REVIEWS

Stem cell-based dendritic cell vaccine development: A review with emphasis on lung cancer treatment

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Abstract

Background: Dendritic cell (DC) vaccines have significant potential in cancer immunotherapy. While autologous DCs can be derived from bone marrow, umbilical cord, and peripheral blood, monocyte-derived DC vaccines are most widely tested in clinical trials. However, producing autologous DC vaccines is labor intensive, has large variations among donors, and may not be feasible for patients with impaired cell function or requiring multiple vaccinations. Human pluripotent stem cells (hPSCs) have unlimited expansion potential while maintaining their pluripotency. They are being tested as a novel cell source to derive DCs for clinical application. Lung cancer is the leading cause for all cancer-related mortality. Efficient treatment of lung cancer by DC vaccines could offer great benefits in cancer immunotherapy. This review uses lung cancer as a case study to discuss the application of DC vaccines.

Results: DC derivation from hPSCs has been demonstrated with high purity and comparable in vitro functions to autologous DCs derived from monocytes. The differentiation can be achieved either by co-culturing hPSCs with OP9 stromal cells or by the formation of three-dimensional embryoid bodies. As the scalable culture system is critical for hPSC-derived DC production, progress in the scalable culture systems for other hPSC-derived cells is reviewed and the use of relevant systems for hPSC-derived DCs is proposed.

Conclusions: hPSCs provide a new source for DC production and have significant implication in DC-based cancer immunotherapy. Their use in clinical trials requires refinement of the culture expansion and differentiation protocols. Development of scalable culture systems is crucial in truly harnessing the potential of the hPSC-based DC immunotherapy in cancer treatment.

Key words

Stem cell, Cancer, Dendritic cell vaccines

1 Introduction

Dendritic cells (DCs) are the antigen-presenting cells inside the human body that stimulate the immune response. For therapeutic purposes, autologous DCs have been derived from human bone marrow, umbilical cord blood, or peripheral blood-derived monocytes, and have been tested as vaccines for cancer treatment. Ex vivo generated DCs loaded with tumor-specific antigens are capable of stimulating powerful antitumor immune responses, and over 150 clinical trials have been reported to explore the safety and efficacy of the DC-based vaccine for various cancer treatments [1,2]. Among

various cancers, lung cancer is the leading cause of mortality for all cancer patients and the long-term survival rate is very poor [3]. As such, application of DC vaccine in treatment of lung cancer patients has attracted significant interest [4-7].

Despite the initial excitement, autologous DC vaccines have several limitations. First, the preparation process is laborious and intensive, and subjects to big donor-to-donor variations. In addition, the cell number acquired from donor is often limited and not suitable for multiple vaccinations and the immediate treatment. While ex vivo cell expansion has been proposed, it is often associated with prohibitive costs for cell processing. For some patients, autologous DC functions might be impaired due to pathology or prior treatment, making autologous DC therapy unfeasible. These limitations have motivated the quest for an alternative DC source. Recently, DC derivation from human pluripotent stem cells (hPSCs) has been successfully demonstrated in multiple studies and is gaining increasing attention in DC-based therapy.

Human pluripotent stem cells, including human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs), can give rise to any cell types from three-germ layers. They are capable of expanding indefinitely in culture without losing their pluripotency [8,9]. In theory, hPSCs can be used to produce unlimited numbers of somatic cells, which makes them an attractive cell source for large-scale production of therapeutic cells. Along with other types of somatic cells such as neuronal cells, cardiomyocyes, β-islet cells, hepatocytes, chondrocytes, and osteoblasts [10], several protocols have been developed to generate DCs from hPSCs [11-15]. These studies produced DCs with expression of DC specific markers, including major histocompatibility complex (MHC)-I, MHC-II, CD40, CD80, CD83, and CD86. The in vitro function of hPSC-based DCs was also demonstrated to be comparable with autologous DCs [14,16]. Similar to the PBMC-derived DCs, hPSC-derived DCs stimulated significant allogeneic T cell proliferation and interferon (IFN)-γ production from antigen specific CD8 + T cells, suggesting their functional potential as an alternative to autologous DCs. As hPSC-based DCs move toward clinical applications, major tasks are to further improve the protocols for efficient DC differentiation from hPSCs and to design scalable culture system that truly harnesses hPSC's potential for the clinical-scale production of the "off-the-shelf" DC vaccines.

This review summarizes the progress of DC immunotherapy and its application to lung cancer treatment. As hPSCs have emerged as an important source for DC immunotherapy, the advancements in DC derivation from hPSCs are discussed. Finally, challenges in the scalable production of DCs from hPSCs for clinical application are discussed with a focus on the impact of process parameters on DC differentiation.

