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OPTIMIZATION OF PRODUCTION OF ANTI-CANCER VACCINES BASED ON DENDRITIC CELLS

Dissertação para obtenção do Grau de Mestre em
Genética Molecular e Biomedicina

Orientadoras: Zélia Silva, PhD, NOVA Medical School
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Optimization of production of anti-cancer vaccines based on dendritic cells

AGRADECIMENTOS

Às minhas orientadoras, Paula Videira, Zélia Silva.
Por me terem deixado entrar na ciência.
Por todos os ensinamentos.
Por todo o carinho, amizade e confiança que não julgava serem sequer possíveis.
Por tudo, Obrigado.

A todo o grupo de Glicoimunologia do CEDOC, sem exceção.

Ao professor Pedro Batista, pela humildade incutida.

Aos meus pais.
À minha avó.
Ao meu sobrinho.

Aos que me puseram a viver.
Silva. Chico. João. Silvia. Sr. Luiz.

A todos os doentes com cancro,
Por vocês. Para vocês.

ABSTRACT

Cancer remains as one of the top killing diseases in first world countries. It's not a single, but a set of various diseases for which different treatment approaches have been taken over the years. Cancer immunotherapy comes as a "new" breath on cancer treatment, taking use of the patients' immune system to induce anti-cancer responses. Dendritic Cell (DC) vaccines use the extraordinary capacity of DCs' antigen presentation so that specific T cell responses may be generated against cancer.

In this work, we report the *ex vivo* generation of DCs from precursors isolated from clinical-grade cryopreserved umbilical cord blood (UCB) samples. After the thawing protocol for cryopreserved samples was optimized, the generation of DCs from $CD14^+$ monocytes, *i.e.*, moDCs, or $CD34^+$ hematopoietic stem cells (HSCs), *i.e*, CD34-derived DCs, was followed and their phenotype and function evaluated. Functional testing included the ability to respond to maturation stimuli (including enzymatic removal of surface sialic acids), Ovalbumin-FITC endocytic capacity, cytokine secretion and T cell priming ability. In order to evaluate the feasibility of using DCs derived from UCB precursors to induce immune responses, they were compared to peripheral blood (PB) moDCs.

We observed an increased endocytosis capacity after moDCs were differentiated from monocyte precursors, but almost 10-fold lower than that of PB moDCs. Maturation markers were absent, low levels of inflammatory cytokines were seen and T cell stimulatory capacity was reduced. Sialidase enzymatic treatment was able to mature these cells, diminishing endocytosis and promoting higher T cell stimulation. CD34-derived DCs showed higher capacity for both maturation and endocytic capacity than moDCs.

Although much more information was acquired from moDCs than from CD34-derived DCs, we conclude the last as probably the best suited for generating an immune response against cancer, but of course much more research has to be performed.

Keywords: Immunotherapy; Dendritic Cells; Umbilical Cord Blood; Hematopoietic Stem Cells; Sialic Acids.

RESUMO

O cancro permanece como uma das doenças que mais mata nos países do primeiro mundo. Não é uma única, mas sim um conjunto de várias doenças para as quais diferentes abordagens a nível de tratamento têm vindo a ser tomadas ao longo dos anos. A imunoterapia surge como uma "nova" lufada em tratamentos contra o cancro, fazendo uso do sistema imunitário dos pacientes para induzir respostas anti-cancro. Vacinas de Células Dendríticas (CDs) usam a extraordinária capacidade de apresentação antigénica das CDs para que respostas de células T específicas contra o cancro possam ser geradas.

