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DC Maturation: A Brief Comparison between Three Different Processes

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Abstract

Dendritic cell (DC) maturation approved to be a pivotal process for initiating immunity. Many protocols were established in order the isolated peripheral blood mononuclear cells (PBMCs) from healthy donors to mature into dendritic cells (DCs). The purpose of this study was to present an effective and reliable DC maturation procedure by comparing three different protocols (Interleukin-4/Tumor Necrosis Factor-appha (IL-4/TNFa) DC protocol, Interferon alpha (IFNa) DC protocol and FAST DC protocol). Whole blood was collected from six healthy donors and PBMCs were isolated by Ficoll gradient centrifugation. The counted cells were incubated with the addition of three different cocktails of supplements for appropriate time period. The final mature DC population was examined either by its phenotypic characteristics under light microscope or by measuring the expression of antigen presenting molecules such as CD80 and CD86 by flow cytometry. It was found that the mature DCs, generated from the IL-4/TNFa DC protocol, expressed higher levels of CD80 and CD86. Furthermore, they sharply exhibited their phenotypic hallmarks.

Keywords

DC Maturation, TNFa, IL-4, IFNa

1. Introduction

DCs are exceptionally powerful initiators of immunity, with the ability to activate an immune response more potently than any other cell in the immune repertoire. Because of this unique hallmark, DCs are now a major focus of laboratory and clinical study and are critical targets in cancer vaccine development [1] [2]. Cancer malignancies are often treated by administering a DC vaccine during the premalignant stages, prior to development of immune suppression [3].

As it is well-known, DCs are antigen-presenting cells of the mammalian immune system. Their main function

is to process antigen and introduce it on the cell surface to the T cells of the immune system. They act as messengers between the innate and adaptive immune systems. Naturally, they are present in those tissues that are in contact with the external environment, such as the skin and the inner lining of the nose, lungs, stomach and intestines. DCs found in the blood are characterized as immature DCs (iDCs) [4]. Once they activated, they migrate to the lymph nodes where they interact with T cells and B cells to initiate and shape the adaptive immune response. The difference between iDCs and mature DCs is that the second one grows branched projections, the dendrites that give the cell its name while the iDCs called veiled cells as they possess large cytoplasmic "veils" rather than dendrites. The DC population is categorized into three main categories, each of which involves antigen presentation. The first category included the antigen presentation and activation of T cells. The second category of DC function is not well established, but it has been suggested that a different class of DCs exist with the hallmark of inducing and maintaining immune tolerance. The third population, known as follicular DCs, appears to work to maintain immune memory in tandem with B cells [5] [6].

Many approaches have been established for the generation of DCs. Culturing CD34+ or CD14+ cells with the presence of cytokines are two well characterized methods [7] [8]. A novel approach to generate DCs *in vivo* includes the usage of Flt3-ligand [9].

The presence or absence of molecules on DC's surface is the key on their definition. They express co-stimulatory molecules including CD80 (B7.1), and CD86 (B7.2), which are upregulated during DC activation. CD86 tends to be a marker of early DC maturation, while CD80 only appears in mature DCs [10].

In order to examine the maturation of DCs, we compared three different populations obtained after performing three different experimental procedures. Monocyte derived DCs were prepared by the elutriation of monocytes from PBMCs followed by incubation in 1) X-VIVO 20 medium containing IL-4 and Granulocyte-macrophage colony-stimulating factor (GM-CSF) for 5 days. The resulting immature DC were further treated by a 2 day culture step with fresh medium X-VIVO 20 medium containing TNFa and GM-CSF, 2) serumfree X-VIVO 20 medium supplemented with IFN-α and GM-CSF for 3 days and 3) RPMI 10% Fetal Bovine Serum (FBS) medium containing GM-CSF and IL-4 for 24 h followed by an another 24 h incubation with TNFa, Interleukin-1 beta (IL-1β), Interleukin-6 (IL-6), prostaglandin 2 (PGE2) and CD40 ligand (CD40L). To evaluate the effectiveness of these DC maturation cocktails the three different populations of mature DCs were compared by measuring the expression of CD80 and CD86 molecules by flow cytometry. We also compared the mature DCs by comparing their phenotypic hallmarks under light microscope.

