece of parafilm and a spacer (approx 0.75 mm).
5. Place another microscope slide on top of the gelatin and clamp the two slides together.
6. Allow the gelatin to solidify on ice, and then remove the slide with the parafilm.
7. Prepare appropriate blocks from the gelatin slab and transfer them to 2.3 *M* sucrose.
8. Rotate the block in 2.3 *M* sucrose for at least 2 h at 4°C.

3.4. Freezing Specimens

The main concern in freezing specimens is to prevent ice crystal formation. A sucrose concentration exceeding 1.8 *M* effectively abolishes ice crystal formation and vitreous ice is formed during freezing ***(27,28)***. The freezing itself is therefore no longer a critical step.

3.4.1. Specimen Holders

Copper or aluminum specimen holders should fit perfectly into the microtome, otherwise the sectioning will be very irregular. Usually these are provided by the microtome manufacturer; however, ordinary rivets seem to perform just as well and are much cheaper.

1. Roughen the top of the specimen holder using sandpaper or a sharp metal point. This improves the "sticking" of the specimen to the surface.
2. Degrease the specimen holders using acetone or 100% alcohol.
3. Afterward check under a binocular microscope whether the specimen holders are perfectly clean and that no remnants of sandpaper or metal grains can be observed. These remnants may ruin an expensive diamond knife. The specimen holders can be reused again after thorough rinsing in distilled water.

3.4.2. Freezing in Liquid Nitrogen

1. Place the specimens on top of a specimen holder and remove the excess of sucrose with filter paper.
2. Plunge specimen and holder into liquid nitrogen (*see* **Note 14**).

3.4.3. Storage of Frozen Specimens

Frozen specimens (on specimen holders) can be stored in liquid nitrogen for months or even years. Prolonged storage in liquid nitrogen freeze dries the specimens to a certain extent. This renders sectioning more difficult. Remount-

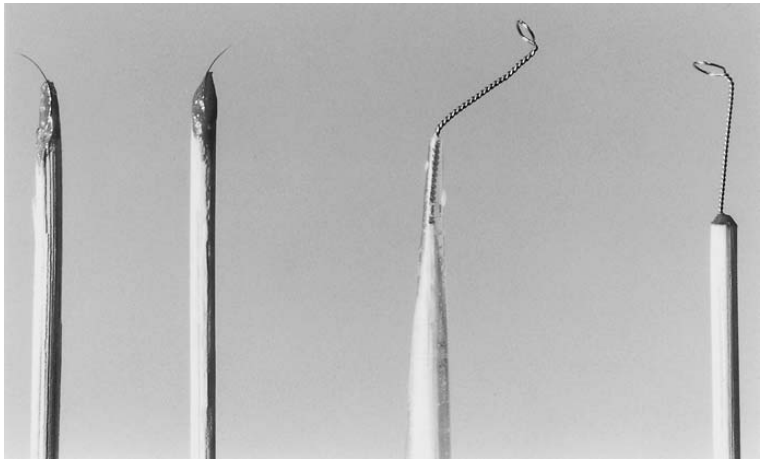


Fig. 2. Tools for section manipulation and retrieval. **(A)** Two wooden sticks with eyelashes mounted on top are shown. These tools can be used to manipulate the sections on the knife. **(B)** Two loops for section retrieval are shown, which can be either bent (left) or straight (right).

ing the specimen, which involves replacing the block in 2.3 *M* sucrose for some time, solves this potential problem.

3.5. Sectioning

3.5.1. Trimming and Semithin Sections

One of the keys to successful ultrathin sectioning is a perfectly trimmed block. The smaller the block, the easier the sectioning will be, but often we have to compromise about the size. The shape of the block is equally important. In contrast to plastics, which are usually pyramid shaped, the ideal cryospecimen surface is a rectangle.

1. Flatten the front of the specimen by sectioning at a relative high temperature (knife, specimen and chamber temperature set at –100 to –90°C).
2. Cut semithin sections of about 0.3 mm using a glass knife (*see* **Note 15**). The sectioning speed can be very high, up to 100 mm/s. The first sections will have a "snowy" appearance and mostly consist of plain sucrose. When shiny colored sections begin to appear, the actual tissue is being sectioned. Do not allow any snowy spots in the semithin section.
3. Once the face of the block is nicely trimmed, retrieve a section from the knife with a 2.3 *M* sucrose droplet in a stainless-steel loop (diameter 3 mm, steel wire diameter 0.25 mm mounted on a 15-cm long bamboo stick) (**Fig. 2**). When the

droplet enters the cold nitrogen atmosphere, it will start "smoking." Once the smoking has stopped, push the almost frozen sucrose droplet against the section. This will stick to the droplet and stretch.
4. Remove the droplet from the nitrogen atmosphere and melt it.
5. Push the droplet with the section faced downward on top of a microscope slide and remove the wire loop. The section will remain on the slide and can be stained for light microscopy with 1% toluidine blue in water. Do not use alcohol or other solvents in the staining solution as the sections will be removed from the slide.
6. Trim the sides of the specimen either using a small piece of a razor blade in an arterial clamp or using the corners of the glass knife or a trimming diamond. In the latter method first the left and right sides are trimmed on the right and left corners of the knife, respectively. Next the specimen is turned 90° and the procedure is repeated. This method is more time-consuming than the first one, but it requires less experience and yields a perfect rectangle (*see* **Note 16**).

3.5.2. Ultrathin Sectioning of Sucrose Cryoprotected Specimens

1. Replace the trimming knife with a perfect glass knife or a diamond knife. The effect of scratches on the knife is shown in **Figure 3**. Make sure that the specimen and knife are secured tightly and set the temperature of specimen, knife, and chamber at –120°C (*see* **Note 17**).
2. Align the knife edge and the block face and approach the knife edge carefully with 0.1 to 0.5 mm steps using back light illumination.
3. With a perfect specimen knife, sections ranging from 20 to 100 nm can be cut. Usually the feed is set between 45 and 60 nm (*see* **Notes 18**,**19**).

3.6. Section Retrieval and Storage

3.6.1. Section Retrieval

Once the cryo-ultramicrotome produces relatively flat, minimally compressed sections, they have to be retrieved from the knife. On modern cryo-ultramicrotomes like the Leica Ultracut S/FCS, ribbons of section can be produced. Usually the sections are "guided" while sectioning with an eyelash on top of a wooden stick (**Fig. 2**).

1. Put two or three sections aside from the knife edge. When the available space is exhausted, stop the sectioning and start picking them up.
2. Dip the wooden stick with a stainless-steel loop (loop diameter 2–2.5 mm, wire diameter 0.3 mm) into a pickup solution. Retracting the wire loop fast out of the pickup solution will result in a large drop. Slow withdrawal will decrease the drop size. The size of the drop is important, because a larger drop will take longer to freeze.
3. If the picking-up solution is 2.3 *M* sucrose, the wire loop filled with a sucrose droplet is introduced into the cryochamber. In our laboratory we routinely use a picking-up solution of 2.3 *M* sucrose supplemented with methyl cellulose ***(10)***. However, section retrieval with this solution requires very flat sections and a lot

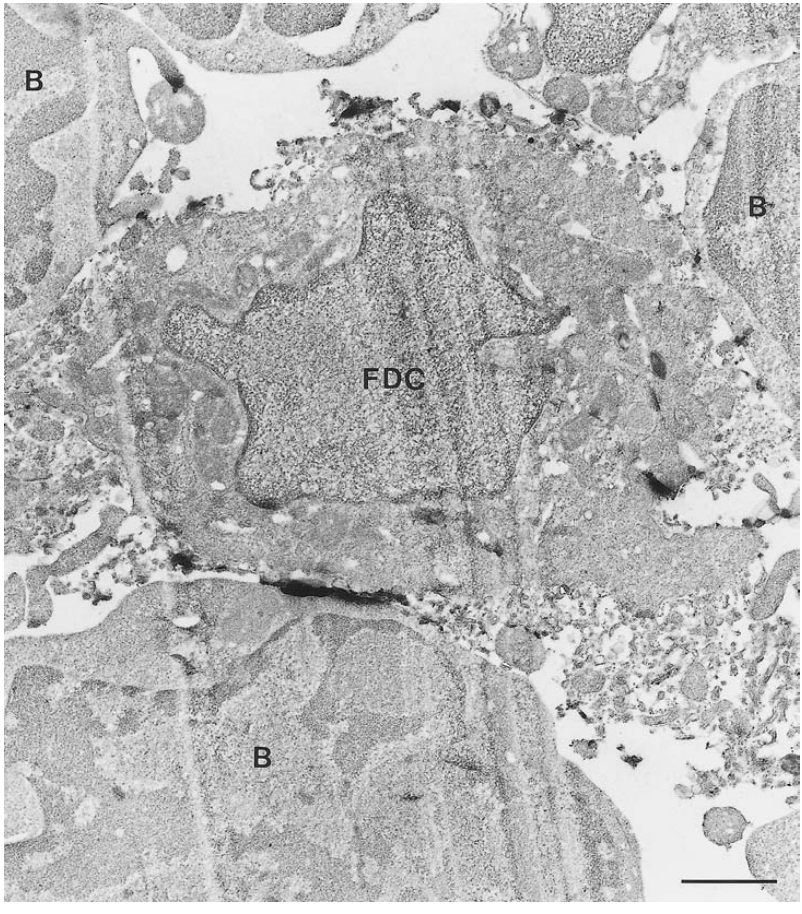


Fig. 3. Effect of scratches on the knife. A follicular dendritic cell (FDC), isolated from human tonsil, is surrounded by several B lymphocytes **(B)**. The micrograph shows light and dark stripes, in the cutting direction. This is due to damages on the knife, which result in irregular thickness of the section. Bar, 1 μm.

of practice (*see* **Note 20**). **Figure 4** shows the difference in morphology as a result of picking-up with either of the solutions.

4. While looking through the binocular, the droplet is placed near the sections. Usually some "smoke" can be seen emerging from the droplet.

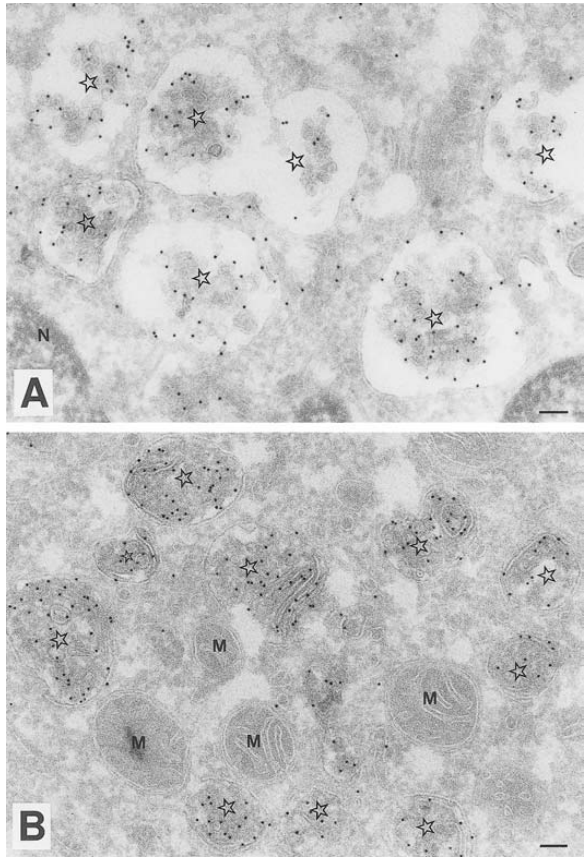


Fig. 4. Influence of different picking-up solutions on morphology. Human DC were generated as described ***(45)***, and fixed in 2% PFA plus 0.2% glutaraldehyde for 2 h. Ultrathin cryosections were either retrieved with 2.3 *M* sucrose **(A)** or a 1:1 mixture of 2.3 *M* sucrose and 2% methyl cellulose **(B)**. Ultrathin cryosection were immunosinglelabeled with antibodies to MHC class II and 10 nm PAG. **(A)** The electron micrograph shows several MHC class II enriched compartments (stars) with internal membrane vesicles. Note that the compartments are dilated, with large electron lucent areas. This is due to over stretching of the section during its retrieval. (N = nucleus). **(B)** Electron micrograph of a similar part of a DC as in **A**, but this section has been retrieved with the 1:1 mixture of 2.3 *M* sucrose and 2% methyl cellulose. Note that the MHC class II compartments (stars) are more intact, and membranes better visualized. M, mitochondrion. Bars, 100 nm.

5. Once the droplet stops "smoking," press the droplet gently on top of two or three sections. Do not try to pick up too many sections, otherwise the surface of the droplet becomes "overcrowded." Looking through the droplet the sections can be seen, and sometimes the stretching of the sections is visible. Soon after that the sucrose solution is frozen.
6. Remove the droplet from the cryo chamber and melt the sucrose by gently breathing on it.
7. Press the droplet with the sections facing the grid on top of a Formvar carbon-coated grid.
8. To evaluate whether the sections are of good quality, the sucrose is removed by floating the grid on distilled water. After approx 5 min the grid is removed from the water, air-dried, and viewed in the electron microscope. If the sections are satisfactory, they can be stored as described in **Subheading 3.6.2.**

3.6.2. Storage of Thawed Cryosections

After the sections are put on top of the grid, they can be stored in one of two ways.

1. The grids can be transferred to 2% solid gelatin (sections facing the gelatin) and kept there for 2–24 h at 4°C without noticeable effect on the sections.
2. Another more recently developed method is to place a droplet of sucrose or methyl cellulose/sucrose on the grids in a closed Petri dish or microscope storage box at 4°C. We have stored sections for more than 6 mo in this way and still an excellent morphology and labeling could be observed.

3.7. Immunogold Labeling

3.7.1. Immuno-Single Labeling

The incubation procedure may vary from lab to lab and from person to person and probably has to be 'fine tuned' for every specimen or antibody under investigation. Therefore, we suggest some alternatives in each step. **Figure 5** shows an immuno-singlelabeling and a schematic representation is given in **Fig. 6**.

1. Transfer grids, which were stored at 4°C in either sucrose or methyl cellulose/sucrose, to 2% gelatin (40°C) for at least 30 min. (If the grids were already on 2% gelatin, the gelatin is melted at 40°C and kept there for 15 min.) The gelatin reduces the background staining by saturating nonspecific binding sites. Alternatively 1% fetal calf serum (FCS) or 1% cold fish gelatin (CFG) can be used.
2. Remove the grids from the gelatin solution and transfer them to PBS/glycine drops of about 100 µL on a parafilm. This is done three times for 2 min, whereby the glycin blocks free aldehyde groups.
3. Incubate for 5 min in PBS/BSA 1% for saturation of nonspecific binding sites. Alternatively, FCS or CFG can be used ***(29–32)***.

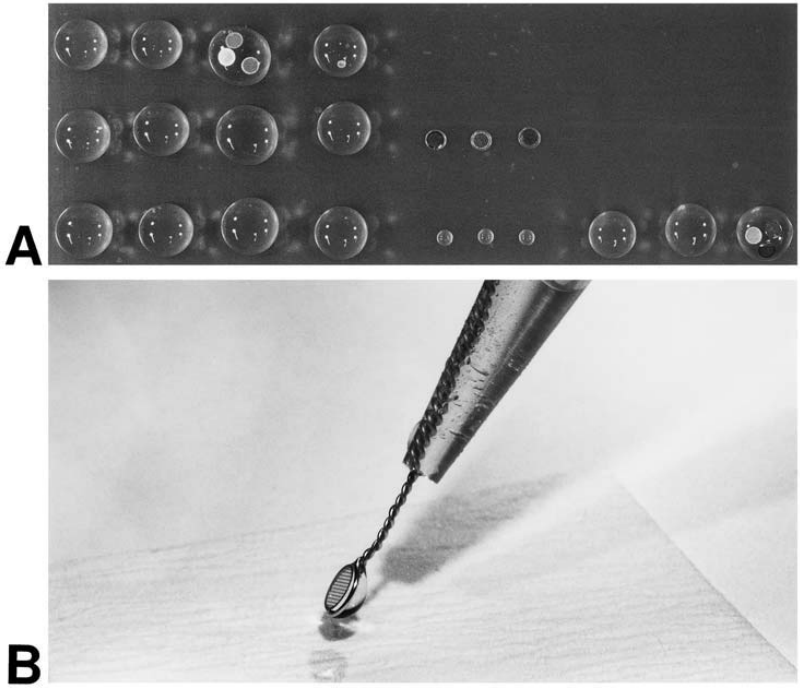
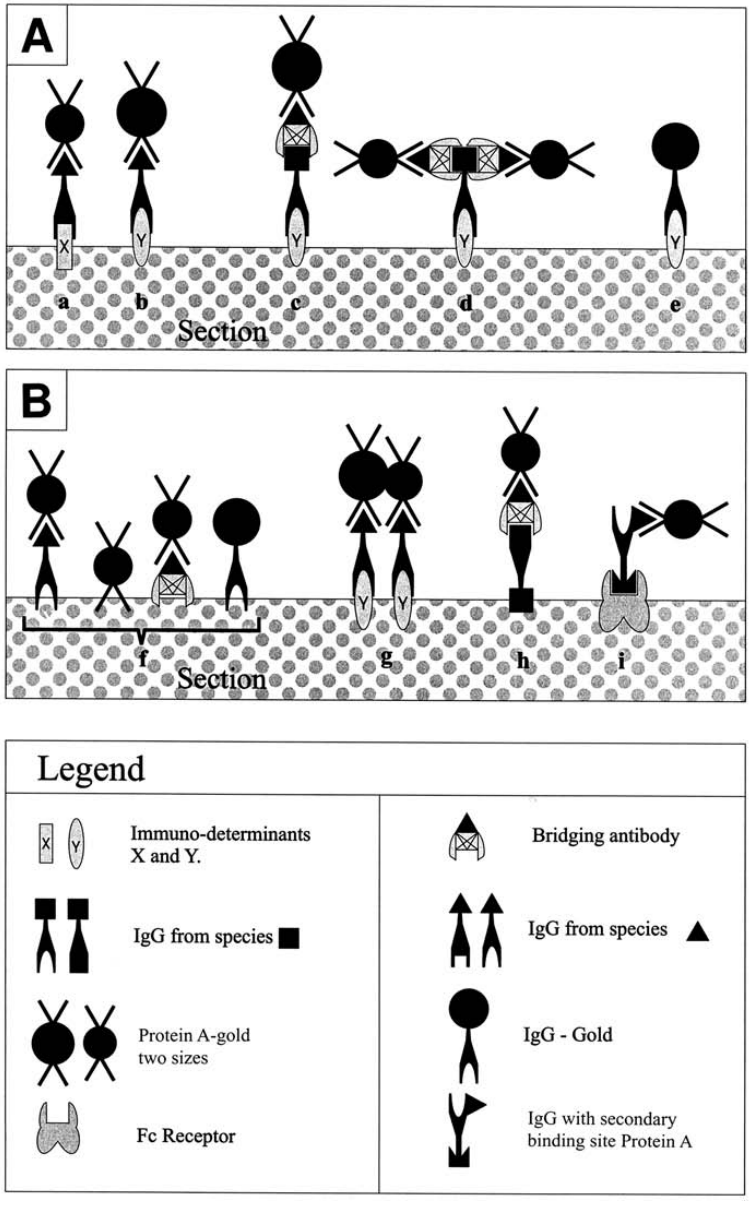


Fig. 5. The different steps in an immunolabeling. An immunolabeling is performed on a piece on parafilm, preferably on a dark surface for better visualization of the grids. **(A)** Grids present are 200 μL drops of PBS/ 50 m*M* glycine, 5 μL drops of antibody (note that each grid is incubated separately), and 200 μL drops of PBS/0.1% BSA. **(B)** The last step in the immunolabeling procedure is the removal of methyl cellulose/uranyl acetate from the grid with a stainless-steel loop.

