

MONOCYTE-DERIVED DENDRITIC CELLS ISOLATION AND PHENOTYPIC CHARACTERIZATION

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ABSTRACT

Dendritic cell (DC) is the most powerful inducers and regulators of immune responses, responsible for interaction within the immune system. The ability of DC to induce or regulate/suppress immune responses led to attention in their immunotherapeutic use in various disease types. The aim of the presented study was to generate *in vitro* CD14+ enriched peripheral blood monocytes (PBMCs) following the culture with human monocyte-derived dendritic cell serum-free differentiation media over ten days with phenotypical analysis and morphological identification for functional studies. *In vitro*, peripheral blood of human donors samples were collected for the purification of blood CD14+ monocytes that represent the most common origin for DC precursors based on ficoll density gradient separation and human monocyte-derived dendritic cell enriched differentiation medium. After PBMCs isolation, the cell viability, cell yield were determined, the monocyte-derived dendritic cell surface marker expression was detected by flow cytometric analysis after staining with specific fluorescence-conjugated monoclonal antibodies. *in vitro* culture and differentiation of human monocytes into DCs and their subsequent maturation into mature DC constitute a critical first step for different downstream analysis ranging from an understanding of both human DC biology and function to their use in clinical diagnostics and therapeutic design for different disease.

Keywords : Monocyte; Dendritic cell; APCs, antigen-presenting cells; Ficoll density gradient separation.

Introduction.

Dendritic cells (DCs) are the most professional antigen-presenting cells (APC) of the mammalian immune system that represent a key mediator of both innate and adaptive immune responses (**Steinman, 2012**). Due to their abilities to pathogen recognition, capturing and processing machinery, migratory capacity and cytokines production that promotes pathogen-specific effector T cell differentiation and activation, growing interest in their use in clinical approaches has been observed. (**Soloff, 2010**).

Currently, there are two principal cell populations of dendritic cells in humans: classical DC (cDC) derived from myeloid precursor cells, e.g. peripheral blood monocytes and plasmacytoid DC subset (pDC) originated from lymphoid precursor (**Bol et al., 2016**).

During development, immature DCs express specific pattern recognition receptors that serve as expression markers and allow for the capture and processing of foreign antigens following infection (**Harman et al., 2013**). Once activated, immature dendritic cell exhibit an increase in the expression of class II MHC and co-stimulatory molecules critical for effective antigen presentation to naïve T cells (**Sabado et al., 2017**). Cytokines produced by DCs can also promote the differentiation of CD4+ T helper cells as part of immune activation (**Bol et al., 2019**).

Human blood DCs populations originate from bone marrow precursors represents approximately 0.1 - 1% of circulating peripheral blood mononuclear cells (PBMCs)(**Macri et al., 2018**). Subsequently, their isolation in enough numbers and purity for functional studies is challenged by their scarcity and a lack of distinguishing markers(**Lo, 2003**). Hence, the main objective of this study is to generate in vitro immature dendritic cells from CD14+ peripheral blood monocytes (PBMCs) by means of a differentiation and maturation process induced by exogenous stimuli (Recombinant Human IL-4, Recombinant Human GM-CSF and Recombinant Human TNF- α) with morphological determination and phenotypic identification of PBMC-derived DCs following culturing in a defined period which represent a crucial step for studying the biology of DCs, their roles in immune responses, and their potential use as immunotherapeutic tool against both malignant cell antigens and infective microorganisms.

Materials and methods

A. Materials

1. Study group:

The study was conducted in children cancer Hospital from September 2018 to June 2019, Egypt. 15 ml of human whole blood was collected from 10 normal human donors ranging from 20-35 years old. all donors signed a written informed agreement for the study.

2. Chemicals and cell culture media:

Blood samples were collected from Ahmed Maher teaching hospital blood bank, Egypt, in a vacutainer containing Heparin as anti-coagulant (Greiner bio-one, Australia) and processed immediately within 2 hours of collection.

