Differentiation of Dendritic Cells from Human Induced Pluripotent Stem Cells

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Abstract

Dendritic cells (DCs) occupy a pivotal role in the immune system by determining the outcome of antigen presentation, either eliciting an aggressive immune response or imposing a state of immunological tolerance. As such, DCs serve as an obvious point of intervention for therapeutic purposes: while targeting DCs to enhance the response to tumour associated antigens (TAAs) remains the goal of cancer immunotherapy, reducing deleterious immune responses is critical for the treatment of autoimmunity and allograft rejection, or in situations in which therapeutic proteins may prove immunogenic upon administration. Functional heterogeneity among DCs suggests that distinct subsets may prove suitable for different applications. In terms of cancer vaccination, for example, much interest has focused on the recently-described subpopulation of $CD141^{+}XCR1^{+}$ DCs, capable of cross-presenting exogenous antigens in an MHC class I-restricted manner, since these may recruit cytotoxic T cells to the eradication of transformed cells. Furthermore, conventional DCs may be rendered pro-tolerogenic upon exposure to various pharmacological agents such as rapamycin and interleukin 10 (IL-10). For all such applications, a well-defined source of patient-derived DCs is highly desirable which is tractable for *in vitro* culture, expansion and manipulation. Given that for many disease states amenable to such strategies for therapeutic intervention, use of the patient's

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own peripheral blood is contraindicated, we have investigated the use of autologous induced pluripotent stem cells (iPSCs) as an alternative source of DCs. Here we describe our recently-published protocols for the derivation of DCs from human iPSCs that yield around 90–95% CD11c$^+$ DCs, of which up to 40% express the CD141$^+$XCR1$^+$ phenotype of the subset capable of antigen cross-presentation. These protocols therefore form a promising foundation for the future clinical application of DCs to immunotherapy.

Introduction

The outcome of the immune response determines the prognosis of many disease states, not merely infectious diseases but cancer, autoimmunity and end-stage organ failure, for which solid organ transplantation remains the treatment of choice. Whereas the eradication of transformed cells or infectious microorganisms requires strategies for vaccination, the induction of immunological tolerance is essential to restrain deleterious immune responses targeted against self components or tissue allografts.

Evidence accrued over several decades has implicated dendritic cells (DCs) as the fulcrum on which the balance between tolerance and immunity pivots. As professional antigen presenting cells, DCs are exquisitely adapted to the capture of exogenous foreign antigens and their processing for presentation to MHC class II-restricted helper T cells (Th cells). Furthermore, recent findings have identified in humans a minor subset of DCs in the peripheral blood and lymphoid tissues, defined by their co-expression of CD141 and XCR1 (Bachem et al. 2010; Crozat et al. 2010). This subset, equivalent to CD8α$^+$ DCs in the mouse, differs from conventional DCs in its ability to acquire exogenous antigens and process them for presentation on MHC class I (Sachamitr and Fairchild 2012). This process of cross-presentation is responsible for eliciting cell mediated immunity by recruiting cytotoxic T lymphocytes (CTL), essential for the eradication of viral infection and transformed cells expressing tumour associated antigens (TAAs). In order to exploit the unique properties of DCs for adoptive immunotherapy, many groups have made use of DCs differentiated from a patient's own peripheral blood monocytes. Indeed, such a source of autologous cells has been used extensively in Phase I clinical trials for cancer immunotherapy (Engell-Noerregaard et al. 2009) and as a therapeutic vaccine for chronic HIV-1 infection (Lu et al. 2003; Garcia and Routy 2011). Although such a source of DCs has yet to be used *in vivo* in regimens for the induction of antigen-specific tolerance, a variety of different pharmacological agents has been used to reduce the immunogenicity of moDCs, including rapamycin, 1α,25-dihydroxyvitamin D_3, dexamethasone, aspirin, IL-10 and TGF-β (Hackstein et al. 2001; Horibe et al. 2008; Unger et al. 2009; Boks et al. 2012). These modulators have been shown to generate DCs whose loss of co-stimulatory molecules, reduced T cell stimulatory capacity, increased expression of immunoinhibitory receptors and increased capacity for induction of regulatory T cells (Tregs), augurs well for applications requiring the establishment of systemic tolerance (Leishman et al. 2011).

