



***In vitro* generation of human monocyte-derived dendritic cells methodological aspects in a comprehensive review**

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Manuscript received on March 15, 2019; accepted for publication on June 1, 2019

How to cite: CECHIM G AND CHIES JAB. 2019. *In vitro* generation of human monocyte-derived dendritic cells methodological aspects in a comprehensive review. *An Acad Bras Cienc* 91: e20190310. DOI 10.1590/0001-3765201920190310.

Abstract: Dendritic cells (DCs) initiate and shape both innate and adaptive immune responses. They are specialized in antigen presentation to naive T cells, thereby orchestrating the T cell immune responses. Human peripheral blood and tissues contain several subsets of phenotypically and functionally distinct DCs, which promote interactions between the external environment and lymphoid organs. Because of the difficulty in purifying these cells, *in vitro* studies only became more frequent when Frederica Sallusto and Antonio Lanzavecchia developed a method to generate DCs from blood monocytes *in vitro*. Nowadays a wide range of biotechnological innovations has allowed the study of DCs and their precursors in the most diverse situations faced by the immune system. As a result of such studies, monocyte-derived dendritic cells (MDDC) are presently used in clinical protocols for the treatment of a variety of diseases, including cancer and human immunodeficiency virus infection. We summarize recent advances in the understanding of methodologies and inputs used in protocols to differentiate DCs from blood monocytes *in vitro*.

Key words: Cytokines, dendritic cell, differentiation, monocytes.

INTRODUCTION

In 1973, Ralph M. Steinman and Zanvil A. Cohn described a new cell type that had a cytoplasm with pseudopodia structures of various sizes and shapes, giving the cell a starry aspect, much like neuronal dendrites (Steinman and Cohn 1973). This work described the cell currently known as a Dendritic cell (DC). In 2011, the importance of this discovery was recognized with the Nobel Prize in Physiology or Medicine (Steinman 2012). However, the study of these cells only had significant advances in the 1990's, when Frederica Sallusto and Antonio

Lanzavecchia first developed a method allowing the generation of DCs from blood monocytes. In this study, monocytes obtained from peripheral blood were differentiated into myeloid DCs in the presence of granulocyte-macrophage colony stimulating factor (GM-CSF) and interleukin (IL)-4, (Sallusto and Lanzavecchia 1994).

DCs are the major antigen presenting cells (APCs) due to their unique ability to activate naive T cells (Steinman and Witmer 1978, Banchereau et al. 2000). DCs are bone marrow derived, originating from both myeloid and lymphoid precursors and can encompass a range of cell types with several phenotypes and functions (Ardavin et al. 1993, Banchereau et al. 2000, Ueno et al. 2007,

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Merad et al. 2013). They are distributed throughout the body but are especially present in regions such as the skin and mucous membranes, promoting interactions between the external environment and lymphoid organs (Steinman 1991, Guermonprez et al. 2002, Granot et al. 2017, Worbs et al. 2017). Due to its migratory patterns, DCs are called “the Sentinels” of the immune system (Stockwin et al. 2000, Randolph et al. 2008, Merad et al. 2013, Worbs et al. 2017).

Generally, these cells are found inside tissues in a so-called “immature” state characterized by a high capacity to capture and process antigens and a limited capacity to stimulate T cells. In the immature state, DCs exhibit low expression of the co-stimulatory molecules CD80 and CD86, as well as a low expression of major histocompatibility complex (MHC) class II molecules. The presence of “danger signals” triggers morphological changes in DCs, inducing a process known as maturation, which is characterized by a reduction of the DC endocytic capacity and the increased expression of MHC class II and co-stimulatory molecules (Banchereau et al. 2000, Joffre et al. 2009, Dalod et al. 2014, Worbs et al. 2017).

Considering this cell type diversity, DCs may be classified according to several criteria, including their location, such as skin, lungs, and lymphoid organs, since their function is intimately linked to the compartment where they are located (Wu and Liu 2007). Within this anatomical classification, it is also possible to separate DCs in two further categories: the migratory DCs, which migrate continuously from peripheral tissues to the draining lymph nodes via the afferent lymphatic vessels, and resident DCs, which are found in the tissues, and when activated, migrate to the draining lymph nodes (Randolph et al. 2008, Segura et al. 2012, Merad et al. 2013, Pulendran 2015). In this regard, DCs are usually classified according to their different abilities to drive the immune response by differential antigen presentation or by

modulating the immune system through cytokine secretion (Joffre et al. 2009, 2012, Kurts et al. 2010, Pulendran 2015). Nowadays DCs are evaluated according to their transcriptional profiles in order to understand the regulation of the development and differentiation of the distinct DC lineages (Belz and Nutt 2012, Hammer and Ma 2013, Merad et al. 2013, Collin and Bigley 2018).

Due to its central role in immune responses, a better understanding of the physiology and function of the DCs will help us to improve the use in clinical protocols to treat several diseases such as cancer and human immunodeficiency virus (HIV) infection (Banchereau et al. 2001, Turville et al. 2002, Kavanagh and Bhardwaj 2002, Gandhi et al. 2016, Garg et al. 2017).

This review presents key information about the generation of human DC from blood monocytes (MDDC). We will analyze alternatives for the purification of precursor cells (meaning the monocytes), the selection of suitable culture medium, appropriate culture medium supplements, growth factors, and cytokines. We will also review the most common molecular markers used to characterize DCs by flow cytometry. The Figure 1 presents a schematic summary of the steps in a monocyte-derived dendritic cell culture.

SOURCES OF MONOCYTES

The first step for monocyte-derived dendritic cell culture is to choose the monocyte source, since these cells are the preferred precursors for *in vitro* MDDC generation. One possibility is to directly collect blood by venipuncture. The advantage of whole blood as a monocyte source is the freshness of the material. However, the disadvantage 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 (Meyer et al. 2005, WHO 2010, Gillio-Meina et al. 2013).

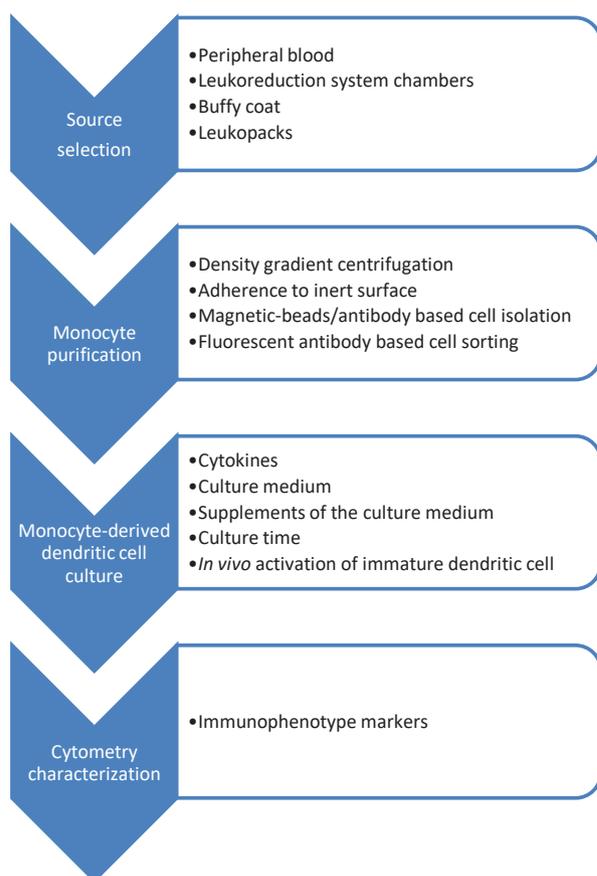


Figure 1 - Scheme of the steps of a MDDC culture.

The use of the cells, which are normally discarded after the processing of donated blood, is a way to skip such a problem. Commonly, after blood donation, the blood bag is centrifuged, which allows the separation of its components into erythrocytes, plasma, and a buffy coat, which are collected into separate bags. The buffy coat is a concentrate of platelets and leukocytes which can be further processed for platelet purification. The remaining material is rich in leukocytes and constitutes an excellent source of monocytes, but is commonly discarded. Processing a 450 mL blood bag usually generates 30–80 mL of buffy coat with approximately 1×10^9 cells (Ito and Shinomiya 2001, Repnik et al. 2003, Meyer et al. 2005, Strasser and Eckstein 2010).

When plasmapheresis is performed in order to obtain distinct blood products, leukocytes are often collected together with platelets as a byproduct and must be removed to avoid immune rejection in the recipient. Therefore, leukocytes are filtered out by leukoreduction and collected into leukoreduction system chambers (LRSC) or leukocyte removal filters also known as buffy cones (Ebner et al. 2001, Dietz et al. 2006, Strasser et al. 2007). The buffy cone usually contains 10 mL of a processed mixture with approximately 1×10^9 cells.

Another possible source for large quantities of leukocytes 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. Usually, leukopaks are collected from healthy donors, but for research purposes it is possible to obtain leukopaks from donors that present certain pathologies such as hematological malignancies and diabetes. 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 7×10^9 peripheral blood mononuclear cells (PBMC), (more information available on: www.allcells.com/products/whole-tissue/leukopak). The Table I shows the percentage of cell types and the amount of cells found in different monocyte sources.

MONOCYTE PURIFICATION

After choosing the source of cells, the next step in MDDC culture is to process the sample

TABLE I
Percentage of cells in whole blood and blood products in health individuals.

	Whole blood (%)	Buffy coat (%)	LRSC (%)	Leukopak (%)
CD4 ⁺ T cells	22.5	24.4	32.39	31,92
CD8 ⁺ T cells	6.8	10.8	15.04	17.13
B cells	5.2	7.2	11.33	12.52
Monocytes	8.4	10.7	20.28	19.08
NK cells	4.4	4.1	10.21	7.65
Eosinophils	3.2	1.3	1.88	1.07
Neutrophils	53.8	34.4	2.89	3.91
PBMC	2x10 ⁶ cells/mL	1x10 ⁹ cells	1x10 ⁹ cells	7x10 ⁹ cells/half

LRSC: Leukoreduction System Chamber. NK: Natural Killer. PBMC: Peripheral Blood Mononuclear Cells. Modified from: <https://www.miltenyibiotec.com/US-en/resources/mac3-handbook/human-cells-and-organs/human-cell-sources/blood-human.html>.