2. Materials and Methods

2.1. Human PBMC Isolation and Counting

In order to fractionate 10 mL of EDTA—treated peripheral blood (Vacutainer K3E 7.2 mg, Bd), a 50 mL conical tube (430291, Corning) with 10 ml Biocol separating solution 1077 (L6115, Biochrom) was used. The isolated cells measured by using NC-100+ NucleoCounter system (Chemometec, Denmark) and were subsequently cultured in 12 well plate (3513, Costar) (1 - 2 × 10^6 cells/well) in RPMI 1640 medium (R0883, Sigma) supplemented with 10% FBS (10270-106, Gibco) and 2 mM L-glutamine (G7513-100ML, Sigma).

2.2. DC Generation

To generate mature DCs the isolated PBMCs incubated 24 h at 37˚C with the presence of 5% CO_2. The day after plating the supernatant was removed and the detached cells (monocytes) were supplemented with the following proinflammatory mediators:

- IL-4/TNFa DC protocol: serumfree medium (X-VIVO 20 medium, BE04-448Q, LONZA) containing 500 U/ml IL-4 (204-IL-010, RnD) and 800 U/ml GM-CSF (04-RHUGM-CSF-300MCG) for 5 days. The resulting immature DC were further treated by a 2 day culture step with fresh medium X-VIVO 20 medium containing 1000 U/ml TNF-α (300-01A, PeproTech) and 800 U/ml GM-CSF.
- IFNa DC protocol: serumfree X-VIVO 20 medium supplemented with 1000 U/ml IFN-α (Interferon-a A/D Human Recombinant, 11200-1, PBL) and 1000 U/ml GM-CSF for 3 days.
- FAST DC protocol: monocytes were cultured with 1000 U/ml GM-CSF and 500 U/ml IL-4 for 24 h followed by an another 24 h incubation with 1000 U/ml TNFa, 10 ng/ml IL-1β (Recombinant Human IL-1β/IL-1F2, 201-LB), 10 ng/ml IL-6 (Recombinant Human IL-6 - 10 µg, RnD), 1 M PGE2 (Prostaglandin E 2—1

mg, P5640, SIGMA) and 500 ng/ml CD40L (Human CD154 (CD40 Ligand) Recombinant Protein, 14-8502, eBioscience).

The final volume in the three wells was 3 ml.

2.3. PBMC's Evaluation

In order to evaluate the PBMC population, approximately 106 cells were incubated in 100 μL of Phosphate Buffer Saline (PBS, P3813, Sigma), pH 7.4, along with FITC-conjugated anti-CD14 antibody (anti-human CD14 FITC, 11-0149-42, eBioscience) at room temperature for 25 minutes at dark. Cells washed once with PBS/2% FBS and then once with PBS and finally analyzed by flow cytometry using FACS Calibur flow cytometer (BD Biosciences).

2.4. DCs Markers Measurements by Flow Cytometry

After decanting the supernatant, the cells were washed once with PBS. In order the cells to be detached, 0.5 ml 0.25% Trypsin-EDTA (1X) (25200-072, Life Technologies), was added and cells were incubated for 5 min in 37˚C, 5% CO_2. The detached cells were washed once with 0.5 ml FBS and 9.5 ml PBS. A further washing with 10 ml of PBS was performed. The washed cells were stained with anti-human CD80 (MHCD8004, Life Technologies) conjugated with R-phycoerythrin (R-PE) and CD86 (MHCD8601, Life Technologies) conjugated with Fluorescein isothiocyanate (FITC). Cells were analyzed on a FACSCalibur flow cytometer (BD Biosciences). Data were analyzed using CellQuest (BD Biosciences; version 3.2.1) software.

