

Chapter 2

Generation of Murine Growth Factor-Dependent Long-Term Dendritic Cell Lines to Investigate Host–Parasite Interactions

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Summary

Substantial progress has been made over the last several years in the development of protocols for the isolation of large numbers of dendritic cells (DCs) from different tissues and their short-term culture. Indeed, several stable DC lines and clones have been established from various tissues of mice and humans, providing useful experimental tools for studying the biology of DCs at both molecular and biochemical levels and for the establishment of new DC-based immunotherapies. In this chapter, we will describe the development of long-term DC lines that maintain the growth factor dependence and their immature functional state, thus providing a unique opportunity to study the mechanisms of the initiation of the immune response to infectious agents.

Key words: Dendritic cell lines, Host–parasite interaction, GM-CSF, Bone marrow, Bacteria, Immune response.

1. Introduction

Dendritic cells (DCs) are distributed especially in tissues that interface with the external environment, such as the skin, the gut, and the lungs (1–3). At these locations, they can perform a sentinel function for incoming pathogens, and have the capacity to recruit and activate cells of the innate immune system upon inflammation (4–6). Uptake of pathogens by DCs induces a state

of activation, which eventually leads to the migration of the antigen-loaded DCs to the lymphoid organs where the cells of the adaptive immune response can be alerted (7).

Until the last decade, the paucity of DCs in most tissues and the lack of specific markers have hampered the study of DC ontogeny, phenotype, and function. Several groups have succeeded in generating large numbers of functional DCs/Langerhans cells in murine or human systems by treating DC precursors with granulocyte-macrophage colony-stimulating factor (GM-CSF) alone or in combination with other growth factors (8–13). However, such DCs could be propagated only for limited periods of time. Growth factor-dependent long-term DC lines from mouse fetal or newborn skin have been established (14, 15). Nevertheless, although these lines possess some properties of DC precursors and maintain an immature phenotype, they cannot be induced to mature *in vitro* (14, 15).

In recent years, immortalization of DCs from mouse tissues, using retroviral vectors carrying immortalizing oncogenes, has successfully been achieved (16–18). The immortalized DC lines are homogeneous, easily grown, and do not require growth factors for their propagation, being extensively used for functional and biochemical DC characterization. Indeed, the cells exhibited phenotypic and functional features of immature DCs, 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* (19–21). However, the inability to induce growth factor arrest in these immortal lines has hampered the complete maturation of DCs upon activation.

In the mouse system, we succeeded in generating long-term growth factor-dependent immature DC lines, derived from adult mouse spleen (22) or mouse bone marrow. In this system, proliferation and survival are strictly dependent upon the presence of exogenous murine GM-CSF (mGM-CSF) and fibroblast-derived growth factors (22). Long-term DCs preserve an immature phenotype. Activating signals, such as living bacteria, Toll-like receptors agonists and cytokines, promote full maturation of these DC lines, such as the D1 cells, mimicking and recapitulating the whole natural process of DC differentiation that occurs in response to activation by pathogens *in vivo*.

This chapter focuses on *in vitro* techniques leading to the establishment of murine long-term DC lines, similar to the D1 cells that have been widely used (23–26). Using a conditioned medium (R1 medium) containing several cytokines, including mGM-CSF produced by GM-CSF-transfected NIH/3T3, we show that DCs can be stimulated to undergo maturation in response to live bacteria, thus allowing investigation of host-pathogen interactions (22, 27).

2. Materials

1. *Iscove's Modified Dulbecco's Medium (complete medium)*. IMDM supplemented with 10% heat-inactivated fetal bovine serum (FBS) of Australian origin by Gibco BRL, 2 mM L-glutamine, 100 IU/mL penicillin, 100 µg/mL streptomycin, and 0.05 mM 2β-mercaptoethanol (*see Note 1*).
2. *DC-GM medium (R1 medium)*. IMDM complete medium supplemented with conditioned medium produced by mGM-CSF-transfected NIH/3T3 fibroblasts (available upon request from P. Ricciardi-Castagnoli) corresponding to 10 ng/mL of mGM-CSF as final concentration. DC medium is stable at 4°C for up to 3 weeks. (For conditioned medium preparation *see Subheading 3.1* and *Note 2*).
3. *Phosphate-buffered saline (PBS)*, pH 7.2–7.4. 154 mM NaCl, 8.1 mM Na₂HPO₄ (7H₂O), 1.9 mM NaH₂PO₄ (H₂O).
4. *Red blood cell (RBC) lysis buffer*. 105 mM NH₄Cl, 10 mM KHCO₃, 0.1 mM Na₄EDTA.
5. *PBS-EDTA*. Solution of PBS supplemented with 2 mM EDTA.
6. *Composition of Luria Bertani (LB) medium*. Tryptone 10 g/L, yeast extract 5 g/L, NaCl 10 g/L.