(Elkord et al. 2005). It is possible to use density gradient media to separate blood components. One of the most commonly used density gradient medium is Ficoll® Paque Plus (GE Healthcare Life Sciences – Catalog number: 17144003). Ficoll® Paque Plus is a synthetic sucrose polymer that ensures the separation of blood components as follows: erythrocytes and polymorphonuclear cells (eosinophils and neutrophils) are denser than Ficoll® Paque Plus and therefore are deposited on the bottom of the tube. Immediately above the erythrocytes a density gradient medium Ficoll® Paque Plus layer forms (density of 1.077 G/mL at 20°C). Above the Ficoll® Paque Plus layer, PBMCs form a layer of cells similar to a cloud, while the plasma is the uppermost layer in the tube (see Figure 2). The PBMC layer includes B and T lymphocytes, monocytes, NK (Natural Killer) cells, and dendritic cells (Meyer et al. 2005). This methodology is highly efficient and recovers around 95% of the mononuclear cells present in the original blood sample (Ito and Shinomiya 2001, Lehner and Holter 2002).

Since monocytes are 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 (Patel et al. 2017). Another distinct characteristic of monocytes is the ability to adhere

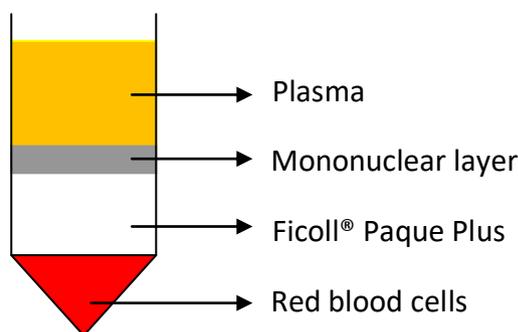


Figure 2 - Schematic diagram of the separation of the components of a blood sample from Ficoll® Paque Plus density gradient medium.

to inert surfaces like plastic, different from other cells present in the PBMC fraction (Patarroyo et al. 1988). Protocols that take advantage of this characteristic usually seed PBMC cells in a plastic flask with the appropriate culture medium (presented in the “Culture Medium” topic) and allow adherence for 2 hours 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.

Another system to isolate monocytes is the use of magnetic beads coated with specific antibodies. Such magnetic bead-based cell isolation methodology can be performed using both a positive or a negative selection approach. In a negative selection approach using the Dynabeads™

Untouched™ Human Monocytes Kit, for example, (Invitrogen, Norway, Catalog number: 11350D), a mixture of biotinylated monoclonal antibodies, each one of them specific against a distinct surface cell marker [anti-CD3 (a typical T lymphocyte antigen), anti-CD7 (a typical T- and NK-lymphocyte antigen), anti-CD16a and anti-CD16b (typical antigens expressed in T lymphocytes, DCs, NK cells, macrophages and granulocytes), anti-CD19 (expressed in B lymphocytes), anti-CD56 (a NK cell surface molecule), anti-CDw123 (a typical plasmacytoid DCs antigen) and anti-CD235a (a typical antigen expressed in erythrocytes and stem cell precursors)] is added to the sample. After that, beads coupled with streptavidin are added. Of note, these beads act as superparamagnetic particles, meaning that they exhibit magnetic properties when placed in a magnetic field. After a short incubation period, the streptavidin-beads will bind to the biotinylated-antibody-labeled cells. A magnet is applied to the sample and the cells linked to the biotinylated-antibody-streptavidin-beads will be attracted and will be immobilized by the magnet whereas cells not linked to any of these antibodies will be free in the supernatant and can be washed out and collected. Since the mixture of antibodies encompasses specificities against all PBMCs, but not against monocytes, this system will capture all non-monocyte cells. Importantly, no binding will occur between antibodies and monocytes, and therefore the isolated cells will be recovered after a relatively mild handling, without any activation mediated by antibodies.

Conversely, using a positive selection approach with anti-human CD14 MicroBeads from Miltenyi Biotec, Germany, (Catalog number: 130-050-201), for example, the cell sample is incubated with an anti-CD14 antibody bound to a magnetic bead. During this incubation period, the anti-CD14 antibody binds to the CD14-positive cells present in the sample and the cell suspension is loaded onto a column which contains a magnet that induces a

high-gradient magnetic field. Thus, the magnetically labeled CD14-positive cells are retained within the column and the unlabeled cells run through. After removing the column from the magnetic field, the magnetically retained CD14-positive cells are eluted. 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” (Joffre et al. 2009) capable of inducing a maturation process on immature DCs (more details in “Activation of Immature Dendritic Cell”). However, according to the manufacturer, the binding of an antibody to CD14 does not trigger signal transduction since CD14 alone lacks a cytoplasmic domain. (Akira and Takeda 2004, Liew et al. 2005, Napolitani et al. 2005). The advantages of using magnetic selection are high purity (>95%) and speed (Delirezh et al. 2013).

Another technical alternative for cell separation involves flow cytometry. Fluorescent antibody-based cell sorting is a specialized type of flow cytometry used in the isolation of a specific cell type from a heterogeneous mixture of cells. The use of specific antibodies labeled with distinct fluorochromes allied to data about cell size and complexity allows the sorting of cells based on the presence or absence of specific molecules on its surface. Moreover, this technique is the only one capable of distinguishing and separating cells with different levels of expression of a given molecule within a population. Thus, through flow cytometry it is possible, for example, to isolate CD14^{high}CD16⁻ cells from a complex sample like total PBMCs, in a single procedure. As a consequence, cells will be subjected to less handling and, therefore, will be exposed to less stress. In this sense, when the objective is the isolation of a cell population defined by multiple simultaneous features (meaning the presence/absence of several surface markers in distinct expression intensities) cell sorter flow cytometry is the technique of choice. However,

drawbacks of this technique include the high cost of both the equipment and the accessories needed to ensure operator biosafety due to aerosols formed during sorting (Holmes et al. 2014).

CYTOKINES AND DCs GENERAL FEATURES

Sallusto and Lanzavecchia (Sallusto and Lanzavecchia 1994) first achieved *in vitro* MDDC generation through medium supplementation with distinct cytokines, in this case, through the combined use of IL-4 and GM-CSF. In the following years, several works characterized different protocols concerning the use of distinct cytokines in order to induce and support *in vitro* monocyte differentiation and maturation in MDDCs. It is of crucial importance to note that different combinations of cytokines will generate MDDCs with distinct characteristics and functions, so we should choose the cytokines that best match the purpose of the work. The cytokines most frequently used for *in vitro* MDDC differentiation and the main characteristics of the generated DCs will be briefly reviewed in the next paragraphs.

Sallusto and Lanzavecchia used the combination of IL-4 and GM-CSF to promote the differentiation of monocytes into DCs. GM-CSF growth factor seems to down-regulate the expression of the macrophage colony-stimulating factor (M-CSF) receptor on monocytes, thereby inhibiting M-CSF induced differentiation of monocytes into macrophages (Suzuki et al. 2004). Similarly, IL-4 exerts its actions in monocytes differentiation by inhibiting macrophage colony formation (Jansen et al. 1989, Relloso et al. 2002). Reports also suggest that IL-4 can activate some properties of monocytes and up-regulate MHC class-II molecules, Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin (DC-SIGN) and co-stimulatory molecules, and down-regulate CD14 (Ruppert et al. 1991, Ulanova et al. 2001, Relloso et al. 2002, Sander et al. 2017). The DC generated

by this protocol, after seven days in culture, show a typical dendritic morphology. The phenotype of this DC is characterized by high membrane expression of MHC class I and class II molecules, CD1a, CD1b, CD1c, Fc γ RII, ICAM-1, CD11b, CD11c, CD40, B7, and CD33. In this work, DCs were also positive for Ii, LFA-1, LFA-3, and CD44. The expression of CD14 was either low or negative and these DCs were also negative for Fc γ RI and Fc γ RIII. They also presented a high capacity to stimulate allogeneic and autologous T cells and a unique ability to stimulate naive T lymphocytes, being efficient at presenting soluble antigens (tetanus toxoid) to a specific T cell (Sallusto and Lanzavecchia 1994).

Sanarico and colleagues (Sanarico et al. 2006), described another methodology for inducing differentiation of MDDCs *in vitro* using GM-CSF, IL-4, and IL-2. The DCs described by this group show the same morphology and phenotype of the DCs generated in the presence of GM-CSF and IL-4. After LPS stimulation, it was observed that there was an up-regulation of activation markers such as human leukocyte antigen (HLA)-ABC, HLA-DR, CD80, CD86, and CD83 but not CD25 (the IL-2 receptor subunit). However, some differences were observed regarding the DCs generated with GM-CSF and IL-4 only, such as a significantly higher secretion of IL-1 β , Tumor Necrosis Factor (TNF)- α , and IL-12p70 in response to LPS stimulation. They also demonstrated the capacity to induce high interferon (IFN) γ secretion by allogeneic naive T cells (Sanarico et al. 2006).

Geissmann and coworkers developed a protocol for generating Langerhans cells (LC) *in vitro*, which are specific DCs found on the human skin (Perussia et al. 1985, Geissmann et al. 1998). They added GM-CSF, IL-4, and transforming growth factor (TGF) β to monocyte culture. The predominant TGF- β isoform expressed in the immune system, TGF- β 1, regulates cell differentiation and survival during Langerhans

cell development (Bauer et al. 2012, Esebanmen and Langridge 2017). TGF- β also induces immature DCs to present an immunosuppressive phenotype thereby ensuring system homeostasis by downregulating the function of these cells (Li et al. 2006, Travis and Sheppard 2014, Esebanmen and Langridge 2017). The Langerhans cells generated by this protocol, in an immature state, exhibit Birbeck granules and express CD1a, E-cadherin, and CLA, but do not express CD83 and CD86. However, the addition of TNF- α and IL-1 induces HLA class II, CD83, and CD86 expression and the loss of E-cadherin expression. These cells are also highly efficient in stimulating the proliferation of allogeneous lymphocytes (Geissmann et al. 1998).

A different combination of cytokines was used to produce MDDC by Takahashi et al. (Takahashi et al. 1997). A combination of GM-CSF and IL-7 gave rise to floating cells with typical DC morphology and some adherent cells developed the appearance of Langerhans cell-like dendrites. The typical DC presents a membrane phenotype with high expression of MHC class I and class II, CD1a, CD11c, CD23, CD40, CD54, CD58, CD80, CD86, and CD95, and decreased CD14 expression (very low or absent). DCs induced by this protocol are also positive for CD21, weakly positive for CD32, and negative for CD16. Compared to PBMCs, the DCs generated by this protocol, also called G7 DC by the authors, are more effective in stimulating autologous and allogeneic T cell proliferation. G7 DCs are more effective in inducing peptide-specific CTL activity than the DCs generated with GM-CSF and IL-4 (Takahashi et al. 1997). The role of IL-7 in monocyte differentiation in DCs is not fully elucidated. Studies on IL-7R $\alpha^{-/-}$ and IL-7 $^{-/-}$ mice demonstrated that IL-7 may play an important role in the development of DCs and plasmacytoid dendritic cells (pDCs) but further investigations are required (Yang et al. 2005, Vogt et al. 2009).

