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IN LABORATORY GENERATION AND MATURATION OF HUMAN MONOCYTE-DERIVED DENDRITIC CELLS FOR CANCER IMMUNOTHERAPY

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ABSTRACT

Dendritic cells (DCs) play a critical role in the regulation of adaptive immune responses, furthermore they act as a bridge between the innate and the adaptive immune systems they have been ideal candidates for cell-based immunotherapy of cancers and infections in humans. The first reported trial using DCs in 1995, since they have been used in trials all over the world for several of indications, including cancer and human immunodeficiency virus infection. Generally, for *in vitro* experiments or for DCs vaccination monocyte-derived dendritic cells (moDCs) were generated from purified monocytes that isolated from peripheral blood by density gradient centrifugation. A variety of methods can be used for enrichment of monocytes for generation of clinical-grade DCs. Herein we summarized up to date understanding of systems and inputs used in procedures to differentiate DCs from blood monocytes *in vitro*.

Keywords: Dendritic Cells, Monocyte-derived Dendritic Cells, Immunotherapy

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INTRODUCTION

For more than a century it has been hypothesized that the immune system can be redirected to target malignant cells and thus cure cancer [1, 2]. In 1973 Steinman discovered a new type of immune cell, the dendritic cell (DCs) [3], which play an important role in the induction of specific immunity. DCs are sentinels of the immune system, as they are deployed throughout the body and monitor their surroundings for antigens and danger signals derived from pathogens or tissue damage. DCs have been clinically used for three decades, with more than 300 completed or ongoing registered clinical trials conducted to test their application for boosting anti-tumor immunity [4]. DCs are the most potent professional antigen-presenting cells (APC) and play critical roles in regulating the innate and adaptive immune responses [5].

In their immature state, DCs mainly reside in lymphoid and peripheral tissues where they recognize and capture antigens and become activated in the presence of foreign pathogens. This activation occurs following stimulation by exogenous danger signals via pattern recognition receptors (PRR) such as Toll-like receptors (TLR) [6, 7] and leads to DC migration to the draining lymph node and the presentation of the processed epitopes to T cells. During the T cell activation, DC engages the T-cell receptor (TCR), secrete specific cytokines and stimulate the immune responses toward TH1, TH2, or Tregs depending on the cytokine environment. Due to their proficiency at antigen cross-presentation (i.e., the presentation to both CD4+and CD8+T cells), DC have been used as vaccine platforms to induce anti-tumor cytotoxic T lymphocyte (CTL) CD8 immune responses [8, 9].

With DC vaccination, mature DCs loaded with tumor antigens ex-vivo are injected into cancer patients to induce tumor-specific effector T-cells that aim to recognize and eliminate cancer cells and induce immunological memory to control tumor growth [10]. In the majority of clinical DC vaccination trials conducted so far, DCs differentiated ex-vivo from monocytes or CD34+progenitors have been used, since naturally circulating DCs (nDCs) are present in the blood but only constitute about 1% of blood mononuclear cells. The most commonly used preparation involves the reinfusion of ex-vivo derived DC pulsed with tumor-associated antigens (TAAs) or tumor cell lysates and stimulated with a defined maturation cocktail. The gold standard maturation cocktail included the pro-inflammatory cytokines TNF-α, IL-1β, and IL-6 in combination with prostaglandin-E2 (PGE2) [11, 12].

While, DC-based vaccinations appeared promising after Sipuleucel-T (Provenge®) approval in 2010, a DC-based immunotherapy for the treatment of advanced prostate cancer [13], however unfortunately, the vaccination against established malignancies has shown partial clinical benefit.

Recently Indian government agency (CDSCO-Central Drugs Standard Control Organization) has approved in 2017 an autologous monocyte-derived and tumor lysate-pulsed mature DC-based vaccine (APCEDEN®) for treatment of four cancer suggestions (prostate, ovarian, colo-rectal and non-small cell lung carcinoma) [14]. Furthermore, the efficacy profile of APCEDEN® therapy demonstrated a survival benefit of >100 d [14]. Various types of DC vaccines have been evaluated in clinical trials so far (table 1). The most commonly used preparation involves the reinfusion of ex-vivo derived DC pulsed with tumor-associated antigens (TAAs) or tumor cell lysates and stimulated with a defined maturation cocktail and clinical trial results demonstrated encouraging outcome along with safe and well-tolerated in patients with solid tumors [15].