Although clinical trials using monocyte-derived DCs (moDCs) for vaccination purposes have been shown to be inherently safe, the efficacy of the approach for securing tumour regression and eradication of HIV-1 has enjoyed only moderate success. Various factors have contributed to this disappointing outcome, including significant donor-to-donor variation in the yield and quality of moDCs which is frequently exacerbated by the very disease states such an approach seeks to address. Exposure to long-term chemotherapy for the treatment of malignancy, for example, has a detrimental impact on the capacity of the bone marrow to replenish populations of blood-borne monocytes. Furthermore, chronic HIV-1 infection has been associated with changes in the functional potential of monocytes and the DCs differentiated from them, while the long term culture of peripheral blood from HIV-1 infected individuals poses significant hazards. Most importantly, however, the limited capacity of moDCs to cross-present exogenously-acquired

antigen significantly restricts the nature of the responses they can elicit, and, in particular, the cell mediated immunity required for viral and tumour clearance.

Given the seminal findings of Takahashi and Yamanaka (2006) and Takahashi et al. (2007), and recent advances in cellular reprogramming that permit the generation of induced pluripotent stem cells (iPSCs) in a patient-specific manner, we reasoned that iPSCs might serve as an alternative source of DCs for immunotherapy. The generation of a pluripotent cell line capable of indefinite self renewal, would provide a potentially unlimited source of DCs on demand that could be subject to extensive quality control prior to administration to patients. Accordingly, we recently described protocols for the directed differentiation of DCs from human iPSCs which were able to generate not only conventional DCs indistinguishable from moDCs, but cells co-expressing CD141 and XCR1 with the functional capacity for antigen cross-presentation (Silk et al. 2012). Unlike their moDC counterparts, this subset was able to elicit primary T cell responses to the TAA, Melan A, among naïve $CD8^+$ CTL from the peripheral blood of a healthy donor. Here we describe how human iPSCs are cultured in the laboratory, expanded as required and guided by growth factors to differentiate down the hematopoietic lineage to ultimately give rise to DCs (ipDCs), a strategy first applied to the differentiation of human embryonic stem cells (hESCs) (Tseng et al. 2009). This protocol may be performed in an animal product-free manner for downstream clinical use and yields around 90–95% $CD11c^+$ ipDCs of which up to 40% constitute the $XCR1^+CD141^+$ population, capable of antigen cross-presentation (Bachem et al. 2010; Crozat et al. 2010).

Methods

Coating Tissue Culture Plates with Matrigel™

To provide cells with a means of attachment, phenol red-free, growth factor-reduced Matrigel™ (BD Biosciences, cat# 356231) is used as a basement matrix for iPSC culture. Matrigel™ may be stored as aliquots at −80 °C. We store Matrigel™ as a 1:2 dilution in ice-cold knockout Dulbecco's Modified Eagle's Medium (KO-DMEM) (Invitrogen, cat# 10829-018). When working with Matrigel™, care should be taken to keep it cold at all times, which involves pre-cooling of tubes and pipettes, in order to prevent the formation of aggregates. Matrigel™ aliquots are thawed on ice and further diluted 1:15 in ice-cold KO-DMEM to make up a final Matrigel™ dilution of 1:30. Care should be taken not to introduce bubbles while mixing the dilutions. The surface of individual wells of a 6-well tissue culture plate (Corning, cat# 3335) are covered with 1 ml/well of diluted Matrigel™ and incubated overnight at 4 °C or for 1 h at room temperature before use. It is essential that Matigel™ covers the entire surface of each well and that no bubbles have been introduced. When Matrigel™ covered plates are stored at 4 °C overnight or longer, they should be sealed with cling film or a similar product to prevent evaporation.

Before use, Matrigel™ is removed and the wells of tissue culture plate optionally washed with Dulbecco's Phosphate-Buffered Saline (DPBS) (Gibco, cat# 14190). This Matrigel™ can be reused at least once by pooling and replating 1 ml per well. Since Matrigel is an animal product, we have also shown that we are able to culture the iPSCs in a Matrigel™-free culture system using xeno-free Corning Synthemax tissue culture plates (Corning, cat# 3979XX1) and thereby proving the clinical applicability of our protocols.

Culture of Human iPSCs

Human iPSCs are cultured in complete mTeSR1 medium (Stemcell Technologies, cat# 05850) on 6-well plates coated with Matrigel™ as described above. mTeSR1 medium is left to warm up to room temperature before use. iPSCs are fed daily, except the day following passaging in order to allow more time for the cells to attach to the substrate.