2.5. Microscopical Overview

After the recommended incubation time of its protocol, the cells were observed an inverted microscope (Primovert, Carl Zeis).

3. Results

3.1. Donor Characteristics and PBMC Numbers

Different amount of peripheral blood was collected from six individuals, seven females and one male who all were healthy. Their age ranged from 22 to 30 years. For monocyte isolation the blood was freshly isolated. To prevent coagulation, the samples were collected in tubes (BD Falcon 12 × 75 mm style 5 mL Polystyrene Round Bottom Tube, 352054, Becton Dickinson Greece) containing ethylene diamine tetraacetic acid and rotated for approximately 20 min before testing. Consent was provided by all donors. The PBMC's concentration ranged from $0.81*10^6$ to $4.42*10^6$ cells/ml depended either on the blood volume or on the fact that was collected from different donors (**Table 1**).

Mononuclear cells were detached to the wells after 24 h of incubation. They are round in shape and different from the mixed PBMC population which was both in suspension and/or detached in a cell culture (**Figure 1**).

Monocytes were stained with CD14-FITC antibody in order to evaluate their purity. Flow cytometric results, concerning the donor D, are indicatively shown below (**Figure 2**). Monocytes are 98.27% positive to CD14 marker as represented on the below histograms.

3.2. DC Evaluation/Donor

DCs population was evaluated after the end of the incubation time of each protocol by observing the formation of dendrites in a culture as well as by analyzing the expression of CD80 and CD86 molecules by flow cytometry.

Donor A and Donor B. Firstly, two different populations from two different donors (donor A and donor B) were evaluated by performing separately the three different protocols mentioned above (IL-4/TNFa DC protocol, IFNa DC protocol and FAST DC protocol). Comparing the formation of dendrites, it was clearly observed the highly percentage of maturation of monocytes into DCs after performing the IL-4/TNFa DC protocol instead of performing either the IFNa DC protocol or the FAST DC protocol (**Figure 3**). Concerning the flow cytometric results, the IL-4/TNFa DCs expressed 6.82% CD80/CD86 when IFNa DCs expressed 2.57% and FAST DCs expressed 2.67% (**Figure 4**).

Table 1. PBMC's numbers and donor characteristics.

Donor ID	Age	Gender	Blood collection (ml)	Total PBMC number (cells)	PBMC concentration (cells/ml)
A	30	F	10	9,900,000	$1.98*10^6$
B	26	F	7.5	8,100,000	$1.62*10^6$
C	22	F	14	16,350,000	$3.27*10^6$
D	29	M	18	44,250,000	$4.42*10^6$
E	22	F	10	3,645,000	$0.81*10^6$
F	22	F	10	4,200,000	$0.84*10^6$

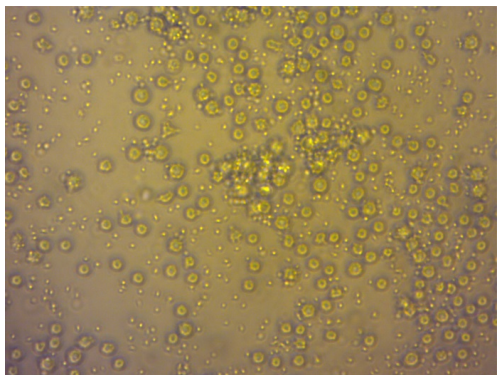

Figure 1. Monocytes detached to the flask after 24 h of incubation. They were isolated from donor E and cultivated in RPMI-1640 medium.