4. Dilute the antibody in PBS/BSA 1% and incubate grids on 5 μL (minimum) drops for 20–60 min. The Ig antibody concentration should be between 2 and 20 mg/mL (*see* **Note 21**).
5. Rinse the grids four times in PBS/BSA 0.1% for 2 min.
6. In case the primary antibody does not react with protein-A gold, a bridging antibody is required (*see* **Note 22**). This is diluted in PBS/BSA 1% and grids are incubated for at least 20 min in the bridging antibody.
7. If a bridging antibody is used, the grids will require four 2 min rinses in PBS/ BSA 0.1%.
8. Incubate grids on protein-A gold diluted in PBS/BSA 1%. Different sizes of protein-A gold can be used. Most commonly, 5, 10, 15, and 20 nm sizes are used (*see* **Note 23**).


A
X
Y
a
b
c
d
e
Section
B
f
g
h
i
Section
Legend
Immuno-determinants X and Y.
IgG from species
Protein A-gold two sizes
Fc Receptor
Bridging antibody
IgG from species
IgG - Gold
IgG with secondary binding site Protein A

9. Rinse grids three times in PBS for 5 min.
10. To stabilize the immuoreaction, incubate grids for 5 min in 1% glutaraldehyde in PBS. In addition, this will denature IgGs, which is required to prevent colabeling in case of a double-labeling.
11. Perform six 1 min rinses with distilled water. The ions in the PBS will cause precipitates during contrast enhancement.
12. Incubate grids for 5 min on uranyl oxalate pH 7.
13. Rinse briefly with methyl cellulose/uranyl acetate pH 4.
14. Incubate grids for 5 min on methyl cellulose/uranyl acetate pH 4 on ice.

3.7.2. Contrast Enhancement and Post-Embedding

In our laboratory we routinely use positive–negative staining for contrast enhancement and embedding of the sections, but other staining methods are also possible ***(33–36)***. The removal of excess methyl cellulose/uranyl acetate is described below.

1. Remove the grids from the drops of methyl cellulose/uranyl acetate with loops having the same or slightly larger diameter than the grids.
2. Blot the excess of fluid by gently pushing the loop sideways over filter paper, so that a thin film is left behind over the section side of the grid.
3. Dry the grid while still on the loop.

3.7.3. Multiple Immunolabeling

Multiple immunolabeling procedures are in fact two or more consecutive single labeling procedures. (***36***,***37***; **Fig. 6**). After stabilization of the

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Fig. 6. *(opposite page)* Schematic representation of valid and non-specific immunolabeling patterns. In **A** examples of valid immunolabelings are shown. Immunodeterminant X is labeled with an antibody, and small protein A-gold particles (PAG) (a), whereas Y is labeled with large PAG (b). In this case both antibodies contain a protein A binding site, e.g., rabbit antibodies. In contrast, when immunodeterminant Y is labeled with an antibody that has no protein A binding site (c) (e.g., rat or mouse IgG1 antibodies), a bridging antibody has to be applied (d). The bridging antibody can occupy several binding sites on one antibody, which leads to the presence of two or more gold particles on one immunodeterminant. Gold particles can also be coupled to the Ig directly (e). The nonspecific immunolabelings are shown in **B**, which can result from binding of any reagents to the section (f). PAG which was applied in the second round of a double-immunolabeling experiment, can bind to the antibody applied in the first step (g). This may happen when the protein A binding site of the first round antibody was not properly fixed by glutaraldehyde. Soluble or membrane bound Igs (h), as expressed in B lymphoblasts, can nonspecifically bind bridging reagents and thus PAG. This binding is dependent on the expression level and type of fixation used. Fc-receptors can bind to the Fc portion of antibodies (i), which are subsequently labeled with PAG on a second protein A binding site. (Most antibodies contain such a second binding site for PAG.)

immunoreaction (**step 10**) the incubation continues from **step 2** onward. However, one should keep in mind that the immunoreagents used in the second labeling can and will react with the previously used immunoreagents. This may generate false-positive labeling patterns, such as pairs or clusters (**Fig. 6**; *see* **Note 24**).

4. Notes

4.1. Materials

4.1.1. Characterization of the Endocytic Pathway

1. Medium: For endocytic experiments medium with relatively low amounts of serum are used to prevent competition of the endocytic marker protein with serum proteins. Naturally, for long uptake or chase times it is better to use a higher percentage of serum. Cold serum-free medium is used to wash the cells in pulse-chase experiments. Different types of endocytic tracers can be used, and the choice is dependent on the cell type and endocytic route, which is being studied. To mark the entire endocytic route, involved in both fluid phase and receptor-mediated uptake, BSA-gold or horseradish peroxidase (HRP) are suitable candidates. PAG can be used as a fluid phase marker. For characterizing Fc-receptor mediated uptake, immune complexes (i.e., anti-ovalbumin(OVA)-IgG/OVA complexes) can be used, and either detected by antibodies against the IgG or the antigen. Alternatively, the antigen can be conjugated to gold particles prior to the experiment. However, one should be very careful using gold-coupled tracers, because many of them are readily endocytosed and transported toward lysosomes. Proper control experiments and the use of low tracer concentrations allow one to distinguish between fluid-phase and receptor-mediated endocytosis. In addition, proteases in the acidic environment of endocytic compartments may degrade part of the antigen, and thereby influence its intracellular localization. To reveal organelles with an acidic content, the weak base 3-(2,4-dinitroanilino)-3-amino-*N*-methyldipropylamine (DAMP) is added to cells for 30 min at 37°C. Then the cells are washed in an excess of cold medium and fixed. DAMP diffuses through membranes and accumulates in acidic compartments, like endosomes and lysosomes. DAMP can be visualized by immunolabeling with DNP antibody, which recognizes DAMP ***(13)***.
2. Buffers are important to maintain the pH within a physiological range ***(38)***. The buffering capacity should be high enough to be able to compensate for pH changes during fixation. Some commonly used buffers like Tris are not suitable as a buffer vehicle in combination with an aldehyde, because the amino groups will quench the aldehyde action. Furthermore, substances can be added to the buffers that favor the preservation of specific cellular components like cytoskeletal elements. A 0.1 *M* PIPES or HEPES buffer can also be used if phosphate interferes with one of the reagents (for example, it precipitates with uranyl acetate if the sections are not rinsed in water). The buffers used to prepare fixa-

tives need to have a good buffer capacity to maintain a pH of about 7.4 during fixation. PIPES 0.2 *M*, pH 7.4 (stock solution), is prepared in distilled water. The pH is set to 7.4 with NaOH while dissolving the PIPES, otherwise it will not be completely solubilized.

3. Mildly fixed specimens may benefit from intracellular support by using long molecules such as polyvinylpyrolidon (PVP;***35***). PVP-sucrose is prepared by dissolving 3 g PVP (MW 20,000, Fluka) in 0.6 mL of 1.1 *M* Na_2CO_3 and 17 mL 2 *M* sucrose (stir overnight).
4. Nowadays cryo-diamond knives are of excellent quality. Although rather expensive they are an overall good value for the money. The sectioning properties of a diamond knife are outstanding and remain good for as long as a year. Even facing and shaping the specimen can be done with "blunt" or "old" diamond knives (thus giving them a second life as trimming knife), with semithin diamond knives or with specially designed trimming diamond knives. Diamond knives (in combination with an ionizer) can save considerable time. However, they must be treated well. Cryo-diamond knives have to be cleaned after and before the sectioning with a Styrofoam rod, which is supplied by the manufacturers together with cleaning instructions. Briefly, shape a triangular tip to the Styrofoam rod using a razor blade. Press the tip gently on the knife edge and move it along the edge avoiding lateral forces. Remove the rod from the edge and repeat this several times, each time moving the rod in the opposite direction. Resharpen the rod, dip it into 100% alcohol, remove the excess of alcohol, repeat the cleaning, and store the knife in its box. If the knife has more persistent stains, use some saliva on the rod before the above-mentioned procedure. If none of these methods works, the knife may be submersed in a 2% Decon (or similar detergent) overnight. When a knife is used every day, cleaning prior to sectioning is not necessary. However, when it is used less frequently, cleaning is necessary. Make sure that the knife is absolutely dry when put into the cryo-chamber.
5. The preparation of a glass knife starts with the glass rods from which they are being made. The glass should be tough and preferably without fluctuations in consistency. When a new batch of glass rods has been received, the dimensions should be checked. The width of the rods must be constant and, moreover, the lateral sides of the rods must not be distorted. If the rods do not meet your requirements, just send them back to the manufacturer. The ideal knife is made according to the Tokuyasu method ***(36)*** using a Leica knife maker. For practical reasons this method has been adapted by Griffiths et al. ***(2,39)*** so it can be used in the laboratory.
6. A number of reports describe in detail the properties of colloidal gold solutions and the various methods to prepare gold conjugated with antibodies, lectins, *Staphylococcus aureus* protein-A or protein G ***(2,18–20)***. The method used in the authors' laboratory has been described in detail elsewhere ***(21,22)***. This method uses a mixture of tannic acid and citrate to reduce Au^{3+}, usually provided as gold trichloride. By varying the concentration of the tannic acid in the mixture, colloidal gold solutions displaying different sizes of gold particles can be obtained. By

this method protein gold solutions have been proven to be of satisfactory quality in terms of uniform sizes that can be prepared reproducibly in the range of 3–15 nm. In the authors' laboratory the different sizes of gold particles conjugated to protein-A (PAG) are prepared four times in the year and are also commercially available ***(22,23,40)***.

4.2. Fixation Procedure

7. Several factors, including concentration, temperature, osmolarity, and length of fixation, can have an effect on the fixation quality. Concentration: When high concentrations of PFA are directly used on cells or tissue, swelling often occurs. Initial fixation should be done with 2% PFA thereafter increasing concentrations can be applied. Keep in mind that the PFA fixation process is reversible and prolonged storage of tissue in buffers without fixative may result in a loss of structural integrity. Temperature: The initial fixation should be performed at room temperature (minimum) or even at 37°C. At these temperatures the structural integrity of the cytoskeleton is maintained. Furthermore the diffusion of the fixative into the tissue or cells is faster and the actual cross linking will be more effective. After the initial fixation the specimens can be stored for longer periods of time at 4°C. Osmolarity: A physiological osmolarity for a fixative would be 360 mOsm. Some fixatives like PFA can increase the osmolarity to a great extent. For example, 8% PFA in a 0.1 *M* phosphate buffer has an osmolarity of 1300 mOsm. However, the effect on cells is not as profound as these figures may suggest. When a fixing agent is added to cells or tissues, the fixative starts to react with amino-moieties on the cells and therefore does not raise the osmolarity as much as anticipated. However, when rather slow reacting substances are used like PFA, osmolarity should be taken into account. Length of fixation: The time during which specimens can be fixed depends on the fixative used, its concentration, and the temperature. Usually 2 h at room temperature is sufficient for fixation. Prolonged fixation does not necessarily improve the ultrastructural preservation, but may affect the antigenicity of immunodeterminants under investigation. Furthermore, prolonged fixation may harden the specimen and affect the sectioning properties.
8. To increase a cell pellet add nonrelevant fixed cells. Importantly, antibodies being used should not react on the nonrelevant cells. Thus, in the case of human DC, a murine T-cell clone is suitable, or if working with mouse DC, human T2 cells can be added. Alternatively, when available, pellets can be supplemented with fixed red blood cells, which are easily identified by EM and also by light microscopy.
9. Sometimes the scraped cells have a tendency to float on the fixative, which makes it more difficult to collect the cells. If this happens, remove the fixative after an appropriate fixation period and rinse three times with PBS/50 m*M* glycine. After the last centrifugation place the pellet in 1% gelatin in buffer (37°C). Remove the cells from the Petri dish with a cell scraper and transfer to an Eppendorf vial for further handling.
10. The most crucial step in the fixation of tissues is speed. The fixative should reach the cells in the tissue of interest within s otherwise the ultrastructural preserva-

tion will not be sufficient and immunodeterminants can be relocated within cells. When possible, a "whole body perfusion" is performed. After sedation with a barbiturate (for example, Nembutal, 20–30 mg/kg body weight), the abdominal cavity is opened and a needle is inserted in the abdominal aorta. An incision is made in the inferior vena cava close to the liver and the perfusion pump is started. First the vascular bed is flushed with PBS 1.5–2 times the blood volume to remove blood cells. To prevent clotting, an anticoagulant can be added. Another possibility is perfusion via the left ventricle. In this case the chest is opened an a needle is inserted into the left ventricle.

4.3. Support of Fixed Samples

11. Gelatin embedded material can be postfixed. A disadvantage of postfixation is that the gelatin is cross-linked to proteins present at the extracellular face of the plasma membrane, thus reducing the labeling efficiency. Mildly fixed specimens may benefit from intracellular support provided by long molecules such as polyvinypyrolidon or polyvinylalcohol (MW 10,000–30,000), which can be added to the cryoprotectant.
12. Alternatively, most of the gelatin can be removed and the specimens and some gelatin are transferred to a microscope slide. They can then be processed as described in **Subheading 3.3.2.**
13. Samples fixed in PFA must be removed from the sucrose within 4 h, owing to the reversibility of this fixation. Glutaraldehyde fixed samples can be left rotating over night.

4.4. Freezing Specimens

14. The sucrose-infiltrated specimens should not be exposed to air for too long while handling them. Air exposure evaporates water from the specimen and the sucrose solution thickens and sucrose crystals may form. Copper and, to a lesser extent, aluminum specimen holders cool down much more quickly than the specimens (particularly when the specimens are relatively big), and crevices may emerge between the holder and the specimen. Crevices may also develop if the top of the holder has not been cleaned properly (owing to grease). During sectioning the specimen will inevitably break away from the holder. It is also possible to remount the specimen, because freezing is no longer a crucial step. In such a case remove the specimen from the holder, put it back in 2.3 *M* sucrose, leave it there for 10 min, and remount.

4.5. Sectioning

15. Thicker sections (>0.3–0.4 mm), cut at a temperature of –120°C, may cause irreversible damage to the specimen. Chips may break away from the specimen or even the entire block when it is removed from the specimen holder.
16. The trimming can also be performed using diamond trimming tools. They allow semithin sectioning and the sides are specially designed for trimming specimens.
17. At –120°C most of 2.3 *M* sucrose infiltrated specimens will give good results using a diamond knife. However, sectioning is possible from –170 to –85°C.

When the specimens are more compact and fixed thoroughly, higher temperatures than –120°C are advisable.

18. One problem is static charging. Owing to the extremely dry atmosphere the knives (diamond knives in particular) will be charged during sectioning. To overcome this difficulty, a regulatable ionizer (Diatome, Bienne, Switzerland) is mounted in the cryochamber. An ionizer also improves the section quality by preventing most of the compression. The use of the ionizer will not always solve the static charging (and thus compression) problem. Diamond knives from different manufacturers behave differently, and it may be very useful to experiment with the ionizer strength and knife clearance angles. The ionizer should be turned off during section retrieval.
19. It is always possible that sectioning does not work. In this case, one should vary the sectioning speed (usually 2 to 5 mm/s but sometimes even 80 mm/s can be used), temperature, feed, knife or replace the specimen itself.

4.6. Section Retrieval and Storage

20. The second solution we use is a 1:1 mixture of 2.3 *M* sucrose and 2% methyl cellulose in water ***(9)***, which is prepared just before use. Keep this mixture at 4°C otherwise white deposits will emerge. The viscosity of this solution is much lower and it freezes more rapidly. The freezing will start at the edge (white rim), at that time the section should be picked up. An instant of a second later the drop will have solidified and the sections cannot be picked up anymore. The sections should be parked somewhere away from the knife edge, because water vapor is emitted from this drop in relatively large quantities thus causing vast ice deposits on the knife. Picking up the sections is easiest from a nonmetallic surface such as the glue of the diamond knife. With this solution the sections do not stretch on the droplet, and are kept as they are just after sectioning. This implies that the section has to be without wrinkles or compression. The operator has to be more experienced in order to use this pickup method but it pays off in the end. The final structure is much better as compared with sections retrieved with 2.3 *M* sucrose. In many cases 2.3 *M* sucrose or sucrose/methyl cellulose are suitable pick up solutions. However, when delicate lipid-rich structures are involved, sucrose-based pickup solutions are not sufficient to retain lipids in a cryo-section. An additional "while picking up" fixation is necessary to preserve lipid-rich structures and the lipids in membranes. Liou et al. used for these purposes a mixture of 2% methyl cellulose and 2% uranyl acetate in water (final concentrations) ***(9)***. The sections must be of outstanding quality in terms of thickness (50 nm) and flatness. A prerequisite for this method is that the specimens are either physically fixed or the buffer vehicle of the primary fixative and sucrose do not form precipitates with the uranyl acetate (PIPES or HEPES buffers).

4.7. Immunogold Labeling

21. Membrane-bound immunodeterminants and adequately fixed soluble immunodeterminants can be submitted to overnight incubations at 4°C in a mois-

turized atmosphere. This procedure may increase the labeling efficiency of antibodies, which give low labeling after 60 min of incubation. Note also that background labeling may increase.

22. In fact three types of labeling procedure can be distinguished:
    a. Primary antibody followed by protein-A-gold ***(18–20)***.
    b. Primary antibody followed by a bridging antibody and protein-A-gold.
    c. Primary antibody followed by IgG-gold ***(41,42)***.
23. PAG is used as the final step in the immuno incubation. This procedure results in a very precise localization in which primary antibody and PAG combine in a 1:1 ratio. Usually the observed background is extremely low: less than 0.5 gold particle/mm^2. Another widely used immunoreagent is IgG coupled to gold. Often the primary antibody to IgG-gold ratio will be greater than 1:1.
24. Possible problems in double-immunolabeling include co-labeling and appearance of two sizes of gold particle in close vicinity. Bridging antibody for a second antibody in a double-labeling is only possible when this bridge does not recognize the first antibody.