2 Characteristics of dendritic cells

Dendritic cells are the most potent antigen presenting cells (APCs) and the only cells that have the ability to induce a primary immune response in resting naive T lymphocytes. DCs were first described by Ralph Steinman in 1973 when observing cells from mouse spleen that adhered to glass or plastic surfaces. In addition to mononuclear phagocytes, granulocytes, and lymphocytes, a subpopulation of cells with striking dendritic shape was noticed [17]. These loosely adherent cells with striking dendritic morphology possess a group of markers to define their phenotype, including (but not limited to) CD45, CD40, CD86, CD83, CD80, MHC-I, and MHC-II [18,19]. However, the capability of studying the mechanism of DC interactions with the immune system was not fully realized until a sufficient number of DCs was generated ex vivo from hematopoietic progenitors, first from bone marrow using granulocyte-macrophage colony stimulating factor (GM-CSF) [20] and later from human blood monocytes cultured in the presence of GM-CSF and interleukin (IL)-4 [21]. Currently CD34 + bone marrow stem cells are thought to be the main source of DCs: DC derivation directly from CD34 + myeloid cells, CD34 + lymphoid cells, and from peripheral blood monocytes originated from CD34 + myeloid cells has been demonstrated [22]. However, DCs were also found in other organs such as skin and liver [23].

DCs from different sources share some phenotype markers but they are often identified as DCs by the following common features. 1) Precursor DCs seeded via the bloodstream to the tissues that can give rise to immature DCs. 2) Immature DCs have the ability to take up antigen by receptor or non-receptor mediated mechanisms. They can degrade antigens in

endocytic vesicles to produce antigenic peptides capable of binding to MHC Class II. 3) In response to danger signals such as inflammatory cytokines, DCs mature and migrate to lymphoid organs where they interact with antigen specific T cells to initiate immune responses. 4) Immature DCs have distinct chemokine receptors, which regulate their traffic into tissue sites in response to inflammatory chemokines. 5) Mature DCs express a high density of MHC class II molecules complexed with antigen that can be recognized by T cells. They also express costimulatory molecules to stimulate T cell proliferation. 6) Mature DCs can produce IL-12 and initiate T-helper type 1 (Th1) responses [22,24]. Among DCs from different sources, monocyte-derived DCs have been the most widely used DCs in clinical trials due to their availability and the ease of isolation and derivation [25].

The unique immune-regulatory functions of DCs make them especially attractive in cancer immunotherapy. Tumor cells are thought to have the ability to evade immune-surveillance and were believed to be inadequate to prime immune responses. However, tumor-associated antigens when presented by DCs have been shown to induce strong immune responses [26,27]. Use of DCs in immunotherapy was initially performed by pulsing the bone marrow-derived DCs with tumor peptide in ex vivo cultures [28]. Animals vaccinated in this study were resistant to challenge with tumor cells expressing the relevant tumor antigen. Motivated by the observation, DCs pulsed with tumor antigens were immediately used for treatment of patients with B-cell lymphoma and melanoma thereafter [29,30]. All patients developed measurable antitumor cellular immune responses. Later, DC vaccines were tested in almost all types of cancers with melanoma as the most frequently treated cancer followed by prostate cancer, renal cell carcinoma, breast cancer along with others including lung cancer [1]. Details in clinical trials grouped by different cancer types can be found elsewhere [31]. In 2010, FDA approved the first dendritic cell-based vaccine Sipuleucel-T (Provenge®) to treat prostate cancer [32]. The list of US trials using DCs can be found at http://www.dendritic.info/currentUsTrials.html. However, it should be noted that comparison of results from different clinical trials is complicated by nonstandard DC and antigen preparations.

The potency of DCs is affected by maturation stage and antigens presented, with the mature DCs having better migration ability and stimulatory effect than immature DCs. The stimulatory ability of monocyte-derived DC vaccines is affected by different maturation protocols [25]. There are three major categories of maturation protocols based on the level of activated canonical pathways: lipopolysaccharide (LPS), CD40 ligand/tumor necrosis factor-α (TNF-α), and IFN dependent maturation. To create a physiological environment for DC maturation, a balanced cocktail of maturation agents should be most representative of various inflammatory states. Such cocktail usually contains IL-1β, TNF-α, IL-6, prostaglandin E2 (PGE2), and IFN-γ. DCs directly injected into tumors have limited immune stimulatory activity unless they are loaded with antigen. Antigen-presentation could be affected by maturation stimulus, but comparison of different protocols is lacking. It also could be affected by antigen preparation method and antigen type. Different antigen presentation methods including irradiation, boiling, or freeze-thaw lysis for DC priming were compared and only DCs loaded with irradiated tumor vaccines stimulated IFN-γ production [33]. Defined antigens such as peptides and undefined antigens such as tumor lysates or apoptotic bodies were loaded to DCs by various methods. Such methods include mature DC pulsing, electroporation of mRNA, proteins, and cell lysates, and adenovirus/retrovirus mediated delivery [23,34-37]. Although the DC loading method is critical for eliciting desirable T-cell responses, there is no gold standard available.

