Chapter 33

Differentiation of Dendritic Cells from Human Embryonic Stem Cells

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Abstract

Improving our understanding of the interactions between human dendritic cells (DCs) and T cells may contribute to the development of therapeutic strategies for a variety of immune-mediated disorders. The possibility of using DCs themselves as tools to manipulate immune responses opens even greater therapeutic avenues. Current methods of generating human DCs are both inadequate and susceptible to high levels of variability between individuals. DCs differentiated from human embryonic stem cells (hESCs) could provide a more reliable, consistent solution. DCs have now successfully been differentiated from hESCs and more recently this has been repeated using protocols that avoid the inclusion of animal products, an important modification for clinical use. We have developed a novel method for the generation of DCs from hESCs in the absence of animal products that does not necessitate a separate embryoid body (EB) generation step. The technique involves the use of four growth factors and their successive removal from culture, resulting in accumulation of DCs with phenotypic, morphological, and immunostimulatory properties comparable to those of classical human monocyte-derived DCs. In addition to the application of hESC-derived DCs in basic research and novel approaches to cancer immunotherapy, they may also play a central role in the field of regenerative medicine. Tolerogenic DCs differentiated from hESCs may be used to persuade the immune system of the recipients of cell replacement therapy to tolerate allogeneic tissues differentiated from the same hESC line. Such an approach may help to address the immunological barriers that threaten to derail the clinical application of hESCs.

Key words: dendritic cells, human embryonic stem cells, ESC, differentiation of human ESC, regulatory T cells, treg, feeder-free culture, Serum-free culture

1. Introduction

Research into the function of dendritic cells (DCs) has identified them as cells of pivotal importance at the interface between innate and adaptive immunity. DCs integrate "danger signals" and down-modulatory cues in order to direct the adaptive immune response. They are considered unique in their ability to prime

Philip H. Schwartz and Robin L. Wesselschmidt (eds.), *Human Pluripotent Stem Cells: Methods and Protocols*, Methods in Molecular Biology, vol. 767, DOI 10.1007/978-1-61779-201-4_33, © Springer Science+Business Media, LLC 2011

T cells, emphasising their critical role in the immune system at the decision-making stage. Strategically, DCs have become key targets for the manipulation of immune responses, for example as vaccines delivering antigen in the desired context to T cells, to promote a robust anti-tumour response in the case of cancer immunotherapy, or to dampen immune responses in the context of transplantation and autoimmunity (1). A reliable source of human DCs would also enable further research into interactions between DCs and regulatory T cells (Tregs). Tregs regulate immune responses in vivo and an absence of functional Treg leads to the development of severe autoimmune conditions (2, 3). A better understanding of how DCs prime and expand Tregs could lead to the generation of therapeutic strategies for autoimmune diseases. Inhibition of Treg, on the other hand, can promote immune responses in cancer and tumour regression (4).

Conventional methods of obtaining human DCs involve culture of human monocytes separated from peripheral blood mononuclear cells (PBMCs) in medium containing granulocyte macrophage-colony stimulating factor (GM-CSF) and interleukin-4 (IL-4). A high level of inter-donor variation creates considerable problems in both in vitro experiments and clinical trials in which DCs are generated from monocytes following patient leukophoresis. Following the successful in vitro differentiation of DCs from mouse embryonic stem cells (mESCs) (5, 6), the potential of applying this protocol to human ESCs was investigated. DCs differentiated from hESCs offer consistency, expandability, and higher yields compared to monocyte-derived DCs (moDCs). Downstream clinical applications necessitate the generation of human embryonic stem cell-derived DCs (esDCs) using culture conditions that exclude the use of animal products. Recently, we have described the successful differentiation of DCs from hESCs under such stringent culture conditions (7). The protocol that we describe avoids a separate embryoid body (EB) generation step but allows EBs to form spontaneously in cultures on ultra-low attachment plates. The four growth factors bone morphogenetic protein-4 (BMP-4), vascular endothelial growth factor (VEGF), stem cell factor (SCF), and GM-CSF are vital in this method and their inclusion results in the emergence of copious numbers of cells with surface marker expression and morphology comparable to moDCs. hESC-derived DCs, differentiated under these culture conditions, efficiently process and present antigen and stimulate vigorous T-cell responses in vitro (7).