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References

1. Larsson, L. I. (1988) *Immunocytochemistry: theory and practice.* CRC Press Inc., Boca Raton, FL.
2. Griffiths, G. (1989) Fine structure immunocytochemistry. Springer-Verlag, NY.
3. Raposo, G., Kleijmeer, M. J., Posthuma, G., Slot, J. W., and Geuze, H. J. (1997) Immunogold labeling of ultrathin cryosections: application in immunology. In *Weir's handbook of experimental immunology.* Herzenberg, L. A., Weir, D. M., Heizenberg, L. A., and Blackwell, C., eds. pp. 208. 1–208.11.
4. Kleijmeer, M. J., Raposo, G., and Geuze, H. J. (1996) Characterization of MHC class II compartments by immunoelectron microscopy. In: *Methods: A Companion to Methods in Enzymology* Academic Press, Inc., vol 10, pp. 191–207.
5. Fernandez-Moran, H. (1952) Application of the ultrathin freezing-sectioning technique to the study of cell structures with the electron microscope. *Arkiv. Fysik.* **4,** 471–491.
6. Bernard, W. and Leduc, E. H. (1967) Ultrathin frozen sections. Methods and ultrastructural preservation. *J. Cell Biol.* **34,** 757–771.
7. Tokuyasu, K. T. (1973) A technique for ultramicrotomy of cell suspensions and tissues. *J. Cell Biol.* **57,** 551–565.
8. Tokuyasu, K. T. and Singer, S. J. (1976) Improved procedures for immunoferritin labeling of ultrathin frozen sections. *J. Cell Biol.* **71,** 894–906.
9. Liou, W. and Slot, J. W. (1994) Improved fine structure in immunolabeled cryosections after modifying the sectioning and pick-up conditions. *Proc. Int. Conf. Electr. Microsc.* **13,** 253,254.

10. Griffith, J., Posthuma, G., Slot, J. W., and Geuze, H. J. Submitted J. Mistochen and Cytochem
11. Roberts, I. M. (1975) Tungsten coating—A method of improving glass microtome knives for cutting ultrathin frozen sections. *J. Microsc.* **103,** 113–119.
12. Michel, M., Gnägi, H., and Müller, M. (1992) Diamonds are a cryosectioner's best friend. *J. Microsc.* **166,** 43–56.
13. McDowall, A. W., Chang, J. J., Freeman, R., Lepault, J., Walter, C. A., and Dubochet, J. (1983) Electron microscopy of frozen hydrated sections of vitreous ice and vitrified biological samples. *J. Microsc.* **131,** 1–9.
14. Kleijmeer, M. J., Oorschot, V. M. J., and Geuze, H. J. (1994) Human resident Langerhans cells display a lysosomal compartment enriched in MHC class II. *J. Invest. Dermatol.* **103,** 516–523.
15. Nijman, H. W., Kleijmeer, M. J., Ossevoort, M. A., et al. (1995) Antigen capture and MHC class II compartments of freshly isolated and cultured human blood dendritic cells. *J. Exp. Med.* **182,** 163–174.
16. Kleijmeer, M. J., Ossevoort, M. A., van Veen, C. J. H., et al. (1995) MHC class II compartments and the kinetics of antigen presentation in activated mouse spleen dendritic cells. *J. Immunol.* **154,** 5715–5724.
17. Tan, M. C., Mommaas, A. M., Drijfhout, J. W., et al. (1997) Mannose receptor-mediated uptake of antigens strongly enhances HLA class II-restricted antigen presentation by cultured dendritic cells. *Eur. J. Immunol.* **27,** 2426–2435.
18. Engering, A. J., Cella, M., Fluitsma, D., et al. (1997) The mannose receptor functions as a high capacity and broad specificity receptor in human dendritic cells. *Eur. J. Immunol.* **27,** 2417–2425.
19. Bendayan, M. (1984) Protein A-Gold electron microscopic immunocytochemistry: Methods, applications, and limitations. *J. Elec. Microsc. Tech.* **1,** 243–270.
20. Slot, J. W. and Geuze, H. J. (1983) The use of protein A-colloidal gold (PAG) complexes as immunolabels in ultrathin frozen sections. In *Immunohistochemistry—IBRO Handbook Series,* Cuello, A. C. (ed.) John Wiley, Chicester, England, pp. 323–346.
21. Roth, J. (1983) The colloidal gold marker system for light and electron microscopic cytochemistry. In *Techniques in Immunocytochemistry,* vol. II. Academic Press, New York, pp. 217–284.
22. Slot, J. W. and Geuze, H. J. (1985) A new method of preparing gold probes for multiple-labeling cytochemistry. *Eur. J. Cell Biol.* **38,** 87–93.
23. Slot, J. W., Geuze, H. J., and Weerkamp, A. J. (1988a) Localization of macromolecular components by application of the immunogold technique on cryosectioned bacteria. Methods Microbiol. **20,** 211–236.
24. Hopwood, D. (1985) Cell and tissue fixation, 1972–1982. Histochem. J. **17,** 389–442.
25. Hopwood, D. (1970) The reactions between formaldehyde, glutaraldehyde and osmium tetroxide, and their fixation effects on bovine serum albumin and on tissue blocks. *Histochemie* **24,** 50–64.
26. Fox, C. H., Johnson, F. B., Whiting, J., and Roller, P. P. (1985) Formaldehyde fixation. *J. Histochem. Cytochem.* **33,** 845–853.

27. Slot, J. W., Geuze, H. J., and Weerkamp, A. J. (1988) Localization of macromolecular components by application of the immunogold technique on cryosectioned bacteria. In *Methods in Microbiology: Electron Microscopy in Microbiology* vol. 20, Mayer, P. F. (ed.), Academic Press, London, pp. 211–236.
28. Dubochet, J., Adrian, M., Lepault, J., and McDowell, A. W. (1985) Cryo-electron microscopy of vitrified biological specimens. *Trends Biochem. Sci.* **10,** 143–146.
29. Geuze, H. J. and Slot, J. W. (1980) Disproportionate immunostaining patterns of two secretory protein in guinea pig and rat exocrine pancreas cells. An immunoferritin and fluorescence study. *Eur. J. Cell Biol.* **21,** 91–100.
30. Kraehenbühl, J. P. and Jamieson, J. D. (1974) Localization of intracellular antigens by immunoelectron microscopy. *Exp. Pathol.* **13,** 1–53.
31. van de Plas, P. and Leunissen, J. L. (1993) Ultrasmall gold probes: characteristics and use in immuno(cyto)chemical studies. *Meth. Cell Biol.* **37,** 241–257.
32. Birrell, G. B., Hedberg, K. K., and Griffith, H. (1987) Pitfalls of immunogold labeling: analysis by light microscopy, transmission electron microscopy, and photoelectron microscopy. *J. Histochem. Cytochem.* **35,** 843–853.
33. Griffiths, G., Brands, R., Burke, B., Louvard, D., and Warren, G. (1982) Viral membrane proteins acquire galactosyl in trans Golgi cisternae during intracellular transport. *J. Cell Biol.* **95,** 781–792.
34. Griffiths, G., McDowell, A., Back, R., and Dubochet, J. (1984) On the preparation of cryosections for immunocytochemistry. *J. Ultrastruct. Res.* **89,** 65–78.
35. Keller, G. A., Tokuyasu, K. T., Dutton, A. H., and Singer, S. J. (1984) An improved procedure for immunoelectron microscopy: ultrathin plastic embedding of immunolabeled ultrathin frozen sections. *Proc. Natl. Acad. Sci. USA* **81,** 5744–5797.
36. Tokuyasu, K. T. (1989) Use of polyvinylpyrrolidone and polyvinylalcohol for cryoultramicrotomy. *Histochem. J.* **21,** 163–171.
37. Bendayan, M. and Stephens, H. (1984) Double labeling cytochemistry applying the protein A-gold technique. In *Immunolabeling for electron microscopy*. Polak, J. M. and Varndell, I. M. (eds.) Elsevier, Amsterdam, pp. 143–154.
38. Geuze, H. J., Slot, J. W., Scheffer, R. C. T., and van der Ley, P. A. (1981) Use of colloidal gold particles in double-labelingimmunoelectron microscopy of ultrathin frozen tissue sections. *J. Cell Biol.* **89,** 653–665.
39. Griffiths, G., Simons, K., Warren, G., and Tokuyasu, K. T. (1983) Immunoelectron microscopy using thin frozen sections: Application to studies of the intracellular transport of semLiki forest virus spike glycoproteins. *Methods Enzymol.* **96,** 466–483.
40. Slot, J. W. and Geuze, H. J. (1981) Sizing of protein A-colloidal gold probes for immunoelectron microscopy. *J. Cell Biol.* **90,** 533–536.
41. De Mey, J. R. (1983) The preparation of immunoglobulin gold conjugates (IGS reagents) and their use as markers for light and electron microscopic immunocytochemistry. In *Immunohistochemistry—IBRO* Cuello, A. C. (ed.) Wiley, UK.
42. Romano, E. L., Stolinski, C., and Hughes-Jones, N. C. (1974) An antiglobulin reagent labeled with colloidal gold for use in electron microscopy. *Immunochemistry* **11,** 521–522.

35

Purification and Characterization of MHC Class II Containing Organelles in Mouse Bone-Marrow-Derived Dendritic Cells

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1. Introduction

The initiation and propagation of the immune responses is dependent on the ability of antigen-presenting cells (APC) to convert proteins into peptides, to load them intracellularly onto major histocompatibility complex (MHC), and then to deliver the peptide–MHC complexes to the plasma membrane in order to stimulate T cells. It is becoming increasingly clear that most of the intracellular compartments that contain MHC class II products in APC simply represent the conventional endosomes and lysosomes that are expressed in all cell types ***(1)***. However, data from cell-fractionation studies, predominantly those using electrophoresis techniques, show that in professional APC, a class of class II-containing endosomes that is specialized for antigen processing may exist. Strong support for this possibility comes from the observation that such specialized structures, designated CIIV, are particularly abundant in mature dendritic cells (DC; **ref *2***).

At this point some clarification on the nature of MHC class II-containing compartments has to be done. In work initially done by Geuze and colleagues with EBV-transformed human B cells, striking electron microscopy images of class II-positive structures were obtained, many of which were endocytic and contained characteristic multivesicular or multilaminar inclusions ***(3)***. Despite their morphological heterogeneity, these structures are collectively designated the MHC class II compartment (MIIC) and viewed as a specialized compartment. Recent work has suggested that most of the class II-positive structures seen by immunocytochemical techniques are indistinguishable from conven-

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tional endosomes and lysosomes and contain lysosomal enzymes such as β-hexosaminidase, cathepsin D, or membrane proteins of the lamp/lgp family ***(4)***. This population of organelles also contains MHC-encoded or -related molecules such as HLA-DM, HLA-DO, in some cases Ii chain, and also members of the CD1 family (B, C, and D) ***(5,6)***. MIIC are typically found in EBV-transformed B cells, macrophages, and immature (tissue) dendritic cells such as epidermal Langerhans cells ***(7)***.

Given initial electron microscopy (EM) findings that many MHC class II-positive cells contained abundant late endosomal–lysosomal structures enriched in class II products (MIIC), Percoll density gradient has been widely used to determine the intracellular distribution of class II molecules, antigens, and immunogenic peptide–class II complexes ***(8–10)***. In cell types found to contain abundant MIIC, appreciable amounts of class II are found in heavy density fractions after Percoll gradient centrifugation ***(10,2)***. A description of a typical protocol for separation of MIIC from immature DC is described below.

The use of additional cell types and alternative gradient procedures provided further support for the possibility that class II can be found in multiple endocytic compartments. In the murine A20 B lymphoblastic cell line, endogenous I-Ad class II molecules do not accumulate in heavy density fractions but are found associated with low density membranes ***(11)***. More recently, free flow electrophoresis (FFE) and a related electrophoretic procedure, density gradient electrophoresis (DGE) ***(12,13)***, have been used for the fractionation of class II-expressing APC. FFE resolves membranes on the basis of surface charge, with lysosomes being more negatively charged than most intracellular membranes. Several years ago, low-density endosomes were found to shift anodally during FFE almost as much as lysosomes, allowing purification of endosomes to near homogeneity ***(14,15)***. Electrophoretic fractionation of mouse A20 B cells revealed the existence of a significant pool of internal class II that was associated with membranes having an entirely different mobility. These class II vesicles (or CIIV) comprised a population that was even more anodally shifted than were the lysosomes, yet were low density by Percoll gradient centrifugation ***(11,10)***. They lacked lysosomal membrane markers (lgp/lamp) despite appearing like classical MIIC by immuno-EM of the isolated fractions. Like MIIC, CIIV contained abundant internal membranes that were also positive for MHC class II. CIIV also contained a small fraction of the total cell pool of transferrin receptor, a classical early endosome marker, suggesting that CIIV might be more closely related to early endosomes than to late endosomes.

From a functional point of view, CIIV in A20 cells were demonstrated to serve as obligatory intermediates in the transport of newly synthesized class II molecules from the TGN to the plasma membrane. Because CIIV is both physi-

cally and functionally distinct from other endocytic and secretory organelles, it seems possible that it comprises a distinct or specialized compartment ***(16)***. Interestingly, in DCs, a population of nonlysosomal CIIV-like vesicles has been identified (DC-CIIV) and shown to be expressed in a developmentally regulated fashion. DC-CIIV appears at a stage of development that correlates with the acquisition of antigen-processing and -presenting capacity, and appear to ferry new peptide-loaded αβ dimers to the plasma membrane ***(2)***.

At the immature phase of their life (residence in the tissues), DC target their MHC class II molecules to conventional lysosomes (MIIC) that can be separated on Percoll density gradients **(Fig. 1)**. The harsh proteolytical environment of lysosomal compartment shorten dramatically the half-life of MHC class II (12 h), which are not efficiently sorted to the cell surface. As a consequence of such an inefficient transport of class II to the cell surface, immature DC are rather poor at antigen presentation. Upon stimulation by inflammatory agents (e.g., LPS), DC migrate to the lymph nodes and, concomitantly, MHC class II transport to the cell surface becomes very efficient ***(17)***. In mature DC, intracellular class II molecules are no longer found in MIIC/lysosomes but they can be detected in another vesicle population (DC-CIIV) using FFE (*see* protocol in **Subheading 3.2.** and **Fig. 1**). The relationship between these two types of compartments is still unclear; however, their purification is a key step to the understanding of antigen presentation and maturation in DC.

2. Materials

2.1. Reagents

1. Percoll (Sigma; P1644).
2. Nycodeenz (Sigma; D2158).
3. (TEA) Recipe?
4. TEA 250: 10 m*M* Triethanolamine (Sigma; T1377), 10 m*M* acetic acid, 1 m*M* EDTA, 250 m*M* sucrose, titrated to pH 7.4 with NaOH.
5. 90% Percoll in 250 m*M* sucrose.
6. Deoxyribonuclease (DNAse) 1 (Sigma; D4527). Prepare a 50 mg/mL stock in TEA.
7. TEA 2.5: 10 m*M* Triethanolamine, 10 m*M* acetic acid, 1 mM EDTA, 2.5 M sucrose, titrated to pH 7.4 with NaOH (*see* **Note 1**).
8. TEA 1.25: 10 m*M* Triethanolamine, 10 m*M* acetic acid, 1 m*M* EDTA, 1.25 *M* sucrose, titrated to pH 7.4 with NaOH.
9. TEA 250: 10 m*M* Triethanolamine, 10 m*M* acetic acid, 1 m*M* EDTA, 250 m*M* sucrose, titrated to pH 7.4 with NaOH.
10. Trypsin (Sigma; T8003).
11. Soybean trypsin inhibitor (Sigma; T9003).
12. Biorad protein assay (Bio-Rad cat. no. 500-0006).

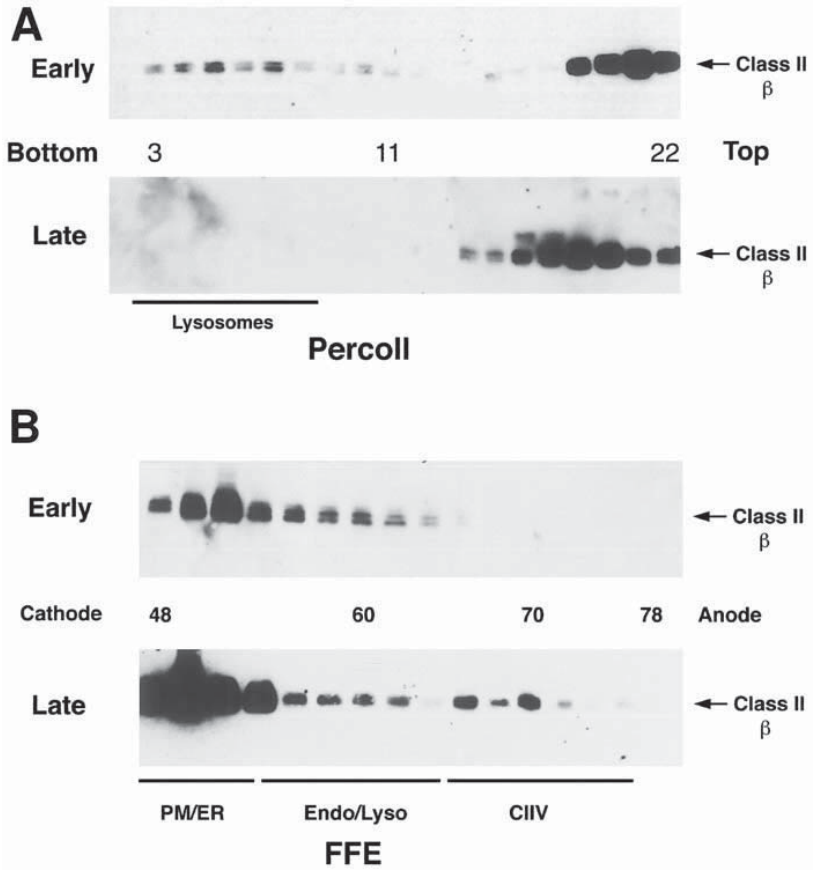

Fig. 1. Characterization of MHC class II compartments in developing DC populations. **Panel A.** Percoll density gradient centrifugation: Postnuclear supernatants of bone marrow cultures enriched in early vs late DC were fractionated by centrifugation in 25% Percoll density gradients under conditions that separate heavy density lysosomes from lower density membranes. Western blot for MHC class II beta chain is shown. Although both early and late cells exhibited class II staining in low density membranes (containing ER, Golgi, and plasma membrane), only early cells exhibited MHC class II in Percoll-dense membranes that cosedimented with the lysosomal markers. **Panel B.** Free flow electrophoresis: Western blots for MHC class II β chain corresponding to the separation profiles of early and late cells by FFE. In early DC, class II molecules were detected in the fractions corresponding to PM (which also contained ER, not shown) as well as in fractions containing endosomes and lysosomes. In late DC populations, class II was detected in the PM/ER fractions, as well as in the endosome/lysosome fractions. Strikingly, a distinct peak of class II was found as having been anodally deflected relative to the major peak of lysosomes; this electrophoretic mobility corresponded to CIIV.