Two different groups used the combination of GM-CSF and IFN- α to generate MDDC *in*

vitro (Santini et al. 2000, Mohty et al. 2003). In both cases, the DCs generated with GM-CSF and IFN- α exhibited a typical DC morphology and show an immature phenotype which demonstrates, after exposure to maturation stimulus (CD40L or LPS), up-regulated expression of co-stimulatory molecules CD40, CD80, CD86, and high expression of CD83, HLA-DR, and MHC class I. The prominent ability of these DCs to secrete IFN- α suggests an ability to promote a Th1 response. Mohty and colleagues also measured the expression of CD1a, blood dendritic cell antigen (BDCA) -4, CD54, CD58, and DC lysosome-associated membrane protein (DC-LAMP) on MDDC, which showed an increase in expression after the maturation stimulus with CD40L for 2 days. The DCs generated by Mohty and colleagues (called as IFN-DCs by the authors) differentially secreted, depending on their maturation stage, large amounts of inflammatory cytokines such as IL-1 β , IL-6, IL-10, TNF- α , and especially IL-18 (which could be detected at both maturational stages). However, these cells did not secrete IL-12p70. In the immature state, IFN-DCs induced a potent autologous antigen-specific immune response. Like the natural type I IFN-producing plasmacytoid DCs, the IFN-DCs expressed a several Toll-like receptors (TLR) including TLR7 and could secrete IFN- α following viral stimulation or TLR7-specific stimulation (Ito et al. 2002, Mohty et al. 2003, Liu 2005).

Santini and coworkers observed that, in response to type I IFN (IFN- α , IFN- α 2b and IFN- β) and GM-CSF treatment, adherent monocytes became non-adherent cells within three days of culture. The loss of adherence was associated with cellular aggregation in large cell clusters. However, a considerable percentage of the cells underwent apoptosis after five days in culture, and it is important to note that TNF-related apoptosis-inducing ligand (TRAIL) and TRAIL receptors (R1 and R2) were upregulated in response to

LPS stimulus (Wang and El-Deiry 2003). The DCs obtained by Santini and coworkers showed a stronger capability to stimulate the proliferation of allogeneic lymphocytes than DCs generated with GM-CSF and IL-4. In particular, DCs generated in the presence of type I IFN and GM-CSF showed a potent ability to take up, process, and present inactivated HIV-1 to autologous T lymphocytes *in vitro* when compared to DCs generated with GM-CSF and IL-4 (Santini et al. 2000, Biron 2001, Ito et al. 2001).

IL-3 cytokines are also used in association with IFN- β or IL-4 in protocols to produce MDDC. (Buelens et al. 2002, Ebner et al. 2002). DCs generated by these protocols acquire a dendritic morphology and show similarities in phenotype. The expression of CD14 molecules is low or absent, but high levels of BDCA-4, and CD11c are found when compared to DCs generated with GM-CSF and IL-4. Only CD1a was differentially expressed, either at low levels or not at all. After a maturation stimulus, DCs upregulated the costimulatory molecules CD40, CD80, CD86, CD83, DC-LAMP, HLA-DR, and MHC class I (Biron 2001, Buelens et al. 2002, Ebner et al. 2002).

The IL-15 cytokine is used in two different protocols to generate MDDC *in vitro* (Mohamadzadeh et al. 2001, Saikh et al. 2001). Saikh and colleagues use only IL-15 to promote monocyte differentiation into DC. The DCs generated by Saikh and colleagues show surface levels of the costimulatory molecules CD86, CD80, CD40, and CD83 that were equivalent to DC obtained from cultures of monocytes treated with GM-CSF plus IL-4 and stimulated with TNF- α . However, the DCs generated with IL-15 did not express CD1a. Another important feature of Saikh's DCs is the ability to stimulate strong allo-responses and produce significant amounts of IFN- γ and IL-12 when compared to DCs generated with GM-CSF plus IL-4 and stimulated with TNF- α (Saikh et al. 2001, Dubois et al. 2002, 2005).

Mohamadzadeh's group differentiated monocytes in the presence of IL-15 plus GM-CSF (Mohamadzadeh et al. 2001). The resulting cells, after six days in culture, had a phenotype positive for CD1a molecule, negative for CD14, and an increased in surface expression of HLA-DR, CD40, CD80, CD86 and CD83. The intracellular expression of DC-LAMP was observed upon LPS activation. The interesting features about this DC are the expression of Langerhans cell markers such as E-Cadherin, Langerin, and chemokine receptor (CCR) 6. Nevertheless, they do not express Birbeck granules like a "genuine" Langerhans cell (Mohamadzadeh et al. 2001, Dubois et al. 2002, 2005).

TNF- α is used together with GM-CSF by Iwamoto and coworkers to induce the differentiation of monocytes into DCs (called TNF-DC by the authors), (Iwamoto et al. 2007). The culture of CD14⁺ monocytes was incubated for seven days in the presence of GM-CSF and TNF- α , inducing a spindle-shaped and adherent morphology. Two days after LPS stimulation, they began to convert to DC-like floating cells with extended dendrites. The TNF-DC expressed low levels of CD14 and substantial levels of HLA-DR, CD40, CD70, CD80, CD86 and CD83, and produced extremely large amounts of TNF (>150 ng/mL at peak). They also produce IL-12/IL-23p40 and IL-23p19/p40, but very little IL-12p70 compared to DC generated with IL-4 and GM-CSF. The TNF-DC had the capacity to induce naive CD4 T cells to produce IFN- γ and TNF- α and stimulated resting CD4 T cells to secrete IL-17 when compared to DC generated with IL-4 and GM-CSF (Chomarat et al. 2003, Wajant et al. 2003, Iwamoto et al. 2007, Brenner et al. 2015). The Table II presents a summary of the cytokines, growth factors and maturation inducing substances used by the different groups described above.

TABLE II
Combination of cytokines, grown factors and maturation stimulus for MDDC culture *in vitro*.

Cytokines	Concentration	Time to add cytokines	Stimulus for maturation	Maturation stimulus added	Stimulation period	Total culture time	References
GM-CSF + IL-4	50 ng/mL ¹⁺ 1000 U/mL ¹	1° day	TNF- α (10 ng/mL) ² CD40L ³	Not mentioned	24 hours 24 hours	Seven days	Sallusto and Lanzavecchia 1994
GM-CSF + IL-7	600 U/mL ⁴⁺ 6 U/mL ⁵	1° day	Not mentioned	Not mentioned	Not mentioned	Seven days	Takahashi et al. 1997
GM-CSF + IL-4 + TGF- β 1	250 ng/mL ⁶⁺ 100 ng/mL ⁷⁺ 10 ng/mL ⁸	1° day ⁹	TNF- α ¹⁰ + IL-1 ¹⁰	Not mentioned	Not mentioned	Seven days	Geissmann et al. 1998
GM-CSF + IFN- α	500 U/mL ¹¹⁺ 1000 U/mL ¹² 100 ng/mL ¹⁴⁺ 500 IU/mL ¹⁵	1° day	LPS (1 μ g/mL) ¹³	3° day	48 hours	Five days	Santini et al. 2000
GM-CSF + IFN- α	100 ng/mL ²⁰ 200 ng/mL ²¹ 100 ng/mL ²³	1° day ¹⁶	LPS (10 μ g/mL) ¹⁷ or Poly(I:C)(15 μ g/mL) ¹⁸ or CD40L ¹⁹	5° day	48 hours	Seven days	Mohity et al. 2003
GM-CSF + IL-15	100 ng/mL ²⁰ 200 ng/mL ²¹ 100 ng/mL ²³	1° day	LPS (100 ng/mL) ²²	Not mentioned	Not mentioned	Six days	Mohamadzadeh et al. 2001.
IL-15	100 ng/mL ²⁴ 1000 U/mL ²⁵	1° day	Not mentioned	Not mentioned	Not mentioned	Seven days	Saikh et al. 2001
IL-3 + IL-4	100 U/mL ²⁴ 1000 U/mL ²⁵	1° day	TNF- α (10 ng/mL) ²⁶⁺ IL-1 β (10 ng/mL) ²⁷⁺ IL-6 (1000U/mL) ²⁸⁺ PGE2 (1 μ g/mL) ²⁹	7° day	48 hours	Nine days	Ebner et al. 2002.
IL-3 + IFN- β	50 U/mL ³⁰ 1000 U/mL ³¹	1° day	LPS (1 μ g/mL) ³² or Influenza virus ³² or Poly[I:C](20 μ g/mL) ³³ CD40L ³⁴	Not mentioned	24 hours 24 hours 48 hours 3 days	Six days	Buelens et al. 2002
GM-CSF + IL-4 + IL-2	50 ng/mL ³⁵ 10 ng/mL ³⁵ 100 U/mL ³⁶	1° day 1° day 1° and 5° days	LPS (200 ng/mL) ³⁷	5° day	48 hours	Seven days	Sanarico et al. 2006
GM-CSF + TNF- α	10 ng/mL ³⁸ 10 ng/mL ³⁸	1° day	LPS (10 μ g/mL) ³⁹	Not mentioned	Several time intervals	Seven days	Iwamoto et al. 2007