We explored previous and recent years published papers in English indexed in Pubmed from 1967-2020, using key words like Cancer immunotherapy, Generation of DCs, Monocytes presence of a cytokine cocktail and growth factors, DC vaccination and Antigen loading/pulsing method. The papers quoted in the references of those articles were also explored. In this review, we have briefed the cellular aspects essential for Monocyte-derived dendritic cells efficacy, the selection of suitable culture medium, appropriate culture medium supplements, growth factors, and cytokines. We will also review the molecular markers used to characterize DCs by flow cytometry and antigen loading of moDCs.

Table 1: Current encouraging clinical trials using personalized DC-based vaccines [15]

NCT number			Indication	Interventions	Phase	Enrolment	Start date	Estimated primary completion date
Tumor Lysate	1	NCT00703105	Ovarian cancer	Ontak (anti-CD25) DC vaccine+ontak	Phase-2	36	2008	2018
	2	NCT01204684	Glioma Astrocytoma Astro-oligodendroglioma Glioblastoma	Autologous tumor lysate-pulsed DC+0.2% resiquimod DC vaccination+polyICLC	Phase-2	60	2010	2018
	3	NCT01635283	Newly diagnosed or recurrent low-grade glioma	Tumor lysate-pulsed autologous DC vaccine	Phase-2	18	2012	2019
	4	NCT01946373	Malignant melanoma	Cyclophosphamide Fludarabine T cells Interleukin-2 DC vaccine	Phase-1	10	2013	2018
	5	NCT01973322	Malignant melanoma stage III, Stage IV	Arm 1: autologous DC loaded with autologous tu lysate (DC vaccine)+RT Arm 2: DC vaccine+IFN-α Arm 3: both arm 1 and 2+RT Arm 4: DC vaccine	Phase-2	24	2013	2019
	6	NCT01957956	Newly diagnosed glioblastoma	Tumor lysate-pulsed autologous dendritic cell vaccine+temozolomide	Early phase-1	21	2013	2016
	7	NCT01808820	Malignant glioma Glioblastoma	Dendritic cell vaccine Tumor lysate Imiquimod Leukapheresis	Phase-1	20	2013	2019
	8	NCT02496520	Advanced solid tumors, sarcoma Central nervous system tumor	Dendritic cells Surgery as needed Chemotherapy as needed Radiation: radiation therapy as needed	Phase 1|2	10	2014	2018
	9	NCT01803152	Sarcoma Soft tissue sarcoma Bone sarcoma	Biological: dendritic cells vaccine Lysate of tumor Gemcitabine Imiquimod Leukapheresis	Phase-1	56	2014	2019
	10	NCT02718391	Malignant melanoma	DC pulsed with autologous tumor lysate	Phase-2	120	2015	2019
	11	NCT02301611	Malignant melanoma	Autologous Tumor Lysate (TL)+Yeast Cell Wall Particles (YCWP)+Dendritic Cells (DC) (TLPLDC Vaccine) Placebo	Phase-2	120	2015	2019
	12	NCT02503150	Metastatic colorectal cancer	Antigen pulsed dendritic cells+chemotherapy Chemotherapy	Phase-3	480	2015	2019
	13	NCT02678741	Metastatic melanoma	TLPLDC vaccine in addition to standard of care checkpoint inhibitor of choice	Phase 1|2	45	2016	2019
	14	NCT03395587	Newly diagnosed glioblastoma	Autologous DC pulsed with autologous tumor lysate	Phase-2	136	2018	2022
	15	NCT03360708	Recurrent glioblastoma	Cytokine-induced killer cells Tumor lysate-pulsed autologous DC vaccine	Early phase-1	20	2018	2022
	16	NCT03014804	Recurrent glioblastoma	Autologous dendritic cells pulsed with tumor lysate Nivolumab	Phase-2	30	2018	2020
RNA Peptide	17	NCT01983748	Uveal melanoma	Autologous DC loaded with autologous tumor RNA	Phase-3	200	2014	2022
	18	NCT02775292	Adult solid neoplasm Childhood solid neoplasm Metastatic neoplasm	Aldesleukin Cyclophosphamide Fludarabine phosphate Nivolumab NY-ESO-1 reactive TCR retroviral vector transduced autologous PBL NY-ESO-1(157-165) peptide-pulsed autologous DC vaccine	Phase-1	12	2017	2019
Tumor Neoantigen	19	NCT01885702	Colorectal cancer	Neoantigen-loaded DC vaccination	Phase 1|2	25	2010	2016
	20	NCT03300843	Melanoma Gastrointestinal Breast Ovarian Pancreatic cancer	DC vaccine loaded with neoantigen coding peptide	Phase-2	86	2018	2027