The protocol was optimised for the preparation of PBMCs from human blood through using Ficoll-Hypaque density gradient centrifugation in addition to using RPMI-10 complete medium (Gibco, United Kingdom) supplemented with penicillin/streptomycin (10 mg/ml), Glutamine (200 mM), 10% FCS, insulin and hydrocortisone.

In vitro culture and differentiation of human monocytes into dendritic cells DCs were achieved by using the Human Monocyte-derived Dendritic Cell Differentiation Kit (R&Dsystem, Minneapolis, MN, USA). This kit enables the generation of active mature DCs from human monocytes in 10 days (Amedei *et al.*, 2011; Sabado and Bhardwaj, 2010).

B. Methods

1. Generation of MoDCs from Freshly Isolated blood:

1.1 Protocol for PBMCs Isolation.

Protocol for dendritic cell generation started with isolation of peripheral blood monocytes (PBMCs) from fresh human whole blood (< 24 hours old) using Ficoll-Hypaque density gradient centrifugation (Gill, 2019). In brief with slight modifications, blood samples were diluted in 1x Balanced Salt Solution (Gibco, UK) at 1:1.4 ratio, 35 mL of diluted cell suspension (blood/PBS mixture) layered over 15 mL of Ficoll-Paque (GE Healthcare, Life Science, Sweden) in a 50 mL conical tube.

For separation of PBMCs from whole blood effectively, Ficoll/diluted blood mix was centrifuged at different speeds for optimization purposes. centrifugation at 1600 rpm (400xg) for 30 min at 20°C with no brakes was approved for further isolation based on PBMCs yield and viability, after centrifugation, four distinct layers were observed which consisted of plasma, peripheral blood mononuclear cells (PBMC), ficoll-paque solution and sedimented red blood cells (RBC) with granulocytes. Upper layer that contains the plasma and most of the platelets discarded, the buffy coat mononuclear cell layer (lymphocytes, monocytes, and thrombocytes) suspended in 5ml of 1x BSS buffer, centrifuged at 1800 rpm for 8 minutes. after centrifugation, RBC lysis was performed to obtain a pure monocyte population. (10X) RBC lysis buffer was prepared by adding 8.3g NH₄Cl (Sigma, Germany), 1.0g KHCO₃ (Sigma, Germany), 1.8ml of 5% EDTA (Sigma, Germany) in 1000 ml of sterile water. RBC lysis was performed once for 3min. The lysis process was stopped using a 1x BSS buffer and centrifuged at 1800 rpm for 5 min with no brakes. After centrifugation, PBMC washed twice in 5ml of 1x buffer. after the first wash, PBMC Centrifuged at 350 xg (1300 rpm for 8 min at room temperature while cells centrifuged at low speed 200 xg (1000 rpm for 10 min for platelet removal. Finally, the PBMC white pellet obtained, resuspended immediately in

RPMI-10 complete medium (Gibco, United Kingdom) for cells counting, purity and viability determination by trypan blue exclusion test.