The availability of hESC-derived DCs may also have an important role in the field of regenerative medicine (8–11). Although studies performed in the mouse suggest that tissues derived from ESCs possess a degree of immune privilege, perhaps by virtue of their origin from blastocysts that are themselves immune privileged, tissues differentiated from ESCs with

more than a single minor histocompatibility mismatch with the recipient are promptly rejected by the immune system (12). This presents a significant barrier to progress, given that tissues differentiated from hESCs will inevitably be allogeneic to the recipients of cell replacement therapy. While immune suppression is conventionally applied under such circumstances, its protracted use is associated with severe side effects, the risks of which may exceed those of diseases amenable to this form of intervention. A promising alternative is, therefore, the administration of tolerogenic DCs differentiated from the same hESC line as the therapeutic cells, thereby coaxing the immune system into accepting the allograft in an antigen-specific manner (8–11). Here we describe protocols for the generation of DCs in culture from well-characterised hESC lines under conditions conducive to their subsequent clinical use.

2. Materials

2.1. Coating Tissue Culture Plates with Matrigel™ Matrix

1. Matrigel™ (phenol red-free, growth factor-reduced, BD Biosciences) thawed on ice.
2. Ice-cold, Knock-Out Dulbecco's Modified Eagle's Medium (KO-DMEM, Invitrogen).
3. 50 mL centrifuge tubes on ice.
4. Culture vessels to be coated with Matrigel™.

2.2. Culture of Human ES Cells and Routine Passage/Harvesting

1. hESC culture medium: XVIVO-10 supplemented with 80 ng/mL recombinant human basic fibroblast growth factor (rhb-FGF) (R&D Systems) and 0.5 ng/mL recombinant human transforming growth factor-β (rhTGF-β) (R&D Systems).
2. XVIVO-10 medium without gentamycin or phenol red (Lonza).
3. Collagenase IV (Invitrogen).
4. Dulbecco's Phosphate-Buffered Saline (DPBS).
5. Cell scrapers or 5-mL pipettes, depending on the culture vessels.
6. Culture vessels coated with Matrigel™.

2.3. Counting hESCs

1. Collagenase IV.
2. Dulbecco's Phosphate-Buffered Saline (DPBS).
3. TrypLE™ Express (Invitrogen) at room temperature.
4. Cell Culture Medium containing 10% FCS (any medium suitable for cell culture may be used).

2.4. Differentiation of ESCs into Dendritic Cells and Feeding of Differentiation Cultures

1. Culture medium for the differentiation of hESCs into DC consists of room temperature XVIVO-15 (with phenol red and gentamycin, Lonza), supplemented with the following:
   (a) 1 mM Sodium Pyruvate
   (b) 1× non-essential amino acids
   (c) 2 mM L-glutamine
   (d) 50 μM 2-mercaptoethanol
   (e) Recombinant human bone morphogenetic protein-4 (rhBMP-4) (R&D Systems) to give a final concentration of 50 ng/mL; 50 ng/mL recombinant human vascular endothelial growth factor (rhVEGF) (R&D Systems); 20 ng/mL recombinant human stem cell factor (rhSCF) (R&D Systems); and 50 ng/mL recombinant human granulocyte macrophage-colony stimulating factor (rhGM-CSF) (R&D Systems). On day 5, BMP-4 is removed from feeding medium, followed by VEGF on day 10 and SCF on day 15.
2. XVIVO-10 medium at room temperature.
3. Dulbecco's Phosphate-Buffered Saline (DPBS).
4. Collagenase IV.
5. Cell scrapers or 5-mL pipettes.

2.5. Differentiation of Monocytes into Immature DCs

1. Room temperature XVIVO-15 supplemented with 50 ng/mL rhGM-CSF and 100 ng/mL recombinant human interleukin-4 (rhIL-4) (R&D Systems).
2. Trypan blue.
3. Dulbecco's Phosphate-Buffered Saline (DPBS).
4. 70 μm cell strainers.

2.6. Maturation of DC Differentiated from hESCs

1. Recombinant human interferon-γ (rhIFN-γ) (R&D Systems).
2. Prostaglandin E_2 (PGE_2) (Sigma).
3. Recombinant human tumour necrosis factor-α (rhTNF-α) (R&D Systems).
4. Recombinant human interleukin-1β (rhIL-1β) (R&D Systems).
5. Recombinant human granulocyte macrophage-colony stimulating factor (rhGM-CSF) (R&D Systems).

