4

Genetic Modification of Dendritic Cells Through the Directed Differentiation of Embryonic Stem Cells

Paul J. Fairchild, Kathleen F. Nolan, and Herman Waldmann

Summary

Recent years have witnessed a progressive acceptance of the dual role played by dendritic cells (DC) in the initiation of immune responses and their specific attenuation through the induction of immunological tolerance. Nevertheless, as terminally differentiated cells of the myeloid lineage, DC share with macrophages an inherent resistance to genetic modification, greatly restricting strategies available for studying their physiology and function. Consequently, little is known of the molecular interactions provided by DC that underlie the critical decision between tolerance and immunity. Embryonic stem (ES) cells are, by contrast, relatively amenable to genetic modification. Furthermore, their propensity for self-renewal, one of the cardinal features of a stem cell, permits cloning at the single cell level and the rational design of ES cell lines, uniformly expressing a desired, mutant phenotype. Here, we describe how another defining property of ES cells, their demonstrable pluripotency, may be harnessed for their directed differentiation along the DC pathway, enabling the generation of limitless numbers of DC faithfully expressing candidate genes of interest. The protocols we outline in this chapter may, therefore, offer new opportunities for dissecting the biology of DC and the molecular basis of their unique properties.

Key Words: Dendritic cell; embryonic stem cell; directed differentiation; tolerance; genetic modification.

1. Introduction

During the past decade, numerous lines of evidence have converged on dendritic cells (DC) as playing a critical role in the establishment and ongoing maintenance of immunological tolerance ***(1,2)***. Although originally considered to be restricted to the induction of central tolerance ***(3)***, their remit is now known to extend beyond negative selection in the thymus to the imposition of dominant tolerance in the periphery, though the polarization of naïve T cells toward a regulatory phenotype ***(4)***. Various studies have suggested that recognition

From: *Methods in Molecular Biology, vol. 380: Immunological Tolerance Methods and Protocols*
Edited by: Fairchild © Humana Press Inc., Totowa, NJ

of antigen by naïve T cells in the absence of full activation signals is the critical parameter in driving their commitment to the regulatory lineage ***(5)***, a scenario epitomized by antigen presentation by immature DC ***(6,7)***. In contrast, other studies have provided evidence consistent with the need for expression by DC of molecules such as B7-H1, inducible costimulator ligand or the enzyme indoleamine 2,3-dioxygenase ***(8–10)*** to set in place a regulatory network. These findings imply that DC play a proactive role in the decision-making process, inducing regulatory T cell development solely though the provision of specific signals. Systematic attempts to resolve this ambiguity have, however, been confounded by the innate resistance of terminally differentiated DC to genetic modification. Although electroporation and lipid-based protocols have proven to be of little value when applied to this cell type, the use of viral vectors has enjoyed some measure of success ***(11)***. Nevertheless, their use has been widely reported to adversely affect the maturation status of DC, either impairing their activation in response to pathogen-associated molecules ***(11)*** or prompting their premature maturation and significantly reducing their life span ***(12)***.

In the light of these limitations, we have devised an alternative approach to the study of DC by deciphering their differentiation pathways from pluripotent embryonic stem (ES) cells through the formation of embryoid bodies (EB), macroscopic structures that recapitulate some of the early events of embryogenesis in vitro ***(13,14)***. The relative ease with which ES cells may be transfected with heterologous genes, together with opportunities for cloning at the single cell level, pave the way for the generation of ES cell lines, constitutively expressing defined transgenes or silencing constructs. Such a permanent resource may serve as a prelude to the directed differentiation of copious DC uniformly expressing the same mutant phenotype ***(15)***. Such an approach may greatly facilitate a systematic investigation of the role played by specific genes in DC function and the capacity of this cell type to refine the balance between tolerance and immunity in response to changes in circumstance. Furthermore, the recent advent of pluripotent ES cell lines of human origin ***(16,17)*** offers prospects for adapting these protocols for the genetic modification of human DC, thereby helping to bridge the species barrier between mouse and man.

2. Materials

2.1. Preparation of Embryonic Fibroblasts

1. Female C57Bl/6 or Rosa 26 mice at day 13 of gestation (*see* **Note 1**).
2. Standard dissection instruments.
3. Scalpel fitted with a sterile no. 23 surgical blade.
4. Water bath warmed to 37°C.
5. Inverted phase-contrast microscope.
6. Phosphate buffered saline (PBS).