Sources of monocytes

It's very impressive for monocyte-derived dendritic cell culture is to select the monocyte source, since these cells are the preferred precursors for *in vitro* moDCs generation. One probability is to directly collect blood by venipuncture. The advantage of whole blood as a monocyte source is the freshness of the material. However, the drawback of using whole blood is the low yield of monocytes, since they represent only 6% of all peripheral blood cells, so using whole blood requires the processing of a large blood volume [16]. Processing a 450 ml blood bag usually generates 30–80 ml of buffy coat with approximately $1x10^9$ cells [17].

Second possible source is a leukopak. This is an enriched leukapheresis product consisting of a variety of blood cells including monocytes, lymphocytes, and erythrocytes. There are two types of leukopaks: one is collected from peripheral blood without any stimulation on the blood donor and the other are obtained from donors who were stimulated with G-CSF (granulocyte colony stimulating factor) to induce leukocyte production and trigger migration of stem cells from bone marrow into the bloodstream [18].

Although the production of leukopaks from such specific donors is not usual, this kind of product can be commercially provided under request. Commercial leukopaks generally contain 80–200 ml of processed material with approximately $7x10^9$peripheral blood mononuclear cells (PBMC), [19] (more information available on: www. allcells. com/products/whole-tissue/leuko-pak). The effect of various DC vaccination parameters on immunological and clinical outcome of vaccination has been studied in numerous small phase I/II clinical trials in cancer patients. Most of these studies have been performed with moDCs, due to their easy differentiation protocol *in vitro* [20].

Separation of monocytes from whole blood

After consider the source of cells the next step in moDCs isolation and culture. In 1968, Boyum introduced a convenient and rapid separation using centrifugation through a Ficoll-sodium metrizoate solution [21]. This separation method takes advantage of cell density differences of the components in whole blood that, when centrifuged in the presence of a density gradient media, exhibits a unique migration pattern through the medium allowing distinct cell populations to be fractionated [22].

Following centrifugation above the FicollPaque layer, PBMCs form a layer of cells similar to a cloud, while the plasma is the uppermost layer in the tube. The PBMC layer includes B and T lymphocytes, monocytes, NK (Natural Killer) cells, and dendritic cells [23]. FicollPaque methodology is highly efficient and recovers around 95% of the mononuclear cells present in the original blood sample [24, 25]. There is a very vital step during isolation of PBMCs from blood, one of them is pipetting and carefully layering of blood over density gradient solution into centrifuge tubes, and a second vital step is centrifugation acceleration and deceleration is very critical.

Isolation of monocytes from whole blood

Since monocytes is the ability to adhere to inert surfaces like plastic, different from other cells present in the PBMC fraction. Monocytes isolation procedure take advantage of this characteristic usually seed PBMC cells in a plastic flask with the appropriate culture medium and allow adherence for 2 h in a humidified incubator. All monocytes will adhere to the culture flask while B and T lymphocytes, NK cells, and DCs will remain non-adherent and can be eliminated as floating cells [26].

Another distinct characteristic of monocytes is the only circulating blood cells to show high expression of CD14 on their membrane, this molecule is widely used as a biomarker for monocytes and as a target for their purification (fig. 1) [27]. It is worthy of note that the CD14 molecule belongs to the lipopolysaccharide (LPS) receptor complex. Recognition through this receptor is interpreted by the cell as a "danger signal" [28] capable of inducing a maturation process on immature DCs.