1.2 Culturing and differentiation of human monocytes into dendritic cells DCs.

After PBMC isolation, 1×10^6 PBMCs/mL resuspended, plated in RPMI-10 complete medium. Mononuclear Cells Incubated for 2 hours at 37°C , 5% CO_2 and >95% humidity. On day 0, the adherent cell fraction washed and the differentiation processes into PBMC-derived DCs started by the addition of Mo-DC Differentiation Medium (Medium allow for the efficient and reliable in vitro maturation of human Monocytes (hMo) into immature as well as fully mature CD14^+ monocyte-derived Dendritic Cells moDCs) supplemented with human IL-4 (**Huber et al., 2018**) and human GM-CSF (**Merad et al., 2013**) and incubated for 3 days at 37°C and 5% CO_2 . On day 3, a medium change was performed by removing half of the media from the well and replenishing with the same volume of fresh differentiation medium. the cells incubated for an additional 2 days at 37°C and 5% CO_2 . On day 5, as in a previous step, the medium was changed, and the cells incubated for an additional 2 days. On day 7, (immature MoDCs stage) immature dendritic cells collected, centrifuged, resuspended, and counted. then immature MoDCs cells (1×10^6 cells) proceeded for detection of cell surface marker CD11c expression by flow cytometry. The cells also examined microscopically for the determination of the morphology of the cells on day 7. To complete the MoDCs maturation process, dendritic cell maturation induced by addition recombinant human $\text{TNF-}\alpha$ (1X) to the cell suspension (**Vopenkova et al., 2012**). the cells incubated for an additional 3 days at 37°C and 5% CO_2 . On day 10, CD14^+ monocyte-derived DC maturation induced with addition of human $\text{TNF-}\alpha$ as maturation agent and incubation the cells at 37°C and 5% CO_2 for an additional 3 days. On day 10, mature dendritic cells can be observed and are ready to be used in the desired application.

Results

1. Viability and morphology

Examination of cultured CD14^+ enriched with human differentiation media over a 7-day (immature) or a 10-day (with maturation) period shows a variation in size and morphological appearance (**Figure1**). Typical yields of immature (at day 7) and mature (at day 10) DC are approximately 60% and 50% respectively of the starting cell number with viability degree 90%.

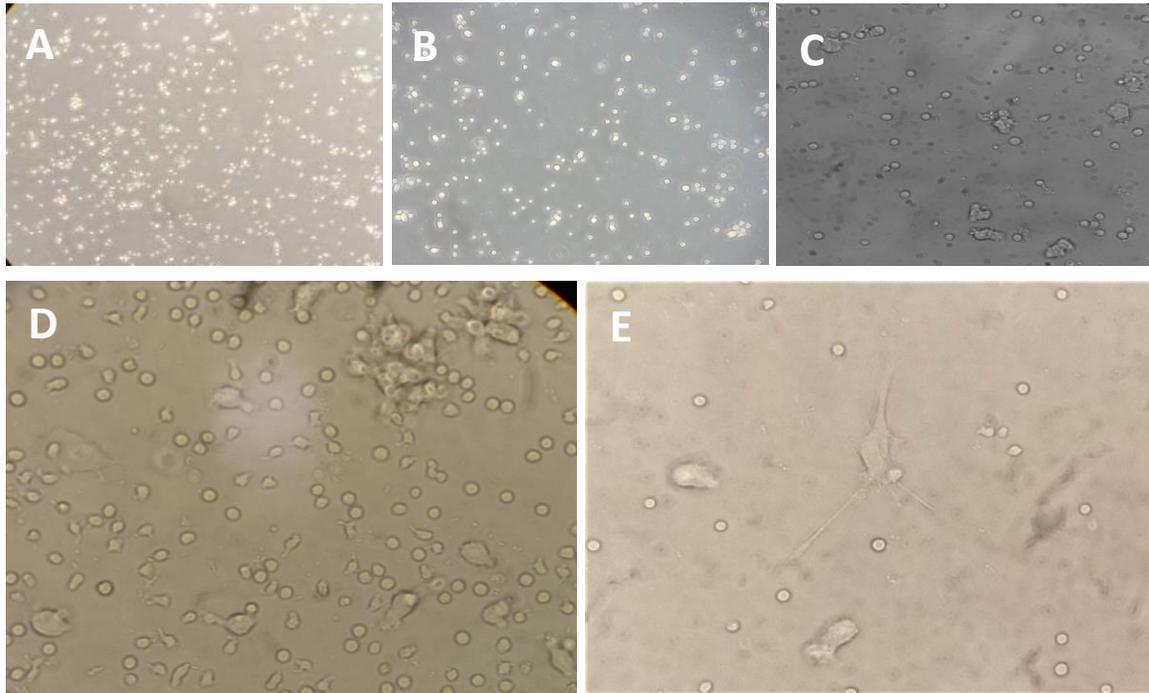


Figure 1 | Morphology of monocyte-derived Dendritic Cells (moDCs) cultured in Differentiation Media for 10 days: A)day 0 ,B)day 3, C)day 5, D)day 7 . Immature moDC appear as irregularly outlined cells, E) Day10: mature monocyte-derived Dendritic Cell (moDC) with multiple dendrites like structures on the surface.