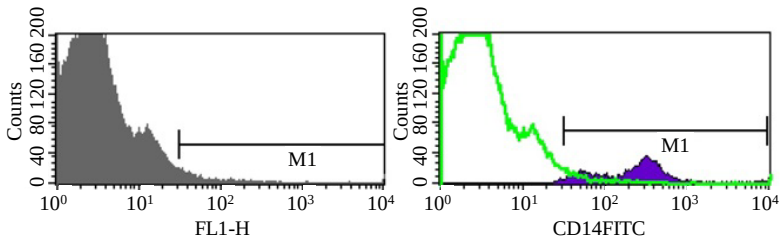

Figure 2. CD14 expression in monocytes isolated from donor D. The cells expressed 98.27% of CD14 marker when compared with a negative (unstained) control. The first (grey) histogram is referred to the unstained (control) sample when the second (purple) histogram is referred to the sample labeled with the CD14 antibody.

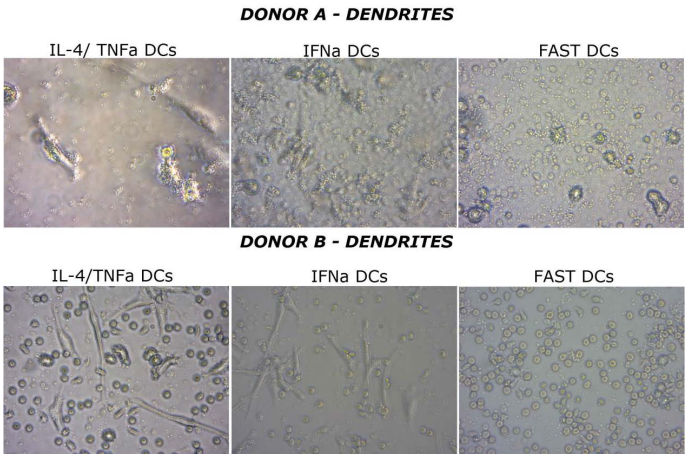

Figure 3. Donor A & B. The above triplicate of photos represents the DCs obtained from the three protocols respectively when the below triplicate is referred to the donor B.

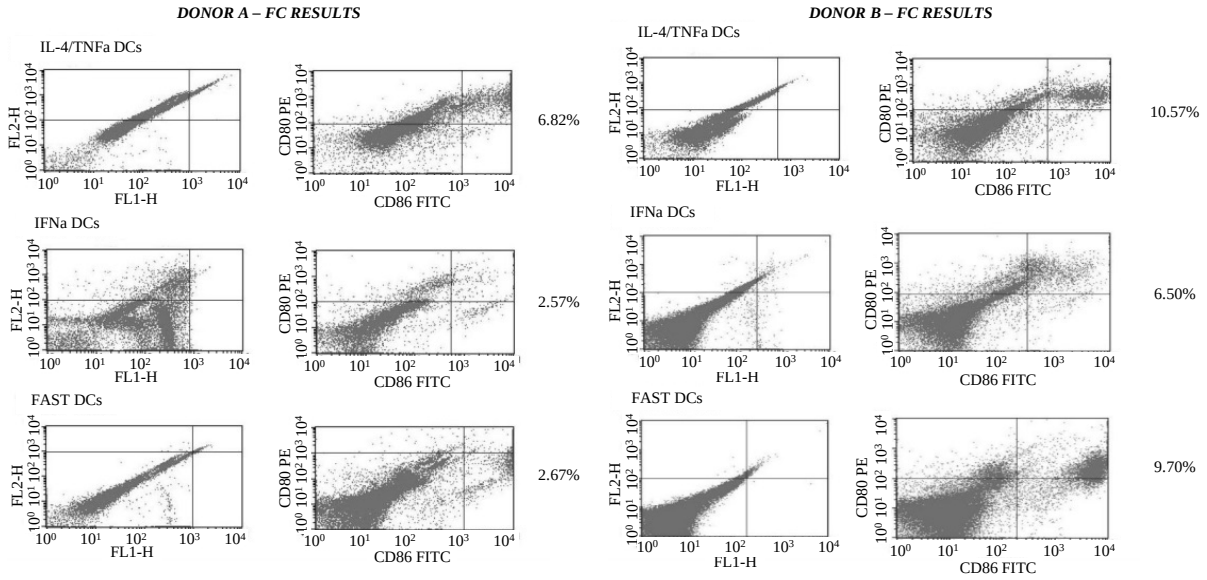

Figure 4. Flow cytometric results for CD80/CD86 markers. Donor A & B. They are represented three different duplicates of photos for each donor. The first photo, from each protocol, represents the unstained sample (control) when the second represents the sample stained with the CD80 and CD86 antibodies. The expression level of the markers (percentage) is referred in red behind each duplicate.