13. Reagents for enzyme assays:
    a. Assay of β-hexosaminidase as a lysosome/MIIC marker.
       Assay buffer: Na citrate-phosphate buffer 4.5: 5.87 g citric acid, 6.26 g Na_2HPO_4 (or 7.85 g Na_2PO_4.2 H2O), 0.1% TX-100, in 500 mL H_2O.
       Stop buffer: 200 m*M* Na_2CO_3 and 110 m*M* glycine. 21.2 g Na_2CO_3 and 8.2 g glycine in 1000 mL H_2O.
       Substrate: 6 m*M* (2.3 mg/mL) in assay buffer of 4-methyl-umbelliferyl-*N* acetyl- β-D glucosaminide (Sigma; M2133).
    b. Assay of alkaline phosphodiesterase as a plasma membrane marker.
       Assay buffer: 100 m*M* Tris-HCl pH 9.0, 40 m*M* $CaCl_2$.
       Stop buffer: 200 m*M* Na_2CO_3 and 110 m*M* glycine. 21.2 g Na_2CO_3 and 8.2 g glycine in 1000 mL H_2O.
       Substrate: 2 mg/mL in assay buffer of thymidine-5'-monophosphate-*p*-nitrophenyl ester (Sigma; T4510).

2.2. Cells

Mouse bone-marrow DCs are typically obtained after sacrifice of 10 mice. DC are differentiated in presence of granulocyte-macrophage colony-stimulating factor (GM-CSF) as described by Kayo Inaba and colleagues ***(18)***. See also Chapter 17 of this volume. After 7 d of culture in RPMI supplemented with 5% FCS and GM-CSF, 5–30 × 10^6 cells are submitted to fractionation in one experiment.

2.3. Equipment

1. Ball-bearing cell cracker (Balch and Rothman, 1985, *Arch. of Biochem. Biophys.*, **240,** 413) available from Berni Tech Engineering Inc. Saratoga, CA.
2. Beckman centrifuge and Ti50 and SW41Ti rotors.
3. Beckman quick seal tubes (16 × 76 mm) cat. no. 342413.
4. Beckman Polyallomer tubes (13 × 89 mm) cat no. 331372.
5. Free Flow electrophoresis "Octopuss," manufactured by Weber GmbH, Munich Germany.
6. Refractometer.
7. Microcentrifuge.
8. Fluorometer.

3. Methods

3.1. Isolation of MIIC/Lysosomes by Percoll Gradient Centrifugation

1. Isolate immature DC (*see* **Note 2**) by sedimentation on 50% serum columns, as described by Kayo Inaba and colleagues ***(18)***.
2. Collect DC by centrifugation at 1200 rpm for 5 min and washed twice with ice-cold PBS.

3. Resuspend DC at 5×10^6 cells/mL in cold TEA 250, supplemented with regular protease inhibitors cocktail.
4. Homogenize DC by several passages through a ball-bearing homogenizer. Homogenization is followed by using phase-contrast microscopy to determine how many passes it takes to lyse the cells but leave the nuclei intact.
5. Spin the homogenate at 3000*g* for 10 min to pellet nucleii and intact cell debris. Collect the supernatant (PNS).
6. Mix the DC PNS with a cold solution of Percoll and TEA 250 to give a final concentration of 26 % Percoll in 12 mL for centrifugation in a Ti50 rotor. For 1 mL of PNS add 3.5 mL of 90 % Percoll and 7.5 mL of TEA 250.
7. Using a Pasteur pipet, load the PNS /Percoll solution in a Ti50 quick seal tube and underlay with a 1 mL solution of 60% Nycodenz in TEA 250. Seal the tube and centrifuge at 18,000 rpm for 1.5 h in a Ti50 rotor. The run should be stopped without the brake.
8. Collect the gradient from the bottom by fractions of 300–500 μL.
9. Determine the position of the MIIC/lysosomes in the gradient by monitoring the β-hexosaminidase activity (**Subheading 3.3.**). The bulk of the activity is generally found in fractions 3, 4, and 5 of the gradient (bottom). In contrast, other intracellular membranes such as the plasma membrane (determined by a peak in alkaline phosphodiesterase activity, *see* **Fig. 1**) are found floating at the top of the gradient (fractions 18 to 22).
10. Collect the membranes, after dilution of the fractions (minimum 10 times in TEA), by centrifugation in a microcentrifuge at maximum speed (*see* **Note 3**). After centrifugation the membranes can be prepared for SDS-PAGE analysis, by resuspending them in sample buffer (*see* **Fig. 1**).

3.2. Isolation of CIIV by Free Flow Electrophoresis (FFE)

1. Isolate 30×10^6 mature DC after incubation with 100 ng/mL of LPS overnight, as described ***(2)***.
2. Collect DC by centrifugation at 1200 rpm for 5 min and wash twice with ice cold PBS.
3. Resuspend DC at 1×10^7 cells/ mL in cold TEA 250.
4. Freshly prepare 2 L of 10X TEA and 4 L of TEA 250 for the FFE.
5. Homogenize DC by several passages through a ball-bearing homogenizer. Use phase-contrast microscopy to determine how many passes it takes to lyse the cells but leave the nuclei intact.
6. Add DNAse at 2 μL/mL to the DC homogenate and leave at room temperature for 10 min.
7. Spin the homogenate is at 3000*g* for 10 min to pellet nucleii and intact cell debris. Collect the supernatant (PNS).
8. Mix the PNS with 1 vol of cold TEA 2.5.
9. Transfer 5 mL of PNS (1.4 *M* sucrose) to a Beckman SW41 tube (14 × 89 mm) on ice. Establish a sucrose step gradient by overlaying the PNS with 5 mL of cold TEA 1.25 followed by 2.5 mL of cold TEA 250 with a Pasteur pipet. This sucrose

step gradient allows for a crude first separation of low density compartments (endosomes, Golgi, plasma membranes) from heavy-density organelles (ER, mitochondria, lysosomes).

10. Load the gradient tubes into a cold SW 41Ti rotor and spin for 1.5 h at 37,000 rpm at 4°C.
11. Two cloudy interfaces can be observed after centrifugation. Collect the top one (0.250–1.25 *M*) containing low-density membranes with a Pasteur pipet. Determine the sucrose density of this fraction by refractometry. Adjust the sucrose density to 0.240 *M* by addition of TEA (sample volume can reach 5 mL).
12. Determine the protein concentration of the samples by standard Bradford assay (Bio-Rad protein assay).
13. Add a concentration of 2–3 ng of trypsin per μg of protein to the low-density membranes and incubate at 37°C for 10 min (*see* **Note 4**).

At this point the FFE should be running with TEA 10X as electrode buffer (2 L) and TEA 250 as running buffer (4 L). The run is generally performed at 110–120 mA (see Note 5).

14. Stop the trypsin digestion by adding 10X excess of soybean trypsin inhibitor.
15. Store the membranes on ice ready to be loaded on the running FFE.
16. The FFE flow and sample loading rate are adjustable. Each machine will have to be calibrated as a starting point, a flow rate of 250 mL/h and a sample loading rate of 3 mL/h can be used (*see* **Note 6**).
17. After the run (1–4 h), collect the fractions by centrifugation at full speed in a microcentrifuge and submit the collected fractions to enzymatic analysis (*see* **Subheading 3.3.** and **Fig. 2**) (*see* **Notes 7** and **8**).
18. CIIV are generally the most anodally shifted organelles and can be detected by immunoblot for MHC class II (*see* **Fig. 1**).

3.3. Enzymatic Assays

One of the keys to a successful subcellular fractionation is a rapid and simple way of determining the nature of the organelles separated. This characterization can be achieved by simple enzymatic assays ***(19)*** or by immunodetection of known specific resident proteins (e.g., rab proteins, lamps, etc.). Two simple protocols, one to detect lysosomes and one to detect plasma membrane, are presented here.

3.3.1. Assay of β-hexosaminidase as a Lysosome/MIIC Marker

From Green et al., 1987 ***(19)***.

1. Mix 30 μL of sample with 50 μL substrate.
2. Incubate 30–60 min at 37°C.
3. Stop by addition of 2 2 mL Stop buffer.
4. Read fluorescence ex.365nm/em. 450 nm.
5. Use buffer only + substrate as blank.

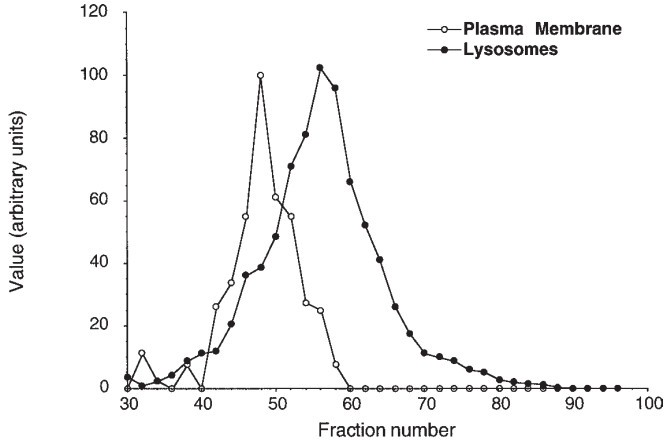

Fig. 2. Enzymatic profile after FFE fractionation of late DC. Separation of plasma membrane and lysosomes in late DC by FFE (96 fractions) is shown. Lysosomes (β-hexosaminidase; peak approx fraction 60) were typically anodally shifted relative to the plasma membrane (Alkaline phosphodiesterase or LFA-1, peak approx fraction 50) by at least 10 fractions. CIIV were found around fractions 66 to 70 (*see* **Fig. 1**).

3.3.2. Assay of Alkaline Phosphodiesterase as a Plasma Membrane Marker

From Green et al., 1987 ***(19)***.

1. Mix 100 µL of sample with 150 µL substrate.
2. Incubate 30–60 min at 37°C.
3. Stop by addition of 2 2 mL Stop buffer.
4. Read colorimetrically at 405 nm.
5. Use buffer only + substrate as blank.

4. Notes

1. TEA 2.5 *M* sucrose preparation takes 1 d to prepare, as it is close to the saturation sucrose concentration and is difficult to get into solution.
2. Mature DC can also be fractionated in the same way, but no MHC class II will be observed in the lysosomes, typical separations profile are shown in **Figs. 1** and **2**.
3. If the last centrifugation step is not efficient enough to collect heavy density lysosomes for analytical purpose, this step can be replaced by a conventional TCA precipitation.
4. Trypsin degradation of the membranes is required to induce an electrophoretic shift. Increased or decreased digestion can be used to influence the FFE separation.
5. FFE has to be started 30 min prior to loading to allow flow, current, and temperature equilibration.

6. FFE flow rate as well as current intensity adjustment can be used to optimize the membrane separation.
7. FFE has 96 collector tubes, assaying one every two samples starting from tube 30 is recommended.
8. The last centrifugation step can be replaced by a conventional TCA precipitation.

Acknowledgments

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References

1. Pierre, P. and Mellman, I. (1998) Exploring the mechanisms of antigen processing by cell fractionation. *Curr. Opin. Immunol.* **10,** 145–153.
2. Pierre, P., Turley, S. J., Gatti, E., et al. (1997) Developmental regulation of MHC class II transport in mouse dendritic cells. *Nature* **388,** 787–792.
3. Peters, P. J., Neefjes, J. J., Oorschot, V., Ploegh, H. L., and Geuze, H. J. (1991) Segregation of MHC class II molecules from MHC class I molecules in the Golgi complex for transport to lysosomal compartments. *Nature* **349,** 669–676.
4. Kleijmeer, M. J., Morkowski, S., Griffith, J. M., Rudensky, A. Y., and Geuze, H. J. (1997) MHC class II compartments (MIICs) in human and mouse lymphoblasts represent conventional endocytic compartments. *J. Cell Biol.* **139,** 639–649.
5. Sanderson, F., Kleijmeer, M. J., Kelly, A., et al. (1994) Accumulation of HLA-DM, a regulator of antigen presentation, in MHC class II compartments. *Science* **266,** 1566–1569.
6. Sugita, M., Jackman, R. M., van Donsselaar, E., et al. (1996) Cytoplasmic tail-dependent localization of CD1b antigen presenting molecules to MIIC. *Science* **273,** 349–352.
7. Kleijmeer, M.J., Oorschot, V. M., and Geuze, H. J. (1994) Human resident Langerhans cells display a lysosomal compartment enriched in MHC class II. *J. Invest. Dermatol.* **103,** 516–523.
8. Pertoft, H., Wärmegård, B., and Höök, M. (1978) Heterogeneity of lysosomes originating from rat liver parenchimal cells. Metabolic relationship of subpopulations separated by density gradient centrifugation. *Biochem. J.* **174,** 309–317.
9. Castellino, F. and Germain, R. N. (1995) Extensive trafficking of MHC class II-invariant chain complexes in the endocytic pathway and appearance of peptide-loaded class II in multiple compartments. *Immunity* **2,** 73.
10. Pierre, P., Denzin, L. K., Hammond, C., et al. (1996) HLA-DM is expressed in conventional and unconventional MHC class II-containing compartments. *Immunity* **4,** 229–239.
11. Amigorena, S., Drake, J. R., Webster, P., and Mellman, I. (1994) Transient accumulation of new class II molecules in a novel endocytic compartment in B lymphocytes. *Nature* **369,** 113–120.
12. Tulp, A., Verwoerd, D., Dobberstein, B., Ploegh, H. L., and Pieters, J. (1994) Isolation and characterization of the intracellular MHC class II compartment. *Nature* **369,** 120–126.

13. Ferrari, G., Knight, A. M., and Pieters, J. (1997) Distinct intracellular compartments involved in invariant chain degradation and antigenic peptide loading of major histocompatibility complex (MHC) class II molecules. *J. Cell Biol.* **139,** 1433–1446.
14. Marsh, M., Schmid, S., Kern, H., et al. (1987) Rapid analytical and preparative isolation of functional endosomes by free flow electrophoresis. *J. Cell Biol.* **104,** 875–886.
15. Schmid, S. L., Fuchs, R., Male, P., and Mellman, I. (1988) Two distinct subpopulations of endosomes involved in membrane recycling and transport to lysosomes. *Cell* **52,** 73–83.
16. Amigorena, S., Webster, P., Drake, J., Newcomb, J., Cresswell, P., and Mellman, I. (1995) Invariant chain cleavage and peptide loading in post-endosomal MHC class II vesicles. *J. Exp. Med.* **181,** 1729–1741.
17. Mellman, I., Turley, S., and Steinman, R. M. (1998) Antigen presentation for amateurs and professionals. *Trends in Cell Biol.* **8,** 231–237
18. Inaba, K., Inaba, M., Romani, N., et al. (1992) Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. *J. Exp. Med.* **176,** 1693–1702.
19. Green, S. A., Zimmer, K.-P., Griffiths, G., and Mellman, I. (1987) Kinetics of intracellular transport and sorting of lysosomal membrane and plasma membrane proteins. *J. Cell Biol.* **105,** 1227–1240.

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Detection of Intracellular Cytokines in Dendritic Cells

Peter Kelleher

1. Introduction

The detection of intracellular cytokines using flow cytometry is a relatively new technique that allows simultaneous labeling of cytokines and cell surface proteins. In contrast with other techniques such as ELISA, bioassays, or PCR that have been used to examine cytokine production in cell cultures, intracellular cytokine labeling allows the investigator to measure cytokine production by individual cells without prior cell sorting. Although one can analyze the production of cytokines by individual cells using ELISPOT, immunohistochemistry, PCR, *in situ* hybridization, and ribonuclease protection assays, these methods are more laborious and technically demanding than intracellular cytokine staining using flow cytometry ***(1)***.

Flow cytometry to detect intracellular cytokine staining has largely been employed in the study of human ***(2–4)*** and murine ***(5,6)*** T lymphocytes. Detection of intracellular cytokines using flow cytometry involves four key components: in vitro stimulation of cells; inhibition of protein secretion; permeabilization of cells to allow labeling with anticytokine antibody; and labeling of cell specific surface proteins. Studies using both human and murine lymphocytes have employed phorbol esters and ionomycin to stimulate cytokine production by T lymphocytes. The addition of monensin or brefeldin A to cell culture medium inhibits the secretion of cytokines from the cell, increases the sensitivity of this technique, and allows a better discrimination between specific anticytokine and background nonspecific staining. Lymphocytes can then be stained for surface-marker expression, fixed, and permeabilized with detergent to allow the entry of anticytokine antibodies into the cells, which can then be detected using flow cytometry.

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The importance of dendritic cells (DC) in initiating immune responses together with recent progress in generating large numbers of these cells from various precursors *(7)* has provided opportunities for the study of cytokine production within this cell population. A number of stimuli including TNFα *(8)*, LPS *(9)*, and anti-CD40 *(10–12)* antibody have been shown to modulate cytokine production by dendritic cells. Interleukin-12 was detected using ELISA in cultures that contained DCs derived from bone-marrow progenitors in the presence of GM-CSF and TNFα *(13)*. The following protocol illustrates how intracellular cytokine staining using flow cytometry may be used to detect cytokine production within individual dendritic cells.

2. Materials

2.1. Cells and Culture Medium

Dendritic cells were derived from bone-marrow progenitors in BALB/c mice using GM-CSF (100 U/mL) and TNFα (50 U/mL). The culture medium consisted of RPMI-1640 supplemented with 10% fetal calf serum (FCS), penicillin 100 U, streptomycin 100 μg/mL, glutamine 2 m*M*, and 10^{-5} *M* 2-mercaptoethanol.

1. GM-CSF (R& D Systems UK). Store at –20°C
2. TNFα (R&D Systems UK). Store at –20°C
3. BALB/c mice Harlan UK Limited, UK.
4. RPMI (Dutch modification, Sigma Chemical Co. UK). When stored at 4°C with added supplements this remains stable for 1 mo.
5. FCS (Gibco-BRL, UK). Store at –20°C.
6. Penicillin (Gibco-BRL, UK). Store at –20°C.
7. Streptomycin (Gibco-BRL, UK). Store at –20°C.
8. Glutamine (ICN Flow UK). Store at –20°C.
9. 2-Mercaptoethanol (Sigma Chemical Co., UK). Store at 4°C.

2.2. Flow Cytometry

FACScan buffer consists of phosphate buffered saline supplemented with 0.02% sodium azide and 1 m*M* EDTA.

1. Monensin (Calbiochem, UK).
2. Phosphate buffered saline.
3. Sodium azide (BDH, UK). Toxic hazard.
4. EDTA (BDH, UK).
5. FACScan flow cytometer (Becton Dickinson Co.)
6. Cytoperm A (Serotec UK). Store at room temperature.
7. Cytoperm B (Serotec UK). Store at room temperature.
8. Streptavidin PE (Pharmingen). Store at 4°C in the dark.