3 Generation of autologous DC vaccines

Autologous DC vaccines require the generation of DCs for each individual patient. Thus, the technology of ex vivo culture of DCs is crucial for the application of DCs in immunotherapy. Following isolation and ex vivo expansion, DCs can be loaded with tumor antigen and be used to vaccinate patients. In current practice, DCs have been generated from proliferating CD34 + cells by GM-CSF and TNF-α, or non-proliferating CD14 + cells (monocytes) by GM-CSF and IL-4. In most clinical applications, DCs are generated from peripheral blood mononuclear cell (PBMC)-derived monocytes since DCs are present in high numbers. Generation of PBMC-derived DCs involves the purification of monocytes from

PBMC, driving monocytes into immature DCs for 5-6 days, and differentiating immature DCs into mature DCs for 24-48 hours. This process is very labor intensive and subject to great variability among donors [38].

To improve the efficiency of DC derivation from monocytes, various strategies have been applied for monocyte purification and ex vivo culture. Monocytes can be enriched by selection via adherence to plastic, density gradient separation, positive and negative selection, and elutriation [38]. Methods of monocyte isolation have no major implications on DC phenotype and function, but impact the feasibility of an integrated closed system. Among different monocyte enrichment methods, elutriation has been recently developed as a cost-effective closed system and is preferred for clinical application [39,40]. During the differentiation of monocytes to immature and mature DCs, DC functions were found to be affected by culture parameters including human plasma proteins, culture medium, growth factors, and granulocyte content of the starting cells from the patient. For example, plasma protein fibrinogen had DC-maturation effects comparable to poly-I: C, TNF-α, and PGE2, but it failed to induce IL-12 production [41]. The DC culture medium appears to have a strong influence on the production of relevant T cell differentiating cytokines. Defined serum-free medium was tested using the commercially available medium AIM-V and XVIVO-15 [42]. Marker expression, migration and stimulatory ability are similar to DCs generated from serum-containing medium. However, IL-12p70 production was lower in serum-free culture. Growth factor type I interferon was used to generate DCs and was compared with growth factors GM-CSF and IL-4. DC differentiation appeared to be faster with type I interferon, but decreased IL-12p70 secretion and increased IFN-α were observed [38]. When monocytes are purified from PBMC, granulocytes could be purified, too. The presence of high granulocyte percentage of more than 16% gradually altered DC quality by inducing significantly lower migratory capacity of the DCs and lower expression levels of CD80, CD40, and CD86 [43]. All these studies indicate that optimal culture parameters are critical to ensure DC generation with appropriate function.

Generation of clinical scale DCs requires scalable culture systems that meet current Good Manufacturing Practice (cGMP) Guidelines. A closed system is preferred to avoid cross contamination during multiple open steps and reduce the cost of maintaining the facility. Quality control should be applied to develop standardized criteria including cell viability, purity, and stimulation activity [44]. To generate mature DCs in large-scale under labor-and cost- effective conditions, roller bottles were tested as culture vessels to replace tissue culture flasks [45]. The roller bottles generated similar numbers of mature DCs from adherent PBMC with fewer culture vessels compared to traditional static flasks. Cell Factories™ was also used for large scale generation of dendritic cells in a closed system [46]. No difference in purity, stimulatory capacity, or IL-12 secretion was observed compared to tissue culture flasks. Another closed system for large scale production of DCs was developed using the Elutra™ cell separation system and cell culture in Teflon bags [39,47]. DCs cultured in bags demonstrated similar phenotype and stimulatory properties compared to DCs cultured in flasks [48]. The bags were widely used since they were easy to connect with cell separation systems and enable a complete closed system production [40,49]. In general, these systems are adequate to produce mature DCs at an order of 10^8 for each batch of processing, but there is difficulty to increase the number further due to the limited number of autologous monocytes.