3. Methods

3.1. Production of Conditioned Medium Containing mGM-CSF

1. NIH/3T3 fibroblasts stably transfected with mGM-CSF (1×10^6 in 7 ml) are plated in 100 × 20 mm tissue-cultured-treated petri dish using IMDM medium supplemented with 5% heat-inactivated FBS of Australian origin by Gibco BRL, 2 mM L-glutamine, 100 IU/mL penicillin, 100 µg/mL streptomycin, and 0.05 mM 2β-mercaptoethanol (*see Note 3*).
2. The conditioned medium is collected from culture after 3 days and centrifuged at $270 \times g$ for 5 min to pellet floating cells and debris. Then, the supernatant is filtered through a 0.2 µm filter and aliquots stored at –20°C.
3. In order to measure the concentration of mGM-CSF, a specific enzyme-linked immunoabsorbent assay should be performed.

3.2. Preparation of Cell Suspensions from Bone Marrow

1. Mice are euthanized using CO₂ asphyxiation followed by cervical dislocation (*see Note 4*).
2. The skin of mouse hind legs is pulled back and the muscles dissected to expose femur and tibia (*see Note 5*).

3. Using a scissors and a pair of tweezers, the knee joint is cut, the muscle along the femur is sliced and the bone is severed from the hip joint. Most of the muscle tissues surrounding the bone should be eliminated with the scissors.
4. After removal of both femurs and tibias, all bones are washed twice in PBS and placed in a 60 × 15 mm petri dish containing 5 mL of IMDM complete medium.
5. Cutting both bone edges using scissors exposes the cavity lumen. Then, the BM is flushed out using a 5-mL syringe and 25-G needle filled with IMDM complete medium. Flush the bone cavity until it appears white.
6. To disaggregate clusters of BM cells, the cell suspension is passed through the syringe and filtered using a cell strainer (pores diameter = 70 μ m) into a 50-mL tube.
7. Cells are recovered by centrifugation and the pellet resuspended in 10 mL R1 medium.
8. Finally, cells are counted using a Bürker chamber.

3.3. Preparation of Cell Suspensions from Spleen

1. Mice are euthanized using CO₂ asphyxiation followed by cervical dislocation (*see Note 4*).
2. Spleens are isolated from mice, placed in a 60 × 15 mm petri dish containing 5 mL of IMDM complete medium, and cut into small pieces. A single cell suspension is obtained by smashing the spleens using a 5 mL-siring plunger until mostly fibrous tissue remains.
3. To avoid cell aggregates, the suspension is filtered through 70- μ m cell strainers.
4. The suspension is transferred to a 15-mL tube and centrifuged for 5 min at 400 × *g*.
5. At this stage, the removal of RBC from the spleen cell suspension is necessary. For this purpose, the pellet is resuspended in 1–2 mL of RBC lysis buffer and incubated at 4°C for 9 min. 10 mL IMDM complete medium is added and the aggregates removed by filtration through 70- μ m cell strainer. The mononuclear cells are recovered by centrifugation at 270 × *g* for 5 min. The supernatant is discarded and the pellet is resuspended in a suitable volume of R1 medium for counting.

3.4. Generation of Mouse Long-Term Growth Factor- Dependent DC Lines

1. Unfractionated cell populations from BM or spleen are plated at a density of 4 × 10⁵ cells/mL in 100 × 20mm suspension culture dishes in R1 medium and incubated at 37°C with 5% CO₂ (*see Note 6*).
2. The culture is fed with fresh R1 medium without the addition of exogenous IL-4 every 3–4 days (*see Note 7*).

3. Usually the first passage of DC-enriched culture is performed around day 7, when the cell density is high, using a solution of PBS-EDTA. Then, the cells are counted and replated at a concentration of $2\text{--}3 \times 10^5$ cells/mL (*see* **Note 8**).
4. Once a week, both suspended and weakly adherent cells are collected using PBS, centrifuged at $270 \times g$ for 5 min, and seeded at a density of $2\text{--}3 \times 10^5$ cells/mL (*see* **Note 9**).
5. After 3 months of continuous culture, in vitro growing cells can be split every 3–4 days using PBS-EDTA.
6. At this point, long-term growth factor-dependent DC lines are established (*see* **Notes 10 and 11**).
7. The established long-term culture retains a characteristic immature DC phenotype, and the cells can be induced to mature in vitro into terminally differentiated DCs upon activation with a number of stimulatory signals, such as LPS, bacteria, or cytokines. In **Fig. 1**, the phenotypical maturation of BM-derived DC lines induced by LPS is shown.