Passage of Human iPSCs

Cells are passaged as clusters of about 0.5 mm diameter every 6–7 days. For passage, wells are washed with DPBS and treated with 1 ml of 1 mg/ml dispase (Stemcell Technologies, cat# 07913) per well for 4–5 min at 37 °C, according to the manufacturers' instructions. Once colonies start to round up at the edges, dispase is removed and cells are washed with DPBS. Cells are then scraped off into mTeSR1 medium containing 10 μM ROCK inhibitor (Reagents Direct, cat# 53-B85) using a cell scraper (Corning, cat# 3010) and gently broken into clusters by pipetting up and down using a 10 ml pipette. Cell clusters are diluted in complete mTeSR1 medium to passage at 1:12 and spread evenly on the Matrigel™-covered 6-well plates by gentle rocking. All cell cultures are incubated in a humidified incubator at 37 °C and 5% CO_2.

Differentiation of hiPSCs

Cell Counting

iPSC cultures are observed in order to identify a typical well which is representative of the confluence and number of cells in other wells: this well is then sacrificed in order to estimate, as accurately as possible, the average number of cells per well. The medium is removed and the wells washed with DPBS. TrypLE™ Express (Gibco, cat# 12604) equilibrated to room temperature is added at 1 ml per well and incubated for 5 min at 37 °C. To deactivate trypsin, medium containing serum is added. Colonies are dislodged and dispersed into a single cell suspension using a 5 ml pipette or 1 ml Gilson pipette. Cells are counted in a haemocytometer and the total number of cells available for differentiation determined by considering the total number of wells. The number of 6-well ultra-low attachment (ULA) plates (Corning, cat# 3471) required to set up an individual differentiation is calculated on the basis of these results, planning to plate 3×10^6 cells per well.

Differentiation Set Up

Medium should be prepared in advance. For the initial harvest, all growth factors are added to complete mTeSR1 medium, while for all following feeds the base medium is supplemented X-VIVO-15 (Lonza, cat# 04-744Q), thereby permitting the gradual transition of cells from mTeSR1 into X-VIVO-15 (Fig. 3.1) which improves cell survival.

To make up the initial differentiation medium, mTeSR1 is supplemented with 50 ng/ml of recombinant human bone morphogenetic protein-4 (BMP-4) (R&D Systems, cat# 314-BP/CF), 50 ng/ml of recombinant human vascular endothelial growth factor (VEGF) (PeproTech, cat# 100-20), 20 ng/ml of recombinant human stem cell factor (SCF) (R&D Systems, cat# 255-SC/CF) and 50 ng/ml of recombinant human granulocyte macrophage-colony stimulating factor (GM-CSF) (PeproTech, cat# 300-03).

In order to set up a differentiation culture, iPSCs are harvested in the same manner used for passaging, but in the absence of ROCK inhibitor. First, colonies are washed with DPBS, followed by dispase treatment. If handling a large number of wells simultaneously, dispase can be incubated at room temperature while working sequentially through the wells. Once colonies start to round up at the edges, dispase is removed and wells are washed with DPBS. Colonies are scraped into mTeSR1 medium, supplemented as described above. The harvested cells are then plated at 3×10^6 cells per well onto ULA plates in a total volume of 4 ml of supplemented mTeSR1.

Feeding Differentiations

After 2 days, the medium is topped up with 2 ml of X-VIVO-15 medium, supplemented with 1 mM sodium pyruvate (PAA Laboratories GmbH, cat# S11-003), 0.1 mM MEM non-essential amino acids (PAA Laboratories GmbH, cat# M11-003), 2 mM L-glutamine (PAA Laboratories GmbH, cat# M11-004), 5 μM 2-mercaptoethanol (Sigma, cat# M7522) and the growth factors BMP-4, VEGF, SCF and GM-CSF, as described above, to produce a total volume of 6 ml. All made up media are filter-sterilised before use. Subsequent feeding