7. B6 medium: Dulbecco's modified Eagle's medium (DMEM) (Invitrogen), 10% (v/v) fetal calf serum (FCS), 2 m*M* L-glutamine (Invitrogen), 50 U/mL penicillin, 50 μg/mL streptomycin (Invitrogen), 5×10^{-5} *M* 2-mercaptoethanol (Promega).
8. Sterile trypsin–EDTA solution: PBS, 0.125% trypsin (v/v) (Invitrogen), 0.02% EDTA (w/v).
9. Dimethyl sulfoxide Hybri-Max® (DMSO) (Sigma).
10. Sterile 90-mm diameter Petri dishes.
11. Sterile 75- and 150-cm^2 tissue culture flasks.
12. Sterile Universal tubes.

2.2. Routine Maintenance of ES Cells

1. Two 75-cm^2 flasks of confluent primary embryonic fibroblasts.
2. ES medium: DMEM, 15% FCS (v/v) (*see* **Note 2**), 2 m*M* L-glutamine, 1 m*M* sodium pyruvate (Invitrogen), 5×10^{-5} *M* 2-mercaptoethanol.
3. PBS and trypsin–EDTA solution.
4. Stock mitomycin C (MMC, Sigma) dissolved in PBS at 1 mg/mL. Being toxic, MMC should be handled carefully and a risk assessment performed.
5. Sterile 25-cm^2 tissue culture flasks.

2.3. Genetic Modification of ES Cells

1. 24-Well plates and 96-well flat-bottomed plates seeded with MMC-treated Rosa 26 embryonic fibroblasts.
2. Inverted phase-contrast microscope.
3. P200 Gilson pipet.
4. Multi-12-channel pipet.
5. Fresh B6 medium (*see* **Subheading 2.1., item 7**).
6. ES medium supplemented with 1000 U/mL of recombinant murine leukemia inhibitory factor (rLIF) (Chemicon).
7. Sterile PBS and trypsin-EDTA solution.
8. Sterile PBS containing 0.1% (w/v) gelatin Type A (Sigma).
9. Endotoxin-free preparation of plasmid DNA.
10. LipofectAMINE Plus™ (Life Technologies) or equivalent transfection reagent.
11. Stock solution of the neomycin analog G418-sulfate (Geneticin; Invitrogen) at 250 mg/mL in DMEM, or an appropriate alternative selection reagent.
12. Trypan blue (Sigma).
13. Six-well plates gelatinized by incubating with 0.1% gelatin (w/v) in PBS for 30 min at 37°C and washing twice with sterile PBS before overlaying with PBS before use.
14. Gelatinized 12- and 25-cm^2 flasks.
15. Polystyrene reagent reservoirs (Corning).

2.4. Directed Differentiation of ES Cells

1. EB maintained in suspension culture for 14 d.
2. P1000 Gilson pipet.
3. Fresh ES medium (*see* **Subheading 2.2., item 2**).

4. Sterile EDTA, 0.2% (w/v) in PBS.
5. Recombinant murine granulocyte/macrophage colony stimulating factor (rmGM-CSF) (R&D Systems) and recombinant murine interleukin-3 (rmIL-3) (R&D Systems).
6. G418-sulfate or an appropriate alternative antibiotic for selection purposes.
7. Lipopolysaccharide (LPS: *Escherichia coli* Serotype 0127:B8) (Sigma).
8. Leukopor tape® (Beiersdorf AG Hamburg, Germany).
9. Sterile 90-mm diameter tissue culture plates (Corning).

3. Methods

3.1. Preparation of Primary Embryonic Fibroblasts

1. To prepare a stock of fibroblasts, arrange timed matings of C57Bl/6 mice, defining the day on which a vaginal plug is observed as d 0 of gestation.
2. Sacrifice the mother on d 13 and carefully remove the embryos, dissecting them from the uterus and surrounding extraembryonic tissues into a Petri dish of ice cold PBS.
3. Decapitate and carefully remove the liver, which serves as the principle hematopoietic organ at this stage of ontogeny.
4. Remove the PBS and replace with 10 mL of trypsin-EDTA. Finely macerate the embryonic tissues using a scalpel fitted with a sterile no. 23 surgical blade.
5. Transfer the tissue to a Universal tube. Rinse the Petri dish with a further 5 mL of trypsin–EDTA and combine with the original suspension.
6. Place in a water bath for 5 min at 37°C.
7. Shake the tube vigorously to mechanically disrupt the tissue and leave for 3 min to allow lipids to rise to the surface and any remaining tissue to sediment under unit gravity.
8. Carefully collect 5–10 mL of cell suspension between the pellet and lipid layer and transfer to a 50-mL conical tube containing 10 mL of complete B6 medium as a source of FCS proteins to inactivate the trypsin.
9. Replace the volume removed with an equivalent volume of trypsin-EDTA and return the Universal to the water bath for a further 5 min.
10. Repeat the extraction protocol two further times (**steps 7–9**), pooling the material obtained from each extraction. When complete, allow the cell suspension to stand briefly and remove any fragments of tissue that settle to the bottom of the tube using a sterile Pasteur pipet. Likewise, carefully remove any fatty deposits from the air–liquid interface and discard them.
11. Spin the tube at 200*g* to pellet the cells and resuspend in fresh B6 medium. Distribute equally among 75-cm^2 tissue culture flasks. Although the number of flasks will vary according to the size of the litter, we routinely seed one flask for every two embryos used.
12. After culture for 1–2 d, the fibroblasts should have produced a near-confluent monolayer of cells. Remove any debris and floating cells by gently pipetting over the surface and replacing with fresh medium (*see* **Note 3**).