3.5. Growth of Bacterial Culture

1. A single colony of DH5 α *E. coli* bacteria is inoculated into 2 mL of LB medium in a loosely capped 15-mL tube. The culture is incubated for ~8 h at 37°C with vigorous shaking. Using a vessel with a volume of at least four times greater than the volume of medium, the starter culture is diluted 1/500 to 1/1,000 into a larger volume of LB medium and grown with vigorous shaking to saturation (12–16 h, overnight). (*see* **Notes 12 and 13**)
2. The bacterial culture is then diluted 1/10 with LB medium and incubated for ~2.5 h at 37°C with shaking. Growth is monitored every 20–30 min by spectrophotometer and stopped in late-log-phase corresponding to OD₆₀₀ ~0.6.
3. The concentration of the bacterial culture can be calculated using the following formula:
$$y = -29 + 1,191 (\text{OD}_{600}) \times 10^6 \text{ bact/mL}$$
 (*see* **Note 14**).
4. The bacterial cells are harvested by centrifugation at $6,000 \times g$ for 2 min at 4°C.
5. The LB medium is removed by aspiration, leaving the bacterial pellet as dry as possible. Bacteria are resuspended in 1 mL of R1 medium without antibiotics per mL of bacterial culture (*see* **Note 15**).

3.6. Bacterial Infection of DCs

1. The day before the infection, DC lines are harvested using a solution of PBS-EDTA and centrifuged at $250 \times g$ for 5 min.
2. After a wash with PBS, DC lines are resuspended in an appropriate amount of R1 medium for counting.

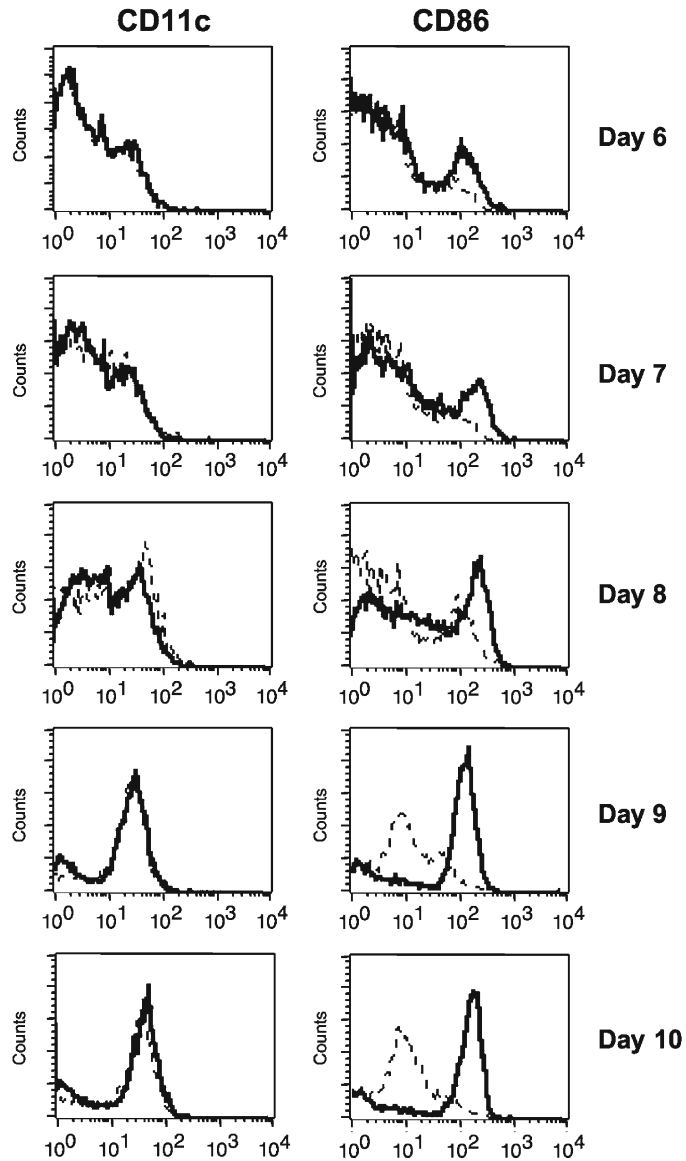


Fig. 1. Analysis of cell surface marker expression in BM-derived DC line cultured in R1 medium. The cells have been harvested at the indicated time and stimulated with LPS (1 $\mu\text{g}/\text{mL}$) for 24 hours. The expression of CD11c and CD86 markers was evaluated by flow cytometry. Dashed histogram: untreated cells; bold histogram: LPS-treated cells.