2 Monocyte-derived dendritic cell surface marker detection.

2.1 Gating strategy of dendritic cells isolate at 5ul APC-conjugated anti CD11c .

Initial gating on dot plot graph using forward and sideways scatters (FS/ SS). Gating was specified to Monocytes expected region (**D**) then cells were examined on quadrant plot in CD11C APC were detected on X axis of the quadrant plot. DCs subsets were defined according to the surface markers expression as CD11C APC positive only. According to our strategy DCs population was in Lower Right (LR) quadrant of the plot, referred to by Purple color and named (**F**). Samples reintroduction to flow cytometry devise to assess purity after centrifugation and resuspending in 1ml PBS Percentage of DCs in MCs mixture regarding to 5ul concentration of monoclonal antibodies was 6.44%. (**Figure 2**).

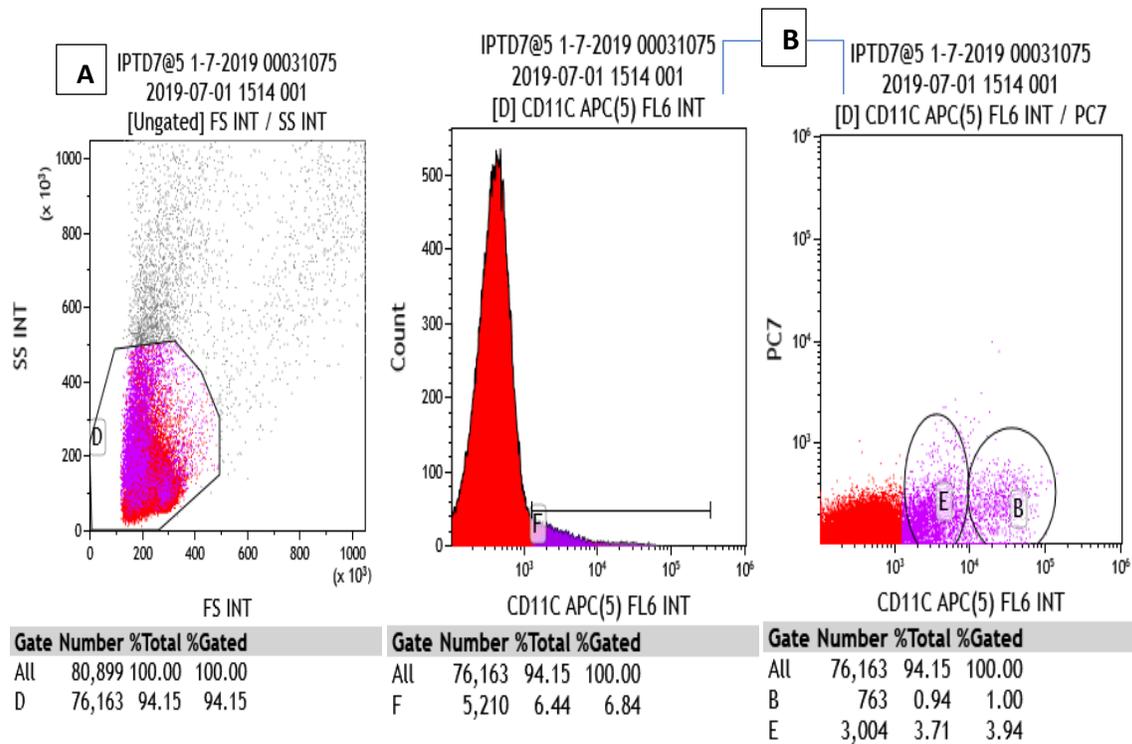


Figure 2 | Illustrates Phenotype analysis for DCs and purity for DCs isolate at 5 µl of anti CD11c antibody [A] Dot plot for initial gating of monocytes region on FS/ SS with extending the gating region to include small and large sized monocytes (D); [B] Detection of DCs. DCs were defined as (CD11C APC positive only) .