¹ Own lab production. ² Reagent supplied by Dr. Manfred Brockhaus (Hoffmann-La Roche, Basel, Switzerland). ³ A soluble chimeric fusion protein between the mouse CD8 α -chain and the human CD40 ligand (CD40L) was a generous gift of Dr. Peter Lane (Basel Institute for Immunology). ⁴ Pepro Tech Inc. (Rocky Hill, NJ) or provided by Prof. Nicos Nicola (The Walter and Eliza Hall Institute of Medical Research, Victoria, Australia). ⁵ Pepro Tech Inc. (Sandooz AG (Bale, Switzerland)). ⁶ Genzyme Corp. (Cambridge, MA). ⁷ R&D Systems, (Minneapolis, MN). ⁸ At days 2 and 4, half of the medium was removed and an equivalent volume of fresh medium, supplemented with GM-CSF, IL-4 and TGF- β 1 was added. ⁹ Concentrations not mentioned. ¹⁰ R&D Systems. ¹¹ C1FN is a synthetic type I IFN produced from recombinant DNA, whose sequence is based on a consensus derived from the amino acid sequences of the most common types of human IFN- α . (C1FN, specific activity of 10⁶ U/mG protein), Amgen. ¹² Leucomax, Novartis (Rueil-Malmaison, France). ¹³ Intron A, Schering-Plough (Levallois-Perret, France). ¹⁴ The medium was replenished with cytokines every 3 days. ¹⁵ LPS from *Escherichia coli*, serotype O26: B6, Sigma-Aldrich (St. Quentin Fallavier, France). ¹⁶ Polyribonucleosinic polyribocytidylic acid-poly(I:C), (Sigma-Aldrich). ¹⁷ Murine L cells transfected with human CD40L were kindly provided by Schering-Plough (Laboratory for Immunological Research, Dardilly, France), used after 75 Gy irradiation (ratio, 1:10). ¹⁸ Leukine, Immunex. ¹⁹ (R&D Systems or Schering-Plough). ²⁰ Sigma-Aldrich. ²¹ Leucomax, Novartis (Rueil-Malmaison, France). ²² Pepro Tech Inc (Rocky Hill, NJ). ²³ Pepro Tech (London, U.K.; sp. act., 10⁷ U/mG). ²⁴ Genzyme (Cambridge, MA; sp. act., 5.10⁷ U/mG). ²⁵ TNF- α (sp. act., 6X10⁷ U/mG) was provided by Dr. G. R. Adolf (Bender, Vienna, Austria). ²⁶ IL-1 β (sp. act., 5X10⁸ U/mG), Genzyme. ²⁷ IL-6 (sp. act., 1X10⁷ U/mG), Genzyme. ²⁸ PGE2 (prostaglandin E), Pharmacia & Upjohn (Buurs, Belgium). ²⁹ R&D Systems Europe (Oxon, United Kingdom). ³⁰ Ares Sero Europe (London, United Kingdom). ³¹ Provided by N. Kuehlm, Aventis, Pasteur Merieux (Val de Reuil, France). ³² Sigma. ³³ DCS were activated by coculture with irradiated 3T6 fibroblasts transfected with the human CD40L gene (CD40L-transfectants) (5x10⁷ mL). ³⁴ EuroClone. ³⁵ R&D Systems (Minneapolis, MN). ³⁶ Sigma-Aldrich (St. Louis, MO). ³⁷ Diaclone Research. ³⁸ LPS from *Escherichia coli* (L4516), Sigma-Aldrich.

CULTURE MEDIUM

The importance of the culture medium in the *in vitro* induction of DCs is undeniable since it should provide a suitable environment (an appropriate pH and all the necessary nutrients and factors) for cell maintenance, growth, and differentiation. There are many different culture media described and used in the extensive literature on MDDC. To make the best choice, it is necessary 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 (Duperrier et al. 2000, Peng et al. 2005, Janetzki et al. 2010).

If the main objective encompasses the clinical (therapeutic grade) use of the MDDCs generated *in vitro*, such as vaccines, the only Food and Drug Administration (FDA) approved culture medium is the AIM-V (GIBCO, Catalog number: 087-0112DK). The complete AIM-V composition is not informed by the manufacturer (GIBCO), although the manufacturer provides some important information (in this sense, it is reported that this medium does not contain serum but contains L-glutamine and antibiotics - 50 µg/mL streptomycin sulfate and 10 µg/mL gentamicin sulfate).

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). As in the previous example, the complete composition is not disclosed. The only information available states that the DC Generation Medium DXF is free from animal-derived components, although it contains human serum albumin. An important point, according to the manufacturer, is that the Monocyte Attachment Medium allows an efficient selection of monocytes from freshly isolated mononuclear cells through differential adherence and immunomagnetic

purification steps. For all media, supplementation with growth factors and cytokines is needed (Challagundla et al. 2015).

Another culture medium used to generate MDDC for research purposes is X-VIVO 15. The complete composition is not reported by the manufacturer (LONZA), so the only information available is that it is chemically defined and serum-free. There are versions with or without L-glutamine, gentamicin, recombinant transferrin and phenol red. As previously stated, all media requires cytokine and growth factor supplementation, depending on the specific goal of the experiment and the initial cell source.

A commonly used medium in cell culture experiments is RPMI-1640. It was developed by Moore and colleagues at the Roswell Park Memorial Institute, hence the acronym RPMI (Moore et al. 1967). The exact RPMI-1640 composition is available and well established, which allows the production of quite similar media by several manufacturers. There are several modified versions of RPMI, some of them lacking specific components, others already supplemented with certain growth factors or other molecules. Nevertheless, as a general rule, supplementation with serum, cytokines, and/or growth factors is required.

SUPPLEMENTS FOR THE CULTURE MEDIUM

The widely used culture medium RPMI-1640 is also the only commercial medium for which the complete formula is available. This enables each researcher to direct the supplementation specifically according to the particularities of each experiment. The most commonly used culture medium supplements (biological or chemical factors) for *in vitro* production of MDDCs will be briefly presented below.

An essential function of culture medium consists in providing cells an environment with a

physiological pH, and thus, buffering agents are essential. Sodium bicarbonate (NaHCO_3) is a commonly used pH regulating agent, since it can be used in cell culture without increased toxicity to the cells. Sodium bicarbonate chemically reacts either as acid or as a base due to its amphoteric characteristic. In solution, sodium bicarbonate produces bicarbonate ions, while the metabolism of cells in culture produces carbon dioxide (CO_2), decreasing the culture medium pH (if the culture medium contains phenol red, the color of the medium will become yellowish). In addition, the incubator also injects carbon dioxide into its atmosphere, thereby increasing the carbon dioxide concentration in the microenvironment and lowering the culture medium pH. Thus, within the incubator microenvironment, a sodium bicarbonate-carbon dioxide buffer balance is established. The carbon dioxide dissolved in the culture medium, either from the incubator or from the cell metabolism, equilibrates with the bicarbonate ions, buffering the medium. The sodium bicarbonate concentration in the medium should be in equilibrium with the carbon dioxide level in the incubator atmosphere. Thus, for media containing 1.5–2.2 g/L of sodium bicarbonate, the incubator must inject 5% of carbon dioxide into its microenvironment. For media containing 3.7 g/L of sodium bicarbonate, the incubator should inject 10% of carbon dioxide. If the sodium bicarbonate concentration is too high in relation to the carbon dioxide level injected by the incubator, the culture medium will alkalize, and its color will turn pink (Barker 1998).

HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) is a buffering agent which is membrane impermeable, chemically and enzymatically stable, demonstrates a limited effect on biochemical reactions, and has very low visible and UV light absorbance. HEPES provides extra buffering capacity when cell cultures require extended periods of manipulation outside of a CO_2 incubator. The HEPES concentration in cell

culture media may vary from 10 mM to 25 mM. Although HEPES-buffered medium exposed to fluorescent light was shown to be cytotoxic, due to the formation of free radicals, this effect is preventable by manipulating the cells under light-shielded conditions.

Serum is a quite ubiquitous supplement in cell cultures. This component will supply mainly growth factors and cytokines to the cells, although generally it is not possible to define the exact types and concentrations of these components in the serum. Many studies use human serum from an AB serum pool to supplement cell cultures. The final concentrations of serum used vary from 1 - 10%. It is also possible to use plasma or even umbilical cord blood serum. Nevertheless, if the cells will not be used in clinical applications, fetal bovine serum is the least expensive choice, and is also easier to obtain. However, it is crucial to observe if the serum was inactivated. This inactivation is necessary due to the presence of proteins from the complement system which could cause cell death (Anton et al. 1998).

Although cell culture procedures are conducted in aseptic environments, any biological contaminant can devastate the cell culture, and therefore the use of antibiotics and antimycotics is sometimes required. The most common antibiotics used in cell cultures are penicillin-streptomycin and gentamicin. Penicillin-streptomycin acts on Gram-positive and Gram-negative bacteria whereas gentamicin is also active against mycoplasma. The recommended concentration for use in cell culture for Penicillin-streptomycin are 50 to 100 I.U./mL penicillin and 50 to 100 $\mu\text{g}/\text{mL}$ streptomycin (Catalog number: ATCC® 30-2300), and 5 to 50 $\mu\text{g}/\text{mL}$ gentamicin (Barker 1998). In 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 (Vatsan et al. 2013).

A widely used antifungal agent is amphotericin B. It acts against both fungi and yeasts. The recommended concentration for use in cell culture is 0.25-2.5 µg/mL (GIBCO, Catalog number: 15290026).

A component often added to culture medium is the amino acid glutamine. This amino acid can lead to citrate production, which is exported to the DC's cytoplasm and acts as a substrate for fatty acids synthesis, an essential process for the induction of DC activation by TLR ligands. Due to its rapid degradation and instability in solution, glutamine supplementation must be performed after careful consideration. The recommended concentration for use in cell culture generally falls in the range of 2-6 mM (GIBCO, Catalog number: 21051024).

Free radicals are a natural side product of the increased metabolism in monocytes and in immature DCs during differentiation and maturation. To avoid the toxic effects of the accumulation of these reactive oxygen species in the culture medium, 2-mercaptoethanol (2-ME) is used as a reducing agent. Due to its instability in solution, 2-mercaptoethanol should be added daily. It is recommended to dilute 2-ME to 50 mM in phosphate-buffered saline (PBS), and add this solution to the cell medium at a final concentration of 50 µM. (GIBCO, Catalog Number: 21985023), (Click 2014).

CULTURE TIME

The time needed to establish a cell line or to induce cell differentiation *in vitro* is quite variable, and several aspects should be taken into consideration. The majority of studies consider that the differentiation process from monocyte to immature DC is completed in five days with an additional period of 48 hours for maturation. However, several works have been demonstrated that *in vitro* development of mature DCs from monocyte precursors does not require more than 2 days of

culture, defined by some as fast DCs (fDC), (Dauer et al. 2003, Obermaier et al. 2003, Ramadan et al. 2004, Massa and Seliger 2013). The data about phenotypic characteristics, immune properties, and the pattern of responses induced for the fDC are still poorly understood (Tanaka et al. 2006, Rojas-Canales et al. 2012, Pavlović et al. 2015).

In vitro MATURATION OF IMMATURE DENDRITIC CELL

In the majority of MDDCs generation protocols, the resulting DCs are in an immature state. An immature dendritic cell (iDC) shows a high capacity to capture and process antigens but has limited capacity to stimulate T cells due to the low expression of the co-stimulatory molecules CD40, CD80, CD86, and HLA-class II. The *in vitro* maturation process is characterized by several events and although different groups may define distinct features, the main events include the downregulation of endocytic/phagocytic receptors, the upregulation of costimulatory molecules CD40, CD80 and CD86, the upregulation of CD58 and CD83, the shift in lysosomal compartments with downregulation of CD68, the upregulation of DC-LAMP, and the changes in HLA-class II molecules (Banchereau et al. 2000, Lutz and Schuler 2002, Iwasaki and Medzhitov 2004).

Some important morphological changes also occur during the DC *in vitro* maturation process, including the loss of adhesive structures, cytoskeleton reorganization, and the acquisition of high cellular motility (see Table III). These changes allow the DCs a better capacity for lymphocyte stimulation (Banchereau et al. 2000, Dalod et al. 2014).