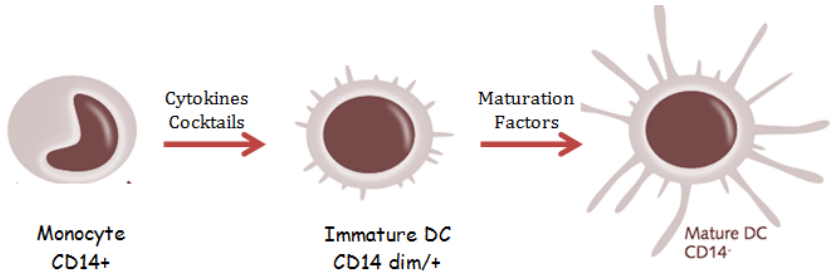

Fig. 1: Generation and maturation of moDCs [27]

Culture medium for monocyte-derived dendritic cell

There are numerous different culture media described and used for moDCs [29]. However, it is essential to consider the general and specific objectives of the experiments, as well to take into consideration the cost of the different supplements to be used. If the main objective is for clinical purpose (therapeutic grade), utilizeonly Food and Drug Administration (FDA) approved culture medium like AIM-V (GIBCO, Catalog number: 087-0112DK). For research purposes, PromoCell, for example, offers three different medium types: DC Generation Medium (Catalog number: C-28050), DC Generation MediumDXF (Catalog number: C-28052), and Monocyte Attachment Medium (Catalog number: C-28051).

Another culture medium used to generate moDCs is RPMI-1640. It was developed by Moore and colleagues at the Roswell Park Memorial Institute, hence the acronym RPMI [30]. The RPMI-1640 composition is available and well established and there are several customized versions of RPMI, some of them lacking specific components, others already supplemented with certain growth factors or other molecules [22, 31].

Serum is a quite ubiquitous supplement in cell cultures. The final concentrations of serum used vary from 1-10%. It is also feasible to use plasma or umbilical cord blood serum. Nevertheless, if the cells will not be used in clinical applications, fetal bovine serum (FBS) is the least expensive choice, and is also easier to obtain. Though cell culture procedures are performed in aseptic environments, however any biological contaminant can demolish the cell culture, therefore the use of antibiotics and antimycotics is at times required. The most frequent antibiotics used in cell cultures are penicillin-streptomycin for Gram-positive and Gram-negative bacteria whereas gentamicin against mycoplasmain culturing cells that are intended for clinical use, the FDA does not recommend the use of penicillin or β-lactams due to the possibility of severe hypersensitivity reactions [32]. A broadly used antifungal agent is amphotericin B. It acts against both fungi and yeasts. Generally, monocyte cultured in RPMI-1640 media supplemented with 1-10% FBS, 2 mmol Glutamax, 100 µg/ml penicillin/streptomycin, 1% non-essential amino acids and 1% sodium pyruvate [31].

The time required to establish monocyte cell differentiation *in vitro* is quite variable. The most of the studies consider that the differentiation process from monocyte to immature DC is terminated in five days with an additional period of 48 h for maturation [33, 34, and 35].

Cytokines for the moDCs

Sallusto and Lanzavecchia [36] are former to achieve *in vitro* moDCs generation with medium supplementation and through different cytokines, combined use of IL-4 and GM-CSF. In the subsequent years, numerous works characterized different protocols regarding the use of different cytokines in order to induce *in vitro* monocyte differentiation and maturation. It is important to know that different combinations of cytokines will generate moDCs with diverse characteristics and functions, thus we should decide the cytokines that good match the cause of the research work.

GM-CSF growth factor appears to down-regulate the expression of the macrophage colony-stimulating factor (M-CSF) receptor on monocytes, thus inhibiting M-CSF induced differentiation of monocytes into macrophages [37]. In the same way, IL-4 applies its actions in monocytes differentiation by inhibiting macrophage colony formation [38, 39]. The DC generated by this procedure, after seven days in culture, demonstrates a typical dendritic morphology.

Sanarico and his groups [40] explain different methodology for inducing differentiation of moDCs *in vitro* using GM-CSF, IL-4, and IL-2. Another combination of cytokines was used to generate moDCs by Takahashi *et al.* [41]. A combination of GM-CSF and IL-7 gave rise to floating cells with characteristic DC morphology and a few adherent cells developed the appearance of Langerhans cell-like dendrites. Santini *et al.*[42], and Mohty *et al.* [43] two different groups used the combination of GM-CSF and IFN-α to produce moDCs *in vitro*. Moreover, in both cases, the DCs generated with GM-CSF and IFN-α showed a typical DC morphology. Iwamoto and co-workers used TNF-α together with GM-CSF to initiate the differentiation of monocytes into DCs (called TNF-DC by the authors), [44].

***In vitro* maturation of moDCs**

Generally *in vivo*, DC maturation is triggered by bacterium, virus, or other microorganism or tissue injury. To induce the DC maturation process *in vitro* it is necessary to give a stimulus that mimics a danger signal [28, 45]. *In vitro*, this can be imitated by incubation with pathogen receptor agonists or a cocktail of proinflammatory cytokines.