2.2 Gating strategy of dendritic cells isolate at 10 µl APC-conjugated anti CD11c .

Initial gating on dot plot graph specified to monocytes expected region (D) then cells were examined on X axis CD11C APC of the quadrant plot. DCs subsets were defined as CD11C APC positive population in Lower Right (LR) quadrant of the plot, referred by orange color and named (G). Samples reintroduction to flow cytometry assess purity (Percentage of DCs in MCs mixture) after centrifugation and resuspending in 1ml PBS regarding to 10µl concentration of monoclonal antibodies was 3.88 %. (Figure 3).

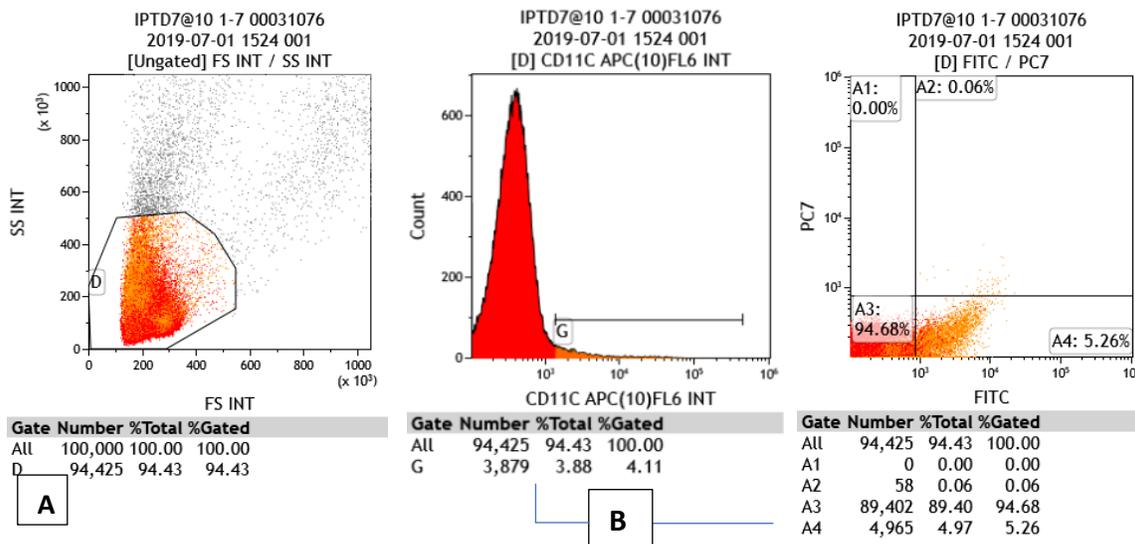


Figure 3 | illustrates phenotype analysis for DCs isolate and purity at 10 μ l of anti CD11c antibody [A] Dot plot for initial gating of monocytes region on FS/ SS with extending the gating region to include small and large sized monocytes (D); [B] Detection of DCs. DCs were defined as (CD11C APC positive only) .

