Chapter 1

Phenotypic Markers and Functional Regulators of Myelomonocytic Cells

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Abstract

In this chapter, there is a description of hematopoietic stem cells, maturation curve and their differentiation into myeloid cells, including phenotypes and transcription factors involved in this process. Further, we discuss myeloid maturation curve from myeloid precursor, monoblast, premonocyte to monocytes, and also monocytes subsets regarding their CD14 and CD16 expressions and related functions in health and disease. In addition, we reason about the differentiation from monocytes either in dendritic cells or in macrophages *in vitro* using differential growth factors; these cells are differentiated from those found *in vivo* being named as monocyte-derived cells. Furthermore, we explore distinguished phenotype of monocytes, macrophages, and dendritic cells monocyte-derived *in vitro*, using confocal microscopy and flow cytometry, in order to display morphological and phenotypic differences among them.

Keywords: myelomonocytic cells, monoblast, promonocytes, dendritic cells, monocytes

1. Introduction

All the cellular elements of blood derive ultimately from the hematopoietic stem cells in the bone marrow. Thus, the blood cells are derived from the common lymphoid progenitor and the myeloid progenitor, apart from the megakaryocytes and red blood cells that are derived from specific progenitors. Particularly, the lymphoid progenitor gives rise to natural killer (NK) cells, T and B lineage cells of the human immune system, while the myeloid progenitor is the precursor of the granulocytes, monocytes, macrophages, and dendritic cells (**Figure 1**).

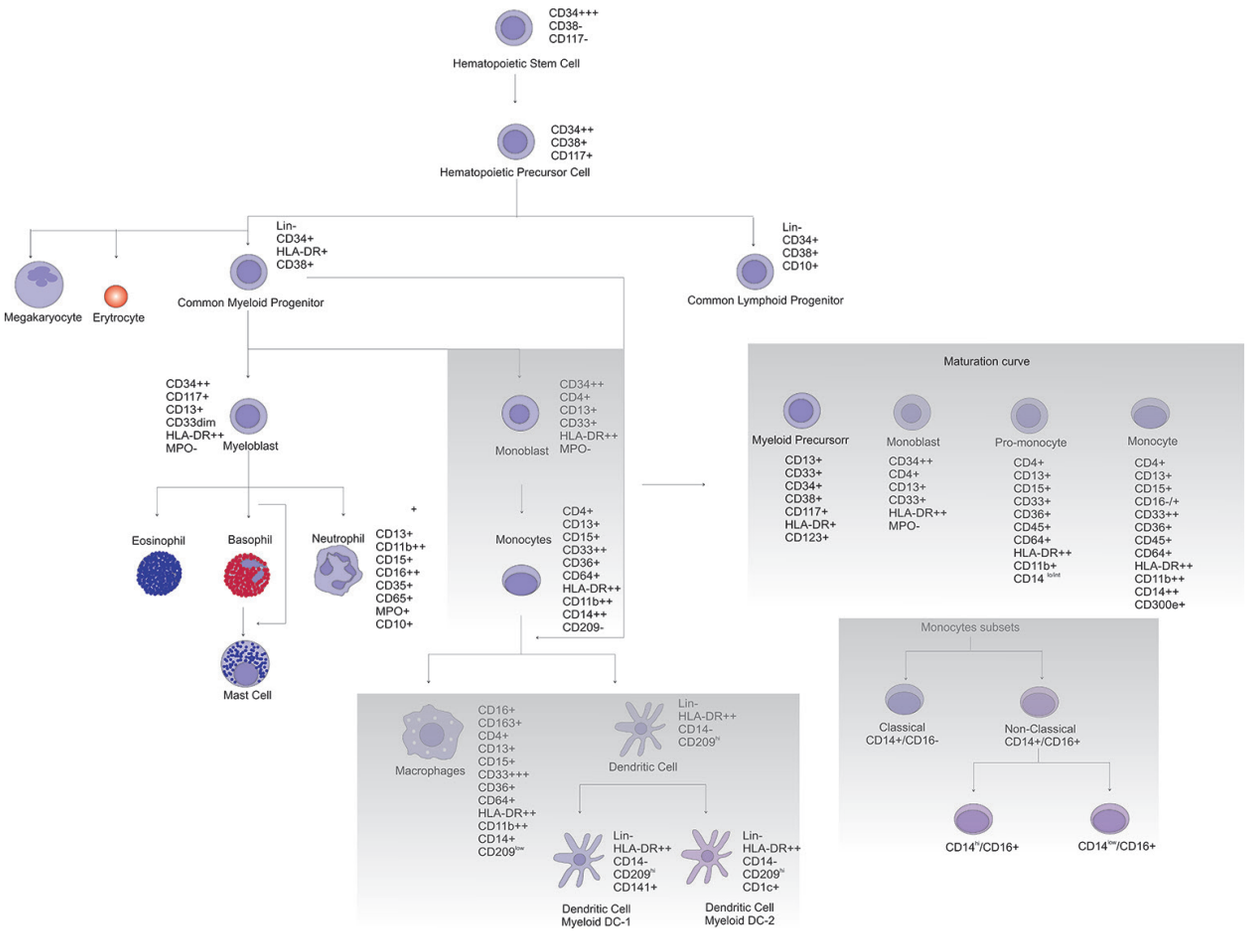

Figure 1. Hematopoiesis—General representation of hematopoiesis with focus on myelomonocitic differentiation [1, 2].