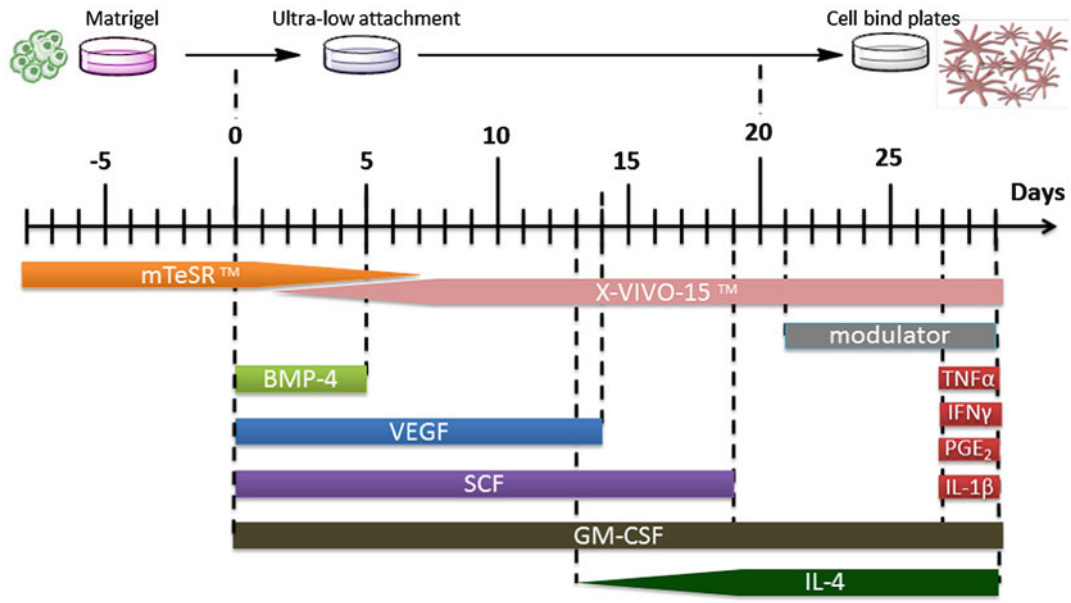

Fig. 3.1 Illustration of the protocol for differentiation of iPSCs into mature ipDCs. iPSCs are expanded in mTeSR1 medium according to the manufacturer's instructions (Stem Cell Technologies). In order to initiate differentiation, iPSCs are harvested as clusters and plated onto ultra-low attachment plates (ULA) in 4 ml per well of mTeSR1 medium supplemented with human recombinant bone morphogenetic protein-4 (BMP-4), vascular endothelial growth factor (VEGF), stem cell factor (SCF) and granulocyte macrophage-colony stimulating factor (GM-CSF). With successive feeds, mTeSR1 is gradually replaced with X-VIVO-15 as the base medium. Differentiation cultures are fed every 2–3 days with medium from which growth factors are progressively removed, as indicated above. On approximately day 13, upon the appearance of macrophage-like cells in cultures, IL-4 is added to medium starting at 25 ng/ml and is gradually increased to 100 ng/ml. Differentiations can be harvested from around day 21 onwards onto Cell Bind plates. From this point onwards, DCs may be treated with different pharmacological agents such as rapamycin or 1α,25-dihydroxy vitamin D_3. After 5 days of culture on Cell Bind plates, DCs can be matured by addition of a maturation cocktail containing GM-CSF, IL-4, IFNγ, TNFα, IL-1β and PGE_2. Cells are harvested and washed after 48 h of maturation and used for subsequent experiments

is performed every 2–3 days by replacing 2–3 ml of old medium with new supplemented X-VIVO-15 medium from which growth factors are successively removed, starting with BMP-4 at day 5, followed by VEGF at day 14 and SCF at day 19 of differentiation [19]. Care is taken not to remove any cells or cell clusters from differentiation cultures during the feeding process.

During the first few days of differentiation, cells cluster together to form embryoid body (EB)-like structures, as shown for days 3 and 7 in Fig. 3.2. Throughout the first few weeks, EBs increase in size and form cyst-like structures as shown at days 7, 10 and 11 (Fig. 3.2). Between day 10 and 20, EBs start to release a large number for cells, among which monocyte like cells can be observed (day 17). From about day 17 onwards, the first DCs, characterised by their cytoplasmic protrusions, appear in the differentiation cultures (Fig. 3.2, day 19). Once macrophage-like cells are observed (days 13–17), 25 ng/ml of IL-4 (Peprotech, cat# 200-04) is added, which is increased stepwise to 100 ng/ml.