13. Once the cells reach confluency, passage them into an equivalent number of 150-cm^2 flasks to provide room for expansion. To do so, remove the overlying medium and replace with 20 mL of PBS. Gently swirl the flask to exclude any traces of medium and FCS, which might inhibit the action of proteases. Replace with 10 mL of trypsin–EDTA and return to the incubator for 3 min. Shake the flask vigorously to release the adherent cells and transfer to a 50-mL conical tube containing 10 mL of B6 medium.
14. Centrifuge for 5 min at 200*g* and resuspend in fresh B6 medium before seeding the appropriate number of flasks.
15. Although the fibroblasts are still subconfluent and are not, therefore, susceptible to contact inhibition, harvest and pool the cells from all flasks. Centrifuge and resuspend in medium supplemented with 10% DMSO (v/v). Prepare vials of feeder cells at 10^7 cells per vial and freeze rapidly on dry ice before storing long-term under liquid nitrogen (*see* **Note 4**).

3.2. Routine Passage of Mouse ES Cell Lines

1. Although different mouse ES cell lines may vary subtly in their properties, we find that they invariably require passaging every third day of culture.
2. Because primary embryonic fibroblasts are required as feeder cells, these must be passaged in parallel. Therefore, thaw a vial of stock fibroblasts in advance and expand to form two confluent 75-cm^2 flasks.
3. The day before passaging the ES cell line, remove the medium from one of the flasks of fibroblasts and replace it with 10 mL of fresh B6 medium, supplemented with MMC at a final concentration of 10 μg/mL. Incubate for 2 h at 37°C to ensure that the cells are mitotically inactivated (*see* **Note 5**).
4. During this period, passage the remaining flask of fibroblasts into two 75-cm^2 flasks so as to encourage expansion and the replenishment of stocks for subsequent use.
5. After 2 h, harvest the MMC-treated cells using trypsin–EDTA (*see* **Subheading 2.1., item 8**), spin and resuspend in fresh B6 medium. Distribute equally among two 25-cm^2 flasks and incubate overnight to encourage the formation of confluent monolayers onto which the ES cells may be passaged.
6. On the day of passaging, the ES cell line should appear as discrete, compact colonies in which the boarders of individual cells are difficult to discern (*see* **Fig. 1A**). Prepare a single cell suspension of the ES cell line by removing the medium and replacing with 10 mL of PBS. Swirl the flask and replace with 5 mL of trypsin–EDTA.
7. Return the flask to the incubator for 3–4 min. Shake vigorously to disaggregate the colonies of ES cells and transfer the cell suspension to a 15-mL conical tube containing 5 mL of complete B6 medium. Centrifuge at 200*g* for 5 min and resuspend in 10 mL of complete ES medium.
8. Seed the two 25-cm^2 flasks with ES cells at two different densities. Although the extent to which the cells should be diluted will need to be determined empirically, we routinely perform a 1:3 and 1:10 dilution. Preparation of stocks of ES cells at two different densities increases the likelihood of ensuring optimal growth conditions.