Myeloid cells represent the major leukocyte population in the peripheral blood. Phylogenically, these cells are the oldest ones found in primitive invertebrates and, in vertebrates, sum to lymphoid cells to constantly supply to all tissues via peripheral blood circulation [1].

The myeloid precursor gives rise to granulocytes and monocytes. Granulocytes are comprised of neutrophils, eosinophils, and basophils. Neutrophils are known as the key effector cells in innate immunity against bacteria and are the first cells to be recruited into local sites on pathogen invasion, providing an immediate defense against infection in tissues. The main role of neutrophils is to isolate, engulf, and kill pathogens using oxidative and nonoxidative mechanisms [1, 2].

Myelomonocytic cells give rise to mature monocytes that are present in circulation and were believed to mature terminally into macrophages in various tissues, where they may display a unique, tissue-dependent morphology and specific functions such as Kupffer cells in the liver or microglia in the brain. Monocytes may also differentiate into dendritic cells in lymphoid organs and Langerhans cells in skin, where they function as professional antigen presenting cells [2].

Monocytes, dendritic cells, and macrophages are the bridge between innate and adaptive immunity, they are a group of cells that are vital for the control of pathogens and for orchestration of a complete immune response, as well as for backing up tissue functions. These

properties make them interesting targets for immune therapy, vaccination, and treatment of autoimmune and inflammatory diseases [3, 4].

However, exactly how many cell types exist in the mononuclear phagocyte system, or whether they establish a family, has been a matter of discussion for many years. Historically, cells of the mononuclear phagocyte system have been referred to as erythrophagocytes, adventitia cells, histiocytes, and several other terminologies until their current terminology was established in 1972 by a bulletin published by the World Health Organization (WHO) [5]. The discovery of a new cell type termed dendritic cells in the 1970s by the Nobel Prize winner Ralph Steinman that was distinct from macrophages added more complexity to the mononuclear phagocyte system classification [6].

Accordingly, it took a while before dendritic cells were fully accepted as a true member of the mononuclear phagocyte system. Over time, there was appreciation that there were not just one but multiple dendritic cells subtypes, each with a specialized role [7]. Nowadays, there are several discussions about macrophages and dendritic cells nomenclature, subsets, and their *in vivo* origin, and how much they are related to macrophages or monocytes.

Thus in this chapter, the principles of hematopoiesis, phenotype, and transcription factors in myelomonocytic lineage will be highlighted, as well as their maturation and differentiation. The contribution of different cytokine environments modulating the monocytic lineage differentiation into subtypes of macrophages or dendritic cells will also be discussed.

2. Hematopoiesis, phenotype, and lineage transcription factors

Blood development in vertebrates includes two hematopoiesis waves: primitive and definitive ones [8]. The primitive wave involves an erythroid progenitor and gives rise to erythrocytes and macrophages during the early embryonic development [9]. The purpose of the primitive wave is to produce red blood cells in order to oxygenate the embryo tissues that experience a fast growth [10]. In mammals, these erythroid progenitor cells first appear in blood islands in the extra-embryonic yolk sac early in development [11]. The primitive wave is transitory, and these erythroid progenitors are not pluripotent and do not have self-renewal ability.

Instead, definitive hematopoiesis occurs later in development, markedly at different periods in different species. Definitive hematopoiesis involves hematopoietic stem cells, which are multipotent cells that can generate all blood lineages of an adult organism. In vertebrates, hematopoietic stem cells are born in the aorta-gonad-mesonephros region of the developing embryo. They migrate to the fetal liver and then to the bone marrow, which is the final site for hematopoietic stem cells in adults [12].

Usually, in order to characterize and quantify hematopoietic stem cells, flow cytometry techniques are commonly used. The immunophenotypic markers CD34 and CD38 are used to characterize and enumerate hematopoietic stem cell (HSC) and progenitors (HPC) as shown in **Figures 1** and **2** [13]. Hematopoietic stem cells are a small population characterized by the expression of CD34+CD38−, and progenitors are recognized by the expression of CD34+CD38+

(**Figure 2A**). HSCs are also CD117−, and during differentiation toward common myeloid and lymphoid progenitors (CMP and CLP), they acquire CD117 and Human Leukocyte Antigen–DR (HLA-DR) expression; and later, as per their lineage commitment, they can or not preserve these markers (**Figure 2B**).