3. Methods

1. Suspend DCs at a concentration of 5–10 × 10^5 cells/mL in 1 mL of RPMI culture medium supplemented with GM-CSF 100 U/mL and TNFα 50 U/mL (*see* **Note 1**).
2. Add 1 mL of culture medium supplemented with monensin 6 μ*M* to DC suspension. The final concentration of monensin should be 3 μ*M* (*see* **Note 2**).
3. Add 1 mL of culture medium without monensin to a second DC suspension. This serves as a negative control.
4. Incubate the 2 mL DC suspensions in a six-well plate for 4–6 h at 37°C with 5% CO_2 (*see* **Note 3**).
5. Add 3 mL of FACScan buffer supplemented with 2% FCS to DC suspensions.
6. Wash the cells by centrifugation at 400*g* for 5 min twice.
7. Suspend the DC solution at a concentration of 10^6 cells/mL in 1 mL of FACScan buffer supplemented with 20% FCS.
8. Leave the DC suspension at room temperature for 10 min.
9. Add Cytoperm A at a concentration of 50 μL/10^6 cells. Keep the cell suspension at room temperature for 15 min (*see* **Note 4**).
10. Wash the DC suspension in FACScan buffer with 20% FCS by centrifugation at 400*g* for 5 min.
11. Resuspend DC suspension in FACScan buffer with 20% FCS at a concentration of 10^6 cells/mL.
12. Add Cytoperm B at a concentration of 50 μL/10^6cells. Keep the cell suspension at room temperature for 15 min.
13. Aliquot 100 μL FACScan buffer into FACS tubes to make a DC suspension at a concentration of 1 × 10^5 cells/mL.
14. Add 5 μL of a biotinylated cytokine antibody to the DC suspension. Leave in the dark for 30 min at room temperature (*see* **Note 5**).
15. Wash the DC suspension in FACScan buffer with 2% FCS by centrifugation at 400*g* for 5 min twice.
16. Add 5 μL of Streptavidin PE to the DC suspensions. Leave in the dark for 20 min. Wash the solution again in FACScan buffer with 2% FCS by centrifugation at 400*g* for 5 min.
17. Add 5 μL of FITC conjugated antibody that labels murine DCs (anti-CD11c, NLDC, 33D1) (*see* **Note 6**). Leave in dark for 30 min. Wash the DC suspension twice in FACScan buffer with 2% FCS by centrifugation at 400*g* for 5 min.
18. Suspend DC pellet in 500 μL FACScan buffer with 2% FCS. Analyse sample on flow cytometer immediately or else add 50 μL of 4% paraformaldehyde to DC suspension. Samples can be stored at 4°C, in the dark, for up to 48 h prior to analysis on the flow cytometer (*see* **Note 7**).

4. Notes

1. If dendritic cells are incubated with monensin for less than 4 h, there may be no need to add GM-CSF and TNFα to cultures. However, cytokines need to be added for time course experiments that last more than 8 h as cell viability drops and nonspecific staining increases.

2. Monensin is suspended in DMSO and stored at –20°C. Monensin was then made up to a concentration of 6 μ*M* in culture medium on the day of the experiment. One can also store monensin in culture medium at a concentration of 6 μ*M* for up to 1 mo at 4°C. A fresh monensin DMSO suspension was prepared every 3 mo.
3. Time course experiments should be performed to determine the optimal incubation period for intracellular cytokine labeling.
4. Reagents need to be used before their date of expiration; experiments have failed because the permeabilization agent used was 1 mo overdue.
5. To determine the specificity of anticytokine labeling, blocking experiments need to be performed. A 100 μL aliquot of fixed and permeabilized DCs, incubated in the presence or absence of monensin was added to a FACScan tube that contained the cytokine antibody and cytokine at equal concentration. The two aliquots were then prepared as described in Subheading 3., steps 14–18. Specificity was demonstrated by showing that the presence of excess cytokine in FACScan tube blocked intracellular cytokine labeling. The level of intracellular staining observed in the blocking experiment was equivalent to that seen with DCs incubated in the absence of monensin. Isotype controls for some cytokine antibodies gave background staining equivalent to DCs incubated in the absence of monensin. As blocking experiments with excess cytokine are expensive, controls for nonspecific cytokine labeling always included DCs incubated in the absence of monensin and isotype antibodies where appropriate. There is considerable variability in the specificity of cytokine antibody staining that commercial companies supply. In addition the performance of cytokine antibody from a single company can vary with different batch numbers. The specificity of antibody staining needs to be determined for each cytokine antibody that the investigator purchases.
6. Although it is possible to add both the cytokine and cell-surface-marker antibodies together, staining for DC surface proteins is more consistent if this procedure is carried out separately from the intracellular staining steps ***(13)***. A variety of different antibodies may be used to label DC with high specificity in the murine system ***(13)***. I found no difference if you labeled first for DCs and then for the cytokine antibody, however given that the half-life of the permeabilizing agent is not stated it is probably prudent to stain for the intracellular cytokine first and then label the DC.
7. The investigator needs to determine the optimal settings on the flow cytometer to analyze intracellular cytokine staining in DCs.

References

1. Carter, L. L. and Swain, S. L. (1977) Single cell analyses of cytokine production. *Curr. Opin. Immunol.* **9,** 177–182.
2. Jung, T., Schauer, U., Heusser, C., Newmann, C., and Rieger, C. (1993) Detection of intracellular cytokines by flow cytometry. *J. Immunol. Methods* **159,** 197–207.
3. Prussin, C. and Metcalfe, D. D. (1995) Detection of intracytoplasmic cytokine using flow cytometry and directly conjugated anti-cytokine antibodies. *J. Immunol. Methods* **188,** 117–125.

4. Picker, L. J., Singh, M. K., Zdraveski, Z., Treer, J. R., Waldrop, S. L., Bergstresser, P. R., and Maino, V. C. (1995) Direct demonstration of cytokine synthesis heterogeneity among human memory/effector T cells by flow cytometry. *Blood* **86,** 1408–1419.
5. Sander, B., Hoiden, I., Andersson, U., Moller, A., and Abrams, J. S. (1993) Similar frequencies and kinetics of cytokine producing cells in murine peripheral blood and spleen. Cytokine detection by immunoassay and intracellular immunostaining. *J. Immunol. Methods* **166,** 201–214.
6. Assenmacher, J. S., Scmitz, J., and Radbruch, A. (1994) Flow cytometric determination of cytokines in activated murine T helper lymphocytes: expression of interleukin-10 in interferon-(and in interleukin-4–expressing cells. *Eur. J. Immunol.* **24,** 1097–1101.
7. Banchereau, J. and Steinman, R. M (1998) Dendritic cells and the control of immunity. *Nature* **392,** 245–252.
8. Winzler, C., Rovere, P., Rescigno, M., Granucci, F., Penna, G., Adorini, L., Zimmermann, V. S., Davoust, J., and Riccardi-Castagnoli, P. (1997) Maturational stages of mouse dendritic cells in growth factor-dependent long term cultures. *J. Exp. Med.* **185,** 317–328.
9. Koide, S. and Steinman, R. M. (1987) induction of murine Interleukin 1: stimuli and responsive primary cells. *Proc. Natl. Acad. Sci.* **84,** 3802–3806.
10. Caux, C., Massacrier, C., Vanbervliet, B., Dubois, B., van Kooten, C., Durand, I., and Banchereau, J. (1994) Activation of dendritic cells through CD40 cross linking. *J. Exp. Med.* **180,** 1263–1272.
11. Cella, M., Scheidegger, D., Palmer-Lehmann, K., Lane, P., Lanzavecchia, A., and Albers, G. (1996) Ligation of CD40 on dendritic cells triggers high production of interleukin-12 and enhances T cell stimulatory capacity: T-T help via APC activation. *J. Exp. Med.* **184,** 747–752.
12. Koch, F., Stanzl, U., Jennewein, P., Janke, K., Heufler, C., Kampgen, E., Romani, N., and Schuler, G. (1996) High level IL-12 production by murine dendritic cells. Upregulation via MHC class II and CD40 molecules and downregulation by IL-4 and IL-10. *J. Exp. Med.* **184,** 741–746.
13. Kelleher, P. and Knight, S. C. (1998) Interleukin-12 increases CD80 expression and the stimulatory capacity of bone marrow-derived dendritic cells. *Int. Immunol.* **10,** 749–755.

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Adenoviral-Vector-Mediated Gene Transfer to Dendritic Cells

Wenru Song and Ronald G. Crystal

1. Introduction

Dendritic cells (DC) are the most potent antigen presenting cells capable of initiating T-cell-dependent immune responses ***(1–5)***. This biologic potential can be harnessed to elicit effective antigen-specific immune responses by transferring the relevant antigens to the DC. Once the DC have been mobilized and purified, the relevant antigens can be transferred to the DC as intact proteins, or as peptides representing specific epitopes, or with gene transfer using sequences of DNA or RNA coding for the pertinent antigen(s) ***(6–15)***. Theoretically, genetically modifying DC with genes coding for specific antigens has potential advantages over pulsing the DC with peptides repeating the antigen or antigen fragment. First, the genetically modified DC may present previously unknown epitopes in association with different MHC molecules. Second, gene transfer to DC ensures that the gene product is endogenously processed, leading to the generation of MHC class I-restricted cytotoxic T lymphocytes (CTL), the effector arm of cell-mediated immune responses. Finally, in addition to genes coding for the antigen(s), genetic modification of the DC can induce genes coding for mediators relevant to generation of the immune response to the antigen(s), further boosting host responses to the antigens presented by the modified DC. Different gene transfer approaches have been explored to genetically modify DC, including retroviral vectors ***(16–18)***, recombinant vaccinia virus vectors ***(19)***, and recombinant adenovirus (Ad) vectors ***(19–23)***. The focus of this chapter is on using recombinant Ad vectors to transfer genes to murine DC. We have used a similar strategy to transfer genes to human DC ***(24)***. As an example of the power of this technology, we will

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describe the use of Ad-vector-modified DC to suppress the growth of tumor cells modified to express a specific antigen.

1.1. Recombinant Adenovirus Vectors

Replication-deficient, recombinant Ad vectors have emerged as promising vehicles for gene transfer both in vitro and in vivo, based on their ability to transfer and express transgenes efficiently in wide replicating and nonreplicating cell types ***(25)***. Ad vectors also can be produced in high titers (10^{12} to 10^{13} pfu/mL). The current widely used Ad vectors are based on Ad serotype 2 and 5. The Ad genome is composed of linear, double-stranded DNA approx 36 kb in length. The gene products are organized into early (E1 through E4) and late (L1 through L5) regions. The first generation of Ad vectors contain deletions of E1 with or without deletion of E3. Additional deletions in E2 or E4 can also be made in Ad vectors. The Ad vector enters the target cells through an interaction of the Ad fiber and penton to two specific receptors, Coxsackievirus and adenovirus receptor (CAR) and $\alpha_v\beta_3$ (or $\alpha_v\beta_5$) surface integrins ***(25,26)***. The Ad is internalized into a cytoplasmic endosome, breaks out and delivers its genome into nucleus, where it functions in an epichromosomal fashion to direct the expression of its transgene product (**Fig. 1**). The Ad vectors in current use can evoke nonspecific inflammation and immune responses to the vector in vivo, and leading to transient transgene expression ***(27)***. Although this may limit the use of these vectors in genetic diseases, it is likely to be beneficial in their use for vaccination against cancer and other disorders.

1.2. DC as Targets for Adenovirus Vectors

Theoretically, direct gene transfer to DC has advantages in generating CTL toward the protein coded by the transgene, in that the gene will be transferred to the DC nucleus, expressed, and presented in the context of class I MHC for the immune system to recognize as self or nonself ***(28,29)***. In this regard, there is emerging evidence that, when Ad vectors expressing heterologous transgenes are administered in vivo, the Ad functions as an "adjuvant" to enhance cellular immune responses against the heterologous transgene product, including the generation of transgene product-specific CTL ***(30–35)***. In vitro DC can easily be infected with Ad vectors and modified to express the transgene carried by the vector ***(19–23)***. Immunization of mice with DC that have been genetically modified ex vivo to express tumor antigens will induce antigen-specific CTL responses and protect mice from a subsequent challenge with tumors expressing the relevant antigen ***(19–21)***. Detailed protocols for DC isolation from murine bone marrow and for Ad vector production are available ***(20,36)***. This chapter will focus on the use of $E1^-E3^-$ Ad vectors expressing the *Escherichia coli* β-galactosidase gene (Adβgal) as an example to illustrate:

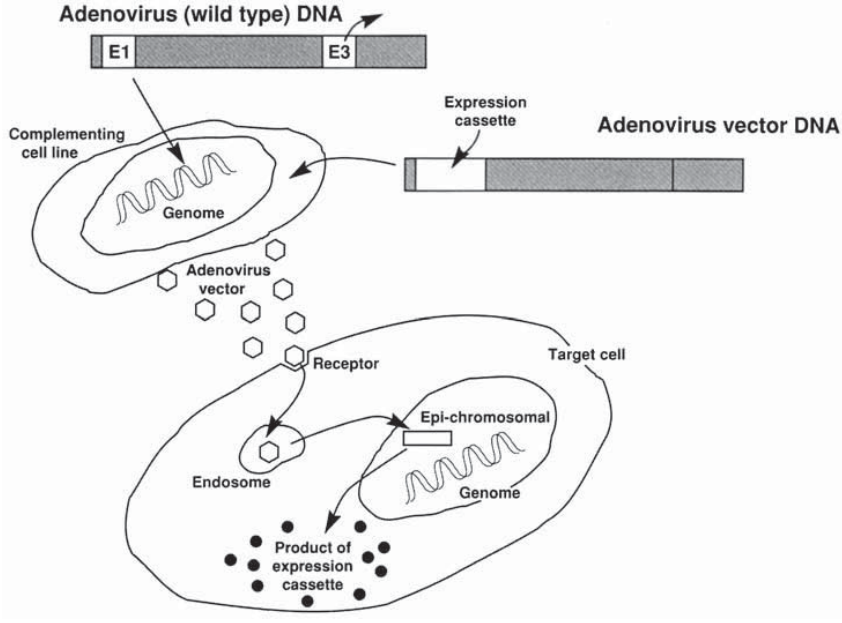

Fig. 1. Adenovirus vector design, production, and gene transfer. Adenoviruses are DNA viruses with a 36 kb genome. The wild-type adenovirus genome is divided into early (E1–E4) and late (L1–L5) genes. All adenovirus vectors administered to humans use adenovirus type 2 or 5 as the base. The ability of the adenovirus genome to direct production of adenoviruses is dependent on sequences in E1. To produce an adenovirus vector, the E1 sequences (and E3 sequences if the space is needed) are deleted. The expression cassette is inserted and the vector DNA transfected into a complementing cell line with E1 sequences in its genome. The adenovirus vector with its expression cassette is $E1^-$ and thus incapable of replicating. The vector binds to the target cell through and interaction of the adenovirus fiber and penton, each to a specific receptor, enters the cell via a cytoplasmic endosome, breaks out and delivers its linear, double-stranded-DNA genome with the expression cassette into the nucleus where it functions in an epi-chromosomal fashion to direct the expression of its product. (Reproduced from **ref. *25*** with permission.)

1. in vitro transduction of DC by Ad vectors and evaluation of gene transfer; and
2. in vivo evaluation of DC genetically modified by an Ad vector in a vaccination strategy using a murine syngeneic colon adenocarcinoma cell line expressing the antigen for which the DC have been genetically modified.

2. Materials

2.1. Transduction of DC In Vitro by Ad Vectors

1. Ad vectors: The replication deficient Ad vectors used are E1a$^-$, partial E1b$^-$, and partial E3$^-$ vectors based on human Ad5 ***(37,38)***. The construction of these Ad vectors has been described previously, including vectors expressing no transgene (AdNull) and the *E. coli* βgal gene (Adβgal) ***(39)***. All Ad vectors are propagated in 293 cells, purified by two rounds of CsCl density centrifugation, dialyzed, and stored at –70°C as previously described ***(37,38)*** (*see* **Notes 1** and **2**). The titer of viral stock is determined by plaque-forming assay using 293 cells ***(40)***. All vector preparations are demonstrated to be free of replication competent adenovirus ***(41)***.
2. DC: Primary bone-marrow-derived DC are obtained from mouse bone marrow precursors. ***(20,42)***. On d 8 of bone-marrow culture, mature, nonadherent cells with the typical morphological features of DC are used for in vitro phenotypic and functional analysis and for transduction by Ad vectors.
3. Media: Complete RPMI media (10% fetal bovine serum, 2 m*M* L-glutamine, 100 μg/mL streptomycin, and 100 U/mL penicillin) supplemented with recombinant murine granulocyte-macrophage colony-stimulating factor (GM-CSF, 100 U/mL; Sigma, St. Louis, MO) and recombinant murine interleukin 4 (IL-4, 20 ng/mL; Genzyme, Farmington, MA) is used for generating DC in culture. Infection of the DC with Ad vectors is carried out using serum-free media (RPMI media with 2 m*M* L-glutamine, 100 μg/mL streptomycin, and 100 U/mL penicillin).

2.2. In Vitro Evaluation of β-Galactosidase Expression by FACS-Gal Assay

1. Staining media: PBS (15 m*M* sodium phosphate, pH 7.3, 150 m*M* NaCl) containing 4% fetal bovine serum, 10 m*M* HEPES, pH 7.3.
2. Fluorescein di-β-galactoside (FDG; Molecular Probes, Eugene, OR). FDG powder is not stable. The 200 m*M* stock solution is made by dissolving 5 mg of FDG in 38 μL of 1:1 H_2O:dimethylsulfoxide. Then dilute the stock solution with sterile water to 2 m*M* working solution and store at –80°C until needed.

2.3. In Vivo Evaluation of Vaccination with DC Modified by Adβ gal

1. Cell lines: CT26.WT (H-2^d) is a clone of the *N*-nitroso-*N*-methyl urethane-induced BALB/c undifferentiated colon adenocarcinoma ***(43)***. CT26.CL25 is a clone of CT26.WT that has been transduced with the *E. coli* β-galactosidase gene (both cell lines kindly provided by N.P. Restifo, NCI, Bethesda, MD) ***(43)***.
2. Media: CT26.WT are grown in complete RPMI media. CT26.CL25 are grown in complete RPMI containing 400 μg/mL G418 (Life Technologies, Inc., Gaithersburg, MD).

3. Hank's balanced salt solution (HBSS): Used to prepare tumor cell or DC suspensions for injection.

3. Methods

3.1. Transduction of DC In Vitro by Ad Vectors

1. Collect DC on d 8 of bone marrow culture into a 50 mL conical tube. Centrifuge for 10 min at 250*g* and resuspend the cell pellet in serum-free infection media to a final concentration of 1–5 × 10^7 cells/mL (in a vol of 0.5–1 mL) (*see* **Note 3**).
2. Add the Ad vector (usually with the titer 10^{10}–10^{11} pfu/mL) at a multiplicities of infection (moi) of 100 and incubate at 37°C for 2 h on a rocker (*see* **Notes 1** and **3**). Gently mix the cell suspension every 10 min.
3. Add fresh media to the tube after 2 h incubation. Centrifuge at 250*g* for 10 min and resuspend the cell pellet and IL-4 to a final concentration of 5 × 10^5 cells/mL.
4. After 24-h culture, collect Ad-vector-transduced DC, centrifuge at 250*g* for 10 min. Resuspend the cell pellet and wash one time with media. Count the cells in preparation for in vitro analysis or for immunization of mice.