To induce the DC maturation process *in vitro* it is necessary to give a stimulus that mimics a danger signal (Matzinger 1994, Gallucci et al. 1999, Joffre et al. 2009, Castiello et al. 2011). It is important to note that in a seven days protocol for MDDC

TABLE III
Characteristics of DCs according to different maturation stages. The characteristics described refers to the expression in the plasma membrane.

Immature DC	Mature DC
High intracellular expression of HLA-II	High membrane expression of HLA-II
High endocytic/phagocytic capacity	Low endocytic/phagocytic capacity
High membrane expression of CCR1, CCR5 and CCR6	Low membrane expression of CCR1, CCR5, and CCR6
Low membrane expression of CCR7	High membrane expression of CCR7
Low membrane expression of CD40, CD54 and CD58	High membrane expression of CD40, CD54, and CD58
Absent/low membrane expression of DC-LAMP	High membrane expression of DC-LAMP
Low membrane expression of CD80, CD86 and CD83	High membrane expression of CD80, CD86, and CD83
High membrane expression of CD68	Low membrane expression of CD68
Induce T cell anergy/tolerance	Great capacity to lymphocyte stimulation

generation, the stimulus is usually given on day 5 and is maintained for 48 hours. In the case of fDCs, the differentiation cocktail already contains substances that will induce cell maturation. The main substances used to promote DC maturation in MDDC cultures are described below (Haenssle et al. 2008, Castiello et al. 2011, Li et al. 2012).

A common substance used to mimic DC activation when provoked by bacteria is LPS, a characteristic component of the wall of Gram-negative bacteria (Raetz and Whitfield 2002). The LPS molecule is bound by the CD14 molecule present on the cell surface and activates the TLR4/MD-2 complex pathway, promoting the secretion of pro-inflammatory cytokines by DCs (Iwasaki and Medzhitov 2004, Park and Lee 2013). Nevertheless, due to its potential toxicity, LPS is only used in research protocols. The majority of protocols aiming for DC maturation use LPS in a concentration range of 10 ng/mL to 10 µg/mL (InvivoGen, Catalog code: tlr1-eklps).

Flagellin is another substance that mimics the danger signal triggered by the presence of Gram-negative and/or Gram-positive bacteria. Flagellin is generally detected by TLR5 whereas intracellular flagellin is detected by NOD-like receptors (NLRs) NLRC4 and NAIP5. The process of DC maturation induced by flagellin results in the activation of the NF-κB signaling pathway and cytokine production

(Akira et al. 2001, Vicente-Suarez et al. 2009, Miao and Warren 2010). It was already been suggested that flagellin could be used as a vaccine adjuvant (Coffman et al. 2010). As in the previous case of LPS use, the flagellin concentrations used in DC maturation protocols widely vary, ranging from 10 ng/mL to 10 µg/mL (Vicente-Suarez et al. 2009), (InvivoGen, Catalog code: tlr1-stfla).

To mimic virus-mediated activation during DC maturation it is possible to use commercially available synthetic polynucleotide sequences such as the double stranded RNA analog polyinosinic-polycytidylic acid [poly(I:C)], the single-stranded RNA analog poly-uridine (polyU), or the single-stranded RNA analog guanosine- and uridine-rich (GU-rich). Whereas poly(I:C) binds to TLR3, the remaining two types of sequences bind to TLR7/TLR8 (Heil et al. 2004, Schlee and Hartmann 2016). Alternatively, the use of compounds derived from imidazoquinoline such as the analog imiquimod can be used to activate immature DCs. Imiquimod is a commercially available compound which selectively binds to TLR7 (Xiao et al. 2016). Poly(I:C) and imiquimod are also used as adjuvants in protocols to develop cancer vaccines (Coffman et al. 2010), (InvivoGen, Catalog code: vac-pic).

Short synthetic single-stranded DNA molecules that contain unmethylated CpG dinucleotides (CpG) are also used as TLR9 agonists to activate

iDCs (Jakob et al. 1998, Hemmi et al. 2000, Akira et al. 2001, Iwasaki and Medzhitov 2004). A modified version of CpGs called CpG-ODN has been developed for clinical use as an adjuvant (Cella et al. 1999, Bauer et al. 2001, Coffman et al. 2010).

The CD40 is a costimulatory molecule expressed on DCs and the interaction with its ligand CD40L (CD154) leads to an activation of the NF- κ B signaling pathway, a key transducer of inflammatory signals in DCs (Schulz et al. 2000, Aggarwal 2003, O'Sullivan and Thomas 2003, Ma and Clark 2009). Transfected cells expressing the CD40L protein can be used in co-culture assays to induce the maturation of iDCs (Buelens et al. 2002, Mohty et al. 2003), whereas, to promote DC activation via CD40 receptor, some commercial alternatives are available. For example, CD40L recombinant protein, which should be used in conjunction with an enhancer for better performance, is already available (Enzo Life Sciences, Catalog number: ALX-850-064).

Interleukins and several other molecules can concomitantly be used to induce DC maturation. A "maturation cocktail" can, for example, contain IL-6, IL-1 β , TNF- α , IFN- γ , and Prostaglandin E2 (PGE2), (Jonuleit et al. 1997). In the cited case, this cocktail enhances the pro-inflammatory effects of TNF- α by creating an inflammatory environment that induces DC maturation. This cocktail generates mature DCs that strongly stimulate the proliferation of allogeneic lymphocytes (Castiello et al. 2011).

TYPICAL MDDC IMMUNOPHENOTYPE MARKERS

The phenotype characterization of the obtained DCs is crucial to demonstrate the success of the MDDC differentiation. However, due to the absence of a consensus concerning which molecules characterize a DC and which markers are expressed in different DC subsets, it is quite common to find

a wide variety of markers used in distinct studies. Nevertheless, the expression of certain molecules is consistently used to indicate successful MDDC differentiation and/or maturation. For example, since a peripheral blood monocyte, the main source for *in vitro* DC generation, expresses high levels of CD14 on its membrane, and MDDC lacks the expression of this same molecule, CD14 is a marker for DC differentiation. Additionally, to determine the success of the iDC maturation process, it is essential 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 can be considerably increased after induction of maturation by LPS (Banchereau et al. 2000, Ueno et al. 2007).

The HLA-II molecule is also constitutively expressed at low levels both in monocytes and DCs. After a maturation stimulus, its expression on DCs is increased. Thus, like co-stimulatory molecules, HLA-II can be used as an indicator of the success of the iDC maturation process (Al-daccak et al. 2004, Neefjes et al. 2011).

CD1 represents a group of antigen-presenting molecules which are responsible for the presentation of lipid antigens and their derivatives. There are five isoforms of CD1 proteins [CD1a, CD1b, CD1c, CD1e (group 1) and CD1d (group 2)]. Myeloid DCs express all five proteins whereas Langerhans cells express only CD1a and CD1e (Mori et al. 2016). In blood monocytes, CD1a, CD1b, and CD1c (BDCA-1) can be upregulated on the cell surface by cytokine cocktails designed to drive DC differentiation *in vitro* (Moody 2006).

A strategy for characterizing a pDC, for example, would encompass the measurement of the CD123 (IL-3R α), CD303 (BDCA-2), and CD304 (BDCA-4) expression levels. This strategy depends on the fact that pDCs present high expression of such molecules in the membrane

(Dzionic et al. 2000, Colonna et al. 2004, Swiecki and Colonna 2015).

Other potential markers are CD207 (Langerin), which is used to characterize Langerhans cells (Merad et al. 2008, Nestle et al. 2009), and CD209 (DC-SIGN), which is expressed on pDCs and MDDCs and is highly expressed on DCs in mucosal tissues. The expression of this last marker is increased after LPS-mediated DC maturation induction (Belz and Nutt 2012).

Finally, in the analyses performed by flow cytometry it is of great importance to evaluate the cellular viability. Substances used to determine cell viability can be divided into two broad groups: substances used in live cells or fixed cells. Calcein AM (BD Biosciences, Catalog Number 564061) is a dye used for staining live cells. The hydrophobicity of the acetomethoxy (AM) derivative of Calcein allows this dye to enter on viable cells. Once inside, intracellular esterases cleave the AM groups off allowing Calcein to fluoresce within the cell. Only viable cells are stained, since dead cells lack esterase activity. Calcein AM is optimally excited at the 495 nm wavelength of light and emits maximally at 515 nm (Chung et al. 2017).

7-Amino-actinomycin D, or 7-AAD, is a nucleic acid dye used to indicate cell viability in flow cytometric assays. The fluorescence is detected in the far red range of the spectrum with a 650 nm long-pass filter (Schmid et al. 1992).

For fixed cells, an alternative may be the reagents BD Horizon FVS from BD Biosciences (Catalog Number 564406). BD Horizon FVS are amine-reactive dyes used to discriminate viable from non-viable cells based on fluorescence intensity. These dyes react by covalently binding the cell surface and intracellular amines, resulting in live cells poorly stained and cells with permeable membranes exhibiting highly fluorescent. Typically, dead cells exhibit a fluorescence intensity 10 to 20-fold greater than live cells stained with the same amount of dye. The advantage of these

dyes is that they can be used in cells fixed with formaldehyde, and can be used in protocols where cell permeabilization occurs (Mahalingaiah et al. 2018).

CONCLUSIONS

Despite several technical challenges, significant progress has been made in recent years concerning the generation of human DCs *in vitro*. Much of the recently acquired knowledge was due to studies using blood monocytes as *in vitro* precursors of DCs. The incorporation of knowledge concerning cellular biology, cytokines, growth factors, transcription profiles of these cells in different stages of maturation with the characterization of the patterns of expression of surface markers has allowed a more comprehensive and accurate description of the DCs present in distinct tissues both in pathological, as well as in healthy situations.

The appropriate combination of cytokines and growth factors that will generate MDDCs with the most suitable characteristics for a given study is already possible. The development of protocols that allow us to differentiate MDDCs into a particular DC subtype as well as to simulate pathological microenvironments is a reality that has not only broadened our knowledge but also allows us to manipulate these cells for use in clinical protocols. The increasing amounts of reagents available to induce maturation of iDCs via a specific receptor also contributes to the production of DCs with specific characteristics, membrane markers, specific profile of cytokine secretion, or a given lymphocyte polarization state.

Another important advance in the study of MDDCs was the use of multiparametric flow cytometry (Hasan et al. 2015). The careful selection of antibodies and the optimal combination of the fluorochromes used to label such specific reagents are key steps in the establishment of a robust panel to analyze MDDCs (Maecker et al. 2004,

Byrd et al. 2015). To succeed in this step, several companies provide specific programs to help set up the analysis panels and protocols to identify the different DC subtypes. International consortia have also been working for the increased standardization of flow cytometry protocols including the EuroFlow Consortium, the Human Immunology Project Consortium (HIPC), the European Network for Translational Immunology Research and Education (ENTIRE) and the Association for Cancer Immunotherapy (CIMT).