3. Methods

It is important to note that, in our experience, it becomes increasingly difficult to direct cells along a haematopoietic route when hESCs have been cultured for more than 40 passages, although DCs have been generated from hESCs at higher passages.

3.1. Culture of hESCs

1. Human ESC lines can be cultured using different methods. We have adapted the culture of H1, H7, and H9 hESC lines to feeder- and serum-free conditions (13, 14) (see Fig. 1). Defined culture conditions are more reliable, have important implications in downstream clinical studies, facilitate scale-up of cultures, and avoid the time and expense required to maintain feeder cells.
2. hESCs are cultured in XVIVO-10 medium supplemented with 80 ng/mL bFGF and 0.5 ng/mL TGF-β on Matrigel™. XVIVO-10 medium is first warmed before adding bFGF and TGF-β (see Note 1).
3. A complete change of medium is performed daily except on the day immediately following either passage of the cells or thawing of the cells.

3.2. Coating Tissue Culture Plates with Matrigel™

1. Culture vessels coated with Matrigel™ need to be prepared in advance of passaging hESCs. To a 10 mL vial of phenol red-free, growth factor-reduced Matrigel™, add 10 mL ice-cold KO-DMEM. Keep Matrigel™ on ice and work quickly. Be careful not to introduce excess bubbles. The diluted Matrigel™ can be aliquoted and stored at –20°C.
2. It is important to avoid the generation of bubbles when handling Matrigel™ to prevent uneven coating of the tissue

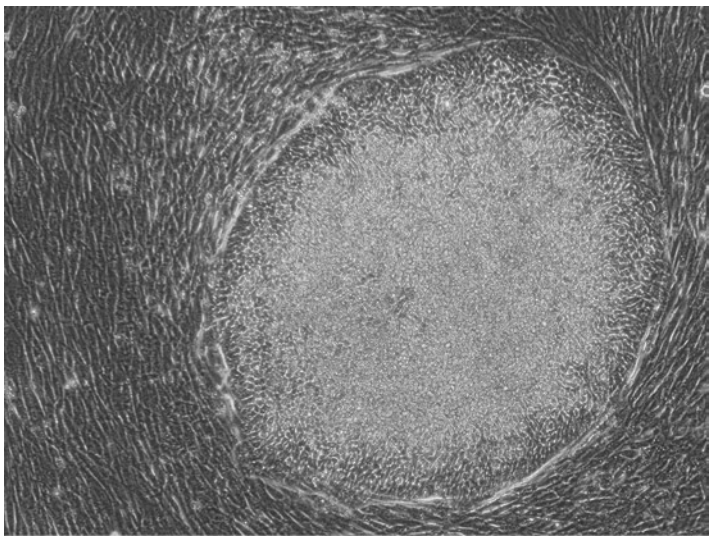

Fig. 1. A typical H1 hESC colony cultured under the feeder and serum-free conditions described in this chapter. Human ESCs have clear borders and at higher magnifications the cells can be seen to have a high nucleus to cytoplasm ratio. The fibroblast-like cells surrounding this colony are not feeder cells but have differentiated spontaneously from the hESCs. This has been reported before in the H1 line and the fibroblast-like cells in these cultures have been shown to support the undifferentiated growth of H1 hESCs (15). Objective magnification: 10×.

culture surface. If Matrigel™ starts to warm, it gels very quickly; for this reason it is best to work with Matrigel™ on ice and keep KO-DMEM ice-cold.

3. Thaw aliquots of Matrigel™ on ice. Transfer Matrigel™ from aliquots into sterile 50 mL centrifuge tubes. To each 1–3 mL of Matrigel™, add 5 mL ice-cold KO-DMEM using a 5-mL pipette and mix thoroughly. Top up with media to give a final volume of 15 mL per 1 mL of thawed Matrigel™ (includes initial volume of thawed Matrigel™ and volume of media used to mix). The final dilution of Matrigel™ is 1:30.
4. A six-well tissue culture plate can be coated with 1 mL per well of diluted Matrigel™, a 25 cm^2 flask with 3 mL and a 75 cm^2 flask with 10 mL. Calculate the volume required according to the surface area of other culture vessels using these volumes as a guide. Tap the sides of culture vessels to distribute the Matrigel™ evenly over the surface.
5. Culture vessels can be coated by leaving them at room temperature for at least 1 h or stored immediately at 4°C. Use parafilm to cover the caps of vented tissue culture flasks and seal tissue culture plates with micro-pore tape and wrap in cling-film to prevent evaporation. Discard culture vessels if Matrigel™ solution is no longer covering the entire surface.