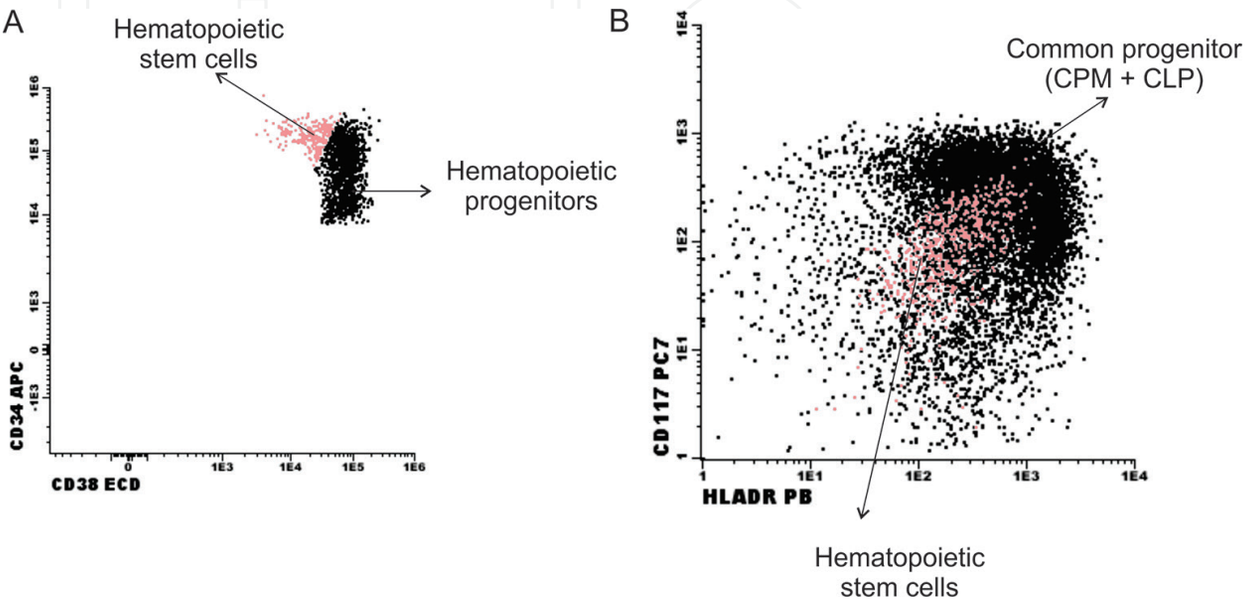

Figure 2. Hematopoietic stem cells differentiation curve—(A) Hematopoietic progenitors CD34+CD38+, hematopoietic stem cells CD34+CD38− (red). **(B)** Common myeloid and lymphoid progenitors (CMP and CLP) are CD34+CD38+CD117+HLA-DR+ (black) (Infinicyt software was used for this analysis, Cytognos).

After hematopoiesis initiation, several decision steps are necessary for HSC pluripotency and quiescence maintenance or specification of lineage commitment [14]. One important checkpoint is the preservation of pluripotency by the combined action of Notch-1, GATA-2, HoxB4, and Ikaros transcription factors. Furthermore, the cell cycle inhibitor p21 is essential to keep a fraction of stem cells in quiescence [15].

During lineage commitment, transcription factors play critical roles at distinct differentiation branches. Concerning the myeloid-lineage commitment, PU.1, an Ets family of transcription factor, seems to play a key role [16]. PU.1-deficient mice lack monocytes and B cells with a greatly reduced number of granulocytes, while overexpression of PU.1 enhances the development of myeloid cells. Consequently, the enhanced expression of PU.1 favors myeloid commitment, while low-to-intermediate expression of PU.1 together with GATA-3 and Ikaros transcription factors commit HSC toward lymphoid lineage [17].

Once the myeloid dominance has been established through increased PU.1 or GATA-1 expression, further transcriptional control determines the commitment along erythroid/megakaryocytic (GATA-1/2 dominance) or myelomonocytic (PU.1 dominance) lineages, while mafB together with PU.1 also play an essential role in monocyte/macrophage differentiation [18]. MafB, c-Maf, and Egr-1 are suggested to promote monocytic differentiation at the cost of granulopoiesis [19].

In addition, the induction of C/EBPα or C/EBPβ modulates the fate of myeloid-committed cells toward granulocytic branches since CCAAT/Enhancer Binding Protein (C/EBP), a basic region leucine zipper DNA-binding protein, is responsible for the transactivation of Granulocyte-Colony Stimulating Factor Receptor (G-CSFR) gene and retinoic acid receptors (RARs) [14]. It has been shown that C/EBP-deficient mice selectively lacks granulocytes and RAR-deficient mice shows a granulocyte differentiation arrested at the myelocyte stage.

Myelomonocytic cells are usually classified based on surface markers and biological responses. The common myeloid progenitor (CMP) is characterized by markers such as CD34 and CD117. These immature cells are able to differentiate into neutrophils, monocytes, macrophages, dendritic cells (DCs), and under pathological conditions or induced by proinflammatory cytokines these cells can also generate a population known as myeloid-derived suppressor cells.

Myeloid-derived suppressor cells are part of the myeloid-cell lineage and a heterogeneous population that is comprised by myeloid-progenitors and precursors cells. In healthy individuals, immature myeloid cells rapidly differentiate toward mature granulocytes, macrophages, or dendritic cells. However, in pathological conditions such as cancer, infectious diseases, trauma, or some autoimmune disorders, a partial impairment in the immature myeloid cells differentiation result in the expansion of this population. Importantly, the activation of these cells in pathological conditions results in an upregulated expression of immune suppressive factors such as arginase, inducible nitric oxide synthase (iNOS), and reactive oxygen species (ROS) increased production. Together, these alterations results in the expansion of immature myeloid cells that possess suppressive activity [20].

3. Monocytes maturation curve and subsets

Regarding phenotypes, monocyte maturation curve can be performed by flow cytometry and can display the differentiation of CD34+/CD117+/CD64−/CD14− myeloid precursor into monoblast CD64+ and further into promonocyte by increased expression of $CD14^{lo/int}$. Additionally, these cells will become full mature monocytes by $CD14^{hi}$ expression (**Figures 1** and **3A**). During monocyte maturation, upregulation of CD64 is followed not only by CD14 but also increased levels of CD33, CD36, and CD300e expression toward mature monocytes (**Figures 1** and **3A**).