Most common material used to mimic DC activation when provoked by bacteria is LPS, a characteristic component of the wall of Gram-negative bacteria [46, 47]. However, due to its possible toxicity, LPS is only used in research protocols.

Flagellin is another substance that mimics the danger signal triggered by the presence of Gram-negative and/or Gram-positive bacteria. Flagellin induced DC maturation results in the activation of the NF-κB signaling pathway and cytokine production [48]. Imiquimod and Poly (I: C) also have been used as adjuvants in protocols to develop cancer vaccines [49]. Short synthetic single-stranded DNA molecules that contain unmethylated CpG dinucleotides (CpG) are also used as TLR9 agonists to activate iDCs [50].

Interleukins and several other molecules can simultaneously be used to induce DC maturation. A "maturation cocktail" canhave IL-6, IL-1β, TNF-α, IFN-γ, and PGE2 [51]. This cocktail augments the pro-inflammatory effects of TNF-α by generating an inflammatory environment that induces DC maturation [45].

However, a cytokine cocktail consisting of tumor necrosis factor (TNFα), interleukin (IL)-1β, IL-6 and PGE2, or monocyte-conditioned medium with TNFα and PGE2 are the most widely used methods for moDC maturation [51, 52].

Although whether this is the best cocktail to induce maturation, still it's controversial because PGE2 may confer immunosuppressive effects [53, 54]. To further induce DC activation, mimicking viral infection, type-I interferons have been added to the cocktail [55]. Furthermore recently, utilize of Toll-like receptor (TLR) ligands [56, 57] or electroporation with mRNA-encoding proteins that induce DC maturation [58] has been investigated.

The immunophenotyping of moDCs

Dendritic cells are so called because of their characteristic cell surface projections that resemble the dendrites of neurons. DCs are a heterogeneous cell population in terms of locations, phenotypes, and immunological functions. Such plasticity DCs allows to differentially shape the immune response when presented with diverse pathogens. DCs, monocytes and macrophages usually comprise the mononuclear phagocyte system. An emerging theme is that components of this system are not as related as was presumed a decade ago. Currently Flow cytometry is frequently used to analyze lymphocyte subsets but synchronized detection of DCs and monocytes is hampered by the lack of a positive lineage marker.

Therefore because of the lack of DC specific markers, it is query whether DCs constitute a separate lineage of cells. The perplexity is related to results showing that DCs can be generated from mature peripheral blood Monocytes as well as from un separated CD34+progenitor cells when cultured in the presence of GM-CSF and TNF-α [59, 60].

The success of the moDCs differentiation can be characterized by phenotype of the DCs. Thus farther expression of certain molecules is typically used to indicate successful moDCs differentiation and/or maturation. Because peripheral blood monocyte, is the main source for *in vitro* DC generation, expresses high levels of CD14 on its membrane, and moDCs lacks the expression of this same molecule.

Furthermore, to find out the success of the iDC maturation process, it is vital to monitor the increased expression of co-stimulatory molecules such as CD40, CD80, and CD86. These molecules are constitutively expressed at low levels in DCs, however, their expression considerably increased after induction of maturation by LPS [61, 62].

On the other hand, Human leukocyte antigen-II (HLA-II) molecule constitutively expressed at low levels in both monocytes and DCs. After a maturation stimulus, its expression on DCs is augmented. Therefore, like co-stimulatory molecules, HLA-II can be used as an indicator of the success of the iDC maturation process [63, 64].

Besides that, in blood monocytes, CD isoforms for example CD1a, CD1b, and CD1c (BDCA-1) can be upregulated on the cell surface by cytokine cocktails designed to drive DC differentiation *in vitro* [65].

Next potential markers are CD207 [66], and CD209 (DC-SIGN), which is expressed on plasmacytoid DCs (pDCs) and moDCs and is greatly expressed on DCs in mucosal tissues. The expression of this last marker is increased after LPS-mediated DC maturation induction [67].