3.3. Routine Passage of hESCs/Harvesting hESCs

1. In our hands, using xeno-free culture conditions, H1 hESCs can be routinely passaged every 4–6 days. hESCs are passaged as clusters of cells using collagenase IV to loosen the colonies from the tissue culture surface and scraping with a cell scraper or 5-mL pipette to dislodge and break up the colonies.
2. Using the same counting method as that used to estimate the number of hESCs (see Subheading 3.4), cells can be seeded at a density of approximately 1×10^5 cells per cm^2. In practice, once culture vessels reach 50% confluency, H1 hESCs can be passaged at a 1:5 dilution (see Note 2).
3. If hESCs are being expanded, the volume of culture medium required can be prepared in advance and the hESCs passaged using supplemented XVIVO-10 medium. If hESCs are being maintained then the volume of unsupplemented room temperature XVIVO-10 used to passage hESCs can be subtracted from the final volume required; TGF-β and bFGF can be added to this amount to give the correct final concentration for culture and used to top up the suspension of harvested hESC clusters.
4. Remove culture medium from hESC cultures and incubate the cells with pre-warmed collagenase at 37°C for the time period calculated while counting hESCs (see Subheading 3.4, step 2). Alternatively, if using a pre-determined dilution, observe cultures after 4–7 min: when the majority of stromal cells, which are differentiated hESCs, have lifted off the tissue culture

surface and hESC colonies are beginning to round at the edges, immediately remove collagenase. Wash gently with DPBS, being careful not to scrape off colonies with the pipette.

5. With XVIVO-10 medium, cover the tissue culture surface and gently scrape off hESC colonies. A 5-mL pipette can be used to scrape the surface of wells from a six-well plate or a cell scraper for flasks. It is critical to maintain clusters of hESC and prevent generation of a single-cell suspension that will result in loss of viability.
6. Using a 5-mL pipette, generate a suspension of hESC clusters.
7. Top up the suspension of hESC clusters to give the correct final volume and concentration of bFGF and TGF-β necessary for culture and pipette into Matrigel™-coated culture vessels (remove Matrigel™ solution immediately before adding hESCs; there is no need to rinse the culture vessel first).
8. Gently rock the culture vessel backwards and forwards and side-to-side to distribute the clusters of hESCs evenly over the tissue culture surface. Incubate at 37°C, 5% CO_2 in a humidified atmosphere (see Note 3).

3.4. Counting hESCs

1. In order to plate hESCs at the correct density for differentiation cultures, it is important to first count them.
2. Remove culture medium from one representative flask or well. Add warm collagenase IV so that the surface is covered and incubate at 37°C for 4–7 min. When the majority of fibroblast-like cells (see Fig. 2) have lifted off the surface and

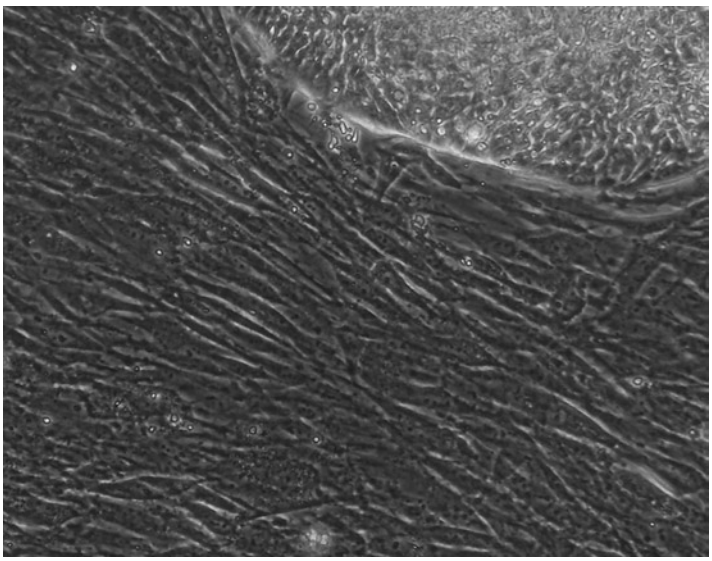

Fig. 2. Fibroblast-like cells surround colonies of H1 hESCs grown under the described feeder and serum-free conditions. Objective magnification: 20×.

colonies of hESC are beginning to round up at the edges, immediately remove collagenase, and gently wash with DPBS (see Subheading 3.3, step 4). Record the time of collagenase incubation as this will be required later.