Discussion

Dendritic cell (DC) are critical regulator of the innate and adaptive immune responses since they are present in different subsets, as either resident or migrating cells, in lymphoid and non-lymphoid organs (Macri *et al.*, 2018). Currently, studies on mouse DC outnumber those on human cells (Cavaliere *et al.*, 2010). however, the comparison between mouse and human models has been slightly misleading due to the basic biological and practical differences between the two models since many experiments performed in the mouse models are difficult to corroborate in humans. Thus, some of the assumptions made about human DC are turned from small animal experiments that have not been confirmed in man and may not necessarily apply (Ju, 2010). moDCs are a subset of DCs widely used in immunological studies as a convenient and easy approach after isolation of mononuclear cells directly from circulation (Fol *et al.*, 2016). Also, using of moDCs instead of CD34+ derived DCs precursor is preferable this is due to the limited number of CD34+ precursors that can be isolated from apheresis products (Sabado and Bhardwaj, 2010). As such, the present study was conducted for generation and phenotypic characterization of human moDCs in vitro from peripheral blood CD14+ monocytes.

The generation of moDCs from a human is a multistep process. Firstly, DC precursors must obtain from the host. Although blood or bone marrow may serve as the initial source, PBMCs are the most common origin (Constantino *et al.*, 2017; Itoh *et al.*, 2002). The present study has used (with minor modification) Ficoll-based purification of mononuclear cells (CD14+ monocytes) from whole blood to serve as a precursor for DCs

(Gill, 2019). For maximum reproducibility of separation, only freshly isolated blood samples (2-4 hours after collection) were used for isolation to ensure high viability of PBMC, blood samples were also diluted in 1x PBS at 1:1.4 ratio to reduce RBC aggregation as well as freeing trapped PBMC, the volumetric ratio of sample to Ficoll was 3:7, Ficoll/diluted blood mix centrifuged at various speeds with optimal centrifugation at 1600 rpm (400xg) for 30 min at 20°C with no brakes based on PBMCs yield (ranges between 0.5-3 x 10⁶ cells per mL blood) with efficient RBC removal, optimal centrifugation temperature of 20°C gives optimal results since higher temperatures (37°C) enhance RBC aggregation with yield reduction of the mononuclear cell count, while lower temperatures (4°C) inhibit aggregation, decreasing purity. To remove platelets from the mononuclear cell fraction, PBMC Centrifuged at low speed 200 xg (1000 rpm for 10 min).

After obtaining the precursor cells (CD14+ monocytes) from 10 individual PBMC samples from healthy blood, PBMC are plated in 6 well plate (1 x 10⁶ cells per well) to expand and differentiate. PBMCs initially cultured in differentiation medium supplemented with granulocyte monocyte colony-stimulating factor (GM-CSF) and interleukin 4 (IL-4). This induces the “immature” DC phenotype. The immature cells subjected to a further differentiation step, to generate the “mature” DC by supplementing the medium with tumor necrosis factor α (TNF- α). The process of generation typically takes 7 days to generate immature DCs, and another 3 days to generate mature DCs (Amedei *et al.*, 2011; Nicolette *et al.*, 2007). Morphology of immature moDC cultured in differentiation media in day 7 appear as irregularly outlined cells, while mature monocyte-derived Dendritic Cell (moDC) in day 10 appears with multiple dendrites like structures on the surface.

Following culture in monocyte-derived differentiation media, day 7 monocyte-derived Dendritic Cell (moDC) stained with different concentration of specific anti CD11 monoclonal antibody. Stained cells were analyzed by using FACSCalibur™ to evaluate immunophenotype form of moDC. Also, to assess the purity of dendritic cells in the sample. At day 7, FACS analysis demonstrates a typical CD11c+ve cells monocyte-derived Dendritic Cell (moDC) with a density 5210 cells/ml represents a purity percentage 6.44% at concentration 5 μ l of APC-conjugated anti CD11c. While staining the monocyte-derived DC with 10 μ l of APC-conjugated anti CD11c demonstrates CD11c+ive moDC with a density 3820 cells/ml represents purity percentage 3.88% which indicates that the lower concentration of APC-conjugated anti CD11c was favorable at staining with more cell density and purity than higher concentration. Continuing culture of moDC in differentiation medium supplemented with a human TNF- α as maturation promoting factor for additional 3 days (day 10) induce the cell maturation that demonstrated an increase in size and acquire a unique morphological appearance with multiple cytoplasmic projections on the moDC surface when compared to their precursor cells such as monocytes or the morphology at day 7 which subsequently ready to be used in downstreaming desired application as in the development of cancer immunotherapies as well as the treatment of autoimmune diseases.