4 Lung cancer treatments

Cancer immunotherapy using DCs has been actively investigated [1,31,36,37] and is an immediate area that can benefit from the advancement in DC immunotherapy. Lung cancer is the most commonly diagnosed malignancy and the leading cause of mortality among all types of cancers. Each year, more than 1 million deaths are contributed by lung cancer worldwide, among which non-small cell lung cancer (NSCLC) accounts for 85% of cases [7]. Although various treatment strategies have been developed in recent years, the long-term survival rate is very low. Surgical resection remains the mainstay of treatment, but the relapse rate is greater than 40%. Other treatments such as chemotherapy and/or localized

irradiation offer only modest improvements in survival. Two year survival rates for stage IIIB and IV NSCLC are 10.8% and 5.4%, whereas five year survival rates are 3.9% and 1.3%, respectively [3]. Thus, there is an apparent urgency for the development of novel treatments for lung cancer patients.

Current vaccine approaches for lung cancer treatment focus on coupling immunogenic adjuvant agents to tumor antigens. When patients are vaccinated, adjuvant agents enhance the antigen presenting cell response to the vaccine, which in turn activates tumor specific T cells. However, most cancer cells can evade the immune system by down-regulation of the expression of target antigens, MHC, costimulatory molecules, or immune cytokines. Lung cancer cells can produce immunosuppressive molecules including transforming growth factor (TGF)-β, PGE2, IL-10, and cyclooxygenase 2 that can affect the presentation of DCs and cytotoxic T lymphocyte effector cells. Several types of lung cancer vaccines are developed based on lung tumor-associated antigens. Allogeneic tumor cell vaccine Lucanix is a mixture of 4 allogeneic NSCLC cell lines genetically modified to suppress TGF-β. Another type is autologous tumor cell vaccine composed of whole tumor cells genetically modified to secrete GM-CSF. Different from allogeneic and autologous tumor cell vaccines, protein-specific vaccines consist only of lung cancer-specific proteins including melanoma-associated antigen a3, epidermal growth factor (EGF), or Mucin 1 [3,50,51]. However, targeting tumor antigens alone or with traditional adjuvants may not elicit a strong enough immune response. It has been proposed that using DCs as a vaccine adjuvant may be an effective way to stimulate antitumor immunity and overcome tolerance.

While DC trials in patients with NSCLC are few in number compared to other types of cancers, recent exciting clinical results in NSCLC patients immunized with autologous tumor cell vaccines support the rationale for further investigation of immunotherapy in patients with NSCLC [4]. Dendritic cells have been loaded with lung cancer specific antigen and stimulate cytotoxic T cell responses [52]. One such trial was performed on patients with stage IA to IIIB NSCLC treated with surgery, chemoradiation, or multimodality therapy [5]. Among the sixteen patients, eleven showed tumor antigen-independent or antigen specific responses. In another trial, a new dendritic cell vaccine into which tumor lysate was loaded by electroporation was used to treat patients with advanced stage III or IV NSCLC [6]. In 5 of 9 patients, the vaccine increased IFN-γ production by CD8 + cells. In yet another trial, five HLA-A2 patients with inoperable stage III or IV NSCLC were treated with DC vaccines pulsed with various tumor specific peptides. Two patients had significant immunologic reaction after the first dose. Two had boosted response after a second dose and one had a mixed response [7]. These promising results encourage future trials with multiple doses in patients with early lung cancer.

A major obstacle to the widespread application of autologous DC vaccines for treating NSCLC is the scale for DC production. For NSCLC clinical trials, each dose of DC vaccine ranged from 5-10 × 10^7 cells. Thus, each batch of autologous DC vaccine could only provide one single dose and multiple production runs would be required if multiple doses are planned. These tedious processes make autologous DC vaccines very costly. For example, the cost of Provenge® for each patient is estimated at about $93,000 (www.dendreon.com). In addition, for some patients, the functions of DCs may be impaired due to prior treatment and autologous DC vaccine is not feasible. Also, some patients at advance stage may need DC vaccine immediately, and waiting for cell production is not an option.