In addition, the differences in monocytes and granulocytes maturation curve can be seen using a combination of CD11b and HLA-DR markers. Granulocytes arise from a precursor that do express HLA-DR and downregulated its expression during maturation, while monocytes arise from a precursor with high expression of HLA-DR and preserve the HLA-DR expression to maturation, while both cells' subsets have an increased expression of CD11b toward maturation (**Figures 1** and **3B**).

Monocytes were originally classified by their physical characteristics, but after flow cytometry advent, monocytes became also recognized by CD14 and CD16 expressions (**Figures 1** and **4A**). Classical monocytes $CD14^{hi}$/CD16− (**Figures 1** and **4B**) are approximately 80% of all monocytes and considered to be better at secreting proinflammatory cytokines, phagocytosis, and ROS production [21]. The nonclassical CD14+/CD16+ cells resemble "resident" tissue macrophages with higher Major Histocompatibility Complex - Class II (MHC-II) expression. CD16+ monocytes

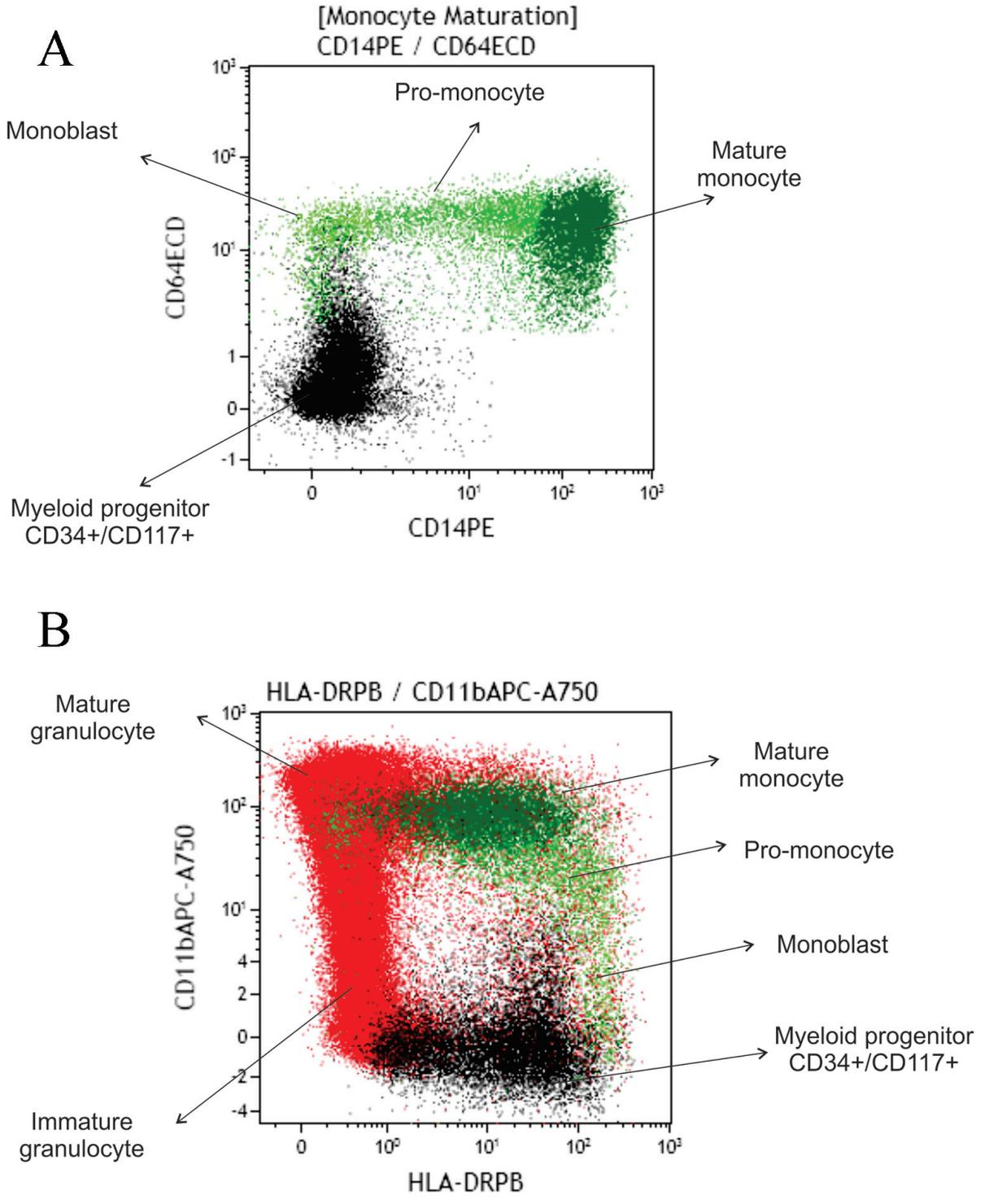

Figure 3. Monocyte and granulocyte maturation curve—(A) Monocyte maturation: this dot plot displays a combination of previously three-gated population and shows myeloid progenitors CD34+/CD117+/CD64−/CD14− (black), monoblast CD64+CD14− and promonocytes CD64+CD14$^{lo/int}$ (light green), mature monocyte CD64+CD14^{hi} (dark green). **(B) Monocyte and granulocyte maturation**: this dot plot displays a combination of previously four-gated population and shows myeloid progenitors CD34+/CD117+/HLA-DR+ (black), monoblast HLADR+CD14− and promonocytes HLA-DR$^{lo/int}$ (light green), mature monocyte HLA-DR+CD14^{hi} (dark green), immature granulocytes HLA-DR−/CD11b^{lo}, and mature granulocytes HLA-DR−/CD11b^{hi} (red) (Kalusa software was used for this analysis, Beckman Coulter).