Great progress has been made in the understanding of the development, differentiation, and function of DCs. However, the major challenge will be to transpose the acquired knowledge into *in vivo* situations to elucidate the DCs physiological behavior. This knowledge will be useful in the design of novel vaccines and DC-based immunotherapies for the prevention and treatment of several human diseases.

ACKNOWLEDGMENTS

This work was supported by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES).

AUTHOR CONTRIBUTIONS

Both authors wrote and revised the manuscript.

REFERENCES

- AGGARWAL BB. 2003. Signalling pathways of the TNF superfamily: a double-edged sword. *Nat Rev Immunol* 3: 745-756.
- AKIRA S AND TAKEDA K. 2004. Toll-like receptor signalling. *Nat Rev Immunol* 4: 499-511.
- AKIRA S, TAKEDA K AND KAISHO T. 2001. Toll-like receptors: critical proteins linking innate and acquired immunity. *Nat Immunol* 2: 675-680.
- AL-DACCAK R, MOONEY N AND CHARRON D. 2004. MHC class II signaling in antigen-presenting cells. *Curr Opin Immunol* 16: 108-113.
- ANTON D, DABADGHAO S, PALUCKA K, HOLM G AND YI Q. 1998. Generation of dendritic cells from peripheral blood adherent cells in medium with human serum. *Scand J Immunol* 47: 116-121.
- ARDAVIN C, WU L, LI CL AND SHORTMAN K. 1993. Thymic dendritic cells and T cells develop simultaneously in the thymus from a common precursor population. *Nature* 362: 761-763.
- BANCHEREAU J, BRIERE F, CAUX C, DAVOUST J, LEBECQUE S, LIU YJ, PULENDRAN B AND PALUCKA K. 2000. Immunobiology of dendritic cells. *Annu Rev Immunol* 18: 767-811.
- BANCHEREAU J ET AL. 2001. Immune and clinical responses in patients with metastatic melanoma to CD34(+) progenitor-derived dendritic cell vaccine. *Cancer Res* 61: 6451-6458.
- BARKER K. 1998. *At the Bench: A Laboratory Navigator*. Cold Spring Harbor Laboratory Press, 465p.
- BAUER M, REDECKE V, ELLWART JW, SCHERER B, KREMER JP, WAGNER H AND LIPFORD GB. 2001. Bacterial CpG-DNA triggers activation and maturation of human CD11c-, CD123+ dendritic cells. *J Immunol* 166: 5000-5007.
- BAUER T, ZAGÓRSKA A, JURKIN J, YASMIN N, KÖFFEL R, RICHTER S, GESSLBAUER B, LEMKE G AND STROBL H. 2012. Identification of Axl as a downstream effector of TGF- β 1 during Langerhans cell differentiation and epidermal homeostasis. *J Exp Med* 209: 2033-2047.
- BELZ GT AND NUTT SL. 2012. Transcriptional programming of the dendritic cell NETWORK. *Nat Rev Immunol* 12: 101-113.
- BIRON CA. 2001. Interferons alpha and beta as immune regulators-a new look. *Immunity* 14: 661-664.
- BRENNER D, BLASER H AND MAK TW. 2015. Regulation of tumour necrosis factor signalling: live or let die. *Nat Rev Immunol* 15: 362-374.
- BUELENS C, BARTHOLOMÉ EJ, AMRAOUI Z, BOUTRIAUX M, SALMON I, THIELEMANS K, WILLEMS F AND GOLDMAN M. 2002. Interleukin-3 and interferon beta cooperate to induce differentiation of monocytes into dendritic cells with potent helper T-cell stimulatory properties. *Blood* 99: 993-998.
- BYRD T, CARR KD, NORMAN JC, HUYE L AND HEGDE M. 2015. Polystyrene microspheres enable 10-color compensation for immunophenotyping of primary human leukocytes. *Cytometry A* 87: 1038-1046.
- CASTIELLO L, SABATINO M, JIN P, CLAYBERGER C, MARINCOLA FM, KRENSKY AM AND STRONCEK DF. 2011. Monocyte-derived DC maturation strategies and related pathways: a transcriptional view. *Cancer Immunol Immunother* 60: 457-466.
- CELLA M, SALIO M, SAKAKIBARA Y, LANGEN H, JULKUNEN I AND LANZAVECCHIA A. 1999. Maturation, activation, and protection of dendritic cells

- induced by double-stranded RNA. *J Exp Med* 189: 821-829.
- CHALLAGUNDLA KB ET AL. 2015. Exosome-mediated transfer of microRNAs within the tumor microenvironment and neuroblastoma resistance to chemotherapy. *J Natl Cancer Inst* 7: djv135.
- CHOMARAT P, DANTIN C, BENNETT L, BANCHEREAU J AND PALUCKA AK. 2003. TNF skews monocyte differentiation from macrophages to dendritic cells. *J Immunol* 171: 2262-2269.
- CHUNG S, NGUYEN V, LIN YL, KAMEN L AND SONG A. 2017. Thaw-and-use target cells pre-labeled with calcein AM for antibody-dependent cell-mediated cytotoxicity assays. *J Immunol Methods* 447: 37-46.
- CIMT-ASSOCIATIONFORCANCERIMMUNOTHERAPY. <http://www.cimt.eu/workgroups/cip/proficiency> (Accessed 12 June 2018).
- CLICK RE. 2014. Review: 2-mercaptoethanol alteration of *in vitro* immune functions of species other than murine. *J Immunol Methods* 402: 1-8.
- COFFMAN RL, SHER A AND SEDER RA. 2010. Vaccine adjuvants: putting innate immunity to work. *Immunity* 33: 492-503.
- COLLIN M AND BIGLEY V. 2018. Human dendritic cell subsets: an update. *Immunology* 154: 3-20.
- COLONNA M, TRINCHIERI G AND LIU YJ. 2004. Plasmacytoid dendritic cells in immunity. *Nat Immunol* 5: 1219-1226.
- DALOD M, CHELBI R, MALISSEN B AND LAWRENCE T. 2014. Dendritic cell maturation: functional specialization through signaling specificity and transcriptional programming. *EMBO J* 33: 1104-1116.
- DAUER M, OBERMAIER B, HERTEN J, HAERLE C, POHL K, ROTHENFUSSER S, SCHNURR M, ENDRES S AND EIGLER A. 2003. Mature dendritic cells derived from human monocytes within 48 hours: a novel strategy for dendritic cell differentiation from blood precursors. *J Immunol* 170: 4069-4076.
- DELIREZH N, SHOJAEEFAR E, PARVINP AND ASADI B. 2013. Comparison the effects of two monocyte isolation methods, plastic adherence and magnetic activated cell sorting methods, on phagocytic activity of generated dendritic cells. *Cell J* 15: 218-223.
- DIETZ AB, BULUR PA, EMERY RL, WINTERS JL, EPPS DE, ZUBAIR AC AND VUK-PAVLOVIĆ S. 2006. A novel source of viable peripheral blood mononuclear cells from leukoreduction system chambers. *Transfusion* 46: 2083-2089.
- DUBOIS S, MARINER J, WALDMANN TA AND TAGAYA Y. 2002. IL-15 α recycles and presents IL-15 In trans to neighboring cells. *Immunity* 17: 537-547.
- DUBOIS SP, WALDMANN TA AND MÜLLER JR. 2005. Survival adjustment of mature dendritic cells by IL-15. *Proc Natl Acad Sci USA* 102: 8662-8667.
- DUPERRIER K, ELJAAFARI A, DEZUTTER-DAMBUYANT C, BARDIN C, JACQUET C, YONEDA K, SCHMITT D, GEBUHRER L AND RIGALD. 2000. Distinct subsets of dendritic cells resembling dermal DCs can be generated *in vitro* from monocytes, in the presence of different serum supplements. *J Immunol Methods* 238: 119-131.
- DZIOŃEK A, FUCHS A, SCHMIDT P, CREMER S, ZYSK M, MILTENYI S, BUCK DW AND SCHMITZ J. 2000. BDCA-2, BDCA-3, and BDCA-4: three markers for distinct subsets of dendritic cells in human peripheral blood. *J Immunol* 165: 6037-6046.
- EBNER S, HOFER S, NGUYEN VA, FÜRHPATER C, HEROLD M, FRITSCH P, HEUFLER C AND ROMANI N. 2002. A novel role for IL-3: human monocytes cultured in the presence of IL-3 and IL-4 differentiate into dendritic cells that produce less IL-12 and shift Th cell responses toward a Th2 cytokine pattern. *J Immunol* 168: 6199-6207.
- EBNER S, NEYER S, HOFER S, NUSSBAUMER W, ROMANI N AND HEUFLER C. 2001. Generation of large numbers of human dendritic cells from whole blood passaged through leukocyte removal filters: an alternative to standard buffy coats. *J Immunol Methods* 252: 93-104.
- ELKORD E, WILLIAMS PE, KYNASTON H AND ROWBOTTOM AW. 2005. Human monocyte isolation methods influence cytokine production from *in vitro* generated dendritic cells. *Immunology* 114: 204-212.
- ESEBANMEN GE AND LANGRIDGE WHR. 2017. The role of TGF- β signaling in dendritic cell tolerance. *Immunol Res* 65: 987-994.
- EuroFlow. 2018. <https://euroflow.org/usr/pub/prlogin.php>. (accessed 03 May 2018).
- ENTIRE - EUROPEAN NETWORK FOR TRANSLATIONAL IMMUNOLOGY RESEARCH AND EDUCATION. 2018. http://www.cost.eu/COST_Actions/bmbs/BM0907?parties (accessed 03 May 2018).
- GALLUCCI S, LOKEMA M AND MATZINGER P. 1999. Natural adjuvants: endogenous activators of dendritic cells. *Nat Med* 5: 1249-1255.
- GANDHI RT ET AL. 2016. Immunization of HIV-1-Infected Persons with Autologous Dendritic Cells Transfected With mRNA Encoding HIV-1 Gag and Nef: Results of a Randomized, Placebo-Controlled Clinical Trial. *J Acquir Immune Defic Syndr* 71: 246-253.
- GARG AD, COULIE PG, VAN DEN EYNDE BJ AND AGOSTINIS P. 2017. Integrating Next-Generation Dendritic Cell Vaccines into the Current Cancer Immunotherapy Landscape. *Trends Immunol* 38: 577-593.
- GEISSMANN F, PROST C, MONNET JP, DY M, BROUSSE N AND HERMINE O. 1998. Transforming growth factor