3. Add room temperature TrypLE™ Express to cover the surface of the dish and incubate at 37°C (for example: 1 mL/well of six-well plate, 5 mL/25 cm² flask). After 5 min, shake the culture vessel in quick, sharp motions to dislodge all the hESC colonies. Pipette to create a single-cell suspension and wash any remaining cells from the tissue culture surface. Quickly add the cell suspension to the same volume of culture medium containing 10% FBS to give a 1:2 dilution and pipette further, if necessary, to achieve a single-cell suspension.
4. The cells are counted without trypan blue exclusion to monitor viability, as trypsin treatment and producing a single-cell suspension will generate significant cell death. It is not possible to distinguish definitively between ESC cells and stromal cells so each cell is counted and used as an estimate of hESC number. Removing the majority of stromal cells during collagenase treatment will alleviate this problem.
5. Trypsin-treated hESCs can be stained for Oct-4, SSEA-4, and Tra-1-60 and analysed by flow cytometry to monitor their pluripotency (see Chapter 16).

3.5. Directed Differentiation of hESCs into Dendritic Cells

1. After counting hESCs, it is possible to calculate how many wells to harvest for differentiation culture and the volume of medium required (see Note 4). Cells are plated at 3×10^6 cells per well of a six-well plate. Prepare XVIVO-15 medium supplemented with BMP-4, VEGF, SCF, and GM-CSF. These growth factors are successively removed from the differentiation culture leaving only GM-CSF in the final feed. Prepare sufficient medium to set up the differentiation culture and for the first feed (6 mL per well of a six-well plate). XVIVO-10, XVIVO-15 and DPBS should be at room temperature.
2. Harvest hESC cultures using collagenase IV treatment for the time period determined when counting hESCs as described in Subheading 3.4, step 2.
3. Wash the cells gently using DPBS as before. Add sufficient XVIVO-10 medium to cover the culture vessel surface and gently scrape off colonies, being careful not to create a single-cell suspension (see Subheading 3.3, step 5).
4. Wash the surface of culture vessels with XVIVO-10 medium to ensure all hESC colonies have been removed.
5. Pool colonies and allow them to settle for 10–20 min at the bottom of a sterile 50 mL conical tube.

6. Gently remove medium without disturbing the loose, settled colonies. Add some of the prepared differentiation medium; a similar volume or less than that used to harvest the colonies. Using a 5-mL pipette, create a suspension of cell clusters.
7. Calculate the volume of differentiation medium required to prepare the determined number of wells. Use 4 mL of medium per well of a six-well plate. Dilute cell clusters to give this final volume.
8. Due to the nature of differentiation, variation is often observed between cultures. For this reason, it is important to be as accurate as possible when pipetting the number of cells per well. Use a 10-mL pipette to prevent further breakdown of cell clusters and keep mixing the suspension as you pipette. It is best to aspirate and dispense 4 mL per well of the suspension, in order to distribute cell clusters as accurately as possible. Pipette into ultra-low attachment six-well plates.
9. Optional: Seal six-well plates with micro-pore tape to reduce potential contamination during long-term culture, and incubate in a humidified 37°C incubator at 5% CO_2.

3.6. Feeding Differentiation Cultures

1. Cultures of hESCs differentiating into DCs need to be fed every 2–3 days. This is particularly important during the early stages of differentiation. In practice, cells can be fed on Mondays, Wednesdays, and Fridays. Every 5 days, a growth factor is removed from the differentiation medium until only GM-CSF remains. Concentrations of the added growth factors are therefore effectively diluted throughout the course of the experiment. BMP-4 is removed from the differentiation culture first, followed by VEGF and then SCF.
2. For the first feed, warm XVIVO-15 containing BMP-4, VEGF, SCF, and GM-CSF and top-up wells with an extra 2 mL, giving a final volume of 6 mL per well. For successive feeds, gently replace 2 mL or 3 mL of culture medium using a 10-mL pipette, being careful not to remove cells or, at later stages of culture, the embryoid bodies (EBs) that spontaneously form (see Note 5). Replace with warm medium containing the appropriate growth factors.
3. Differentiation cultures contain significant debris during the early stages of differentiation due to high levels of cell death, which is normal during differentiation, and due to the inability of intermediates to adhere to the ultra-low attachment (ULA) surface. Around days 14–19 of culture, small, round, non-adherent haematopoietic cells should start to appear and later to accumulate (see Fig. 3). From day 19 onwards, "monocyte-like" cells should become apparent. These cells look morphologically like human blood monocytes and