Donor C and Donor D. Secondly, another two different populations isolated from two different donors (donor C and donor D), were evaluated by performing separately two maturation protocols (IL-4/TNFa DC protocol and FAST DC protocol). The resulting IL-4/TNFa DCs were more efficient than the FAST DC population (**Figure 5**) when the flow cytometric results shown 9.68% and 9.63% expression of CD80/CD86 respectively for donor C and 2.84% and 2.09% respectively for donor D (**Figure 6**).

Donor E and Donor F. Thirdly, the final two different populations isolated from two different donors (donor E and donor F) were evaluated by performing only the IL-4/TNFa DC protocol only. The resulting mature DCs were clearly observed in **Figure 7**. The CD80/CD86 expression was 18.44% for donor E and 15.62% for donor F (**Figure 8**).

The total flow cytometric-based results were listed in **Table 2**. The percentages referred to each donor separately.

4. Discussion

DC vaccination has shown to be a very promising model in treating cancer. According to its classification, mature and not immature DCs are responsible for antigen presentation and T cell activation. The immune response is generated by presenting the captured antigen to cells of the adaptive immune system. DCs have shown to be the most efficient antigen presenting cells (APCs) because they have the ability to elicit very low numbers of T cells to respond [11].

The maturation of DCs is tended to be a simple concept that is rendered complex by the likelihood that not all mature (or activated) DCs are equivalently immunogenic. The previous process belongs to the *in vivo* studies where they differentially respond to every environmental signal and differentiate into mature DCs that can efficiently launch immune responses [12] [13].

The present study attempted to focus on the DC maturation process *in vitro*. For that reason, three different processes were followed. The first protocol included the usage of TNFa and GM-CSF supplements in the presence of X-VIVO medium. [14] [15]. It has been shown by the scientific community that the role of GM-CSF in DC's development appears situation—as well as subset-specific. Under steady-state conditions, GM-CSF supports migratory DC development, whereas its effect on other subtypes c (according to their localization, phenotype, and immune function) is either redundant or even detrimental [16].

The effect of GM-CSF is strongly correlated with other cytokines including IL-4 in the context of physiologic or pathologic conditions. Various cell populations such as T helper 2 lymphocytes, CD3/CD56 bright natural

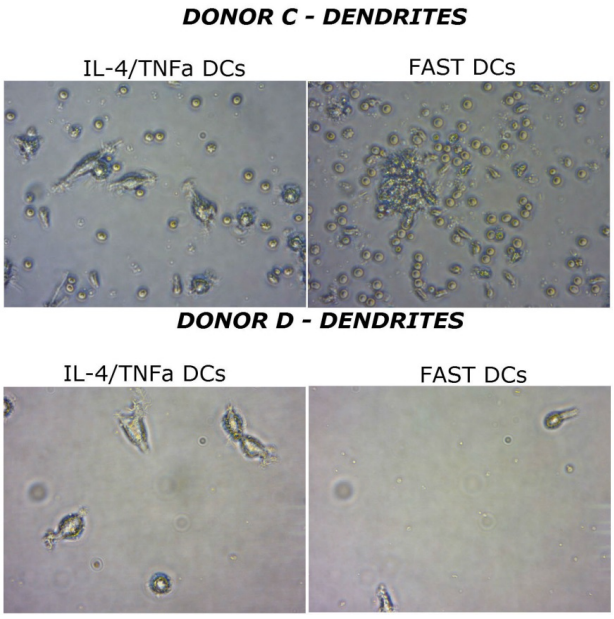

Figure 5. Donor C & D. The above duplicate of photos represents the DCs obtained from the two protocols respectively when the below duplicate is referred to the donor D.