Neste trabalho, reportamos a geração *ex vivo* de CDs a partir de percursores isolados de amostras de nível clínico de Sangue de Cordão Umbilical (SCU) criopreservado. Depois do protocolo de descongelamento ter sido otimizado, a geração de CDs a partir de monócitos $CD14^+$, isto é, moCDs, ou de células estaminais hematopoiéticas $CD34^+$ (CEH), isto é, CDs CD34-derivadas, foi seguida e o seu fenótipo e funcionalidade avaliados. As experiências funcionais incluíram a capacidade de resposta a estímulos de maturação (incluindo a remoção enzimática de ácidos siálicos de superfície), endocitose de ovalbumina-FITC, secreção de citocinas e capacidade de *priming* de células T. De maneira a avaliar a viabilidade do uso de CDs derivadas de percursores em SCU para induzir respostas imunes, estas foram comparadas a moCDs derivadas de sangue periférico (SP).

Observámos uma capacidade endocítica aumentada, depois das moCDs terem sido diferenciadas dos monócitos, mas quase 10 vezes inferior à capacidade endocítica de moCDs de SP. Os marcadores de maturação estavam ausentes, baixos níveis de citocinas inflamatórias foram encontrados e a capacidade de estimulação de células T foi reduzida. O tratamento enzimático com sialidase foi capaz de maturar estas células, diminuindo a endocitose e promovendo uma maior estimulação de células T. Já as CDs CD34-derivadas mostraram uma maior capacidade para maturarem e endocitarem, em comparação com as moCDs.

Apesar de muito mais informação ter sido recolhida a partir das moCDs em contraste com as CD34-derivadas, concluímos que estas últimas provavelmente serão as mais adequadas para gerar uma resposta imune contra o cancro, mas claro que muito mais pesquisa será indubitavelmente necessária.

Palavras-Chave: Imunoterapia; Células Dendríticas; Sangue de Cordão Umbilical; Células Estaminais Hematopoiéticas; Ácidos Siálicos.

The work developed until the present date is part of a QREN (Quadro de Referência Estratégico Nacional) project, Ref. 38870: aDVANCe – Desenvolvimento de novas vacinas anti-cancro a partir de Células Dendríticas. It has originated:

One provisional patent application:

Videira PA, Silva M, Marques GS, Ferro T, Silva Z, Monteiro MB, Gonçalves M, Takodoro C, van Kooyk Y, van Vliet S, Matos M, inventors; Crioestaminal - Saúde e Tecnologia SA, assignee. A novel dendritic cells population, method of production and use thereof. European Patent Application PPP51856/15. 2015 Jun 30.

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LIST OF ABBREVIATIONS

7-AAD	7-amino-actinomycin D
APC	Allophycocyanin
BSA	Bovine Serum Albumin
CFSE	Carboxyfluorescein succinimidyl ester
DCs	Dendritic Cells
DMSO	Dimethyl sulfoxide
FBS	Fetal Bovine Serum
FITC	Fluorescein Isothiocyanate
Flt-3L	FMS-related tyrosine kinase 3 Ligand
FSC	Forward Scatter
GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor
HES	Hydroxyethyl starch
HSCs	Hematopoietic Stem Cells
IL-2/4/6/10/12	Interleucin-2/4/6/10/12
IPST	Instituto Português do Sangue e da Tranplantação (Portuguese Blood Institute)
LPS	Lipopolysaccharide
MFI	Median Fluorescence Intensity
MHC I	Major Histocompatibility Complex Class I
MHC II	Major Histocompatibility Complex Class II
moDCs	Monocyte-derived dendritic cells
NK	Natural Killer cells
PBMCs	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
PE	Phycoerythrin
PerCP	Peridinin Chlorophyll Protein

PRR	Pattern Recognition Receptors
RBCs	Red Blood Cells
SCF	Stem Cell Factor
SSC	Side Scatter
Tc	Cytotoxic T lymphocytes
TCR	T Cell Receptor
Th	T helper cell
TLRs	Toll-Like Receptors
TPO	Thrombopoietin
Treg/s	T regulatory/suppressor cells
UCB	Umbilical Cord Blood

“THE MEDIATOR
BETWEEN HEAD AND HANDS
MUST BE THE HEART!”