Progress in human pluripotent stem cells (hPSCs) has provided a new cell source that can potentially overcome these drawbacks. Figure 1 compares the production scheme for autologous DC vaccines and hPSC-based DC vaccines. For autologous DC vaccines, three production runs are required to treat each patient. In contrast, one production run of hPSC-based DC vaccine could supply sufficient cells for 30 patients receiving the same dosage. In addition, the "off-the-shelf" feature can reduce cost and provide vaccines for the patients with impaired DC function or for those who need immediate treatment. DCs differentiated from hPSCs thus provide a new platform for cancer immunotherapy.
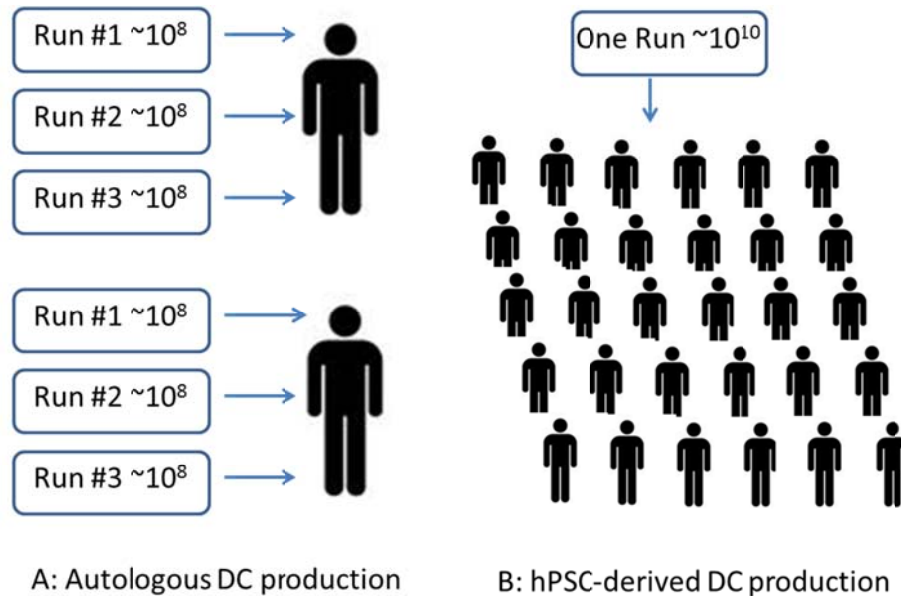
Figure 1. Comparison of production scheme for autologous DC vaccines and hPSC-derived DC vaccines. Each run of autologous DC production consists of monocyte purification and ex vivo culture to produce one lot of DC vaccine. Run #2 and Run #3 are the repeat process of Run #1. Each run of hPSC-derived DC production consists of hPSC expansion and DC differentiation to produce one lot of DC vaccine

5 Dendritic cell derivations from hPSCs

Human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs) were initially derived in 1998 and 2007, respectively [8, 9]. They have unlimited ability of self-renewal and pluripotency to differentiate into any cell type in the body, providing unlimited number of somatic cells for cell therapy, drug discovery, and cancer therapy. Compared to hESCs, hiPSCs can be derived from individual patients by reprogramming factors and provide personalized medicine as well as the "off the shelf" allogeneic medicine. Human ESC-derived oligodendrocyte progenitor cells entered Phase I clinical trials in 2010 (www.geron.com). Human ESC-derived cardiomyocytes (Cytiva™ cardiomyocyte, www.gelifesciences.com) and hiPSC-derived cardiomyocytes (iCell® cardiomyocyte, www.cellulardynamics.com) are commercially available for drug testing. Various blood components have been generated from hPSCs, including red blood cells, platelets, leukocytes, T cells, and B cells [53,54]. Dendritic cells have been derived from hESCs since 2004 [11]. Recently, they were also derived from hiPSCs [15]. Table 1 summarizes different methods of differentiating hPSCs into DCs. In general, there are two types of methods: co-culturing hPSCs with the OP9 stromal cell line and the formation of three-dimensional embryoid body (EB) in suspension culture [55-57].

DCs were derived from hESCs by co-culturing with the OP9 cell line, which was established from M-CSF-deficient mouse bone marrow stromal cells, to facilitate initial hematopoietic differentiation. In this process, hESCs were plated onto an OP9 feeder cell layer in the presence of fetal bovine serum (FBS) [12]. Then the dissociated myeloid cells were grown in suspension in the presence of GM-CSF and FBS. The cells were then grown in serum-free medium in the presence of GM-CSF and IL-4. The resulting DCs were immature and lacked CD83 expression. Another OP9 co-culture system had some minor differences [58]. After the initial co-culture of hESCs with OP9 stromal cells, the isolated non-adherent cells were seeded onto fresh OP9 layer in the presence of GM-CSF and M-CSF instead of putting them in suspension. The further differentiation to DCs was driven by GM-CSF and IL-4. About 50% of cells with DC markers were achieved. This protocol has been adapted to differentiate mouse ESCs into DCs [59].