3.2. In Vitro Evaluation of Gene Transfer by FACS-Gal Assay

1. Resuspend DC in staining media, count the cells, and then adjust the cell concentration to 10^7cells/mL.
2. Aliquot 50 μL of DC into a 4 mL tube and place the tube in a 37°C water bath for 10 min.
3. Add to the cells an equal volume (50 μL) of 2 m*M* FDG ***(44)*** that has been prewarmed to 37°C and mix rapidly and thoroughly to generate a mild hypotonic shock.
4. Return the cells to a 37°C water bath for exactly 1 min. The staining procedure relies on an osmotic shock of cells during the 1 min at 37°C. FDG is taken up into the cells by passive osmotic loading.
5. Stop the FDG loading at the end of 1 min by adding 2 mL of 4°C staining media with 1 μg/mL propidium iodide. The uptake of FDG is stopped and the substrate and products are locked in the cells by a rapid dilution into cold isotonic staining medium. This one step brings the cells back to iso-osmotic conditions, stops the loading, and freezes the cell membrane, thereby locking the substrate and products inside the cells.
6. Keep the cells on ice until ready to perform flow cytometry. The enzymatic conversion of FDG to fluorescein proceeds even though the cells are on ice. Any amount of enzyme will hydrolyze all of the available substrate, given enough time, yielding a homogeneous fluorescence distribution representing the amount of FDG loaded per cell.
7. Example: An example of Ad-mediated gene transfer and expression in bone-marrow DC demonstrates effective gene transfer as assessed by the FACS-gal assay (**Fig. 2**) (*see* **Notes 4** and **5**).

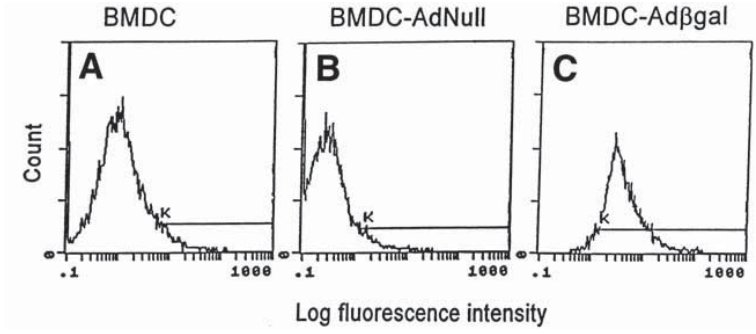

Fig. 2. Adenovirus vector-mediated gene transfer and expression of βgal in bone-marrow DC (BMDC) in vitro. The primary murine DC were infected in vitro with Adβgal or AdNull control vector at moi of 100 for 2 h. Twenty-four hours later, βgal expression was quantified by flow cytometry using fluorescein di-β-galactoside. For all panels, the *y*-axis reflects DC number and the *x*-axis reflects log fluorescein intensity. The percentage of βgal-expressing DC was determined by the right shift of the curve along the K gate. **(A)** Uninfected BMDC. **(B)** BMDC infected with AdNull. **(C)** BMDC infected with Adβgal. (Reproduced from **ref. *20*** with permission.)

3.3. In Vivo Evaluation of DC-Adβgal Vaccination

3.3.1. Immunization of Mice with DC Modified Genetically with Adβgal (DC-Adβgal)

1. Twenty-four hour post in vitro infection of DC with Adβgal, the Adβgal-transduced DC, and control AdNull (a control vector identical to Adβgal but with no transgene) -transduced DC (DC-AdNull) are collected and centrifuged at 250*g* for 10 min. Wash twice with HBSS and count. Resuspend the cell pellet to a final concentration of 10^6cells/mL.
2. Inject subcutaneously (×1) 10^5 Ad-vector-modified DC suspended in 100 μL HBSS in lower left quadrant of the abdominal wall of Balb/c mice (*see* **Note 6**).

3.3.2. Protection of DC-Immunized Mice from a Lethal Tumor Challenge

1. Immunize mice as in **Subheading 3.3.1.**
2. Two weeks later, prepare the syngeneic βgal-expressing murine colon carcinoma cell CT26.CL25 to a final concentration of 2×10^5 cells/mL in HBSS for tumor challenge (*see* **Note 7**).
3. Inject 500 μL (10^5 cells) CT26.CL25 via the jugular vein into both naive and Ad-vector-modified DC immunized mice.
4. Control groups include naive mice challenge with CT26.CL25 and DC-Adβgal-immunized mice challenged with parental non-βgal-expressing CT26.WT.
5. Follow up survival of mice.

3.3.3. Suppression of Preestablished Tumor by DC Immunization

1. Prepare βgal-expressing tumor CT26.CL25 and parental non-βgal-expressing CT26.WT to a final concentration of 6×10^4 cells/mL (*see* **Note 7**).
2. Inject 500 μL (3×10^4 cells) CT26.CL25 or CT26.WT via the jugular vein into naive mice.
3. Three days later, tumor-bearing mice are either left untreated or treated with subcutaneously with DC-AdNull or DC-Adβgal as in **Subheading 3.3.1.**
4. Follow up survival of mice.

Notes 8 and **9** give details of results to expect with both the tumor prevention and tumor treatment models.

4. Notes

4.1. Transduction of DC In Vitro by Ad Vectors

1. Handling of the Ad vectors is classified at P2 containment and should always be carried out in laminar flow hoods. All solid waste should be autoclaved, and liquid waste treated with bleach. To avoid contamination, it is better to use different laminar flow hoods and incubators for viral work and for routine cell culture.
2. Repeat freeze-thawing of Ad vector leads to a decrease of virus titer. Therefore, small aliquots (1020 μL/tube) for storage of virus stock are recommended.
3. For maximizing the transduction efficiency of DC by Ad vectors, infection of DC in suspension should be kept in a minimum volume (0.5–1 mL) at a cell concentration of $1–5 \times 10^7$cells/mL in a 50 mL conical tube. A moi of 100 is sufficient to transduce >90% DC if transgene expression is quantified by sensitive assays 24 h post infection (**Fig. 2**). If the moi is too high (>1000), Ad-vector infection has a cytopathic effect on DC. A moi of 100–300 is recommended depending on the Ad vector used (*see* **Subheading 4.2.**).

4.2. In Vitro Evaluation of Gene Transfer by FACS-Gal Assay

4. Normal untransduced DC exhibited little background activity from endogenous β-galactosidase.
5. Transduction efficiency of DC by Adβgal correlates well with the moi used. Expression of βgal is readily detectable (>90%) by sensitive FACS-gal assay in DC infected with Adβgal at a moi of 100. Parallel studies carried out using other moi demonstrate 17% DC cells expressing βgal with a moi of 30 and 96% at a moi of 300. In contrast, the less sensitive X-gal histologic staining assay for βgal ***(19,20)*** suggests that only 10 to 30% of DC are positive at an moi of 100.

4.3. In Vivo Evaluation of DC-Adβgal Vaccination

6. Comparison of subcutaneous (SQ), intravenous (IV), and interperitoneal (IP) routes for Ad-modified DC immunization demonstrated that IV and SQ are equally effective, with IP less effective. In regard to dose of Ad-modified DC for immunization, for both the SQ and IV routes, immunization of DC at $10^4–10^6$ cells/mouse yields similar results.

7. To achieve consistent tumor growth rate in an animal model in vivo, tumor cells should be collected in the log growth phase, and the cells should be well suspended.
8. In the tumor prevention model in which mice received 10^5 CT26.CL25 cells 2 wk post Ad-modified-DC immunization, the mice without DC immunization usually succumb to advanced pulmonary tumor 2–3 wk after the IV tumor challenge. In contrast, DC-Adβgal-immunized mice have a marked prolonged survival ($p < 0.0001$), with >80% of mice survived up to 8 mo post tumor challenge ***(20)***.
9. In the tumor treatment model in which mice received 3×10^4 CT26.CL25 cells 3 d prior to Ad-modified-DC immunization, tumor-bearing mice that were not treated with DC immunization died 3–4 wk post tumor challenge. In contrast, tumor-bearing mice that were treated with DC-Adβgal had a marked prolonged survival ($p < 0.0001$), with >20–30% of mice surviving up to 8 mo post tumor challenge ***(20)***.

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References

1. Grabbe, S., Beissert, S., Schwarz, T., Granstein, R. D. (1995) Dendritic cells as initiators of tumor immune responses: a possible strategy for tumor immunotherapy? *Immunol. Today* **16,** 117–121.
2. Schuler, G. and Steinman, R. M. (1997) Dendritic cells as adjuvants for immune-mediated resistance to tumors. *J. Exp. Med*. **6,** 1183–1187.
3. Steinman, R. M. (1991) The dendritic cell system and its role in immunogenicity. *Annu. Rev. Immunol.* **9,** 271–296.
4. Young, J. W. and Inaba, K. (1996) Dendritic cells as adjuvants for class I major histocompatibility complex-restricted antitumor immunity. *J. Exp. Med.* **183,** 7–11.
5. Banchereau, J. and Steinman, R. M. (1998) Dendritic cells and the control of immunity. *Nature* **392,** 245–252.
6. Grabbe, S., Bruvers, S., Gallo, R. L., Knisely, T. L., Nazareno, R., and Granstein, R. D. (1991) Tumor antigen presentation by murine epidermal cells. *J. Immunol.* **146,** 3656–3661.
7. Mayordomo, J. I., Zorina, T., Storkus, W. J., et al. (1995) Bone marrow-derived dendritic cells pulsed with synthetic tumour peptides elicit protective and therapeutic antitumour immunity. *Nature Med.* **1,** 1297–1302.
8. Ossevoort, M. A., Feltkamp, M. C., van Veen, K. J., Melief, C. J., and Kast, W.M. (1995) Dendritic cells as carriers for a cytotoxic T-lymphocyte epitope-based peptide vaccine in protection against a human papillomavirus type 16–induced tumor. *J. Immunother. Emphasis. Tumor. Immunol.* **18,** 86–94.

9. Boczkowski, D., Nair, S. K., Snyder, D., and Gilboa, E. (1996) Dendritic cells pulsed with RNA are potent antigen-presenting cells in vitro and in vivo. *J. Exp. Med.* **184,** 465–472.
10. Celluzzi, C. M., Mayordomo, J. I., Storkus, W. J., Lotze, M. T., and Falo, L. D. (1996) Peptide-pulsed dendritic cells induce antigen-specific, CTL-mediated protective tumor immunity. *J. Exp. Med.* **183,** 283–287.
11. Hsu, F. J., Benike, C., Fagnoni, F., et al. (1996) Vaccination of patients with B-cell lymphoma using autologous antigen-pulsed dendritic cells. *Nature Med.* **2,** 52–58.
12. Mayordomo, J. I., Loftus, D. J., Sakamoto, H., et al. (1996) Therapy of murine tumors with p53 wild-type and mutant sequence peptide-based vaccines. *J. Exp. Med.* **183,** 1357–1365.
13. Paglia, P., Chiodoni, C., Rodolfo, M., and Colombo, M. P. (1996) Murine dendritic cells loaded in vitro with soluble protein prime cytotoxic T lymphocytes against tumor antigen in vivo. *J. Exp. Med.* **183,** 317–322.
14. Porgador, A., Snyder, D., and Gilboa, E. (1996) Induction of antitumor immunity using bone marrow-generated dendritic cells. *J. Immunol.* **156,** 2918–2926.
15. Zitvogel, L., Mayordomo, J. I., Tjandrawan, T., et al. (1996) Therapy of murine tumors with tumor peptide-pulsed dendritic cells: Dependence on T cells, B7 costimulation, and T helper cell 1-associated cytokines. *J. Exp. Med.* **183,** 87–97.
16. Henderson, R. A., Nimgaonkar, M. T., Watkins, S. C., Robbins, P. D., Ball, E. D., and Finn, O. J. (1996) Human dendritic cells genetically engineered to express high levels of the human epithelial tumor antigen mucin (MUC-1) *Cancer Res.* **56,** 3763–3770.
17. Reeves, M. E., Royal, R. E., Lam, J. S., Rosenberg, S. A., and Hwu, P. (1996) Retroviral transduction of human dendritic cells with a tumor-associated antigen gene. *Cancer Res.* **56,** 5672–5677.
18. Aicher, A., Westermann, J., Cayeux, S., Willimsky, G., Daemen, K., Blankenstein, T., Uckert, W., Dorken, B., and Pezzutto, A. (1997) Successful retroviral mediated transduction of a reporter gene in human dendritic cells: feasibility of therapy with gene-modified antigen presenting cells. *Exp. Hematol.* **25,** 39–44.
19. Brossart, P., Goldrath, A. W., Butz, E. A., Martin, S., and Bevan, M. J. (1997) Virus-mediated delivery of antigenic epitopes into dendritic cells as a means to induce CTL. *J. Immunol.* **158,** 3270–3276.
20. Song, W., Kong, H. L., Carpenter, H., et al. (1997) Dendritic cells genetically modified with an adenovirus vector encoding the cDNA for a model antigen induce protective and therapeutic antitumor immunity. *J. Exp. Med.* **186,** 1247–1256.
21. Wan, Y., Bramson, J., Carter, R., Graham, F., and Gauldie, J. (1997) Dendritic cells transduced with an adenoviral vector encoding a model tumor-associated antigen for tumor vaccination. *Hum. Gene Ther.* **8,** 1355–1363.
22. Dietz, A. B. and Vuk-Pavlovic, S. (1998) High efficiency adenovirus-mediated gene transfer to human dendritic cells. *Blood* **91,** 392–398.
23. Mulders, P., Pang, S., Danuull, J., et al. (1998) Highly efficient and consistent gene transfer into dendritic cells utilizing a combination of ultraviolet-irradiated adenovirus and poly(L-lysine) conjugates. *Cancer Res.* **58,** 956–961.

24. Frey, B. M., Hackett, N. R., Bergelson, J. M., et al. (1998) High-efficiency gene transfer into *Ex vivo* expanded human hematopoietic progenitors and precursor cells by adenovirus vectors. *Blood* **91,** 2781–2792.
25. Crystal, R. G. (1995) Transfer of genes to humans: Early lessons and obstacles to success. *Science* **270,** 404–410.
26. Bergelson, J. M., Cunningham, J. A., Droguett, G., Kurt-Jones, E. A., Krithivas, A., Hong, J. S., Horwitz, M. S., Crowell, R. L., and Finberg, R. W. (1997) Isolation of a common receptor for coxsackie b viruses and adenoviruses 2 and 5. *Science* **275,** 1320–1323.
27. Wilson, J. M. (1996) Adenoviruses as gene-delivery vehicles. *N. Engl. J. Med*. **334,** 1185–1187.
28. Bevan, M. J. (1995) Antigen presentation to cytotoxic T lymphocytes in vivo. *J. Exp. Med.* **182,** 639–641.
29. Germain, R. N. (1994) MHC-dependent antigen processing and peptide presentation: providing ligands for T lymphocyte activation. *Cell* **76,** 287–299.
30. Chen, P. W., Wang, M., Bronte, V., Zhai, Y., Rosenberg, S. A., and Restifo, N. P. (1996) Therapeutic antitumor response after immunization with a recombinant adenovirus encoding a model tumor-associated antigen. *J. Immunol.* **156,** 224–231.
31. Flanagan, B., Pringle, C. R., and Leppard, K. N. (1997) A recombinant adenovirus expressing the simian immunodeficiency virus Gag antigen can induce long-lived immune responses in mice. *J. Gen. Virol.* **78,** 991–997.
32. Juillard, V., Villefroy, P., Godfrin, D., Pavirani, A., Venet, A., and Guillet, J. G. (1995) Long-term humoral and cellular immunity induced by a single immunization with replication-defective adenovirus recombinant vector. *Eur. J. Immunol*. **25,** 3467–3473.
33. Rodriques, E. G., Zavala, F., Eichinger, D., Wilson, J. M., and Tsuji, M. (1997) Single immunizing dose of recombinant adenovirus efficiently induces CD8+ T cell-mediated protective immunity against malaria. *J. Immunol.* **158,** 1268–1274.
34. Song, W., Kong, H.-L., Traktman, P., and Crystal, R. G. (1997) Cytotoxic T lymphocyte responses to proteins encoded by heterologous transgenes transferred in vivo by adenoviral vectors. *Hum. Gene Ther*. **8,** 1207-1217.
35. Xiang, Z. Q., Yang, Y., Wilson, J. M., and Ertl, H. C. (1996) A replication-defective human adenovirus recombinant serves as a highly efficacious vaccine carrier. *Virology* **219,** 220–227.
36. Inaba, K., Inaba, M., Romani, N., et al. (1992) Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. *J. Exp. Med.* **176,** 1693–1702.
37. Rosenfeld, M. A., Siegfried, W., Yoshimura, K., et al. (1991) Adenovirus-mediated transfer of a recombinant alpha 1-antitrypsin gene to the lung epithelium in vivo. *Science* **252,** 431–434.
38. Rosenfeld, M. A., Yoshimura, K., Trapnell, B. C., et al. (1992) In vivo transfer of the human cystic fibrosis transmembrane conductance regulator gene to the airway epithelium. *Cell* **68,** 143–155.

39. Hersh, J., Crystal, R. G., and Bewig, B. (1995) Modulation of gene expression after replication-deficient, recombinant adenovirus-mediated gene transfer by the product of a second adenovirus vector. *Gene Ther*. **2,** 124–131.
40. Graham, F. L., Smiley, J., Russell, W. C., and Nairn, R. (1977) Characteristics of a human cell line transformed by DNA from human adenovirus type 5. *J. Gen. Virol.* **36,** 59–74.
41. Crystal, R. G., McElvaney, N. G., Rosenfeld, M. A., et al. (1994) Administration of an adenovirus containing the human CFTR cDNA to the respiratory tract of individuals with cystic fibrosis. *Nat. Genet*. **8,** 42–51.
42. Inaba, K., Inaba, M., Deguchi, M., Hagi, K., Yasumizu, R., Ikehara, S., Muramatsu, S., and Steinman, R. M. (1993) Granulocytes, macrophages, and dendritic cells arise from a common major histocompatibility complex class II-negative progenitor in mouse bone marrow. *Proc. Natl. Acad. Sci. USA* **90,** 3038–3042.
43. Wang, M., Bronte, V., Chen, P. W., et al. (1995) Active immunotherapy of cancer with a nonreplicating recombinant fowlpox virus encoding a model tumor-associated antigen. *J. Immunol.* **154,** 4685–4692.
44. MacGregor, G. R., Nolan, G. P., Fiering, S., Roederer, M., and Herzenberg, L. A. (1991) Use of E. coli lacZ (β-Galactosidase) as a reporter gene. In *Gene transfer and expression protocols*. Murray, E. J. (ed.). The Humana Press Inc., Totowa, NJ, pp. 217–235.

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Stimulation of Anti-HIV-1 Cytotoxic T Lymphocytes by Dendritic Cells

Xiaoli Huang, Zheng Fan, Lian Zheng, and Charles R. Rinaldo, Jr.