Metropolis, 1927

1 | INTRODUCTION

1.1 | Immunology and Immune System.

One could not start an assay about the use of dendritic cells without getting acquainted with the most basic immunologic principles first. And, what is immunology then? In its first paragraph, Abbas's Cellular and Molecular Immunology states *immunity* as a semantic derivation from the Latin word *immunitas,* which meant protection from legal prosecution, something that was offered to roman senators during their tenures in office. Historically, it represented protection from (infectious) disease and, to the biological structures, cells, and molecules responsible for it we now call "immune system", able to generate a response against foreign substances occurring in the body (Abbas *et al.*, 2012).

We can divide the subject of immune system into two complementary concepts: innate and adaptive immunity. The first one refers to non-specific defense mechanisms against strange structures appearing in the body. They include physical and tissue barriers like MALT and SALT (Mucosa- and Skin-Associated Lymphoid Tissue), cells and certain chemicals that recognize, protect us from, and neutralize certain foreign cells or structures sharing "understandable patterns". Our body is able to counter-attack this pathogenic patterns without the need of learning how to, opposing to the second concept: adaptive immunity. Adaptive immunity will only comprise responses against very specific sets of structures – antigens. If recognized, the antigen will be processed and presented to lymphocytes in a very sophisticated process. Simply put, B lymphocytes will in turn produce molecules to help improving immunity against that antigen, the so called antibodies, and T lymphocytes will proliferate and eliminate every cell containing that antigen. If differing between innate and adaptive immunity is a goal, we could say that while adaptive is able to retain memory to those specific antigens, this is not applicable in innate immunity (Alberts *et al.*, 2002).

1.2 | Cells of the immune system.

Lymphocytes, monocytes, macrophages, dendritic cells, neutrophils, eosinophils, basophiles, natural killer cells. All of them different sets of cells with different abilities and functions but always developing from a single cell type primarily residing on the bone marrow, the hematopoietic stem cell (Eaves, 2015). **FIGURE 1.1** represents the process of hematopoiesis, or the formation, development, and differentiation of blood cells, deriving from a pluripotent set of cell progenitors.

Lymphocytes, comprising both T and B cell families are needed to initiate an adaptive immune response upon exposure to foreign antigens. While the first are to acquire their mature status in the

thymus, B lymphocytes will achieve it in the bone marrow (when talking about adult mammals) (Male *et al.*, 2006).

T lymphocytes (or T cells) are a functionally diverse family represented by three main groups: T cytotoxic (Tc), T helpers (Th) and T regulatory/suppressor (Treg/Ts). Cytotoxic T cells are known to express CD8 molecules, which is used to help distinguishing these cells from other T cell subsets. Those molecules will form a complex with the T cell receptor – TCR on the periphery of the cell, providing it with the ability to interact with cells presenting an antigen via MHC class I during an adaptive immune response. Once these cells are able to perform their duty of recognizing and killing pathogenic antigen presenting cells, they will be fully equipped with acidic and potent enzyme containing granules and FasL on its membrane, leading to the activation of both intrinsic and extrinsic apoptosis pathways on these pathogenic cells (Abbas *et al.*, 2012).