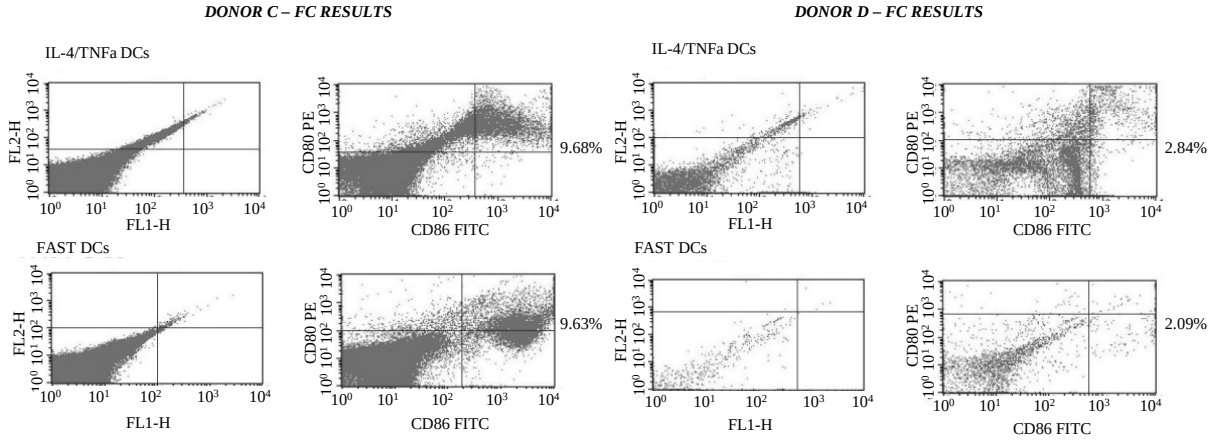

Figure 6. Flow cytometric results for CD80/CD86 markers. Donor C & D. They are represented two different duplicates of photos for each donor. The first photo, from each protocol, represents the unstained sample (control) when the second represents the sample stained with the CD80 and CD86 antibodies. The expression level of the markers (percentage) is referred in red behind each duplicate.

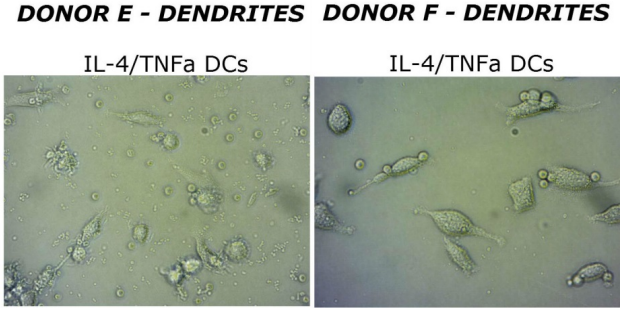

Figure 7. Donor E & F. The above duplicate of photos represents the DCs obtained from the IL-4/TNFa DC protocol for donor E and F respectively.

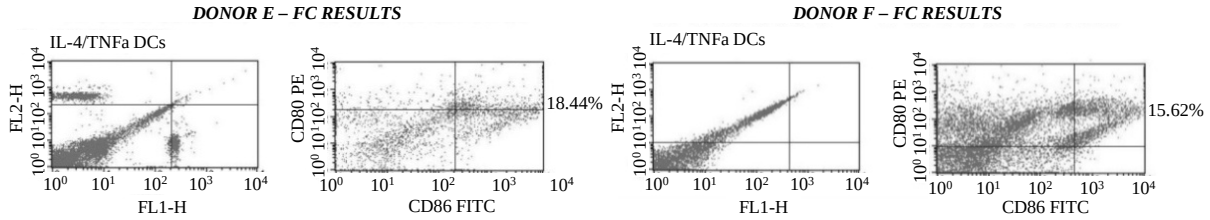

Figure 8. Flow cytometric results for CD80/CD86 markers. Donor E & F. They are represented a different duplicate of photos for each donor. The first photo, from each protocol, represents the unstained sample (control) when the second represents the sample stained with the CD80 and CD86 antibodies. The expression level of the markers (percentage) is referred in red behind each duplicate.