- beta 1, in the presence of granulocyte/macrophage colony-stimulating factor and interleukin 4, induces differentiation of human peripheral blood monocytes into dendritic Langerhans cells. *J Exp Med* 187: 961-966.
- GILLIO-MEINA C, CEPINSKAS G, CECCHINI EL AND FRASER DD. 2013. Translational research in pediatrics II: blood collection, processing, shipping, and storage. *Pediatrics* 131: 754-766.
- GRANOT ET AL. 2017. Dendritic Cells Display Subset and Tissue-Specific Maturation Dynamics over Human Life. *Immunity* 46: 504-515.
- GUERMONPREZ P, VALLADEAU J, ZITVOGEL L, THÉRY C AND AMIGORENA S. 2002. Antigen presentation and T cell stimulation by dendritic cells. *Annu Rev Immunol* 20: 621-667.
- HAENSSELE H, BUHL T, KNUDSEN S, KRUEGER U, ROSENBERGER A, REICH K AND NEUMANN C. 2008. CD40 ligation during dendritic cell maturation reduces cell death and prevents interleukin-10-induced regression to macrophage-like monocytes. *Exp Dermatol* 17: 177-187.
- HAMMER GE AND MAA. 2013. Molecular control of steady-state dendritic cell maturation and immune homeostasis. *Annu Rev Immunol* 31: 743-791.
- HASAN M ET AL. 2015. Semi-automated and standardized cytometric procedures for multi-panel and multi-parametric whole blood immunophenotyping. *Clin Immunol* 157: 261-276.
- HEIL F, HEMMI H, HOCHREIN H, AMPENBERGER F, KIRSCHNING C, AKIRA S, LIPFORD G, WAGNER H AND BAUER S. 2004. Species-specific recognition of single-stranded RNA via toll-like receptor 7 and 8. *Science* 303: 1526-1529.
- HEMMI H ET AL. 2000. A Toll-like receptor recognizes bacterial DNA. *Nature* 408: 740-745.
- HOLMES KL, FONTES B, HOGARTH P, KONZ R, MONARD S, PLETCHER CHJR, WADLEY RB, SCHMID I AND PERFETTO SP. 2014. International Society for the Advancement of Cytometry cell sorter biosafety standards. *Cytometry A* 85: 434-453.
- ITO T, AMAKAWA R, INABA M, IKEHARA S, INABA K AND FUKUHARA S. 2001. Differential regulation of human blood dendritic cell subsets by IFNs. *J Immunol* 166: 2961-2969.
- ITO T, AMAKAWA R, KAISHO T, HEMMI H, TAJIMA K, UEHIRA K, OZAKI Y, TOMIZAWA H, AKIRA S AND FUKUHARA S. 2002. Interferon-alpha and interleukin-12 are induced differentially by Toll-like receptor 7 ligands in human blood dendritic cell subsets. *J Exp Med* 195: 1507-1512.
- ITO Y AND SHINOMIYA K. 2001. A new continuous-flow cell separation method based on cell density: principle, apparatus, and preliminary application to separation of human buffy coat. *J Clin Apher* 16: 186-191.
- IWAMOTO S, IWAI S, TSUJIYAMA K, KURAHASHI C, TAKESHITA K, NAOE M, MASUNAGA A, OGAWA Y, OGUCHI K AND MIYAZAKI A. 2007. TNF-alpha drives human CD14+ monocytes to differentiate into CD70+ dendritic cells evoking Th1 and Th17 responses. *J Immunol* 179: 1449-1457.
- IWASAKI A AND MEDZHITOV R. 2004. Toll-like receptor control of the adaptive immune responses. *Nat Immunol* 5: 987-995.
- JAKOB T, WALKER PS, KRIEG AM, UDEY MC AND VOGEL JC. 1998. Activation of cutaneous dendritic cells by CpG-containing oligodeoxynucleotides: a role for dendritic cells in the augmentation of Th1 responses by immunostimulatory DNA. *J Immunol* 161: 3042-3049.
- JANETZKI S ET AL. 2010. Performance of serum-supplemented and serum-free media in IFN gamma Elispot Assays for human T cells. *Cancer Immunol Immunother* 59: 609-618.
- JANSEN JH, WIENTJENS GJ, FIBBE WE, WILLEMZE R AND KLUIN-NELEMANS HC. 1989. Inhibition of human macrophage colony formation by interleukin 4. *J Exp Med* 170: 577-582.
- JOFFRE O, NOLTE MA, SPÖRRI R AND REIS E SOUSA C. 2009. Inflammatory signals in dendritic cell activation and the induction of adaptive immunity. *Immunol Rev* 227: 234-247.
- JOFFRE OP, SEGURA E, SAVINA A AND AMIGORENA S. 2012. Cross-presentation by dendritic cells. *Nat Rev Immunol* 12: 557-569.
- JONULEIT H, KÜHN U, MÜLLER G, STEINBRINK K, PARAGNIK L, SCHMITT E, KNOP J AND ENK AH. 1997. Pro-inflammatory cytokines and prostaglandins induce maturation of potent immunostimulatory dendritic cells under fetal calf serum-free conditions. *Eur J Immunol* 27: 3135-3142.
- KAVANAGH DG AND BHARDWAJ N. 2002. A division of labor: DC subsets and HIV receptor diversity. *Nat Immunol* 3: 891-893.
- KURTS C, ROBINSON BW AND KNOLLE PA. 2010. Cross-priming in health and disease. *Nat Rev Immunol* 10: 403-414.
- LEHNER M AND HOLTER W. 2002. Endotoxin-free purification of monocytes for dendritic cell generation via discontinuous density gradient centrifugation based on diluted ficoll-paque plus. *Int. Arch Allergy Immunol* 128: 73-76.
- LI DY, GU C, MIN J, CHU ZH AND OU QJ. 2012. Maturation induction of human peripheral blood mononuclear cell-derived dendritic cells. *Exp Ther Med* 4: 131-134.
- LI MO, WAN YY, SANJABI S, ROBERTSON AK AND FLAVELL RAL. 2006. Transforming growth factor-beta

- regulation of immune responses. *Annu Rev Immunol* 24: 99-146.
- LIEW FY, XU D, BRINT EK AND O'NEILL LA. 2005. Negative regulation of toll-like receptor-mediated immune responses. *Nat Rev Immunol* 5: 446-458.
- LIU YJ. 2005. IPC: professional type 1 interferon-producing cells and plasmacytoid dendritic cell precursors. *Annu Rev Immunol* 23: 275-306.
- LUTZ MB AND SCHULER G. 2002. Immature, semi-mature and fully mature dendritic cells: which signals induce tolerance or immunity? *Trends Immunol* 23: 445-449.
- MA DY AND CLARK EA. 2009. The role of CD40 and CD154/CD40L in dendritic cells. *Semin Immunol* 21: 265-272.
- MAECKER HT, FREY T, NOMURA LE AND TROTTER J. 2004. Selecting fluorochrome conjugates for maximum sensitivity. *Cytometry A* 62: 169-173.
- MAHALINGAIAH PK, PALENSKI T AND VAN VLEET TR. 2018. An *In Vitro* Model of Hematotoxicity: Differentiation of Bone Marrow-Derived Stem/Progenitor Cells into Hematopoietic Lineages and Evaluation of Lineage-Specific Hematotoxicity. *Curr Protoc Toxicol* 1: e45.
- MASSA C AND SELIGER B. 2013. Fast dendritic cells stimulated with alternative maturation mixtures induce polyfunctional and long-lasting activation of innate and adaptive effector cells with tumor-killing capabilities. *J Immunol* 190: 3328-3337.
- MATZINGER P. 1994. Tolerance, danger, and the extended family. *Annu Rev Immunol* 12: 991-1045.
- MERAD M, GINHOUX F AND COLLIN M. 2008. Origin, homeostasis and function of Langerhans cells and other langerin-expressing dendritic cells. *Nat Rev Immunol* 8: 935-947.
- MERAD M, SATHE P, HELFT J, MILLER J AND MORTHA A. 2013. The dendritic cell lineage: ontogeny and function of dendritic cells and their subsets in the steady state and the inflamed setting. *Annu Rev Immunol* 31: 563-604.
- MEYER TP, ZEHNTER I, HOFMANN B, ZAISSERER J, BURKHART J, RAPP S, WEINAUER F, SCHMITZ J AND ILLERT WE. 2005. Buffy Coats (FBC): a source of peripheral blood leukocytes recovered from leukocytodepletion filters. *J Immunol Methods* 20: 150-166.
- MIAO EA AND WARREN SE. 2010. Innate immune detection of bacterial virulence factors via the NLR4 inflammasome. *J Clin Immunol* 30: 502-506.
- MOHAMADZADEH M, BERARD F, ESSERT G, CHALOUNI C, PULENDRAN B, DAVOUST J, BRIDGES G, PALUCKA AK AND BANCHEREAU J. 2001. Interleukin 15 skews monocyte differentiation into dendritic cells with features of Langerhans cells. *J Exp Med* 194: 1013-1020.
- MOHTY M, VIALLE-CASTELLANO A, NUNES JA, ISNARDON D, OLIVE D AND GAUGLER B. 2003. IFN-alpha skews monocyte differentiation into Toll-like receptor 7-expressing dendritic cells with potent functional activities. *J Immunol* 171: 3385-3393.
- MOODY DB. 2006. TLR gateways to CD1 function. *Nat Immunol* 7: 811-817.
- MOORE GE, GERNER RE AND FRANKLIN HA. 1967. Culture of normal human leukocytes. *JAMA* 199: 519-524.
- MORI L, LEPORE M AND DE LIBERO G. 2016. The Immunology of CD1- and MR1-Restricted T Cells. *Annu Rev Immunol* 34: 479-510.
- NAPOLITANI G, LEPORE M AND DE LIBERO G. 2005. The Immunology of CD1- and MR1-Restricted T Cells. *Nat Immunol* 6: 769-776.
- NEEFJES J, JONGSMA ML, PAUL P AND BAKKE O. 2011. Towards a system understanding of MHC class I and MHC class II antigen presentation. *Nat Rev Immunol* 11: 823-836.
- NESTLE FO, DI MEGLIO P, QIN JZ AND NICKOLOFF BJ. 2009. Skin immune sentinels in health and disease. *Nat Rev Immunol* 9: 679-691.
- OBERMAIER B, DAUER M, HERTEN J, SCHAD K, ENDRES S AND EIGLER A. 2003. Development of a new protocol for 2-day generation of mature dendritic cells from human monocytes. *Biol Proced Online* 5: 197-203.
- O'SULLIVAN B AND THOMAS R. 2003. CD40 and dendritic cell function. *Crit Rev Immunol* 23: 83-107.
- PARK BS AND LEE JO. 2013. Recognition of lipopolysaccharide pattern by TLR4 complexes. *Exp Mol Med* 6: 45-66.
- PATARROYO M, PRIETO J, BEATTY PG, CLARK EA AND GAHMBERG CG. 1988. Adhesion-mediating molecules of human monocytes. *Cell Immunol* 113: 278-289.
- PATEL AA ET AL. 2017. The fate and lifespan of human monocyte subsets in steady state and systemic inflammation. *J Exp Med* 214: 1913-1923.
- PAVLOVIĆ B, TOMIĆ S, ĐOKIĆ J, VASILJIĆ S, VUČEVIĆ D, LUKIĆ J, GRUDEN-MOVSESIJAN A, ILIĆ N, MARKOVIĆ M AND ČOLIĆ M. 2015. Fast dendritic cells matured with Poly (I:C) may acquire tolerogenic properties. *Cytotherapy* 17: 1763-1776.
- PENG JC, THOMAS R AND NIELSEN LK. 2005. Generation and maturation of dendritic cells for clinical application under serum-free conditions. *J Immunother* 28: 599-609.
- PERUSSIA B, FANNING V AND TRINCHIERI G. 1985. A leukocyte subset bearing HLA-DR antigens is responsible for *in vitro* alpha interferon production in response to viruses. *Nat Immun Cell Growth Regul* 4: 120-137.
- PULENDRAN B. 2015. The Varieties of Immunological Experience: Of Pathogens, Stress, and Dendritic Cells. *Annu Rev Immunol* 33: 563-606.