are subdivided into CD14^{hi}/CD16+ and CD14^{lo}/CD16+ (**Figures 1** and **4B**). CD14^{hi}/CD16+ monocytes express highest levels of phagocytosis; MHC-II and accessory molecule expression are also higher compared with CD14^{lo}/CD16+ (**Figures 1** and **4C**) [21]. Functional data and gene arrays suggest that CD14^{hi}/CD16+ monocytes share more common pattern with CD14^{hi}/CD16− monocytes than with CD14^{lo}/CD16+ [22].

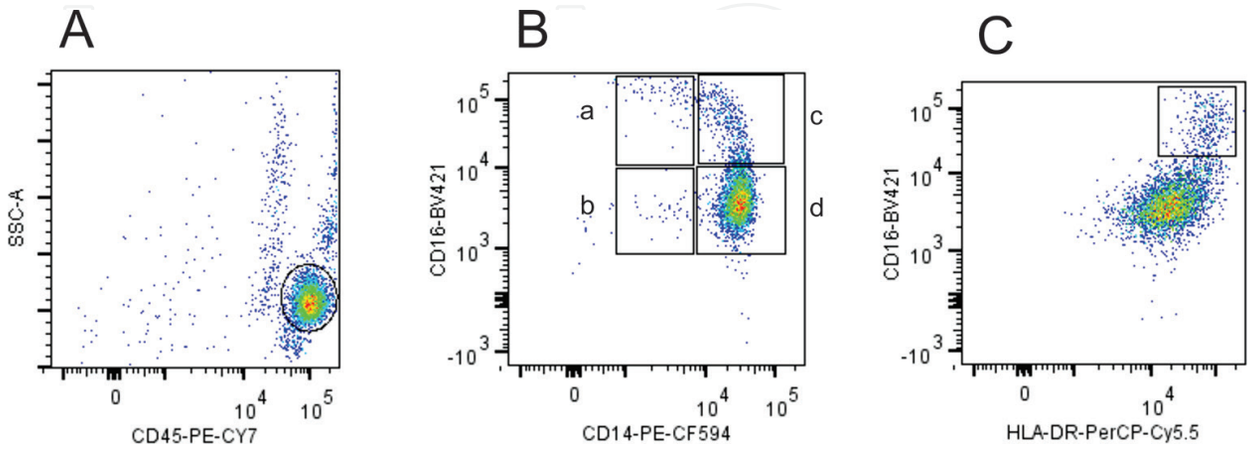

Figure 4. Monocytes (CD14+) sorted from human peripheral blood—(A) SSC vs CD45. **(B)** Monocytes subpopulations (a) CD14^{lo}/CD16+ (b) CD14^{lo}/CD16− (c) CD14^{hi}/CD16+ and (d) CD14^{hi}/CD16−. **(C)** CD14^{hi} (c+d)/CD16+ are HLA-DR^{hi} (FlowJo software was used for this analysis, TreeStar).

These monocyte characteristics subsets have recently been reported as a signature diagnostic for chronic myelomonocytic leukemia (CMML). Researchers compared the population of monocytes among healthy bone marrow donors, patients with reactive monocytosis, another hematologic malignancy, and CMML patients, which demonstrate a characteristic increase in the fraction of CD14+/CD16− cells compared with the other samples [23].

In addition, the development and biological significance of monocyte subsets remain a matter of active investigation, as well as their respective functions and developmental relationships. CD161 is another important marker that also defines monocytes subsets. These CD161 subsets seem to be expanded in a variety of clinical situations, including autoimmune diseases, bacterial and viral infections, asthma, stroke, and coronary artery disease [24–29]. In addition, there is a new emerging technology known as tissue macrophage scanning (TiMaScan), which is a sensitive intra-tissue total body scanning. This new technology promises to accurately detect and define monocyte and macrophage subsets in blood and tissues not only in homeostatic or traumatic injuries but also in cancer [30, 31].

Monocytes, macrophages, and myeloid DCs are members of the mononuclear phagocyte system that exhibit several functions during immune responses. Historically, these cells have been grouped together because although monocytes have their unique functions as mononuclear phagocytic cells, they were also considered as precursors of macrophages and myeloid DCs [32].

Monocytes and macrophages are critical effectors and regulators of inflammation involved in innate immune response, the immune system for immediate support. On the other hand, DCs

are a bridge between innate and adaptive immunity, because they initiate and regulate the highly pathogen-specific adaptive immune responses and play a central role in the development of immunological memory and tolerance. These cells can display significant heterogeneity in phenotype and function according to the tissue of residence. Dendritic cells are described as distinct lineage specialized in antigen presentation, initiation, and control of immunity, contributing to development of the immune response to pathogens, vaccines, and tumors [33].