3. Cells are plated at a concentration of $2\text{--}3 \times 10^5/\text{mL}$ in suspension petri dishes and incubated at 37°C 5% CO_2 for 16–18 h.
4. The R1 medium is removed and replaced with fresh R1 medium without antibiotics (*see Note 16*).
5. DC lines are infected with *E. coli* bacteria at a bacteria:DC ratio of 10:1 and incubated at 37°C 5% CO_2 for 1 h (*see Note 17*).

6. At this point, the medium is discarded and the petri dishes washed gently with PBS. Finally, fresh R1 medium supplemented with gentamicin (50 $\mu\text{g}/\text{mL}$) and tetracycline (10 $\mu\text{g}/\text{mL}$) is replated, and the cell culture is incubated at 37°C 5% CO_2 for the desired time.
7. Bacteria are efficient in upregulating the expression of DC maturation surface markers, such as CD80, CD86, MHC class II, and CD40. In **Fig. 2**, the upregulation of costimulatory molecules induced by bacteria in DC lines derived from BM and spleen is shown, as assessed by FACS analysis.

4. Notes

1. To optimize DC growth conditions, different batches of sera should be tested prior to use. In order to avoid phagocytosis of debris by DCs, it is optimal to filter complete medium using 0.2- μm filter.

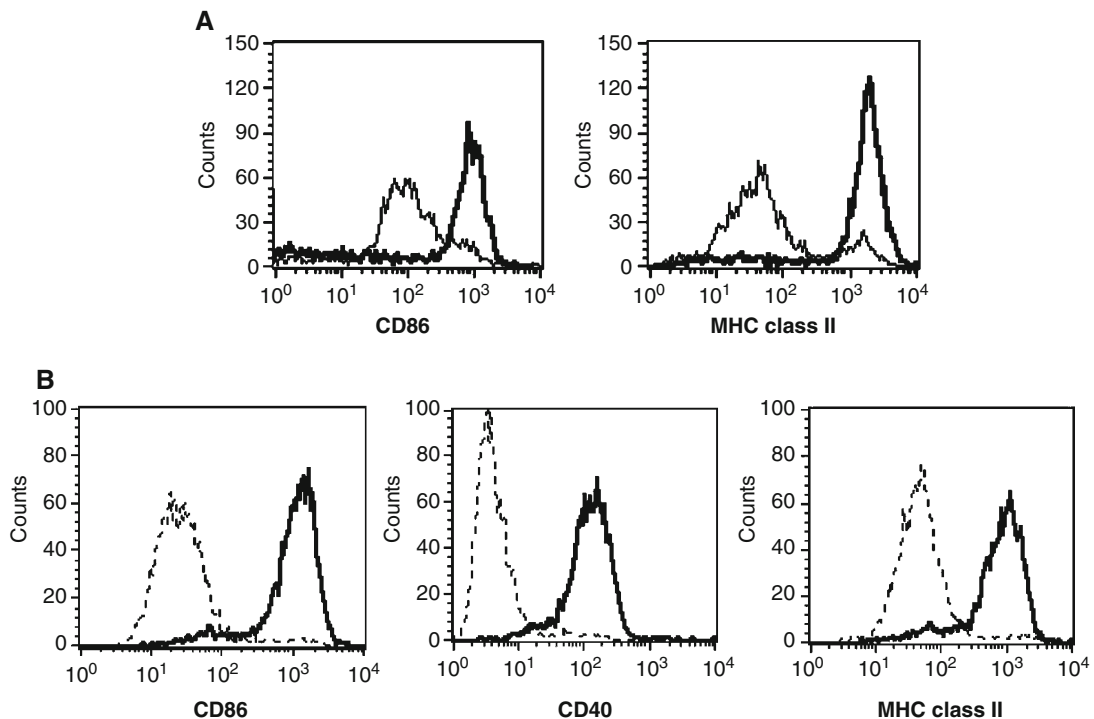


Fig. 2. Phenotypical maturation of BM- and spleen-derived long-term GM-CSF-dependent DCs. BM- (**A**) and spleen-derived (**B**) immature DCs were stimulated with DH5 α *E. coli* bacteria (moi: 10) for 1 h, and the expression of CD86, CD40, and I-A MHC class II cell surface markers was measured by flow cytometry. Dashed and bold histograms show untreated cells and cells challenged with DH5 α *E. coli* bacteria, respectively.