- RAETZ CR AND WHITFIELD C. 2002. Lipopolysaccharide endotoxins. *Annu Rev Biochem* 71: 635-700.
- RAMADAN G, KONINGS S, KURUP VP AND KEEVER-TAYLOR CA. 2004. Generation of Aspergillus- and CMV- specific T-cell responses using autologous fast DC. *Cytotherapy* 6: 223-234.
- RANDOLPH GJ, OCHANDO J AND PARTIDA-SÁNCHEZ S. 2008. Migration of dendritic cell subsets and their precursors. *Annu Rev Immunol* 26: 293-316.
- RELLOSO M, PUIG-KRÖGER A, PELLO OM, RODRÍGUEZ-FERNÁNDEZ JL, DE LA ROSA G, LONGO N, NAVARRO J, MUÑOZ-FERNÁNDEZ MA, SÁNCHEZ-MATEOS P AND CORBÍ AL. 2002. DC-SIGN (CD209) expression is IL-4 dependent and is negatively regulated by IFN, TGF-beta, and anti-inflammatory agents. *J Immunol* 168: 2634-2643.
- REPNIK U, KNEZEVIC M AND JERAS M. 2003. Simple and cost-effective isolation of monocytes from buffy coats. *J Immunol Methods* 278: 283-292.
- ROJAS-CANALES D, KRISHNAN R, JESSUP CF AND COATES PT. 2012. Early exposure of interferon- γ inhibits signal transducer and activator of transcription-6 signalling and nuclear factor κ B activation in a short-term monocyte-derived dendritic cell culture promoting 'FAST' regulatory dendritic cells. *Clin Exp Immunol* 167: 447-458.
- RUPPERT J, FRIEDRICH D, XU H AND PETERS JH. 1991. IL-4 decreases the expression of the monocyte differentiation marker CD14, paralleled by an increasing accessory potency. *Immunobiology* 182: 449-464.
- SAIKH KU, KHAN AS, KISSNER T AND ULRICH RG. 2001. IL-15-induced conversion of monocytes to mature dendritic cells. *Clin Exp Immunol* 126: 447-455.
- SALLUSTO F AND LANZAVECCHIA A. 1994. Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor alpha. *J Exp Med* 179: 1109-1118.
- SANARICO N, CIARAMELLA A, SACCHI A, BERNASCONI D, BOSSÙ P, MARIANI F, COLIZZI V AND VENDETTI S. 2006. Human monocyte-derived dendritic cells differentiated in the presence of IL-2 produce proinflammatory cytokines and prime Th1 immune response. *J Leukoc Biol* 80: 555-562.
- SANDER J ET AL. 2017. Cellular Differentiation of Human Monocytes Is Regulated by Time-Dependent Interleukin-4 Signaling and the Transcriptional Regulator NCOR2. *Immunity* 47: 1051-1066.
- SANTINI SM, LAPENTA C, LOGOZZI M, PARLATO S, SPADA M, DI PUCCHIO T AND BELARDELLI F. 2000. Type I interferon as a powerful adjuvant for monocyte-derived dendritic cell development and activity *in vitro* and in Hu-PBL-SCID mice. *J Exp Med* 191: 1777-1788.
- SCHLEE M AND HARTMANN G. 2016. Discriminating self from non-self in nucleic acid sensing. *Nat Rev Immunol* 16: 566-580.
- SCHMID I, KRALL WJ, UITTENBOGAART CH, BRAUN J AND GIORGI JV. 1992. Dead cell discrimination with 7-amino-actinomycin D in combination with dual color immunofluorescence in single laser flow cytometry. *Cytometry* 13: 204-208.
- SCHULZ O, EDWARDS AD, SCHITO M, ALIBERTI J, MANICKASINGHAM S, SHER A AND REIS E SOUSA C. 2000. CD40 triggering of heterodimeric IL-12 p70 production by dendritic cells *in vivo* requires a microbial priming signal. *Immunity* 13: 453-462.
- SEGURA E, VALLADEAU-GUILEMOND J, DONNADIEU MH, SASTRE-GARAU X, SOUMELIS V AND AMIGORENA S. 2012. Characterization of resident and migratory dendritic cells in human lymph nodes. *J Exp Med* 209: 653-660.
- STEINMAN RM. 2012. Decisions about dendritic cells: past, present, and future. *Annu Rev Immunol* 30: 1-22.
- STEINMAN RM AND COHN ZA. 1973. Identification of a novel cell type in peripheral lymphoid organs of mice. I. Morphology, quantitation, tissue distribution. *J Exp Med* 137: 1142-1462.
- STEINMAN RM AND WITMER MD. 1978. Lymphoid dendritic cells are potent stimulators of the primary mixed leukocyte reaction in mice. *Proc Natl Acad Sci USA* 75: 5132-5136.
- STOCKWIN LH, MCGONAGLE D, MARTIN IG AND BLAIR GE. 2000. Dendritic cells: immunological sentinels with a central role in health and disease. *Immunol Cell Biol* 78: 91-102.
- STRASSER EF AND ECKSTEIN R. 2010. Optimization of leukocyte collection and monocyte isolation for dendritic cell culture. *Transfus Med Rev* 24: 130-139.
- STRASSER EF, WEIDINGER T, ZIMMERMANN R, RINGWALD J AND ECKSTEIN R. 2007. Recovery of white blood cells and platelets from leukoreduction system chambers of Trima Accel and COBE Spectra plateletpheresis devices. *Transfusion* 47: 1943-1944
- SUZUKI H ET AL. 2004. Activities of granulocyte-macrophage colony-stimulating factor and interleukin-3 on monocytes. *Am J Hematol* 75: 179-189.
- SWIECKI M AND COLONNA M. 2015. The multifaceted biology of plasmacytoid dendritic cells. *Nat Rev Immunol* 15: 471-485.
- TAKAHASHI K, HONEYMAN MC AND HARRISON LC. 1997. Dendritic cells generated from human blood in granulocyte macrophage-colony stimulating factor and interleukin-7. *Hum Immunol* 55: 103-116.
- TANAKA F, YAMAGUCHI H, HARAGUCHI N, MASHINO K, OHTA M, INOUE H AND MORI M. 2006. Efficient induction of specific cytotoxic T lymphocytes to tumor rejection peptide using functional matured 2 day-cultured

- dendritic cells derived from human monocytes. *Int J Oncol* 29: 1263-1268.
- TRAVIS MA AND SHEPPARD D. 2014. TGF- β activation and function in immunity. *Annu Rev Immunol* 32: 51-82.
- TURVILLE SG, CAMERON PU, HANDLEY A, LIN G, PÖHLMANN S, DOMS RW AND CUNNINGHAM AL. 2002. Diversity of receptors binding HIV on dendritic cell subsets. *Nat Immunol* 3: 975-983.
- UENO H ET AL. 2007. Dendritic cell subsets in health and disease. *Immunol Rev* 219: 118-142.
- ULANOVA M, TARKOWSKI A, HAHN-ZORIC M AND HANSON LA. 2001. The Common vaccine adjuvant aluminum hydroxide up-regulates accessory properties of human monocytes via an interleukin-4-dependent mechanism. *Infect Immun* 69: 1151-1159.
- VATSAN RS, BROSS PF, LIU K, THEORET M, DE CLARO AR, LU J, HELMS W, NILAND B, HUSAIN SR AND PURI RK. 2013. Regulation of immunotherapeutic products for cancer and FDA's role in product development and clinical evaluation. *J Immunother Cancer* 29: 1-5.
- VICENTE-SUAREZ I, BRAYER J, VILLAGRA A, CHENG F AND SOTOMAYOR EM. 2009. TLR5 ligation by flagellin converts tolerogenic dendritic cells into activating antigen-presenting cells that preferentially induce T-helper 1 responses. *Immunol Lett* 125: 114-118.
- VOGT TK, LINK A, PERRIN J, FINKE D AND LUTHER SA. 2009. Novel function for interleukin-7 in dendritic cell development. *Blood* 113: 3961-3968.
- WAJANT H, PFIZENMAIER K AND SCHEURICH P. 2003. Tumor necrosis factor signaling. *Cell Death Differ* 10: 45-65.
- WANG S AND EL-DEIRY WS. 2003. TRAIL and apoptosis induction by TNF-family death receptors. *Oncogene* 22: 8628-8633.
- WHO - WORLD HEALTH ORGANIZATION. 2010. Guidelines on Drawing Blood: Best Practices in Phlebotomy. http://www.euro.who.int/__data/assets/pdf_file/0005/268790/WHO-guidelines-on-drawing-blood-best-practices-in-phlebotomy-Eng.pdf?ua=1 (accessed 12 April 2018).
- WORBS T. 2017. Dendritic cell migration in health and disease. *Nat Rev Immunol* 17: 30-48.
- WU L AND LIU YJ. 2007. Development of dendritic-cell lineages. *Immunity* 26: 741-750.
- XIAO Q, LI X, SUN D, YIH, LUX AND NIAN H. 2016. TLR7 Engagement on Dendritic Cells Enhances Autoreactive Th17 Responses via Activation of ERK. *J Immunol* 197: 3820-3830.
- YANG GX, LIAN ZX, KIKUCHI K, MORITOKI Y, ANSARI AA, LIU YJ, IKEHARA S AND GERSHWIN ME. 2005. Plasmacytoid dendritic cells of different origins have distinct characteristics and function: studies of lymphoid progenitors versus myeloid progenitors. *J Immunol* 175: 7281-7287.