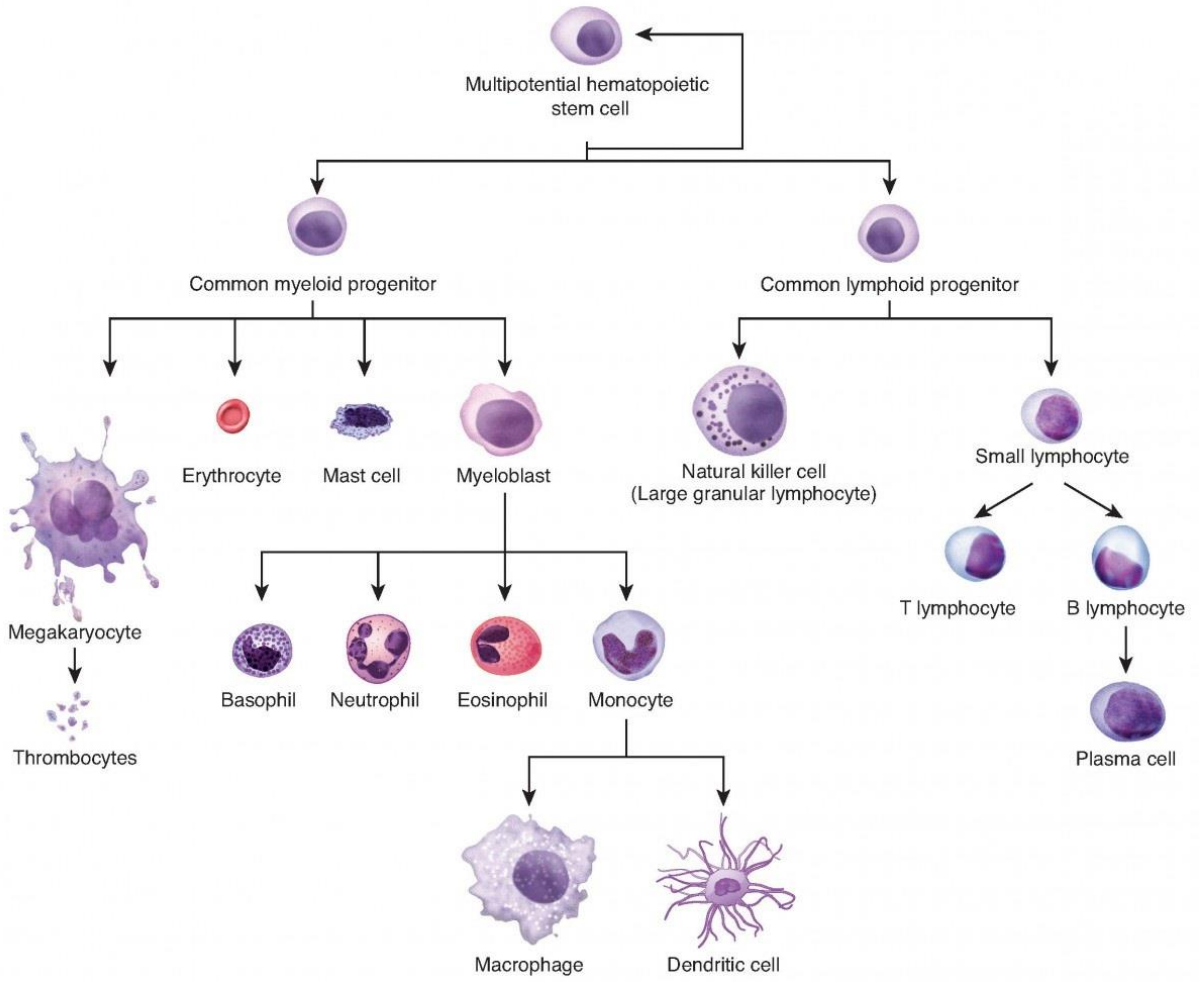

FIGURE 1.1 | Simple representation of hematopoiesis. The scheme portrays the different types of leucocytes (white blood cells), known to have their origin in a self-renewing population of hematopoietic stem cells. Image was adapted from https://www.boundless.com/biology/textbooks/boundless-biology-textbook/gene-expression-16/regulating-gene-expression-in-cell-development-117/mechanics-of-cellular-differentation-465-13121/, retrieved on July 8th 2015.