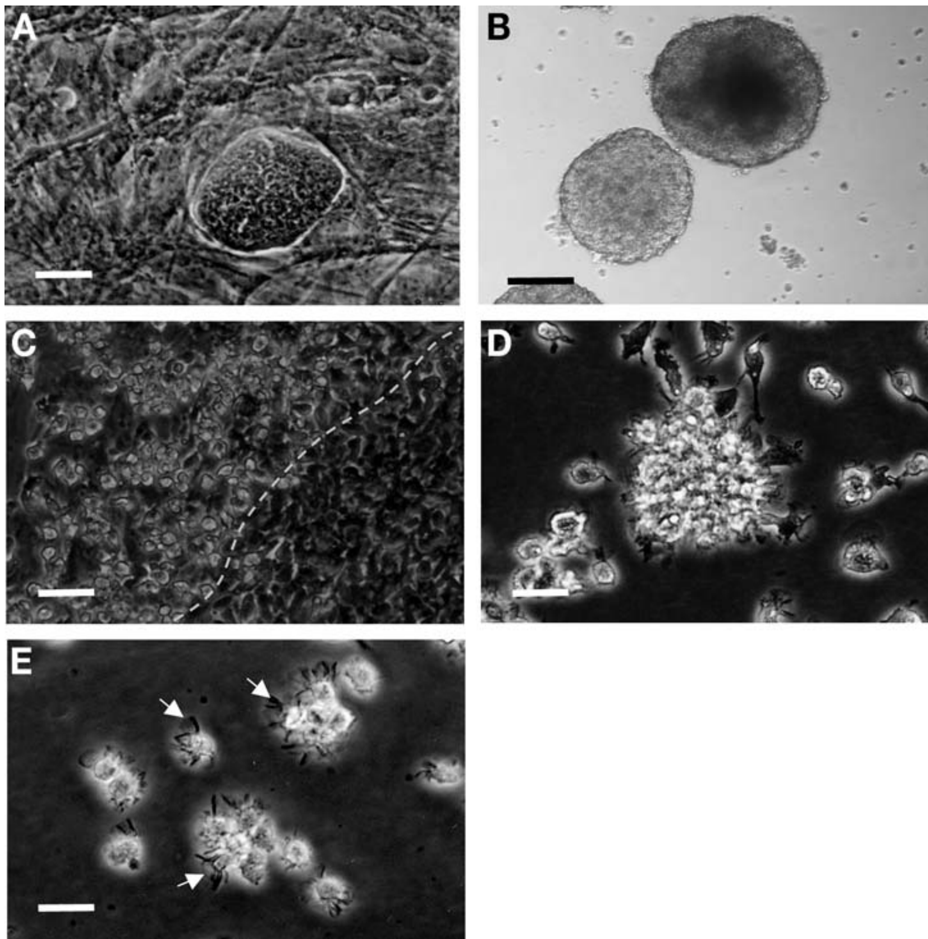

Fig. 1. Sequential stages during the directed differentiation of embryonic stem (ES) cells along the dendritic cell pathway. **(A)** Colony of undifferentiated ES cells cultured on a monolayer of primary embryonic fibroblasts. Bar = 30 µm. **(B)** EB formed from the proliferation of ES cells cultured in single cell suspension for 14 d. Bar = 500 µm. **(C)** Immature dendritic cells (DC) accumulating around the edge of an EB (indicated by the broken line). Bar = 30 µm. **(D)** Typical cluster of immature DC, lightly adherent to tissue culture plastic. Bar = 10 µm. **(E)** DC induced to mature following transient exposure to lipopolysaccharide. Note the many dendrites and veils of cytoplasm (arrows). Bar = 10 µm.

3.3. Transfection of ES Cells

Although various methods may be used for the stable transfection of mouse ES cells, we prefer the use of lipid-based approaches, which do not place the cells under detectable stress and have obtained good results with both

LipofectAMINE Plus and Fugene. Whichever reagent is chosen, however, preparatory titration of the plasmid DNA and adherence to the manufacturer's instructions throughout is strongly recommended. The choice of selection system is largely restricted to the use of the neomycin resistance gene due to the availability of embryonic fibroblasts from Rosa 26 mice that are inherently neomycin resistant and which may, therefore, be used for the routine passage of stable transfectants.

1. In preparation for transfection, it is necessary to determine the optimal concentration of the neomycin analog, G418, that will select for transfectants while not compromising the viability of Rosa 26 fibroblasts. Although the sensitivity of ES cell lines to G418 may vary, we have found 600 µg/mL to be optimal for all lines tested so far.
2. In advance of transfection, the ES cells should be weaned away from primary embryonic fibroblasts by serial passage onto gelatinized flasks in complete ES medium, further supplemented with 1000 U/mL of rLIF to maintain their pluripotency. This results in the progressive dilution of the fibroblasts and preferential expansion of the ES cell line.
3. Harvest the cells with trypsin-EDTA and plate 10^5 cells into each well of a gelatinized six-well tissue culture plate in 4 mL of ES medium containing rLIF.
4. Incubate for 48 h to permit adherence of the cells and the formation of colonies. Monitor carefully until the ES cells reach approx 40% confluency (*see* **Note 6**).
5. Perform the transfection using the preferred reagent according the manufacturer's instructions, designating a single well for "mock" transfection in which the plasmid DNA is omitted. Incubate the ES cells in ES medium supplemented with rLIF for 24–48 h before adding G418 at a predetermined concentration.
6. Monitor the cultures over the ensuing few days until no viable cells remain in the mock transfected well. Screen the remaining wells for colonies of ES cells that have survived selection as evidence of their incorporation of the plasmid DNA.
7. When the surviving colonies have become established, harvest all wells using trypsin-EDTA and pool to form a polyclonal cell line, which may be expanded in a gelatinized 25-cm^2 flask in ES medium supplemented with G418 and rLIF.
8. When ready for passaging, prepare frozen stocks of the transfected cell line for future use and screen for expression of the transgene using an appropriate assay. In the event that monoclonal antibodies are available specific for the gene product, this may involve the use of flow cytometry of the development of a specific enzyme-linked immunosorbent assay. Alternatively, functional assays or an appropriate PCR-based strategy may be required. Once expression of the transgene has been confirmed, the polyclonal cell line may be used for cloning at the single cell level.