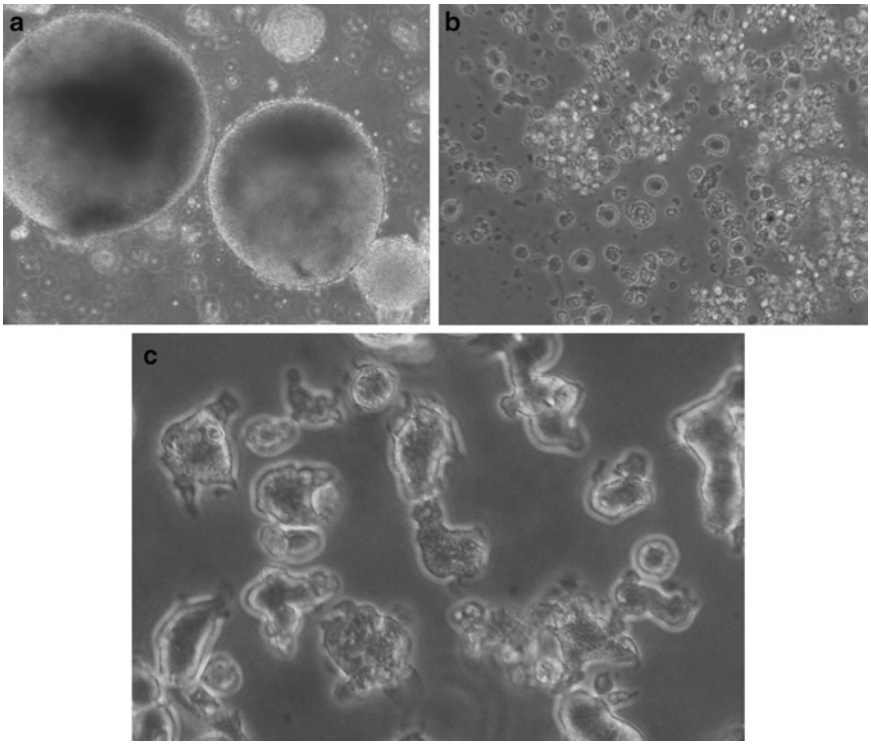

Fig. 3. Different stages of differentiation of H1 hESCs into DCs. (**a**) Embryoid bodies form early on in differentiation cultures. Objective magnification: 10×. (**b**) A significant amount of debris is seen in the initial stages of differentiation. In this image, haematopoietic cells are starting to appear. Objective magnification: 20× (**c**) Monocyte-like cells are evident from day 19 onwards. Objective magnification: 40×.

express high levels of CD14 as well as other myeloid markers, such as CD11b. Cells with monocytic morphology accumulate in number towards the later stages of culture.

4. Monocytes are usually ready to be harvested for DC differentiation between days 30–35. The cultures can be monitored for the appearance of monocytes and their percentages assessed using CD14 expression as determined by flow cytometry.

3.7. Differentiation of Monocytes into iDCs

1. Monocytes are harvested by gently pipetting cultures using a pipettor set on slow and a 10-mL pipette. The aim is to remove the monocytes, which are non-adherent, while leaving adherent macrophages in the culture plate (see Note 6). Transfer cells to 50-mL centrifuge tubes. EBs can be left to settle at the bottom of the tube (approximately 2–5 min) and removed using a pipette before cells also start to pellet.
2. Once the EBs have been removed, the cell suspension can be passed through a 70 μm cell strainer. This excludes any large clumps of cell debris (mostly created by EBs breaking up). Rinse the cell strainer with DPBS.