T helpers are $CD4^+$ and CD4 molecules will in turn help in the reconnaissance of an antigen being presented via MHC class II. One could say the functions of these cells to be more diverse than those of a Tc. And why? The name "helper" may lift the veil a little, but the reason is the fact that they aid other cells performing their role: when stimulating antibody production by B cells and microbicide function of macrophages, when proliferating and differentiating Tcs or even when helping the activation of innate immune cells. Such functions are closely related and supported by the production of specific cytokines by specific Ths (Abbas *et al.*, 2012)

Tregs are other subpopulation with self-tolerance and immune homeostasis related functions, able to suppress a variety of physiopathological responses against self and non-self antigens (Sakaguchi, 2004).

Activating a naive mature T lymphocyte (a cell that has never been in contact with a pathogenic antigen) into a functional effector cell is not a simple process and it is generally accepted that three different and complementary signals are needed: recognition of an antigen that is being presented to it by a dendritic cell (or other specialized antigen presenting cells such as macrophages and B cells), via MHC class I or II, that links to TCR on the T cell's membrane; the second signal, costimulation, is an interaction between other proteins on each of these two cell types. Activated antigen presenting cells will express CD80/86 that by interaction with CD28 on T cell membrane will lead to the activation of the T cell. CD28 has also inhibitory members in its family, CTLA-4 as an example, regulating the time of an immune response. Of course the story is more complicated than that and other sets of important molecules are critical in costimulation. And such is the case of PD-1, PDL-1, ICOS, and ICOS-L; the third signal, represented by cytokines, is also needed for achieving a successful T cell clonal expansion. IL-2, for example, is known to promote clonal expansion of T cells and is also related with their differentiation into effector or memory cells. After this long meeting with dendritic cells, happening in the secondary lymphoid organs, T cells will proliferate in response to specific antigens, facilitating its elimination. This way, providing immunity against them in the best scenario, where all the signals are present (Abbas *et al.*, 2012).

B cells are the ones famous for antibody production, helping us in the recognition of pathogens due to specific antigen binding. There is more than one class of antibodies in which IgG (our most abundant antibody in the plasma) is only part of. These molecules are surface receptors expressed by B cells, and some of them will appear in the soluble form, allowing other cells to recognize and bind specific antigens. Every single B cell produces a particular antibody, and after contacting its specific antigen the B cell will proliferate, differentiate into a plasma cell and produce great quantities of antibodies in the soluble form (Male *et al.*, 2006)

Other type of cells are Natural Killer (NK cells), having the ability to recognize and destroy aberrant cells without the need of a previous activation step. Neutrophils, eosinophils and basophiles are called granulocytes because of their multilobuled nucleus. The first are known for their phagocytic capacity, the second for the release of the content of their granules into the extracellular space and the

third by the release of heparin and histamine, substances promoting blood flow by preventing clotting and enabling vasodilatation (Abbas *et al.*, 2012).

Because of the importance of dendritic cells and monocytes to this thesis, they and their close relatives, macrophages, will be developed on the next chapter.

1.3 | Monocytes to dendritic cells and macrophages.

Monocytes are known to circulate in the blood, bone marrow and spleen and by the fact that they do not proliferate in a steady state. Their adhesion and chemokine receptors mediate migration from blood to tissues during an infection. (Geissmann *et al.*, 2010) During inflammation monocytes can differentiate into macrophages or dendritic cells, something that will probably be dependent on the inflammatory milieu and pathogen-associated pattern-recognition receptors (Serbina *et al.*, 2008). Current models propose that blood monocytes, many macrophage subsets and the majority of DCs originate *in vivo* from hematopoietic stem cell-derived progenitors with myeloid restricted differentiation potential (Geissmann *et al.*, 2010). Under normal homeostatic conditions monocytes develop from precursors in the bone marrow, and as **FIGURE 1.1** depicts originate from the common myeloid progenitor. Monocyte development has been shown to be influenced by cytokines as the evidence of monocytes expressing macrophage colony stimulating factor (M-CSF) receptor and that administration of exogenous M-CSF leads to monocytosis and that of mice deficient in the production of M-CSF have been reported to have lower number of monocytes both circulating and in the bone marrow (Mitchell *et al.*, 2014). Monocyte heterogeneity is something well studied in mice but also recognized in humans with CD14 and CD16 being usually used to separate two subsets: $CD14^{hi}$ $CD16^{-}$ as inflammatory and $CD14^{+}$ $CD16^{hi}$ as patrolling monocytes (Mitchell *et al.*, 2014). We now know that not all types of DCs and tissue macrophages are originated from monocytes. The inflammatory type is referred to as the one giving rise to DCs/macrophages in a variety of infection models. These monocytes are thought to contribute to the repopulation and/or emergence of DCs/macrophages in certain infected sites.