3.4. Cloning of ES Cells

Although ES cell lines may be maintained in gelatinized dishes in medium supplemented with rLIF, it is inadvisable to culture them long-term in this way because they have a tendency to become teratocarcinoma cells, which lose any

capacity for subsequent differentiation. We therefore recommend transferring stable transfectants of the ES cell line onto primary embryonic fibroblasts at the first opportunity.

1. In preparation for cloning the ES cell line, prepare MMC-treated Rosa 26 fibroblasts (*see* above) and distribute into the wells of 96-well flat-bottomed plates. We find that a confluent 75-cm^2 flask of feeder cells is sufficient for seeding two 96-well plates, which should be incubated overnight in B6 medium to promote the formation of monolayers.
2. Remove the medium by aspiration and overlay each well with 50 µL of ES medium.
3. Harvest the polyclonal ES cell line that has been cultured in gelatinized flasks and prepare a single cell suspension. Perform a viability count using trypan blue exclusion as an appropriate readout and resuspend the cells to 2×10^5 cells per milliliter in ES medium.
4. Transfer 100 µL of cell suspension (approx 2×10^3 cells) to a tube containing 900 µL of medium, mix thoroughly and transfer 150 µL of this suspension (approx 300 cells) to a polystyrene reservoir containing 30 mL of ES medium.
5. Using a 12-channel multipipet, transfer 100 µL to each well of the two 96-well plates seeded with fibroblasts, such that each well receives, on average, a single ES cell.
6. Prepare 20 mL of ES medium containing 2.4 mg/mL of G418 and distribute 50 µL to each well, thereby yielding a final concentration of G418 of 600 µg/mL.
7. Culture the plates for 4 d before screening individual wells for colonies using inverted phase contrast microscopy. Exclude any wells containing multiple colonies.
8. Once colonies have become established, expand into 24-well plates seeded with MMC-treated Rosa 26 fibroblasts. To harvest individual colonies, remove medium from the relevant wells by aspiration and overlay with 100 µL of PBS. Replace with 50 µL of trypsin–EDTA and incubate at 37°C for 3 min.
9. Harvest the cells by gentle pipetting with a P200 Gilson pipet and transfer the entire cell suspension to a designated well of the 24-well plate, containing 2 mL of complete ES medium to neutralize the effect of the trypsin. The wholesale transfer of the contents of individual wells is preferable to centrifugation, which risks losing a significant proportion of the small number of ES cells in each colony.
10. Once individual clones have become established, they may be expanded to prepare frozen stock and screened for expression of the transgene (*see* **Fig. 2**). Ultimately, those clones proving positive should be tested for their capacity to sustain DC differentiation.

3.5. Production of Embryoid Bodies

1. As a prelude to the production of EB, the ES cells should be weaned away from primary embryonic fibroblasts by serial passage onto gelatinized 12-cm^2 flasks in