Human dendritic cells subsets found *in vivo* are described in the literature as two main groups, plasmacytoid DCs (pDCs) and *classical* or *myeloid* DCs. Classical or myeloid DCs have been further subdivided into two subsets on the basis of their CD141 expression (also known as BDCA3) and CD1c (also known as BDCA1) [34, 35]. It has been shown that the gene-expression profiles and functions of human CD141+ DCs and CD1c+ DCs resemble those of mouse cDC1s and cDC2s, respectively [36].

Regarding transcription factors that regulate monocytes and DCs differentiation, there are several differences between mice and humans, but also similarities, which should be taken into consideration [37]. A straight comparison between human and mouse can be made due to the presence of Interferon Regulatory Factor 8 (IRF8) deficiency in both species. While human biallelic IRF8 mutation leads to complete loss of blood and skin DCs and monocytes derived cells, the autosomal dominant IRF8 mutation results in absence of CD1c expression and presence of a population CD11c+CD1c− not seen in a healthy control blood [37, 38]. In mice, IRF8 is required for the development of CD8+CD103+ DCs and plays a role in monocyte development through Interferon Regulatory Factor (IRF) interaction with Krüppel-like Factor 4 (KLF4) [39].

In contrast, human macrophages are found throughout body tissues [40]. During HSC transplantation, dermal macrophages in the recipient show prolonged survival and delayed replacement compared with dermal DCs, which is consistent with the impression that macrophages are also self-maintaining in humans. Furthermore, patients carrying a mutation in GATA-2 lack blood monocytes and all conventional DCs subsets, yet they have normal numbers of Langerhans cells and macrophages in skin and lungs, respectively, suggesting that these populations development may also occur independent of monocytes and DCs [41].

Another important characteristic of macrophages, which should be mentioned, is their polarization in two phenotypes M1 and M2, inflammatory macrophages are called M1, whereas those that decrease inflammation and favor tissue repair are called M2 macrophages. Later, findings regarding granulocyte macrophage colony-stimulating factor (GM-CSF) and macrophage colony-stimulating factor (M-CSF) effects in macrophages led to the independent inclusion of these as M1 and M2 stimuli, respectively. The polarized M2 phenotype, in a tumor microenvironment, has been named tumor-associated macrophages (TAMs) and is associated to tumor progression and to a poor prognosis [42].

4. Cytokines, growth factors, and *in vitro* models of monocyte differentiation into dendritic cells and macrophages

It is possible to differentiate either dendritic cells or macrophages *in vitro* from monocytes using differential growth factors, and these cells are differentiated from those found *in vivo* by being named as monocyte-derived cells. A number of growth factors have been shown to influence

monocyte development and differentiation into macrophages, and the best-recognized growth factor is the macrophage colony-stimulating factor (M-CSF). Its significance involves the observation that circulating monocytes express the M-CSF receptor [43, 44] and administration of M-CSF drives monocytosis [45–47].

Moreover, mice deficient either in the production of Macrophage-Colony Stimulating Factor (M-CSF) or in the Macrophage-Colony Stimulating Factor Receptor (M-CSFR) have been reported to have decreased numbers of monocytes in the bone marrow and/or in circulation [48]. Homeostatic control of monocyte/macrophage development has been proposed to result from the modulation of M-CSF levels by differentiated cells of the mononuclear phagocyte system, mature mononuclear phagocytes express high levels of the M-CSFR, and M-CSF is produced continuously by stromal cells, and the addition of IL-3 to cultured bone marrow cells enhances the activity of M-CSF [49].

Additional growth factors, including granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-4, are able to influence monocyte development and differentiation during inflammation. *In vitro* GM-CSF supports monocyte expansion and differentiation [50, 51]. Unexpectedly, GM-CSF deficient mice show minimal perturbation of hematopoiesis and no decrease in circulating monocyte numbers compared with the control [52]. However, *in vivo*, GM-CSF is not produced at high levels under homeostatic conditions; instead, it is upregulated during inflammation [50, 51]. This suggests that, in contrast to M-CSF, GM-CSF primarily contributes to monopoiesis during inflammatory states. Accordingly, M-CSF and GM-CSF drive different differentiation platforms, with M-CSF stimulation leading to a homeostatic phenotype, and GM-CSF stimulation leading to monocytes with an inflammatory phenotype. In addition, GM-CSF was the first growth factor shown to efficiently promote dendritic cells development *in vitro* and has been used to induce dendritic cell differentiation from human monocytes, as well as human and mouse hematopoietic progenitor cells [53, 54].

IL-4 has been argued to drive both tissue-resident macrophage [55, 56] and monocyte [57] expansion during type 2 inflammation. IL-4 in combination with GM-CSF drives inflammatory dendritic cells *in vitro* [53]. Studies in both Signal Transducer and Activator of Transcription 6 (STAT6) [57] and IL-4R [55] deficient mice indicate that IL-4-dependent signaling does not contribute to monocyte development during homeostasis.