For hESC-DC based on EB formation, the first study generated EBs in suspension with the differentiation induced by FBS [11]. This method was adapted from DC differentiation from mouse ESCs by EB formation [60,61]. After 10-20 days, EBs were transferred to tissue culture plates and cultured in the presence of growth factors such as stem cell factor (SCF), Flt3 ligand, and thrombopoietin (TPO). To further commit the hematopeitic stem cells to DC progenitors, IL-3, GM-CSF, and IL-4 were added. About 25% DCs indicated by expression of MHC-II and CD86 were observed. A similar method was tested in the presence of bone morphogenetic protein (BMP-4) during EB stage in the presence of FBS [13]. The yield and purity of DCs were improved to 2 DCs per hESC and more than 80% CD11c positive cells respectively. But CD83 expression was less than 50%. A serum-free EB-based protocol was developed using four growth factors including vascular endothelial growth factor (VEGF), SCF, BMP-4, and GM-CSF in XVIVO-15 medium to generate DCs [14,16]. After driving monocyte precursors into immature DCs with GM-CSF and IL-4 and incubating with maturation cocktail, about 75% of cells expressed DC markers CD86 and CD 83. The expression of IL-12p70 was promoted by genetic modification to enhance the stimulation potency of hESC-derived DCs. DCs derived from hESCs in these studies exhibited similar stimulatory functions compared to PBMC-derived DCs.

An OP9 co-culture protocol was also used to derive DCs from hiPSCs [15]. The purity, however, was lower with less than 50% CD86 positive cells and low expression of CD83. It was obvious that the protocol needs to be improved for generation of DCs from hiPSCs. To address the regulatory requirement, a xeno-free culture protocol was tested to derive DCs from hiPSCs in the same study. Serum-free medium AIM-V and Peprogrow III were used to culture hiPSCs on a fibronectin-coated surface in the presence of BMP-4. The cells were then dissociated and firmly adherent cells were removed by incubation with the fibronectin-coated surface. The non-adherent cells were cultured in myeloid cell medium and differentiated into DCs with GM-CSF and IL-4. This protocol, however, only worked for two out of five human iPSC clones. More work still needs to be done to develop efficient xeno-free protocols for clinical applications. And the DC differentiation from hiPSCs needs to be extended into multiple cell lines.

The use of hPSC-based DC vaccine in clinical trials requires large-scale culture systems. Because of hPSC's unlimited proliferation potential, it is expected that an order of 10^{10} cells will be produced in one production run [62]. Although no scalable culture system has been reported for hPSC-DCs, the systems used for other hPSC-derived cells offer great insight for development of scalable hPSC-DC culture systems.

6 Scalable culture systems for hPSC-derived cells

The scalable culture system for DC production from hPSCs depends on DC differentiation protocols. For the two types of hPSC-DC protocol, the EB-based protocol has a higher potential to be scaled up in suspension culture than the OP9 co-culture protocol since it does not need surface for cell adherence. But scale up of the OP9 co-culture protocol is also possible with microcarrier technology [63]. Scalable dynamic culture systems for hPSCs and their derivatives have been explored recently [62,64,65]. The progress and strategies for EB-based dynamic cultures and microcarrier-based dynamic cultures are discussed in the following sections.

6.1 Embryoid body-based scalable culture

EBs are three-dimensional structures that recapitulate the early stage of embryonic development. Under spontaneous differentiation, EBs comprise the cells from three-germ layer lineages (endoderm, mesoderm, and ectoderm) [66,67]. Structurally, it was found that the EB shell consists of an extracellular matrix (ECM) layer comprised of collagen I, a cellular layer with tight cell-cell adhesions, and a collagen IV lining indicative of a basement membrane [68]. The presence of the basement membrane and the compact cell layer could hinder the diffusive transport of the regulatory molecules as the EBs develop with time. However, the diffusion limitation can be mitigated in the dynamic culture systems.

A few scalable culture systems for hPSC-derived EBs have been developed since 2004 [69]. Table 2 summarizes different scalable culture systems for hPSC-derived EBs and their target lineages. The initial bioreactor culture was performed in slow turning lateral vessels (STLVs) for spontaneous EB formation [70]. The low shear stress rotating vessels supported cell expansion, although massive EB agglomeration was observed. Higher EB forming efficiency (1.5-2 fold) was observed in the STLVs than in static cultures [71]. A modified STLV system was also tested with incorporation of perfused and dialysis chamber to remove waste and supply nutrients [72]. Later STLVs were compared with spinner flasks for spontaneous differentiation [73], where shear stress, initial clump size, and seeding density were found to be important parameters. Spinner flasks with glass ball bulb-shaped impellers reached 6.4-fold expansion in 10 days compared to 1.2 fold in STLV. The potential of spinner flasks was further explored. Another study used pre-formed hESC EBs to seed spinner flasks for spontaneous differentiation [74]. Hematopoietic progenitors were evaluated and about 5-6% CD34 + CD31 + cells were detected. To control initial clump size, EBs from micropatterned hESCs were cultured in spinner bioreactor with controlled oxygen tension for cardiomyocyte differentiation [75]. Hypoxia (4% oxygen tension) enhanced cardiac-related genes and the percentage of contracting EBs was about 49% in this bioreactor system compared to 19% in static culture. However, only 3-5% of dissociated EB cells expressed cardiomyocyte markers. A rotary orbital culture system was tested for cardiomyocyte differentiation from mouse ESCs. This culture system led to more homogeneous EB size distribution and higher purity of cardiomyocytes (10-15% vs. 4-6%) compared to static culture [76,77]. Due to the agglomeration phenomenon in EB culture, EB size has been controlled by encapsulation with agarose or alginate [78,79], or by agitation speed [80]. The better differentiation in these bioreactor cultures than in static cultures might be due to a more homogeneous environment, enhanced diffusion, and exposure to shear stress. The shear stress might play an important role in modulating specific lineage differentiation from ESCs.