On days 20–24, monocytes and DCs are harvested by gentle pipetting, leaving behind adherent macrophages in the culture dish. The cell suspension is passed through a 70 μm cell strainer (BD Falcon, cat# 352350) to remove cellular debris, washed with DPBS and plated at $1–1.5 \times 10^6$ monocytes per well of a 6-well Cell

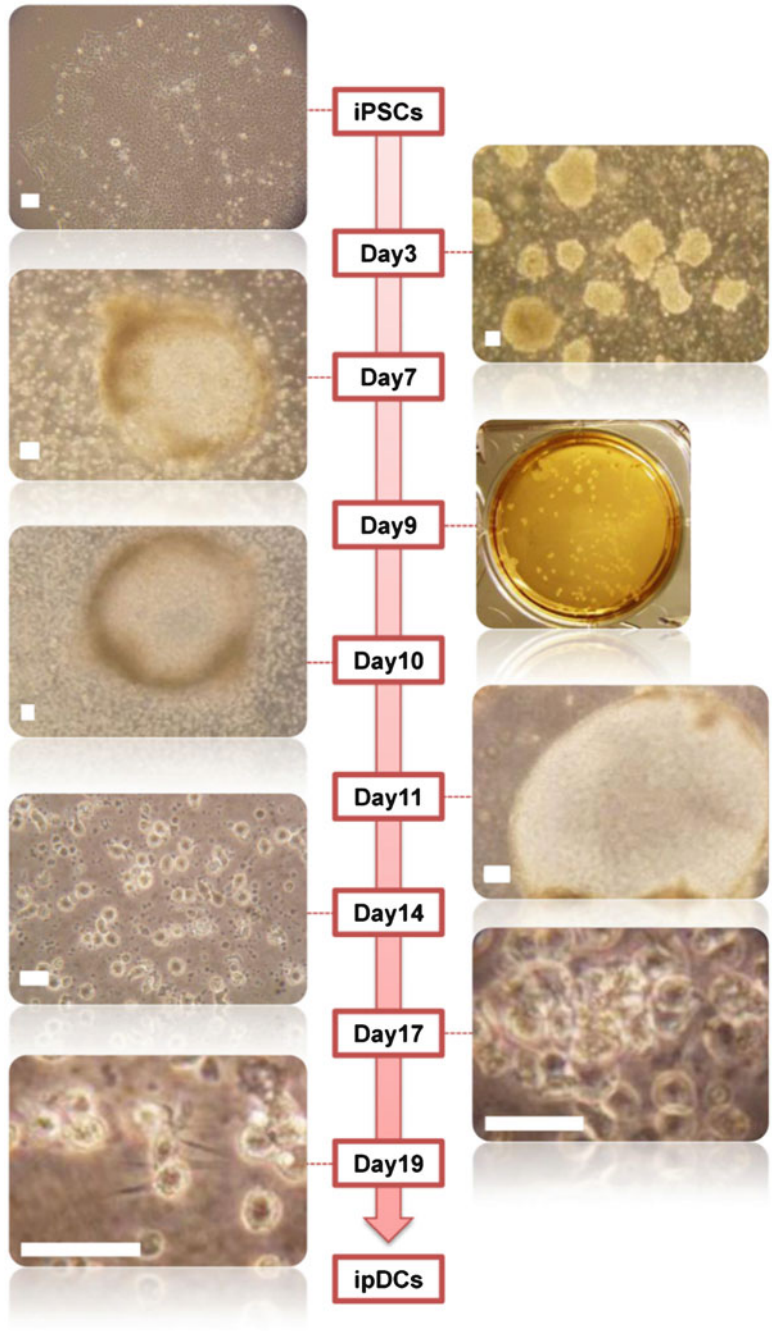

Fig. 3.2 Photomicrographs illustrating the differentiation process from iPSCs into DCs. During the first few days of differentiation, cells cluster together to form embryoid body (EB)-like structures, as shown for day 3. Throughout the first few weeks, EBs increase in size and form cyst-like structures as shown for days 7, 10 and 11. Between days 10 and 20, EBs start to release a large number for cells, among which monocyte-like cells can be observed (day 17 left). At around day 17, the first DCs, characterised by their cytoplasmic protrusions, can be observed in the differentiation cultures (day 17 and 19). *Scale bars* represent 100 μm

Bind plate (Corning, cat# 3335) in a total volume of 4 ml of X-VIVO-15 supplemented with 50 ng/ml GM-CSF and 100 ng/ml IL-4.