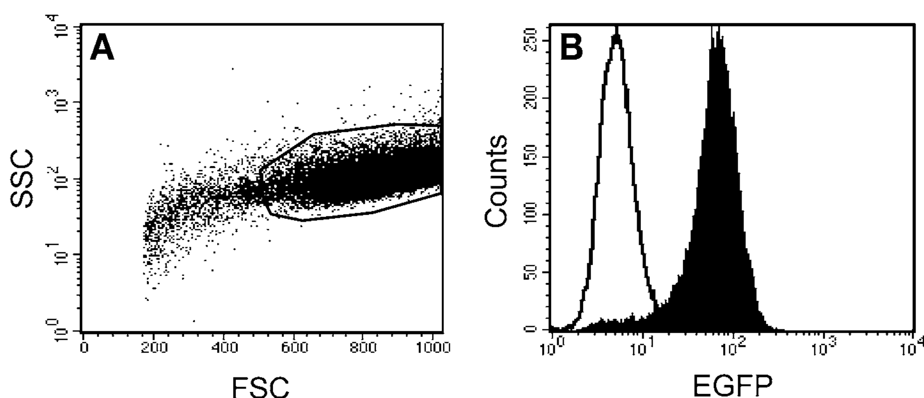

Fig. 2. Expression of the reporter gene (EGFP) by mouse embryonic stem (ES) cells following cloning at the single cell level. **(A)** Scatter profile of ES cells showing the gating procedure adopted. **(B)** EGFP expression by transfectant (filled histogram) compared with mock transfected ES cell line (open histogram).

complete ES medium, further supplemented with G418 and 1000 U/mL of rLIF to maintain their pluripotency (*see* **Note 7**). This results in the progressive dilution of the fibroblasts, which might otherwise interfere with the formation of EBs.

2. Once the burden of fibroblasts has been significantly reduced, prepare a single cell suspension of the ES cells and determine their number and viability using trypan blue exclusion.
3. Resuspend the cells in ES medium containing G418 to a density of 4×10^5 cell/mL and transfer 1 mL of cell suspension to two 90-mm diameter dishes of bacteriological plastic in a total of 20 mL of medium lacking an exogenous source of rLIF. This low density of cells is intended to prevent their aggregation, thereby ensuring that each EB is derived from the proliferation and differentiation of a single ES cell.
4. Return cultures to the incubator for a total of 14 d. EBs may be observed with the naked eye as early as 4 d after seeding the dish with ES cells and appear as small spherical structures, floating freely in the medium. By day 14 of culture, EBs may become large macroscopic structures, often several millimeters in diameter (*see* **Fig. 1B** and **Note 8**).
5. Carefully monitor the medium for the depletion of nutrients during the course of the culture period. Should the medium become acidified, transfer the EB to a 15-mL conical tube and allow them to settle under unit gravity for 2 min. Carefully draw the EB into a sterile Pasteur pipet and transfer them to a dish containing fresh ES medium.

3.6. Directed Differentiation of Dendritic Cells

1. To further direct the differentiation of ES cells along the DC lineage pathway, prepare differentiation medium consisting of complete ES medium further supplemented

with 200 U/mL of rmIL-3 and 25 ng/mL of rmGM-CSF. An appropriate concentration of G418 should also be added to the medium to prevent loss of the transgene during differentiation.

2. Harvest the EB after 14 d in suspension culture (*see* **Note 9**), allowing them to settle in a 50-mL conical tube.
3. Gently draw the EB into a sterile Pasteur pipet and distribute approx 20–30 of these structures into 90-mm tissue culture dishes in 35 mL of differentiation medium (*see* **Note 10**).
4. Seal the dishes with Leukopor tape and incubate at 37°C, 5% CO_2 (*see* **Note 11**). Take care not to disturb the cultures for at least 48 h to encourage the adhesion of EB to the tissue culture plastic and the outgrowth of terminally differentiated cell types.
5. Regular observation of cultures will reveal the chaotic nature of the differentiation process, many different cell types becoming apparent over time, of which cardiomyocytes are undoubtedly the most easily identified by virtue of their propensity to contract rhythmically *in situ*. DC may become visible as early as day 4–5 of culture although the kinetics of their appearance may vary considerably between ES cell lines and even different passages of the same line. Typically DC appear around the very perimeter of the colonies derived from adherent EB (*see* **Fig. 1C**), frequently forming a distinctive “halo” of cells. Extensive proliferation results in their accumulation and the formation of clusters, highly reminiscent of immature DC differentiated from cultures of mouse bone marrow progenitors (*see* **Fig. 1D**). The terminally differentiated DC, which are stably immature at this stage, may eventually become confluent (*see* **Note 12**).
6. Care should be taken to ensure that cultures do not become depleted of nutrients. When feeding cultures, remove all exhausted medium, leaving behind the lightly adherent DC, and carefully replace with fresh differentiation medium.
7. Because DC differentiated from ES cells adhere strongly to one another when pelleted, forming clumps that are difficult to dissociate without harming the cells, we routinely centrifuge the cell suspension in PSB containing EDTA. To do so, discard the medium, removing the last traces using a Pasteur pipet, and overlay with 9 mL of PBS.
8. Use a 1-mL Gilson pipet and moderate force to expel medium over the surface of the dish and dislodge the lightly adherent cells, which may be seen as a “cloud” when tilting the dish forward (*see* **Note 13**).
9. Transfer the cell suspension to a 15-mL conical tube and add 1 mL of 0.2% EDTA in PBS to yield a final concentration of 0.02%. Centrifuge the cell suspension for 5 min at 200*g* before using the cells in immunological assays, designed to investigate the impact of transgene expression on DC function.