Table 2. Percentage of CD80/CD86 expression/donor.

Donor ID	% CD80/CD86 EXPRESSION		
	IL-4/TNFa DCs	IFNa DCs	FAST DCs
A	6.82	2.57	2.67
B	10.57	6.50	9.70
C	9.68	Not tested	9.63
D	2.84	Not tested	2.09
E	18.44	Not tested	Not tested
F	15.62	Not tested	Not tested

killer cells, mast cells and keratinocytes have been demonstrated to synthesize the cytokines required for monocytic differentiation into DCs, including GM-CSF and IL-4. From the established cytokine cocktails that can induce differentiation of human monocytes into DCs *in vitro*, the most authentic and established cytokine combinations may be GM-CSF and IL-4 [17]-[22].

In order to be investigated the potential role of TNFa in DC biology, A W T van Lieshout *et al*. inhibited its function by using a p55 soluble TNFa receptor (PEGsTNFRI). They have found that the neutralisation of TNFa during dendritic cell maturation leads to the development of semi-mature dendritic cells [23] [24]. The above results are well fitted with the results exported by our experiments. The DCs obtained from the IL-4/TNFa protocol were well matured and form dendrites in their culture after s specific incubation time.

Due to the central role played by IFNs in the initiation of innate and adaptive immune responses, the effects on maturation of DCs have been widely studied. It has been shown that the IFNa induces differentiation of monocytes into DCs leading to the secretion of large amounts of IL-12 [25] [26].

This function is characterized by upregulation of MHC-II and CD86. DCs that undergo maturation in response to IFNa may be less kinetically restricted in the acquisition and processing of Ag for presentation to T cells [27].

The morphology of antigen-presenting dendritic cells (DC) is characterized by the possession of numerous long arborizing processes known as dendrites. This formation may contribute to the remarkable efficiency with which they take up, process and present antigen to T cells [28]. Well-formatted dendrites have been defined in DCs obtained from IL-4/TNFa protocol. The other two protocols gave dendrites sporadically.

DCs also express costimulatory molecules including CD80 (B7.1), and CD86 (B7.2), which are upregulated during DC activation. CD86 tends to be a marker of early DC maturation, while CD80 only appears in mature DC. In addition, the more immuno-deficient phenotype of the CD86 knockout mouse has generated a strong perception that CD86 is the more important costimulator of T cell activation [29]. The higher level of CD80+/CD86+ DCs was found in IL-4/TNFa DCs where we had the best well formatted dendrites.

5. Conclusion

It has been shown that by performing the three different protocols for DCs maturation (IL-4/TNFa, IFNa and FAST DC protocol), we obtained DCs population. The best well formatted dendrites were shown in IL-4/TNFa DC cultivation where we had the higher level of CD80 and CD86 markers.

Author Contributions

S.H.C. conceived the study, designed experiments and interpreted the results; M.T. developed analytical tools and wrote the paper; N.C. analyzed data and wrote the paper.

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List of Abbreviations

DC: Dendritic Cell
PBMCs: Peripheral Blood Mononuclear Cells
IL-4: Interleuki-4
TNFa: Tumor Necrosis Factor-Alpha
IFNa: Interferon Alpha
iDCs: immature DCs
GM-CSF: Granulocyte-Macrophage Colony-Stimulating Factor
FBS: Fetal Bovine Serum
IL-1β: Interleukin-1 Beta
IL-6: Interleukin-6
PGE2: Prostaglandin 2
CD40LCD40: Ligand
PBS: Phosphate Buffer Saline
FITC: Fluorescein Isothiocyanate
R-PE: R-Phycoerythrin