The two main colony-stimulating factors involved in monopoiesis, M-CSF and GM-CSF, have opposing polarizing properties. *In vitro*, M-CSF supports the development of cells with anti-inflammatory profile that is characterized by production of IL-10 and CCL2 but not IL-12 or IL-23 [58]. On the other hand, culture of either bone marrow or purified monocytes with GM-CSF leads to upregulation of MHC class II as well as induction of IL-12 and IL-23, but minimal IL-10 production [59]. Based on these data, it has been discussed that M-CSF stimulation represents a homeostatic/M2 pathway for monocyte development [60]. *In vivo*, GM-CSF has been shown to induce an inflammatory DC/M1-like phenotype in monocytes in a variety of models [61].

Macrophages *in vitro* monocyte-derived, using M-CSF, are distinguished as larger and vacuolar cells, been very effective at apoptotic cells, cellular debris and pathogens clearance, and can be differentiated from DCs, monocytes-derived with GM-CSF in combination with IL-4 *in vitro* by the CD14 expression and from monocytes by CD209 (DC-SIGN) expression. By contrast, DCs are defined with stellate morphology that can efficiently present antigens

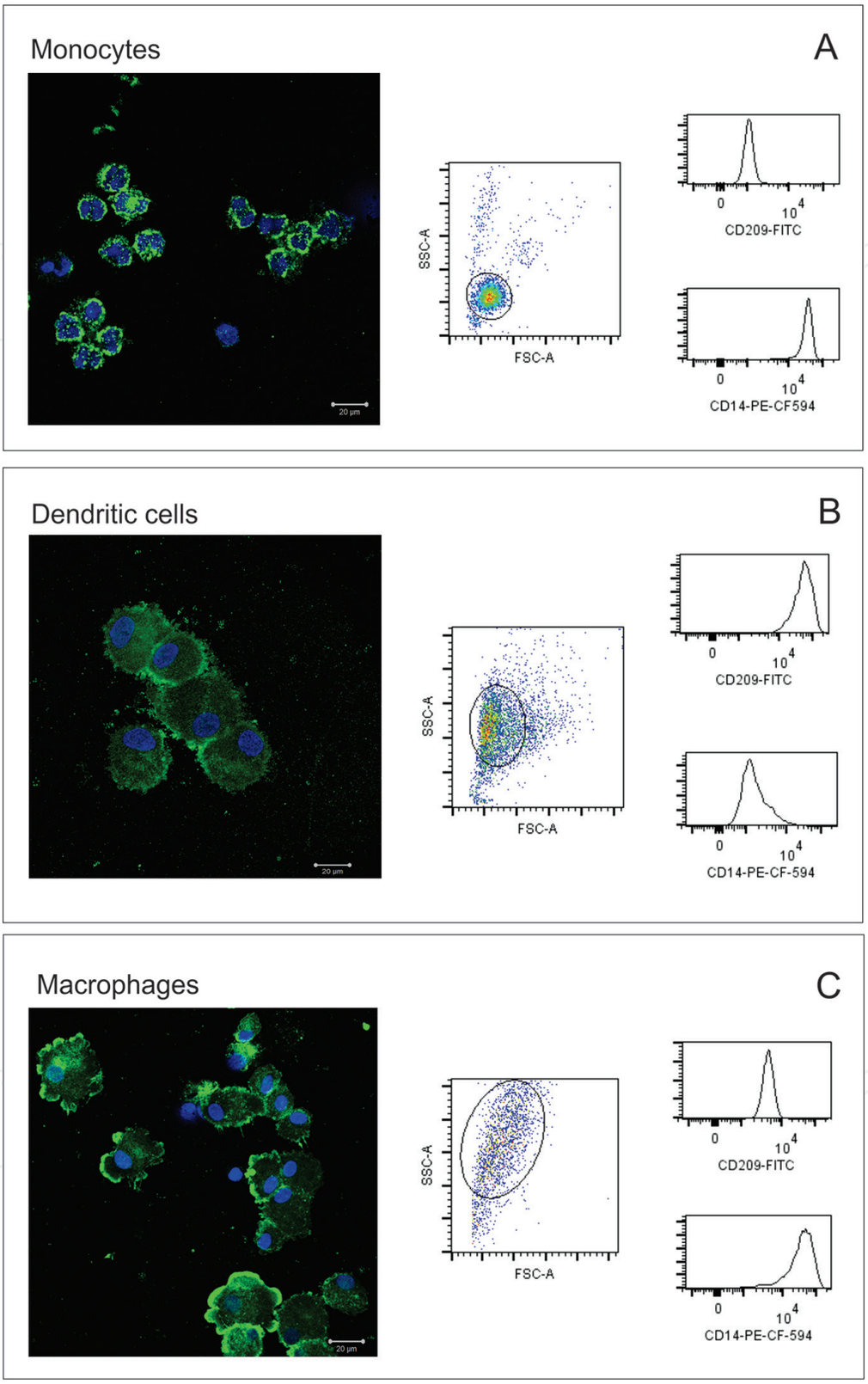

Figure 5. Monocytes, dendritic cells, and macrophages morphology and phenotype—To confocal microscopy analysis, all cells were stained with pan-actin (green) in order to show differences in morphology and cytoskeleton, and the nucleus were stained with DAPI (blue): (A) monocytes CD14+/CD209−, (B) dendritic cells CD14−/CD209^{hi}, (C) macrophages CD14+/CD209^{int} (FlowJo software was used for this analysis, TreeStar).

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through MHC molecules and activate naïve T cells. DCs derived from monocytes lack CD14 and acquire CD209 expression (**Figures 1** and **5**).