Although the scalable dynamic EB cultures demonstrated higher purity and efficiency compared to static cultures, the final purity of the differentiated cells was still low. To meet the clinical requirements without further purification, further optimization of scalable dynamic culture systems to improve the purity of the desired lineage is still required. The poor differentiation in current large scale systems is mainly due to 1) specific lineage differentiation protocols are not optimal with the majority focusing on spontaneous differentiation induced by FBS; 2) operational parameters including shear stress, initial seeding parameters, and physicochemical environment control still need to be optimized. More efficient differentiation protocols in combination with optimized operational parameters are expected to improve the purity of the desired lineage.

6.2 Microcarrier-based scalable culture

Demonstration of scalable production of hPSC-based DCs using the OP9 co-culture system may rely on microcarrier technology. Microcarriers are plastic or glass beads with a diameter of 100-200 μm and cell-compatible surfaces. These beads can be readily suspended in a stirred bioreactor and have been widely used in large scale culture of anchorage-dependent mammalian cells for recombinant protein production [62]. While hESC cultures have also been demonstrated in laboratory scale microcarrier bioreactors [65], the widespread use and extensive experience with large scale microcarrier suspension culture will facilitate its adaption to clinical scale hESC expansion. For generation of hPSC-based DCs, microcarriers can support the initial attachment of OP9 cells and the subsequent DC differentiation from hPSCs can be achieved on the same microcarriers. Alternatively, extracellular protein responsible for interactions between hPSCs and OP9 cells can be identified and coated onto the microcarriers to simplify the culture system. For example, the surface of microcarriers can be modified with a fibronectin coating and directly used to support hPSC differentiation to DCs.

Growth of hPSCs or their derivatives on microcarriers has been demonstrated since 2008, although mouse ESCs were grown on microcarriers earlier [81]. Table 3 summarizes different microcarrier culture systems for hPSCs and their derivatives. In the initial study, Phillips et al. [82] used Hillex II beads pre-incubated in conditioned medium to support hESC growth. A net 14-fold expansion was achieved over 6 passages, but the expansion ratio decreased with passage number. A cylinder-shaped cellulose microcarrier coated with Matrigel™ was found to support hESC growth for more

than 10 passages [83]. More than 2-4 fold higher cell density was achieved on microcarriers compared to two-dimensional (2-D) culture. Microcarrier Cytodex™ 3 coated with Matrigel™ or grown with mouse embryonic fibroblasts supported hESC expansion up to 11 passages [84]. Cytodex™ 3 microcarriers were also used to grow hESCs without any coating [85]. Although a longer lag phase was observed, about 2-fold higher cell yield was achieved compared to 2-D culture. The initial seeding parameters, especially the initial agitation program, were optimized for Cultispher-S microcarriers to allow cell attachment to microcarriers without coating [86].

Table 1. Methods to differentiate DCs from hPSCs

DC generation method	hPSC Line	Characterization	References
Co-culture with OP9 stromal cells (one-step co-culture)	H1, H9	MHC-I, MHC-II, CD80, CD86, lack CD83 expression	[12]
Co-culture with OP9 stromal cells (two-step co-culture)	KhES-1, KhES-3	MHC-I, MHC-II, CD86, low CD80, express CD83 (about 50%)	[58]
Co-culture with OP9 stromal cells (modified one-step co-culture)	Human iPSC (in house)	MHC-I, MHC-II, CD86 (<50%), low CD83	[15]
Xeno-free adherent culture with fibronectin-coated surface	Human iPSC (in house)	Generated DCs from 2 out of 5 iPSC clones	[15]
EB-based suspension culture with serum	H1	About 25% of cells expressed MHC-II, CD86, low CD83	[11]
EB-based suspension culture with serum and BMP-4	HLA-A2+ H9	More than 80% CD11c+ cells, MHC-I, MHC- II, CD86, low CD80, express CD83 (<50%)	[13]
EB-based serum-free culture and four growth factors	H1, H14	MHC-I, MHC-II, CD86, CD83 (>70%)	[14,16]