DC Maturation and Pharmacological Treatment

At this stage, monocytes differentiating into DCs may be treated with pharmacological agents in order to generate functionally-modulated DCs. A variety of agents have been used to generate DCs with tolerogenic properties. In the past, our laboratory has focused on the use of rapamycin for the generation of modulated DCs derived from human embryonic stem cells (hESCs). The protocol involves the treatment of harvested ESC-derived monocytes with 5–10 ng/ml of rapamycin on day 3 prior to DC maturation on day 5.

For maturation, DCs are treated for 48 h using a maturation cocktail consisting of 50 ng/ml of GM-CSF, 100 ng/m IL-4, 20 ng/ml IFNγ (R&D Systems, cat# 285-IF/CF), 50 ng/ml TNFα (R&D Systems, cat# 210-TA/CF), 10 ng/ml of IL-1β (R&D Systems, cat# 201-LB/CF) and 1 μg/ml PGE_2 (Sigma, cat# P6532). This cocktail is made up as a 9× concentration in X-VIVO-15 and added as 500 μl per well. On day 7, DCs are harvested by gentle pipetting, and are washed prior to their use in experiments to prevent any carry-over of pharmacological agents or maturation cytokines. Maturation can be assessed by up-regulation of MHC class II and co-stimulatory molecules, such as CD86 (Fig. 3.3).

Discussion

While the use of DCs for immunotherapeutic intervention has been shown, in principle, to offer fine control over the outcome of pathogenic immune responses, limitations associated with the source of the DCs most commonly employed, have reduced the efficacy of such an approach. Here we have described protocols for the differentiation of DCs from patient-specific iPSCs, conventionally derived from a small punch biopsy taken from the skin. These so-called ipDCs contain a subpopulation defined by their co-expression of CD141 and XCR1, recently demonstrated to harbour the capacity for antigen cross-presentation, a property highly desirable for eliciting CTL responses to transformed or virally-infected cells. Such a novel source of DCs may, therefore, find application in situations in which the patient's own peripheral blood is inadequate as a source of monocytes, from which DCs are traditionally derived, such as the impact of long-term exposure to chemotherapy or chronic HIV-1 infection.

While the cross-presentation capacity of ipDC augurs well for their use in regimens for vaccination, our protocols are equally amenable to the pharmacological manipulation of ipDCs. Agents such as rapamycin, 1α,25 dihydroxyvitamin D_3 and IL-10 have been widely shown to enhance the tolerogenicity of moDC (Leishman et al. 2011), and have proven equally useful for modulating the function of hESC-derived DCs in our hands (Silk et al. 2011). The potential to reduce DC immunogenicity in favour of a tolerogenic phenotype may prove valuable in a variety of settings in which the immune response is itself pathogenic, rather than protective. Whereas the high precursor frequency of alloreactive T cells is likely to prove too great a barrier to the induction of transplantation tolerance using DCs alone, the effective treatment of various congenital conditions would be greatly facilitated by the induction of tolerance to single defined proteins whose therapeutic administration may overcome a patient's own endogenous deficiency. This scenario is most commonly encountered in the case of the lysosomal storage diseases (LSDs) in which the absence of a single enzyme from the lysosomal pathway results in the accumulation of waste products and consequent toxicity among critical cell types. Although LSDs, such as Pompe disease, may be treated by replacement of the missing enzyme, the induction of a robust immune response leading to clearance of the enzyme from the patient's circulation, prevents its efficient targeting to tissues (Banugaria et al. 2011, 2012). Whereas the use of DCs to establish immunological tolerance to individual enzymes in advance of their administration may prove