Antigen loading of moDCs

Our immune system is able to differentiate between self, non-self and eradicate damaged cells. To evade elimination by immune responses, tumors, cancerous cells not only acquire the capability to prevent immune recognition, but also create an immunosuppressive environment and actively hijack immune cells to aid in tumor progression [68, 69]. Reactivating the immune system to treat patients with cancer was proposed at the end of the nineteenth century and cancer immunotherapy has further developed ever since [70, 71]. DCs loaded ex-vivo with specific TAAs or whole tumor lysate to generate an immune response aiming for cancer-cell elimination and recently *in vivo* loading of DCs is being exploited [72, 73].

Thus, to induce a tumor-specific immune response, DCs should be loaded with tumor antigens. The most extensively used techniques for antigen loading of DCs vaccines are pulsing DCs with or tumor lysate, MHC-binding peptides of TAAs, corresponding long peptides or proteins, or TAA-encoding mRNA. All antigen-loading techniques have their own advantages and disadvantages and not any technique has proven to be superior to the others thus far. However recently, there is a great effort made in improving existing DC vaccines and developing new ones. Moreover, DCs loading together MHC class-I and class-II epitopes appears favourable for the quality of the induced immune response [74]. Various new approaches include genetically engineered DCs that express TAAs or display enhanced immunostimulatory properties or explore *in vivo* antigen loading of DCs with freshly released TAAs due to chemotherapy or immunogenic tumor-cell death [75-77].

DISCUSSION

A dendritic cell for immunotherapy using ex-vivo generated moDCs in patients with cancer was first explored over two decades ago [78]. A various clinical trial [79] has established the safety and ability of moDCs immunotherapy to induce anti-tumor responses [80, 81]. Therefore, based on the known difficulties of isolating and generating moDCs from human blood, here we provide and discussed comprehensive information for the generation of moDCs.

In order to study the biology of DCs, and their roles in immune responses, and their potential use for the treatment of certain diseases, and methods to generate mouse DCs *in vitro* have been well described by Shortman, [82]. DCs have unique features that have made them an ultimate choice for antitumor vaccines. They are considered as the most effective APC accountable for primarily sensitizing naive T cells to specific antigens [83]. DCs are 100-times more potent than APCs, B cells and monocytes, in inducing T-cell proliferation [84, 85]. Furthermore, DCs play a central role in the establishment of immunologic memory [86]. In compare with monocytes and B cells, DCs are able to use soluble protein antigens to sensitize naive T cells *in vitro* [84]. Using these soluble proteins, DCs have successfully sensitized CD4+ [87] and CD8+T cells inducing antigen-specific cytotoxic T lymphocytes (CTLs) [88, 89]. This potential capacity gives developers of DC-based vaccines a wider range of potential antigen targets that can be effectively used to sensitize T cells. With respect to their use against cancer, the ability of DCs to prime T cells to attack tumor cells has been demonstrated *in vitro* [90] as well as in various animal models [91, 92]. Another, studies in 1990s, by Sallusto and Lanzavecchia, [36] confirmed that human monocytes differentiate into DCs *in vitro* by culturing with GM-CSF and IL-4.

Moreover, injection of moDCs and pulsed with exogenous antigens used to rapidly expands human T cell immunity [93]. Furthermore moDCs pulsed with certain TAAs can frequently expand CTLs and elicit regression even in advanced cancer [94]. The GM-CSF *in vitro* cultured DC is the most common DC type used in studies of mouse and human DC biology, and for immunotherapy using DC vaccines [82]. The differentiation of monocytes *in vivo* [95] and under mimicked physiological conditions [96] has previously been confirmed. In addition to playing a role in activating the immune system, DCs can also induce immune tolerance, which is a potential barrier to a successful vaccine strategy. Evidence has suggested that DCs that are not fully matured will be prone to inducing tolerance [97, 98].

CONCLUSION

The medical and scientific community established marvellous efforts in the understanding DCs and their precursors. As a result of such efforts, moDCs are presently used in clinical protocols for the treatment of a variety of diseases, including cancer. Despite these achievements, gaps are still exist in terms of the how monocyte are derived and what best methods to be used to isolate and culture

them *in vitro*, and how to achieve a high yield and purity of the isolated monocytes. As well as appropriate combination of cytokines and growth factors that will generate moDCs and transcription profiles of these cells in different stages of maturation. This review presented key information in relation to the generation of human moDCs from blood monocytes. Furthermore moDCs manufacturing strategies need to be reproducible, robust, and inexpensive with the ultimate goal of providing safe, high-quality products.

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AUTHORS CONTRIBUTIONS

All the authors have contributed equally.

CONFLICT OF INTERESTS

Declared none

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