In conclusion:

This study provides one of the approaches used to generate and characterize of human DC in vitro from peripheral blood CD14⁺ monocytes by using differentiation and maturation promoting factors (GM-CSF, IL-4 and TNF- α) in 10 days. Although ex vivo methods have been developed for generating DCs, there is no current acclamation on the optimal ex vivo method. This is an important issue for future research to directly compare all these methods of DC generation. Also studying of ex vivo-generated monocyte-derived DCs stimulation to antigen-specific T cell responses at different aspects is also needed to be developed.

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عزل خلايا الدندرتك المشتقة من وحيادات النواه وتوصيف النمط الظاهري.

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المخلص العربي

تعتبر خلايا الدندرتك dendritic cells واحده من اهم الخلايا الازمه لتنشيط وتنظيم الاستجابات المناعية ، حيث تعتبر حلقة الوصل بين الاستجابة المناعية الفطرية والمتواسطه بالخلايا. أدت قدرة خلايا الدندرتك على حث أو تنظيم / قمع الاستجابات المناعية إلى الانتباه في استخدامها كأحد الأدوات القوية للعلاج المناعي في أنواع الأمراض المختلفة.

كان الهدف من هذه الدراسة المقدمه هو إنتاج خلايا الدندرتك عبر اشتقاقها من خلايا أحادية النوي في الدم المحيطي CD14+ enriched peripheral blood monocytes (PBMCs) بعد استزراعها باستخدام وسط التمايز الغذائي تحتوي علي عوامل لتعزيز النضج ومتابعة النمو والتمايز علي مدار عشرة أيام ثم تحديد النمط المظهري (من خلال الكشف علي بعض المستقبلات السطحيه للخلايا) والتعرف المورفولوجي لها. **معملياً**: تم جمع عينات الدم المحيطي من المتبرعين الاصحاء وذلك لتنقية وتمايز الخلايا احاديه النواه (CD14 +Ve) والتي تمثل المنشأ الأكثر شيوعاً لسلائف خلايا الدندرتك وذلك اعتمادا علي استخدام الفيكول للفصل متدرج الكثافه لوحيادات النوي عن طريق الطرد المركزي ثم استخدام وسط التمايز الغذائي لتوليد خلايا الدندرتك خلال فتره زمنييه محدد. **بعد عزل PBMCs** ، تم تحديد حيوية وإنتاجية الخلايا ، تم الكشف أيضا عن وجود بعض المستقبلات السطحيه المميزه للخلايا الدندرتك المشتقة من الوحيادات عن طريق استخدام تحليل التدفق الخلوي (flowcytometric analysis) بعد صبغ وتحضين الخلايا مع أضداد وحيدة النسيلة متخصصه لبعض المستقبلات السطحيه.

أخيراً ، تشكل عمليه زراعة وتمايز الخلايا الأحادية النوي إلى DC ونضجها اللاحق إلى خلايا الدندرتك معمليا خطوة أولى حاسمة لمختلف التحليلات المتتاليه والتي تتراوح بين فهم كل من بيولوجيا خلايا الدندرتك البشرية ووظيفتها إلى استخدامها في التشخيص السريري وكذلك لتصميم علاجات مناعيه متخصصه لمختلف الأمراض.

الكلمات المفتاحية : الخلايا الأحادية النوي. الخلايا الدندرتك ، الخلايا العارضة للمستضد. فصل الفيكول متدرج الكثافه.