Regarding phagocytosis, monocytes and macrophages are highly phagocytic cells, in contrast to DCs that according to maturation status lose their phagocytic ability and become the most efficient antigen-presenting cells. Differences in expression related to functional status

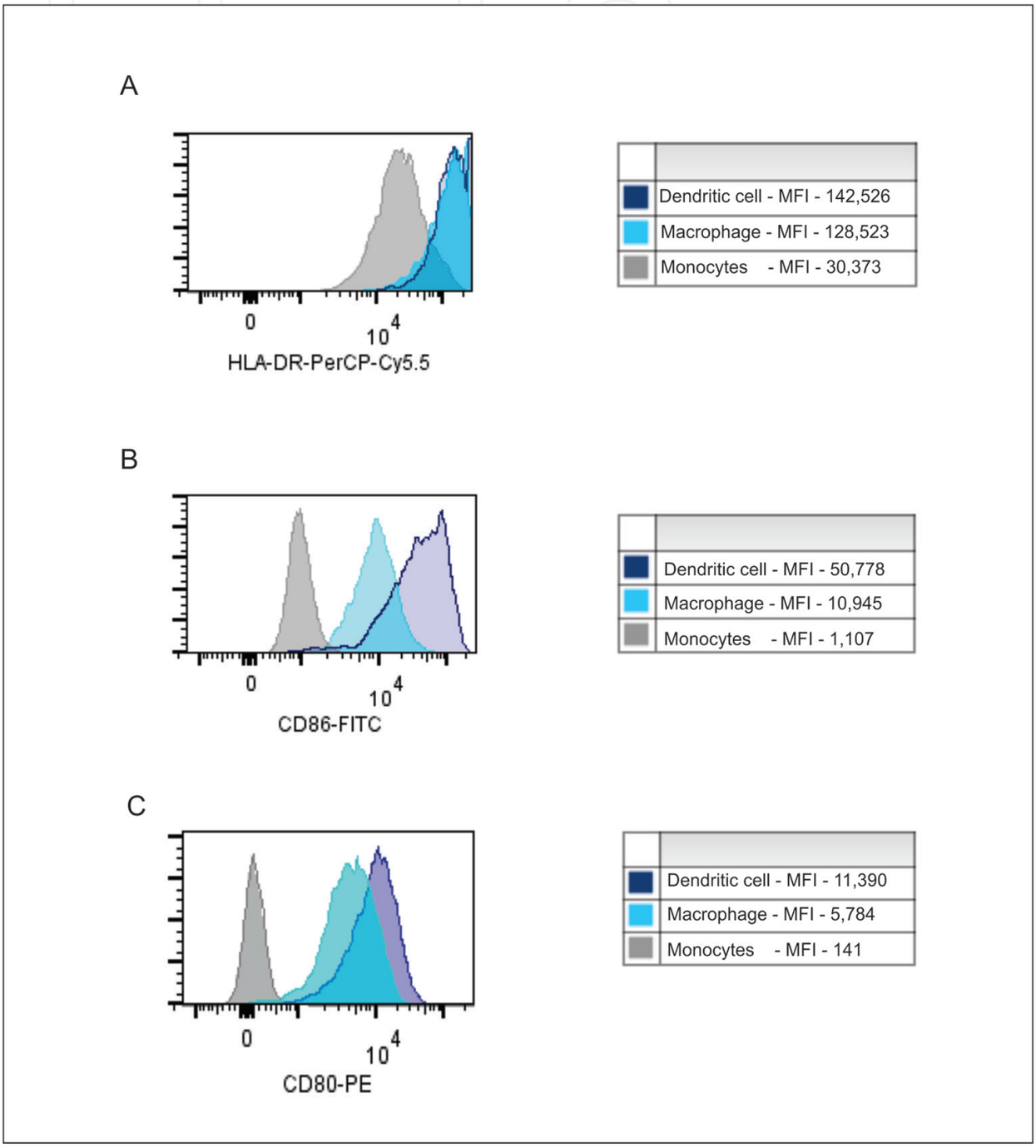

Figure 6. Monocytes, dendritic cells, and macrophages expression of HLA-DR, CD80, and CD86—(A) HLA-DR, (B) CD86, (C) CD80 increasing expression in media fluorescence intensity (MFI) (FlowJo software was used for this analysis, TreeStar).

of these cells are shown in **Figures 5** and **6**. DCs have increased expression in HLA-DR, CD80 and CD86 (molecules related to antigen presentation efficiency) compared with macrophages and monocytes (**Figure 6**).

5. Concluding remarks

Herein, we have presented the principles of hematopoiesis, transcription factors in myelomonocytic lineage phenotype, as well as their maturation and differentiation, and these topics have been the target of several studies for over a century using a variety of model systems. The human hematopoiesis understanding it is very important; this fundamental knowledge allowed scientists and physicians to identify diseases and their causes, leading to the development of new therapies.

In addition, we have discussed the contribution of different cytokine/growth factors' environment, modulating the monocytic lineage differentiation into subtypes of macrophages or dendritic cells and their development *in vitro*. Similarities and differences between cells found *in vivo* with the ones generated *in vitro* are very important for the development of new study models. Furthermore, the comprehension about growth factors and how to use them to modulate cells can favor their application in developmental hematology and immunology. These topics are very important for the continuous development of knowledge.

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