3.7. Maturation of Dendritic Cells

1. Although ES cell–derived DC are stably immature for prolonged periods in culture, they may be induced to mature by transient exposure to LPS. To initiate their

maturation program, harvest the DC and plate overnight onto fresh tissue culture plates in differentiation medium.

2. Add LPS (*E. coli* serotype 0127:B8) at a final concentration of 1 µg/mL and return to the incubator.
3. After overnight culture, the majority of cells will have adhered strongly to the plastic and begun to spread out. Remove all medium and replace with fresh differentiation medium that lacks any traces of LPS, before incubating again overnight.
4. Observation under an inverted phase-contrast microscope the following day will reveal floating cells displaying highly dendritic morphology, a cardinal feature of mature DC (*see* **Fig. 1E**). To obtain a substantially pure population of mature DC, harvest the medium without pipetting over the surface of the dish and centrifuge. Because, upon maturation, the DC lose their propensity for aggregation, the addition of EDTA is unnecessary at this stage.
5. Feed cultures with fresh differentiation medium and return to the incubator, examining for the release of further cohorts of mature cells over the ensuing few days (*see* **Note 14**).

4. Notes

1. We prefer C57Bl/6 mice as a source of embryonic fibroblasts except when culturing ES cells under selection conditions following transfection. When using the neomycin analog G418-sulfate, for selection purposes, it is necessary to use primary embryonic fibroblasts from Rosa 26 mice, because these are intrinsically neomycin-resistant.
2. Batches of FCS may vary enormously with respect to their capacity to support ES cell growth and directed differentiation of ES cells along the DC pathway. We recommend screening samples from different suppliers against both of these criteria and ordering the best batch in bulk.
3. At this stage of the protocol, embryonic fibroblasts are only lightly adherent. Care should be taken, therefore, when trying to dislodge debris by expelling medium onto the surface of the monolayer.
4. Batches of fibroblasts may vary enormously with respect to their viability after thawing, their doubling time and the total number of passages they can sustain. We recommend comparing batches by defining the length of time taken to reach confluency after seeding a 75-cm^2 flask with a single vial of fibroblasts and the number of passages they can undergo before falling quiescent. This information can be extremely valuable when planning the maintenance of ES cell lines and the best time to replace existing flasks of feeder cells from frozen stocks.
5. Although we favor the use of mitomycin C, the availability of a ^{60}Co radiation source provides an inexpensive alternative approach to rendering feeder cells mitotically inactive. The delivery of 30 Gy is sufficient to prevent their proliferation.
6. Although protocols for the use of other cell types frequently suggest performing transfection once cells have reached 80% confluency, we find that mouse ES cells, plated at this density, have a tendency to overgrow and die prematurely, before

succumbing to G418 toxicity. A cell density of 40% therefore provides adequate scope for expansion during the course of transfection and selection.

7. We recommend the use of 12-cm^2 tissue culture flasks at this stage of the protocol as a result of the need to supplement the culture medium with a source of rLIF, which constitutes a major expense. The use of 12-cm^2 flasks requires as little as 4 mL of complete medium but still yields sufficient ES cells for the generation of copious EB.
8. EB vary enormously in size and morphology, even when cultured from a homogenous suspension of ES cells. In general, however, EB may be characterized as either "simple" or "cystic," the latter being distinguished by the development of a fluid-filled cavity, which may increase significantly in size, sometimes reaching up to 5 mm in diameter. Importantly, we have yet to observe any difference in the ability of either type of EB to sustain the differentiation of DC.
9. Although we have been able to generate DC from EB that have been cultured for as little as 4 d before plating, their appearance is significantly delayed and yields are comparatively low. Likewise, EB cultured in suspension for 21 d prior to plating may be permissive, but yields of DC decrease progressively beyond this time point. For this reason, we routinely use EB cultured for 14 d as the starting material for directed differentiation.
10. Given the inevitable investment of time and resources in the generation of genetically modified ES cells, there can be significant psychological pressure not to waste any material, with the result that too many EB may be seeded per plate. This temptation should, however, be strongly resisted, even if a significant proportion of the EB is discarded, because crowding of the EB provides too little space for DC to develop.
11. We find that sealing dishes with leukopor tape helps to maintain sterility during prolonged incubation periods of up to 1 mo. This is particularly important when repeatedly handling dishes, because even the most experienced worker will occasionally deposit medium around the rim of the dish during routine observation, which leaves the culture especially vulnerable to fungal infection.
12. Although ES cell-derived DC remain stably immature with time, showing none of the phenotypic changes associated with maturation, they appear to undergo predicable changes in morphology during prolonged periods in culture. In particular, the cells become enlarged, rounded, and highly vacuolated, as though actively sampling the surrounding milieu. Importantly, such morphological changes do not appear to affect the immunostimulatory function of the cells in vitro.
13. After harvesting DC, we routinely feed the culture with fresh medium supplemented with rmGM-CSF and rmIL-3, to permit the emergence of a further cohort of cells. It is, in fact, possible to obtain at least three batches of DC from the same culture before the cells fall quiescent.
14. When first challenged with LPS, the DC become uniformly and transiently adherent to tissue culture plastic before gradually releasing over the course of the