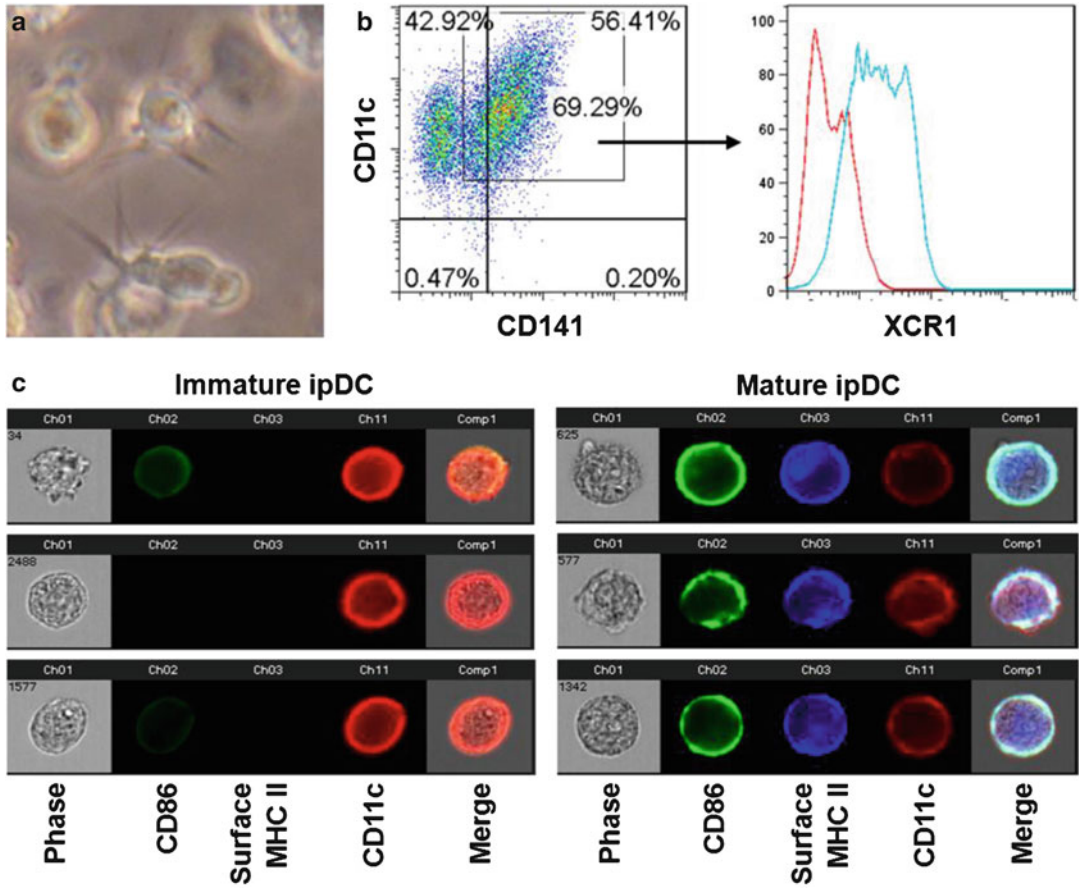

Fig. 3.3 (**a**) ipDCs featuring very long protrusions and dendrites. The generation of cells displaying this morphology coincides with the first appearance in cultures of cross-presenting DCs. (**b**) Differentiation cultures give rise to around 70% $CD11c^{+}CD141^{+}$ DCs amongst which cells express the cross-presentation marker XCR1 (blue histogram) which is a chemokine receptor for XCL1 (the red histogram denotes background staining with an isotype-matched control antibody) (Figure courtesy of Dr. Kate Silk). (**c**) iPSC were differentiated into ipDCs for a total of 30 days. On day 28, some cells were treated with GM-CSF, IL-4, IFNγ, TNFα, IL-1β and PGE_2 to induce their maturation while some were left untreated (immature). Image stream analysis of the expression of CD11c, MHC class II and CD86 demonstrates efficient maturation as immature cells express low or no CD86 and MHC class II, with the exception of a very low percentage of spontaneously matured cells, while matured ipDCs up-regulate both CD86 and MHC class II

feasible, the most severe cases of Pompe disease manifest in infanthood when the availability of peripheral blood monocytes is extremely restricted: under such circumstances, the establishment of an iPSC line from such individuals and its use as a potentially-unlimited source of DCs, offers an attractive alternative which is only minimally invasive.

Quite apart from being endowed with the capacity for antigen cross-presentation, the use of iPSCs as a source of autologous DCs has numerous advantages over conventional moDCs. As a cell line capable of indefinite self-renewal, iPSCs provide a potentially unlimited supply of DCs for repeated administration which are not prone to the same batch-to-batch variation observed among moDC and may be subject to rigorous quality control prior to use. Furthermore, their derivation using protocols that are free of animal products, makes them fully compatible

with downstream clinical use. Given that iPSCs are relatively tractable candidates for genetic modification, unlike fully differentiated DCs, the establishment of an iPSC line may enable the modulation of DC function through the forced expression of co-stimulatory or inhibitory molecules, thereby tailoring their activities to the individual needs of the patient. Once current regulatory hurdles surrounding the *in vivo* administration of cell types differentiated from iPSCs have been resolved, the use of autologous ipDCs may prove efficacious in the treatment of a broad spectrum of disease states.

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