1. Introduction

Dendritic cells (DC) are highly specialized antigen-presenting cells (APC) in both primary and secondary T-cell responses. This may be related to their expression of high levels of MHC class I and II antigens ***(1–4)***, costimulatory molecules CD40, CD80, and CD86 ***(5,6)***, and production of cytokines such as interleukin-12 (IL-12) ***(7–9)***, IL-6 ***(10)***, and interferon α (IFN-α) ***(10,11)***. Moreover, DC can process antigens in various forms by different pathways and induce high levels of both $CD4^+$ and $CD8^+$ T-cell reactivity ***(12)***.

The maintenance of anti-human immunodeficiency virus type 1 (HIV-1) memory cytotoxic T-cell (CTLp) reactivity may be important in preventing development of the acquired immunodeficiency syndrome (AIDS) ***(13–15)***. Most studies use HIV-1 antigen-presenting autologous Epstein-Barr virus (EBV) transformed B-lymphocyte cell lines (B-LCL) to activate the CTLp ***(16)***, which are less potent antigen-presenting cells (APC) than DC ***(17)***. We recently demonstrated that DC from HIV-1-infected subjects can be infected with recombinant VV vectors expressing the three major structural proteins of HIV-1 IIIb: p24 group-specific Ag (Gag), polymerase (Pol), and gp 160 envelope (Env) ***(18)***. These DC can stimulate equal or higher levels of MHC class I-restricted, anti-HIV-1 CTLp than can similarly treated, autologous B-LCL. DC pulsed with peptides representing HIV-1 CTL epitopes for particular HLA phenotypes stimulate higher levels of anti-HIV-1 CTLp responses than do DC infected with VV-HIV-1 constructs. Delivery of HIV-1 Gag, Pol, and Env proteins to DC by a cationic liposome, lipofectin, also stimulated HIV-1 specific CTLp responses ***(19)***. Additionally, allogeneic, MHC class I-matched DC stimulated anti-HIV-1 CTLp activity in cells from HIV-1-infected subjects

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(18,19). Use of such strategies to enhance the ability of DC to stimulate T cells could elicit broader and more effective CTL responses in HIV-1 therapeutic and prophylactic vaccine initiatives.

DC are a distinct lineage that arise from $CD34^+$ cell progenitors in bone marrow *(1,20,21)*. Less than 1% of mature DC are present in peripheral blood mononuclear cells (PBMC). However, studies have now shown that DC can be generated in vitro from monocytes and $CD34^+$ cells by appropriate cytokines. These cultured DC have proven to be useful in studies of antigen-presenting function of DC.

DC are more potent APC than are B-LCL and macrophages *(17,18)*. In this chapter we describe how DC can be prepared as stimulators for anti-HIV-1 CTL by infection with recombinant VV vectors expressing Gag, Pol, and Env, pulsing with peptides representing HIV-1 CTL epitopes or loading with HIV-1 Gag, Pol, and Env proteins by a cationic liposome. Other methods for stimulating antigen presentation by DC include nucleic acid transfection and other viral vectors, and are not included in this chapter. The preparation of immortalized B-LCL cells as target cells for DC stimulated cytotoxic T lymphocytes (CTL) is then described. As target cells, B-LCL can be infected with a VV vector or pulsed with HIV-1 synthetic peptide. The VV constructs that we use to infect B-LCL for the detection of HIV-1 CTL are: vAbT 141, containing the HIV-1 BH 10 *gag* coding sequence for p55 (VV-Gag); vAbT 204, containing the full-length HxB2 *pol* gene, including the reverse transcriptase, protease, and integrase (VV-Pol); vPE11, expressing the *env*-coding region of HIV-1 strain BH10 minus the signal sequence (VV-Env). This is used to minimize the expression of native gp160 on the surface of the target cells, which can be a target for non-CTL, antibody-dependent cell cytotoxicity mediated by $CD16^+$ cells. The NYCBH strain of VV (VV-Vac), expressing β gal but no HIV-1 genes, is used as the control virus. Finally, the detection and quantification of CTL by bulk lysis assay or limiting dilution precursor frequency analyses is described.

2. Materials

2.1. Culture of Blood DC

1. Hank's balanced salt solution (HBSS; Life Technologies, Grand Island, NY).
2. AIM V medium (serum-free lymphocyte medium; Life Technologies).
3. Recombinant human IL-4 (Schering-Plough, Kenilworth, NJ; stock: 10^4 U/mL).
4. Recombinant human GM-CSF (Schering-Plough; stock: 10^4 U/mL).
5. Six-well flat-bottom cell culture plate (Falcon; Becton Dickinson, Franklin Lakes, NJ).

2.2. Infection of DC with Recombinant VV Vectors

1. RPMI 1640 medium (Gibco-BRL, Grand Island, NY).
2. Heat-inactivated fetal calf serum (FCS) (Hyclone, Logan, UT).
3. Psoralen (Sigma; stock: 100 μg/mL in PBS; may have to heat at 56°C to complete solubilization. Store at 4°C or room temperature, protect from light).
4. VV-GPE (vAbT 408 containing the combined *gag/pol* and *env* coding sequences; Therion Biologics, Cambridge, MA; stock: 8×10^6 plaque forming units [pfu] per vial for infection of 2×10^6 DC as an input virus-to-cell multiplicity of 4 to 1).
5. Long-wave UV light (GLO-Mark Systems, Inc, Upper Saddle River, NJ).
6. 15-mL conical polypropylene centrifuge tubes (Falcon, Becton Dickinson).
7. 60 × 15 mm Petri dish (Falcon, Becton Dickinson).
8. Round-bottom 96-well plates (Falcon, Becton Dickinson).

2.3. Pulsing DC with HIV-1 CTL Peptide Epitopes

1. RPMI 1640 medium (Gibco-BRL).
2. Heat-inactivated fetal calf serum (FCS) (Hyclone).
3. Psoralen (Sigma; stock: 100 μg/mL in PBS; may have to heat at 56°C to complete solubilization. Store at 4°C or room temperature, protect from light).
4. HIV-1 synthetic peptides (either commercially prepared or prepared by local peptide synthesis facility; stock concentration: 1×10^4 μg/mL). The peptides are chosen based on epitope specificity according to HLA phenotype expression ***(22)***.
5. Long-wave UV light (GLO-Mark Systems).
6. 15-mL conical polypropylene centrifuge tubes (Falcon, Becton Dickinson).
7. 60 × 15-mm Petri dishes (Falcon, Becton Dickinson).
8. Round-bottom 96-well plates (Falcon, Becton Dickinson).

2.4. Delivery of HIV-1 Proteins to DC by Cationic Liposomes

1. RPMI 1640 medium (Gibco-BRL).
2. AIM-V medium (Life Technologies).
3. Heat-inactivated FCS (Hyclone).
4. Psoralen (Sigma; stock: 100 μg/mL in PBS; may have to heat at 56°C to complete solubilization. Store at 4°C or room temperature, protect from light.).
5. Lipofectin [DOTMA (*N*-[1-{2,3-dideyloxy}porpyl] -*N*, *N*, *N*-trimethy) (ammonium chloride)/ DOPE (dioleoyl-phophotidylethanolamine); Gibco-BRL; stock: 1000 μg/mL].
6. HIV-1 Gag, Pol, Env, or mock control proteins (Protein Synthesis, Meriden, CT; stock: 100 μg/mL).
7. Long-wave UV light (GLO-Mark Systems, Inc.).
8. 15-mL conical polypropylene centrifuge tubes (Falcon, Becton Dickinson).
9. 60 × 15-mm Petri dishes (Falcon, Becton Dickinson).
10. Round-bottom 96 microwell plates (Falcon, Becton Dickinson).

2.5. Preparation of Target Cells

1. NYCBH strain of VV (VV-Vac; Therion Biologics, Cambridge, MA).
2. vAbT 141 (VV-Gag; Therion Biologics).
3. vAbT 204 (VV-Pol; Therion Biologics).
4. vPE11 (VV-Env; B. Moss, National Institutes of Health, Bethesda, MD).
5. HIV-1 synthetic peptides (prepared by your local peptide synthesis facility or a commercial source; stock concentration: 1×10^4 μg/mL).
6. RPMI 1640 medium (Gibco-BRL).
7. Heat-inactivated FCS (Hyclone).
8. Chromium ($Na_2\ ^{51}CrO_4$, New England Nuclear, Boston, MA).
9. Round-bottom 15-mL polypropylene tube (Falcon, Becton Dickinson).

2.6. Detection of Memory CTL

1. RPMI 1640 medium (Gibco-BRL).
2. Heat-inactivated FCS (Hyclone).
3. Recombinant interleukin-2 (rIL2) (Chiron, Emeryville, CA; stock concentration: 10^4 U/mL).
4. Chromium ($Na2^{51}CrO_4$, New England Nuclear).
5. Triton X-100 (1% v/v, Sigma Chemical Co.).
6. 15-mL conical tubes (Falcon, Becton Dickinson).
7. Round-bottom 96-well plates (Falcon, Becton Dickinson).
8. 1.2-mL micro tubes (USA/Scientific, Ocala, FL).
9. Gamma counter (MINAXIγ, Packard, Downers Grove, IL).

2.7. Limiting Dilution Precursor Frequency Analysis (CTLp-LDA)

1. RPMI 1640 medium (Gibco-BRL).
2. Heat-inactivated FCS (Hyclone).
3. Recombinant interleukin-2 (rIL2, Chiron, Emeryville, CA; stock concentration: 10^4 U/mL).
4. Chromium ($Na_2{}^{51}CrO_4$, New England Nuclear).
5. 15-mL conical tubes (Falcon, Becton Dickinson).
6. Round-bottom 96-well plates (Falcon, Becton Dickinson).
7. LumaPlate-96 (Packard Instrument Company).
8. Microplate Scintillation and Luminescence Counter (Packard Instrument Company) or gamma counter (MINAXIγ, Packard).

3. Methods

3.1. Culture of Blood DC

1. Collect blood into heparinized tubes (*see* **Note 1**).
2. Mix the peripheral blood with an equal volume of HBSS and layer 20 mL over 10 mL of Ficoll-Hypaque. Centrifuge at 600*g* for 20 min.
3. Harvest the PBMC from the interface and wash three or four times with HBSS at room temperature.

4. Resuspend the cells at 10×10^6 cells per mL in AIM V medium and place 2 mL of suspension into each well of a six-well flat-bottom plate. Incubate for 2 h at 37°C in 5% CO_2 and a humidified atmosphere (*see* **Note 2**).
5. Wash the cell cultures gently several times with 2 mL of HBSS and a 2 mL pipet to remove nonadherent cells.
6. Add 2 mL of AIM V medium containing 1000 U/mL of hIL-4 and 1000 U/mL hGM-CSF to each well of adherent cells. Culture the adherent cells at 37°C in 5% CO_2 and a humidified atmosphere for 7–14 d (*see* **Note 3**).
7. Add fresh hIL-4 and hGM-CSF at 2–3-d intervals.
8. On a hemocytometer determine the viability and number of the cultured DC by trypan blue exclusion and typical DC ruffled membrane morphology, respectively ***(11)*** (*see* **Note 3**).

3.2. Infection of DC with Recombinant VV Vectors

1. Transfer 1×10^6 autologous cultured blood DC to a 15 mL conical centrifuge tube and spin at 450*g* for 10 minutes at room temperature.
2. Discard the supernatant and resuspend the DC in 1 mL RPMI containing antibiotics (complete medium) with 5% FCS.
3. Add VV-GPE at an input virus-to-cell multiplicity of 4 to 1 to the conical centrifuge tube and gently mixed with the DC. Centrifuge the mixture at 700*g* for 30 min at room temperature.
4. Discard the supernatant carefully, resuspend the cell pellet, and wash with 10 mL complete medium containing 5% FCS.
5. Resuspend the DC in 1 mL complete medium with 15% FCS and incubate for 16 h at 37°C and 5% CO_2.
6. After 16 h incubation transfer the DC suspension to a 60 × 15 mm Petri dish and mix with 3.5 mL of cold complete medium containing 5% FCS and 0.5 mL psoralen (stock concentration: 100 μg per mL, final concentration of psoralen is 10 μg/mL).
7. Expose the mixture to long-wave UV light irradiation for 5 min to inactivate the VV and any residual, endogenous HIV-1 and contaminating non-DC cells (*see* **Notes 4** and **5**).
8. Wash the DC with warm complete medium containing 5% FCS and count to determine the number of viable irradiated-VV-infected DC (*see* **Note 6**).
9. Resuspend the irradiated-VV-infected-DC at 25,000 cells/mL in complete medium containing 15% FCS.
10. Add 100 μL of this cell suspension to each well of a round-bottom 96-well plate containing responder cells (250,000 cells/mL, i.e., 25,000 cells in 100 μL per well) at a responder-to-stimulator cell ratio of 10:1 (*see* **Note 7**).

3.3. Loading DC with HIV-1 CTL Peptide Epitopes

1. Transfer 1×10^6 autologous cultured blood DC to a 15-mL conical polypropylene centrifuge tube and spin at 450*g* for 10 min at room temperature (*see* **Note 4**).

2. Discard the supernatant and resuspend the DC in 1 mL RPMI containing antibiotics (complete medium) with 5% FCS. Add 10 μL of HIV-1 synthetic peptide at a final concentration of 100 μg/mL and gently mix with the DC.
3. Incubate the mixture for 2 h at 37°C in a 5% CO_2 atmosphere and then wash with 10 mL of complete medium containing 5% FCS.
4. Resuspend DC in 1 mL complete medium with 5% FCS. Transfer the suspension to a 60 × 15-mm Petri dish and mix with 3.5 mL cold complete medium containing 5% FCS and 0.5 mL psoralen (stock concentration: 100 μg/mL; final concentration of psoralen will be 10 μg/mL).
5. Expose the mixture to long-wave UV light irradiation for 5 min to inactive any residual, endogenous HIV-1, and contaminating non-DC (*see* **Note 5**).
6. Wash DC with warm complete medium containing 5% FCS and count to determine the number and viability.
7. Resuspend the HIV-1-peptide-pulsed-DC at 25,000 cells/mL in complete medium containing 15% FCS and add 100 μL of this cell suspension to each well of round-bottom 96-well plates containing responder cells (250,000 cells/mL, i.e., 25,000 cells in 100 μL/well) at a responder-to-stimulator cell ratio of 10:1 (*see* **Note 7**).

3.4. Delivery of HIV-1 Proteins to DC by Cationic Liposomes

1. Mix 15 μL of lipofectin with 200 μL of HIV-1 protein (Gag, Pol, Env), or with 6 μL mock control protein (which equals the baculovirus antigen concentration in the recombinant protein) in a either a 15-mL conical centrifuge tube or a well of a round-bottom microwell plate (*see* **Note 8**).
2. Incubate for 10 min at room temperature.
3. Add 1×10^6 autologous cultured blood DC in 800 μL AIM-V medium to the lipofectin-protein mixture. The final concentration of DC in this mixture is 1×10^6/mL (*see* **Notes 4** and **9**).
4. Incubate for 4 h at 37°C in a 5% CO_2 atmosphere.
5. After incubation, wash DC with 10 mL complete medium containing 5% FCS and resuspend in 1 mL complete medium with 5% FCS.
6. Transfer the suspension to 60 × 15-mm Petri dish and mix with 3.5 mL cold complete medium containing 5% FCS and 0.5 mL psoralen (stock concentration: 100 μg/mL, final concentration of psoralen will be 10 μg/mL).
7. Expose the mixture to long-wave UV light irradiation for 5 min to inactivate any residual, endogenous HIV-1 and contaminating non-DC cells (*see* **Note 5**).
8. Wash DC with warm complete medium containing 5% FCS and count to determine the number and viability of HIV-1-peptide-pulsed DC.
9. Resuspend the HIV-1-protein-pulsed-DC at 25,000 cells/mL in complete medium containing 15% FCS.
10. Place 100 μL of this cell suspension into each well of round-bottom 96-well plate containing responder cells (250,000 cells/μL, i.e., 25,000 cells in 100 μL/well) at a responder-to-stimulator cell ratio of 10:1.

3.5. Preparation of Target Cells

3.5.1. Target Cells Infected by VV Vector

1. Place 3×10^6 autologous B-LCL target cells in 1.5 mL complete medium in a 15-mL round-bottom polypropylene tube (*see* **Note 10**). Add VV at an input multiplicity of 4 to 1 and spin at 700*g* for 30 min at room temperature.
2. Wash the VV-infected B-LCL in 10 mL of cold complete medium with 5% FCS.
3. Label the VV-infected B-LCL with 150 μCi of $Na_2{}^{51}CrO_4$ in 1 mL complete medium containing 15% FCS for 16 h at 37°C in a 5% CO_2 atmosphere.
4. Wash the B-LCL three times with cold complete medium containing 5% FCS.
5. Determine the cell count and viability of target cells by trypan blue dye exclusion. Target cells should have a viability of >80%.
6. The cytotoxic activity of the effector cell population is quantitated based on the amount of radioactivity released by the target cells.

3.5.2. Target Cells Pulsed with HIV-1 Synthetic Peptide

1. Resuspend 3×10^6 autologous B-LCL target cells in 1.5 mL complete medium in a 15-mL round-bottom polypropylene tube and pulse with HIV-1 synthetic peptide (100 μg/mL) for 2 h at 37°C in 5% CO_2 atmosphere (*see* **Note 11**).
2. Label the peptide pulsed B-LCL with 150 μCi of $Na_2{}^{51}CrO_4$ in 1 mL complete medium containing 15% FCS for 16 h at 37°C in a 5% CO_2 atmosphere.
3. Wash the B-LCL three times in cold complete medium containing 5% FCS.
4. Determine the number and viability of the target cells by trypan blue dye exclusion. Target cells should have a viability of >80%.
5. The cytotoxic activity of the effector cell population is quantitated based on the amount of radioactivity released by the target cells (*see* **Note 12**).

3.6. Detection of Memory CTL Reactivity by Bulk Lysis Assay (CTLp-Bulk Assay)

Memory cytotoxic T lymphocyte (CTLp) reactivity can be detected by bulk lysis in the standard chromium release assay. Cytotoxic activity is assessed in triplicate at three effector/target (E:T) cell ratios, e.g., 40:1, 20:1, and 10:1 (higher or lower E:T ratios can also be used). A high degree of activation is demonstrable by this antigen-specific method in CTL**p** assays at low E/T ratios ***(16,23,24)***. The CTL**p**-bulk assay is less labor intensive and costly than the CTLp-LDA, but is only semiquantitative.

1. Obtain PBMC from fresh blood or cryopreserved PBMC (*see* **Note 13**) ***(16)***.
2. Mix 1×10^6 PBMC at a 10 to 1 ratio with 0.1×10^6 VVgpe-infected DC, peptide-pulsed DC or lipofectin-protein treated DC as stimulators.
3. Resuspend the stimulator-responder cell mixtures in 8 mL complete medium containing 15% FCS, rIL2 (100 U/mL) (*see* **Note 14**).
4. Seed 200 μL of stimulator-responder cell mixture into 96-well round-bottom microtiter plates and culture for 2 wk.