ensuing few days. These may be harvested in successive waves, or allowed to accumulate in the medium for several days before harvesting. For unexplained reasons, only a proportion of the original cells appears capable of assuming a classic nonadherent dendritic morphology, the majority remaining strongly adherent through out.

References

1. Steinman, R. M., Hawiger, D., and Nussenzweig, M. C. (2003) Tolerogenic dendritic cells. *Annu. Rev. Immunol.* **21,** 767–811.
2. Morelli, A. E. and Thomson, A. W. (2003) Dendritic cells: regulators of alloimmunity and opportunities for tolerance induction. *Immunol. Rev.* **196,** 125–146.
3. Fairchild, P. J. and Austyn, J. M. (1990) Thymic dendritic cells: phenotype and function. *Int. Rev. Immunol*. **6,** 187–196.
4. Fairchild, P. J. and Waldmann, H. (2000) Dendritic cells and prospects for transplantation tolerance. *Curr. Opin. Immunol.* **12,** 528–535.
5. Waldmann, H., Chen, T. -C., Graca, L., et al. (2006) Regulatory T cells in transplantation. *Sem. Immunol*. **18(2)**:111–119.
6. Jonuleit, H., Schmitt, E., Schuler, G., Knop, J., and Enk, E. H. (2000) Induction of IL-10-producing, non-proliferating $CD4^+$ T cells with regulatory properties by repetitive stimulation with allogeneic immature human dendritic cells. *J. Exp. Med.* **192,** 1213–1222.
7. Mahnke, K., Qian, Y., Knop, J., and Enk, A. H. (2003) Induction of $CD4^+CD25^+$ regulatory T cells by targeting of antigens to immature dendritic cells. *Blood* **101,** 4862–4869.
8. Chen, L. (2004) Co-inhibitory molecules of the B7-CD28 family in the control of T-cell immunity. *Nat. Rev. Immunol.* **4,** 336–347.
9. Munn, D. H., Sharma, M. D., Lee, J. R., et al. (2002) Potential regulatory function of human dendritic cells expressing indoleamine 2,3-dioxygenase. *Science* **297,** 1867–1870.
10. Mellor, A. L. and Munn, D. H. (2004) IDO expression by dendritic cells: Tolerance and tryptophan catabolism. *Nat. Rev. Immunol*. **4,** 762–774.
11. Jenne, L., Schuler, G., and Steinkasserer, A. (2001) Viral vectors for dendritic cell-based immunotherapy. *Trends Immunol*. **22,** 102–102.
12. Morelli, A. E., Larregina, A. T., Ganster, et al. (2000) Recombinant adenovirus induces maturation of dendritic cells via an NF-κB-dependent pathway. *J. Virol.* **74,** 9617–9628.
13. Fairchild, P. J., Brook, F. A., Gardner, R. L., et al. (2000) Directed differentiation of dendritic cells from mouse embryonic stem cells. *Curr. Biol.* **10,** 1515–1518.
14. Fairchild, P. J., Nolan, K. F., and Waldmann, H. (2003) Probing dendritic cell function by guiding the differentiation of embryonic stem cells. *Meth. Enzymol.* **365,** 169–186.

15. Fairchild, P. J., Nolan, K. F., Graça, L., and Waldmann, H. (2003) Stable lines of genetically modified dendritic cells from embryonic stem cells. *Transplantation* **76,** 606–608.
16. Thomson, J. A., Itskovitz-Eldor, J., Shapiro, S. S., et al. (1998) Embryonic stem cell lines derived from human blastocysts. *Science* **282,** 1145–1147.
17. Reubinoff, B. E., Pera, M. F., Fong, C. Y., Trounson, A., and Bongso, A. (2000) Embryonic stem cell lines from human blastocysts: somatic differentiation in vitro. *Nat. Biotechnol.* **18,** 399–404.