Table 2. Scalable culture systems for hPSC-derived EBs

Culture system	hPSC Line	Differentiation	References
STLV	H9.2	Spontaneous differentiation induced with FBS	[70]
Perfused and dialyzed STLV	VUB01, H9, HUES-9 OCT-4GFP	EB formation in hESC medium without FGF2 for neural differentiation, neural gene and protein expressed	[72]
STLV; Erlenmeyer flask; Spinner flask with glass ball impeller; Spinner flask with paddle impeller	H9.2	Spontaneous differentiation induced with FBS	[73]
Spinner flask	H9, H1	Spontaneous differentiation induced with FBS, detect CD34+CD31+ cells, 5-6%; CD34+CD45+ cells, 1-2.5%	[74]
Spinner bioreactor with oxygen control	H9, HES2	Spontaneous differentiation induced with FBS, detect cardiomyocyte marker, 3-5% α-actinin+ cells	[75]
Rotary orbital culture	Murine ESC D3 line*	Spontaneous differentiation induced with FBS, detect cardiomyocyte marker, 10-15% α-sarcomeric actin+ cells	[77]

*Included for different hydrodynamics of rotary orbital cultures from other bioreactors

Lineage specific differentiation has also been demonstrated for hPSCs grown on microcarriers. Definitive endoderm cells were differentiated from hESCs grown on microcarriers coated with Matrigel™ [87]. The differentiation was induced by treatment with Activin A and Wnt3a in low serum medium. About 84% of cells on microcarriers expressed definitive endoderm markers FOXA and SOX17, while only 23% of control cells expressed those markers. Differentiation into

cardiomyocytes in suspended microcarrier culture was also explored [88]. Human ESC clumps were seeded onto different types of laminin-coated microcarriers together with 2-D controls. Microcarrier TOSOH-10 had the highest cardiomyocyte purity at more than 20% and the highest cardimyocyte to hESC yield at 0.6. The use of microcarriers for hPSC differentiation into other cell types still needs to be explored.

Table 3. Microcarrier-based culture systems for hPSCs or their derivatives

Group	Microcarriers	hPSC Line	Surface Modification	References
Microcarriers for hPSC expansion	Hillex II (trimethyl ammonium-coated polystyrene)	ESI-017, weaned into single-cell culture over 5 passages	No coating, pre-incubated in conditioned medium	[82]
	Cellulose microgranular cylindrical shape microcarrier	HES-2, HES-3	Matrigel™ coating	[83]
	Cytodex™ 3 (collagen coated dextran)	H9	No coating	[85]
	Cytodex™ 3 (collagen coated dextran)	H1, H9	Seeded with mouse embryonic fibroblasts, or Matrigel™ coating	[84]
	Cultispher-S (crosslinked gelatin)	SHEF-3 hESC	No coating, pre-incubated in culture medium	[86]
Microcarriers for hPSC differentiation	Collagen-coated microcarrier from Hyclone	H1, H9, differentiation into endoderm	Matrigel™ coating	[87]
	TOSOH-10 (Hydroxylated metacrylate)	HES-3, H1, differentiation into cardiomyocytes	Laminin coating	[88]

Both EB-based scalable cultures and microcarrier-based scalable cultures can be applied to generate DCs from hPSCs. However, the operational parameters for scalable production of hPSC-derived cells are yet to be optimized for DCs. Different culture media, growth factors, optimal EB size, and shear stress signaling may be required. Microcarrier type and surface property need to be optimized to favor DC differentiation. The control of physiochemical environment may vary from lineage to lineage. Although much work needs to be done, the advances summarized above suggest that production of hPSC-based DCs in large scale culture systems is possible.

7 Conclusions

Autologous DC vaccines have been under investigation for their use on cancer treatment and have shown promising results in several NSCLC trials. The limitations of autologous DCs, such as available cell number, donor variability, and high cost of cell processing can be potentially addressed by hPSC-derived DCs. Several protocols have been developed to generate DCs from hPSCs with high purity and in vitro functions comparable to autologous DCs. In future studies, the adaption of these protocols in EB-based and microcarrier-based scalable culture systems and the fulfillment of regulatory requirements will play an important role in the eventual use of hPSC-derived DCs in clinical trials.

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