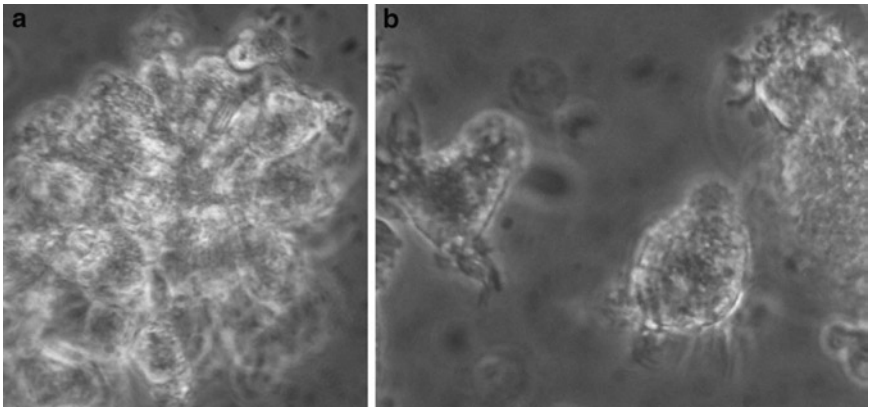

Fig. 4. Immature DCs differentiated from H1 hESCs. (**a**) Immature DCs frequently form tight clusters of cells. Typical veils of cytoplasm can be seen on DCs at the edge of the cluster. (**b**) Immature DC morphology showing veils of cytoplasm characteristic of DCs. Objective magnification: 40×.

3. Monocytes can then be washed by centrifuging at 200 × *g* for 5 min at 4°C. Discard the cell supernatant and resuspend cells in XVIVO-15 medium supplemented with 50 ng/mL GM-CSF and 100 ng/mL IL-4.
4. Pipette 1–1.5 × 10^6 monocytes per well of a six-well ULA plate and incubate for 6–8 days to differentiate monocytes into iDCs (see Note 7) (see Fig. 4).

3.8. Maturation of DCs from iDCs

iDCs differentiated from hESCs can be matured using a cocktail of cytokines including TNFα, IFNγ, PGE_2, and IL-1β. This can be made up in medium already supplemented with GM-CSF and IL-4 and added to cultures of iDCs for the last 48 h. Alternatively, IL-4 can be removed by washing the cells and replacing medium with the maturation cocktail (GM-CSF must be included throughout) (see Notes 8 and 9).

4. Notes

1. Human bFGF is extremely heat labile and therefore addition to pre-warmed medium instead of warming medium that has already been supplemented with bFGF prolongs its half-life in culture.
2. When cells are initially thawed and are therefore more fragile, dilutions of 1:3 or 1:4 can be used, depending on the appearance and recovery of the cells.
3. Under these culture conditions, fibroblast-like stromal cells, differentiated from hESCs are seen. Evidence has shown that these fibroblast-like cells support the pluripotent growth of

hESCs; however, it is necessary to prevent them from overwhelming the cultures.

4. It is best not to move the cultures for 48 h to allow hESCs to adhere to the Matrigel™-coated surface.
5. EBs become cystic and have a tendency to float, making it particularly difficult to avoid aspirating them with the pipette. Haematopoietic cells can often be found inside these cystic EBs when examining the cultures under a microscope, so they are highly likely to be a source of haematopoietic cells in the differentiation cultures. It is, therefore, important to avoid losing these EBs as much as possible when routinely feeding cultures. It is also likely that cells in the differentiation cultures are producing growth factors that may promote the differentiation process and therefore we try not to completely remove the conditioned medium. Also try not to excessively disturb the differentiating cells by only removing medium from the top of cultures.
6. DCs normally weakly adhere to tissue culture plastic whereas it is known that macrophages can stick to the surface of plates that have not been tissue culture treated (for example bacteriological petri dishes). This is because macrophages attempt to phagocytose the surface and therefore can usually only be removed at temperatures below 37°C (to remove macrophages, it is usual to add cold DPBS and incubate at 4°C for a short period of time). By pipetting the surface gently and using reagents at room temperature, it is possible to select for non-adherent or weakly adherent DCs while leaving behind any macrophages, which are stuck to the surface.
7. By counting large cells using a haemocytometer it is possible to distinguish between monocytes and precursor cells when establishing cultures.
8. Monocyte-derived DCs (moDCs) are commonly matured by treating them with the toll-like receptor 4 (TLR4) ligand lipopolysaccharide (LPS). (TLRs bind to pathogen-associated molecular patterns (PAMPs) that are conserved between microbes. TLRs are important in alerting the immune system to the presence of pathogens in the internal milieu.) However, DCs differentiated from H1 hESC do not express TLR4 and are, therefore, unable to respond to stimulation with LPS. For this reason, we have used a maturation cocktail of cytokines in order to mature DCs differentiated from hESC.
9. In our hands, hESC-derived DCs already produce IL-6; for this reason IL-6 is not included in the maturation cocktail.

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