5. Add fresh complete medium and rIL2 added every 5 d.
6. On d 14 harvest the responder cells from each well, combine, and wash and use as effector cells in bulk lysis assay.
7. Resuspend target cells (*see* **Subheadings 3.5.1.** and **3.5.2.**) at a concentration of 10^5cells/mL in complete medium containing 10% FCS and screen for $Na_2{}^{51}CrO_4$ incorporation before adding to the plates with effector cells (*see* **Note 15**).
8. Place 100-μL aliquots of target cells in each well of a round-bottom 96-well microtiter plate. Prepare control cultures in parallel, consisting of either target cells alone incubated in 100 μL for spontaneous release or 100 μL of 1% Triton X-100 for maximum release (*see* **Note 16**).
9. Perform the CTL_p-bulk assay in triplicate at three effector/target (E/T) cell ratios, e.g., 40:1, 20:1, and 10:1.
10. Add 100 μL of effector cells to the target cells in the 96-well plates at the desired E/T ratios.
11. Centrifuge the plates at 50*g* for 3 min at room temperature and incubate for 4 h at 37°C in a 5% CO_2 atmosphere.
12. After incubation carefully transfer 100 μL of cell-free supernatant from each well into 1.2-mL micro tubes and assess radioactivity in a gamma counter. Determine the average counts per minute (cpm) from the triplicate cultures.
13. Calculate the percentage of specific lysis using the formula: % lysis = 100 × [(experimental cpm – spontaneous cpm)/(maximum cpm – spontaneous cpm)]. Lytic activity is presented either as % lysis of HIV-1 expressing and non-HIV-1 expressing target cells, or % specific lysis, i.e., (% lysis of VV-HIV-1 Ag-expressing target cells) – (% lysis of VV-infected, non-HIV-1 Ag-expressing target cells). For the peptide-specific lysis assay, the % specific lysis is calculated similarly using target cells pulsed with HIV-1 peptides compared with mock-treated target cells. According to the cpm from three E/T ratios, the data can also be expressed as lytic units (LU) ***(25)***. One LU is the number of effector cells required to achieve 20% specific lysis of 5×10^3 target cells. LU are calculated per 10^7 effector cells, and expressed as arithmetic means (± SEM).

3.7. Detection of Memory CTL Reactivity by Limiting Dilution Precursor Frequency Analysis (CTLp-LDA)

Memory cytotoxic T lymphocyte reactivity can be detected by a CTL**p**-LDA. Cytotoxic activity is assessed in 24 replicate wells of 96-well round-bottom microtiter plates at seven dilution of responders, e.g., 250, 500, 1,000, 3,000, 6,000, 12,000, and 16,000 cells/well. The precursor frequency method has the advantage of being a more precise quantitative measure of CTL**p** activity, even though it is more labor intensive and costly than the CTL**p**-bulk assay.

1. Seed PBMC in complete medium containing 15% FCS at 250, 500, 1,000, 3,000, 6,000, 12,000, 16,000 cells and medium only (control wells) per well in 24 replicate wells of 96-well round-bottom microtiter plates ***(13,26)*** (*see* **Note 14**).
2. Add to each well 100 U/mL of IL2, and stimulator cells (1600 stimulator cells per well) from either VVgpe-infected DC, peptide-pulsed DC, or lipofectin-protein treated DC preparations.
3. Culture the cells for 14 d at 37°C in a 5% CO_2 atmosphere, with fresh complete medium containing 15% FCS and rIL2 added every 5 d.
4. On d 14, divide the cells in culture and transfer to two new wells. Adjust the volume to 100 µL with complete medium containing 15% FCS.
5. For target cells, autologous B-LCL (3×10^6) are infected with VV vectors or pulsed with HIV-1 synthetic peptides (*see* **Subheading 3.5.**), and labeled with 150 µCi of $Na_2{}^{51}CrO_4$ in 1 mL complete medium containing 15% FCS for 16 h at 37°C in 5% CO_2 atmosphere.
6. Wash the B-LCL three times with cold complete medium containing 5% FCS. Monitor the cell counts and viability by trypan blue dye exclusion.
7. Resuspend the target cells at a concentration of 10^5 cells/mL in complete medium containing 10% FCS.
8. Screen the target cells for $Na_2{}^{51}CrO_4$ incorporation before adding to the plates with effector cells (*see* **Note 16**).
9. The spontaneous release of the target cells should then be determined. Aliquot cells into round-bottom 96-well microtiter wells at 100 µL/well. For a maximum cpm, transfer 12.5 µL of each target cell solution, e.g., B-LCL alone, B-LCL-vac, B-LCL-gag, B-LCL-pol, B-LCL-env, or B-LCL-peptide to a LumaPlate-96 in duplicate wells and count by a Microplate Scintillation and Luminescence Counter (Packard Instrument Company) (*see* **Note 17**).
10. Add 10^4 target cells in 100 µL to 24 replicate wells of effector cells and incubate for 4 h at 37°C in 5% CO_2 atmosphere.
11. Carefully transfer 25 µL of cell-free supernatant from each well to LumaPlate-96 plates and measure radioactivity in a Microplate Scintillation and Luminescence Counter (*see* **Note 18**). The fraction of nonresponding wells is the number of wells in which the ^{51}Cr release does not exceed the mean spontaneous release plus 10% of the incorporation (total ^{51}Cr release – spontaneous ^{51}Cr release) divided by the number of wells assayed. The precursor frequency is estimated by the maximum-likelihood method ***(27)*** with a statistical program provided by S. Kalams (Massachusetts General Hospital, Boston, MA). CTLp activity is expressed as either the precursor frequency and 95% confidence intervals per 10^6 PBMC, or as net precursor frequency per 10^6 PBMC, i.e., the number of CTLp/10^6 PBMC specific for HIV-1 Ag minus the number of CTLp/10^6 PBMC specific for non-HIV-1 expressing target cells.

4. Notes

4.1. Culture of Blood DC

1. All procedures are to be carried out using sterile tissue culture techniques with sterile solutions and equipment.
2. If cell number is low, 12-well or 24-well flat-bottom plates can be used.
3. The cell phenotype can also be assessed by flow cytometry as the % positive for the particular marker and as the mean fluorescence intensity (MFI) of this expression, calculated based on the decade $\log_{10}$ scale ***(18)***. Approximately 70–80% of the cultured cells from both HIV-1-seronegative and HIV-1-seropositive subjects are DC based on dendritic morphology and expression of HLA-DR with MFI of ++, and lack of expression of CD3, CD14, CD16, CD19, and CD56. They also express other markers that are characteristic of DC, i.e., CD80 (approx 30%, MFI = +), CD86 (approx 80%, MFI = +), and CD40 (approx 90%, MFI = +), with a much lower number expressing CD1a (1%). These are therefore defined as "immature," monocyte-derived DC based on current criteria ***(21)***.
4. DC can be cultured from $CD14^+$ monocytes that are isolated by positive selection with anti-CD14 monoclonal antibody coated microbeads (MACS, Miltenyi Biotec, Auburn, CA). For 7 d in IL-4 and GM-CSF, then treated with CD40 ligand (2.5 μg/ml, Immunex, Seattle, WA) for 2 d to augment maturation of the DC.

4.2. Infection of DC with Recombinant VV Vectors and 4.3. Pulsing DC with HIV-1 CTL Peptide Epitopes

5. The distance from light bulb to the Petri dish should be approx 10 cm. This procedure results in more than 95% reduction in PFU in the VV preparation compared to mock-treated control. The cultured DC (either immature or mature) can be used without psoralen-UV treatment, as similar stimulation of T cells occurs with either the treated or untreated DC.
6. VV expression in the DC should be monitored by immunofluorescence staining for the particular HIV-1 protein (if reagents are available) or for β galactosidase (β gal) using standard staining technology. The DC should express >70% HIV-1 antigen or β gal as a surrogate measure of their expression of the CTL peptides.
7. If DC number is low, responder-to-stimulator cell ratio can be increased up to 100 to 1 or even 1000 to 1 for a comparable anti-HIV-1 CTLp reactivity, according to our unpublished data.

4.4. Delivery of HIV-1 Proteins to DC by Cationic Liposomes

8. If two or three HIV-1 proteins are delivered to DC together, lipofectin has to be mixed with HIV-1 protein separately in round-bottom microwells (96-well plate) or tubes (if the volume is more than 200 μL) and then transferred to a 15 mL conical centrifuge tube after 10 min incubation.
9. The final concentration of DC in lipofectin-protein mixture is 1×10^6/mL, i.e., if the DC number is as low as 1×10^5, 1.5 μL of lipofectin is incubated with 20 μL HIV-1 protein and then mixed with 1×10^5 DC in about 80 μL AIM-V medium. Also the volume of AIM-V added is dependent on the number of proteins to be delivered.

4.5. Preparation of Target Cells

10. B-LCL should be in log-phase growth and be given a partial medium exchange (50%) with fresh, complete medium containing 10% FCS twice a week. An acceptable viability of B-LCL is more than 80%. All B-LCLs and VV pools should be negative for mycoplasma, as determined by use of a nucleic acid probe (Genprobe, San Diego, CA).
11. For the most accurate measure of CTL lysis of these targets, several additional concentrations of peptide, higher and lower than this concentration, should be used to pulse the B-LCL.
12. In our experience, these targets yield the highest levels of lysis by CTL *(18)*.

4.6. Detection of Memory CTL Reactivity by Bulk Lysis Assay (CTLp-Bulk Assay)

13. All solutions and materials coming into contact with cells must be sterile, and proper sterile technique must be used accordingly.
14. Addition of gamma irradiated, allogeneic PBMC as feeder cells enhances the outgrowth of $CD8^+$ cells in DC-stimulated cultures *(18,19)*. However, addition of such feeder cells is not required for activation of $CD8^+$ T cells by DC.
15. The optimal $Na_2{}^{51}CrO_4$ incorporation of target cells is more than 2000 cpm per 100 μL of target cell solution.
16. Spontaneous release of target cells should average less than 20%, otherwise the assay may be too insensitive for acceptable results.

4.7. Detection of Memory CTL Reactivity by Limiting Dilution Precursor Frequency Analysis (CTLp-LDA)

17. The optimal $Na_2{}^{51}CrO_4$ incorporation of target cells is more than 2000 cpm per 100 μL of target cell solution detected in a gamma counter (MINAXIγ, Packard).
18. Spontaneous release of target cells should average less than 25%, otherwise the assay may be too insensitive for acceptable results. If using a gamma counter (MINAXIγ, Packard) for radioactivity counting, 50 μL of target cell solution are transferred into 1.2-mL tubes in duplicates as a maximum cpm.
19. After 25 μL of cell-free supernatant are transferred from each well to LumaPlate-96 plates, keep the LumaPlate in the hood until dry, and then count radioactivity.

4.8. Detection of Memory $CD8^+$ T Cell IFN-γ Production by Single Cell Enzyme Immunoassay (ELISPOT)

20. We have recently developed an ELISPOT for enumeration of IFN-γ production *(28).* This assay uses many of the same, basic procedures as described above for CTL assays, but is more sensitive.

References

1. Steinman, R. (1991) The dendritic cell system and its role in immunogenicity. *Annu. Immunol.* **9,** 271–296.

2. Banchereau, J. and Steinman, R. (1998) Dendritic cells and the control of immunity. *Nature* **392,** 245–252.
3. Romani, N., Gruner, S., Brang, D., et al. (1994) Proliferating dendritic cell progenitors in human blood. *J. Exp. Med.* **180,** 83–93.
4. Bhardwaj, N., Bender, A., Gonzalez, N., Bui, L. K., Garrett, M. C., and Steinman, R. (1994) Influenza virus-infected dendritic cells stimulate strong proliferative and cytolytic responses from human $CD8^+$ T cells. *J. Clin. Invest.* **94,** 797–1002.
5. Caux, C., Vanbervliet, B., Massacrier, C., et al. (1994) B70/B7-2 is identical to CD86 and is the major functional ligand for CD28 expressed on human dendritic cells. *J. Exp. Med.* **180,** 1841–1847.
6. Lanier, L. L., O' Fallon, S., Somoza, C., et al. (1995) CD80 (B7) and CD86 (B70) provide similar costimulatory signals for T cell proliferation, cytokine production, and generation of CTL. *J. Immunol.* **154,** 97–105.
7. Macatonia, S. E., Hosken, N. A., Litton, M., et al. (1995) Dendritic cells produce IL-12 and direct the development of Th1 cells from naive $CD4^+$ T cells. *J. Immunol.* **154,** 5071–5079.
8. Kang, K., Kubin, M., Cooper, K. D., Lessin, S. R., Trinchieri, G., and Rook A. H. . (1996) IL-12 synthesis by human Langerhans cells. *J. Immunol.* **156,** 1402–1408.
9. Heufler, C., Koch, F., Stanzl, U., et al. (1996) Interleukin-12 is produced by dendritic cells and mediates T helper 1 development as well as interferon-gamma production by T helper 1 cells. *Eur. J. Immunol.* **26,** 659–665.
10. Ghanekar, S., Zheng, L., Logar, A., et al. (1996) Cytokine expression by human peripheral blood dendritic cells stimulated in vitro with HIV-1 and herpes simplex virus. *J. Immunol.* **157,** 4028–4035.
11. Ferbas, J. J., Toso, J. F., Logar, A. L., Navratil, J. S., and Rinaldo, Jr., C. R. (1994) CD4+ blood dendritic cells are potent produced of IFN-α in response to in vitro HIV-1 infection. *J. Immunol.* **152,** 4649–4655.
12. Lanzavecchia, A. (1996) Mechanisms of antigen uptake for presentation. *Curr. Opin. Immunol.* **8,** 348–354.
13. Rinaldo, C. R., Huang, X-L., Fan, Z., et al. (1995) High levels of anti-human immunodeficiency virus type 1 (HIV-1) memory T-lymphocyte activity and low viral load are associated with lack of disease in HIV-1–infected long-term nonprogressors. *J. Virol.* **69,** 5838–5846.
14. Klein, M. R., van Baalen, C. A., Holwerda, A. M., et al. (1995) Kinetics of Gag-specific cytotoxic T lymphocyte responses during the clinical course of HIV-1 infection: a longitudinal analysis of rapid progressors and long-term asymptomatics. *J. Exp. Med.* **181,** 1365–1370.
15. Miedema, F. and Klein, M. R. (1996) AIDS pathogenesis: a finite immune response to blame? *Science* **272,** 505–508.
16. Huang, X. L., Fan, Z., Liebmann, J., and Rinaldo, C. (1995) Detection of human immunodeficiency virus type 1–specific memory cytotoxic T lymphocytes in freshly donated and frozen-thawed peripheral blood mononuclear cells. *Clin. Diagn. Lab. Immunol.* **2,** 678–686.

17. Croft, M. (1994) Activation of naive, memory and effector T cells. *Curr. Opin. Immunol.* **6,** 431–439.
18. Fan, Z., Huang, X-L., Zheng, L., et al. (1997) Cultured blood dendritic cells retain HIV-1 antigen-presenting capacity for memory CTL during progressive HIV-1 infection. *J. Immunol.* **159,** 4973–4979.
19. Zheng, L., Huang, X-L., Fan, Z., Borowski, L., and Rinaldo, C. (1999) Delivery of liposome-encapsulated HIV type 1 proteins to human dendritic cells, for HIV-1 specific memory cytotoxic T lymphocyte responses. *AIDs Res. Hum. Retro Vir.* **15,** 1011–1020.
20. Galy, A., Travis, M., Cen D., and Chen, B. (1995) Human T, B, natural killer and dendritic cells arise from a common bone marrow progenitor cell subset. *Immunity* **3,** 459–473.
21. Bell, D., Young, J. W., and Banchereau, J. (1999). *Dendritic Cells. Adv. Immunol.* **72,** 255–324.
22. Korber, B., Moore, J., Brander, C., Koup, R., Haynes, B., and Walker, B. (1997) HIV Molecular Immunology Database 1997. Los Alamos National Laboratory, Los Alamos, New Mexico.
23. Lubaki, M. N., Egan, M. A., Siliciano, R. F., Weinhold W. J., and Bollinger, R. C. (1994) A novel method for detection and ex vivo expansion of HIV type 1–specific cytotoxic T lymphocytes. AIDS Res. *Human Retroviruses* **10,** 1427–1433.
24. van Baalen, C. A., Klein, M. R., Geretti, A. M., et al. (1993) Selective in vitro expansion of HLA class I-restricted HIV-1 gag-specific $CD8^+$ T cells: cytotoxic T-lymphocyte epitopes and precursor frequencies. *AIDS* **7,** 781–788.
25. Pross HF, Baines, M. G., Rubin, P., Shragge, P., and Patterson, M. S. (1981) Spontaneous human lymphocyte mediated cytotoxicity against tumor target cells. IX. The quantitation of natural killer cell activity. *J. Clin. Immunol.* **1,** 51–56.
26. Koup, R. A., Pikora, C. A., Luzuriaga, K., et al. (1991) Limiting dilution analysis of cytotoxic T lymphocytes to human immunodeficiency virus gag antigen in infected persons: in vitro quantitation of effector cell population with p17 and p24 specificities. *J. Exp. Med.* **174,** 1593–1598.
27. Fazekas de St. Groth, S. (1982) The evaluation of limiting dilution assays. *J. Immunol. Meth.* **49,** R11–R23.
28. Huang, X-L., Fan, Z., Kalinyak, C., Mellors, J. W., and Rinaldo, C. R., Jr. (2000) $CD8^+$ T-cell gamma interferon production specific for human immunodeficiency virus type 1 (HIV-1) in HIV-1-infected subjects. *Clin. Diagn. Lab. Immunol.* **7,** 279–287.

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METHODS IN MOLECULAR MEDICINE™

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Dendritic Cell Protocols

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It is now apparent that dendritic cells not only play important roles in the body's immune system through their complex interactions with T cells, B cells, and other cell types, but also possess distinct functional attributes that enable them to assume different roles in that system. In *Dendritic Cell Protocols,* Stephen P. Robinson, MD, PhD, and Andrew Stagg, PhD, have brought together a wide range of time-proven methods for studying these so-called "veiled" cells. Many of these readily reproducible techniques deal with the problem of obtaining sufficient dendritic cells for analysis, whether by isolation from a wide variety of tissues or from various progenitor cell populations. Other methods describe in step-by-step fashion the techniques commonly used for analyzing aspects of dendritic cells, ranging from cell migration to antigen uptake and T cell stimulation. Variant methods that have been successful in other laboratories have been included to expand experimental possibilities. In addition, a few techniques explore the practical challenges involved in using dendritic cells in a clinical setting to develop novel immunotherapeutics.

State-of-the-art and highly practical, *Dendritic Cell Protocols* provides both experienced and novice investigators with powerful tools to illuminate the complex biology of these foundation cells—cells that shape the evolution of acquired immune responses, play important roles in innate immunity, and promise the development of powerful new immunotherapeutics.

Features

- **State-of-the-art collection of laboratory techniques to study dendritic cells**
- **Readily reproducible techniques described in step-by-step detail to ensure success**
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