2. The ideal final concentration of mGM-CSF in the conditioned medium produced by mGM-CSF-transfected NIH/3T3 should be around 30 ng/mL.
3. In order to produce optimal conditioned medium, mGM-CSF-transfected NIH/3T3 fibroblasts should be plated starting from a semiconfluent culture.
4. In general, 8–12 weeks old mice yield sufficient numbers of DCs, precursors, or progenitor cells, and organs of sufficient size to be easily manipulated. Mice younger than 6 weeks may yield fewer cells or to be too small to manipulate easily, and mice older than 12 weeks may be more expensive with no additional gain in cell yield. The use of specific pathogen-free mice is recommended to avoid pathogen-specific effect. To minimize the effect of the anesthetizing or euthanizing agents that may perturb DC function, CO₂ asphyxiation followed by cervical dislocation is used to kill mice.
5. Both femurs and tibias should be removed to isolate maximal numbers of mononuclear cells from a mouse. Femurs provide 80% of total BM cells.
6. DC lines tend to adhere strongly to plastic. Thus, the use of suspension culture dishes is highly recommended to obtain and culture DC lines.
7. Half of the medium is collected and the floating cells harvested by centrifugation. Then, the cell pellet is resuspended in fresh R1 medium and replated.
8. Cellular growth is monitored daily by microscopy. Proliferating cells are visible in suspension as clusters with veiled morphology.
9. Cell types other than DC precursors also respond to mGM-CSF and grow in R1 medium. For example, macrophages differentiate and strongly attach to the petri dish. These are discarded at each passage by harvesting only cells in suspension. Other major contaminants of nonadherent DCs are granulocytes (especially when starting from BM) visible as clusters of round cells from day 2. However, the majority of granulocyte contaminants tend to die in culture and are absent by the time DCs are harvested. It is often necessary to wash the cell pellet with PBS, to discard debris and dead cells.
10. In order to monitor the differentiation process of DCs, cell surface marker expression is analyzed by flow cytometry. Generally, it is found that the approaches described earlier generate CD11c⁺ DCs at 80–95% purity.
11. The DC lines once established could be continuously maintained for years but should not be kept in culture. As these DC lines are not clonally selected, it is necessary to prepare frozen stocks of the cells kept as a backup storage. The cell

growth is strictly dependent on the presence in the culture medium of mGM-CSF. Indeed, growth factor deprivation leads to cell growth arrest and cell death.

12. Bacterial cultures should always be grown from a single colony picked from a freshly streaked plate. Subculturing directly from glycerol stocks, agar stabs, and liquid cultures is a poor microbiological practice. Inoculation from plates that have been stored for a long time may also lead to liquid culture containing different bacteria. The desired clone should be streaked from a glycerol stock onto a freshly prepared agar plate such that single colonies can be isolated. This procedure should then be repeated to ensure that a single colony is picked.
13. To avoid bacterial death, LB medium should be prewarmed at 37°C before bacteria inoculation. It is often convenient to grow the starter culture during the day and the larger culture overnight for harvesting the following morning.
14. Usually, $OD_{600} = 0.6$ corresponds to 6.85×10^8 bacteria/mL. Because the relationship between the OD_{600} and the number of viable cells per mL varies substantially from strain to strain, it is essential to calibrate the spectrophotometer. This can be achieved by plating serial dilutions of a bacterial culture onto LB agar plates in the absence of antibiotics. The counted colonies are used to calculate the number of bacteria per mL, which is then set in relation to the measured OD_{600} values.
15. The supernatant can be conveniently withdrawn with a disposable pipette tip attached to a vacuum line. Use gentle suction to avoid drawing the pellet into the pipette tip. Any droplets of liquid adherent to the walls of the tube should be removed by vacuum. It is essential to ensure that the bacterial pellet is completely dispersed.
16. It is critically important to remember to not add antibiotics to the RI medium at this stage to avoid bacterial death.
17. The amount of bacteria used to challenge DC lines can vary depending on the aim of the experiment. It is possible to use lower bacteria:DC ratios and to extend the incubation period to 16–18 h.

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