Macrophages are often viewed as terminally differentiated monocytic cells, seen as phagocytes infiltrating tissues during inflammation with pro and anti-inflammatory functions. They contribute to the clearance of apoptotic cells and the production of growth factors and are equipped with a range of pathogen recognition receptors, contributing to an efficient phagocytosis and induction of production of inflammatory cytokines. Tissue resident macrophages, on the other hand, cover various subsets whose origin and function are poorly understood. Examples of these tissue resident macrophages are dermal, splenic marginal zone macrophages and microglia (Davies & Taylor, 2015; Geissmann *et al.*, 2010).

There is a class of cells with specialized functions in antigen presentation, capable of generating an adaptive immune response against that antigen, as suggested before. This class is usually termed as APCs (Antigen Presenting Cells). Within this group, there is one that is highlighted by its elegance in doing so. These are known as dendritic cells. It took from 1868 with German physician Paul Langerhans up to 1973 with Ralph Steinman and Zanvil Cohn to understand that the cells first described by Langerhans were in fact a new class of distinctive white blood cells. In this way beginning a modern era of dendritic cell science and eventually giving Steinman half a Nobel in 2011 (Steinman, 2015)

When the body is challenged by infections or even in the steady state, dendritic cells will travel from body surfaces to lymphoid tissues, alerting awaiting T cells to the presence of an injury or infection and presenting them with antigens, directing an immune response. And this is what was called a missing link in immunology, since the bridge between the appearance of an infection and the activation of the acquired immunity was not well defined. DCs can patrol the body and seek for foreign invaders, being bacteria, viruses or toxins, capture and process them, culminating in the presentation of those antigens in the cell surface and activating vigorous responses from lymphocytes. Nowadays research is also turning to the comprehension of another seemingly opposite DCs' responsibility, induction of immune tolerance – the silencing of dangerous immune cells which would eventually attack the body's own tissues (Mellman & Steinman, 2001; Banchereau & Steinman, 1998)

There are distinctive DC subsets with distinctive functional and phenotypic characteristics. This heterogeneity arises from their ontogeny, maturation status and functional capacity. Profiling of the cell surface molecules can help us to get isolated populations but since all of these factors are not that well elucidated and because some of them are overlapping, the sub-setting is far from completed (but new efforts are being put into it) (Keller, 2001; Liu & Nussenzweig, 2010; Guilliams *et al.*, 2014). Myeloid DCs coming from monocytes, Langerhans cells, interstitial DCs and plasmacytoid DCs already show us enough variability when we talk about DCs. Specifically (and to the interest of this thesis), monocyte derived DCs (moDCs) have long been studied and long been able to be produced *in vitro* (Sallusto & Lanzavecchia, 1994). *In vivo,* they arise during the process of inflammation and transendotelial migration of blood monocytes. This physiological process that leads to differentiation into DCs can be reproduced *in vitro* if monocytes are put in culture with Granulocyte Macrophage Colony-Stimulating Factor (GM-CSF) and Interleucin-4 (IL-4) cytokines (Keller, 2001).

DCs can appear in two different states, mature and immature (**FIGURE 1.2**). In the immature state DCs are known to have splendid capacities in terms of capture and processing of antigens but a lower ability in stimulating T lymphocytes. This statement is supported by a low expression of costimulatory molecules such as CD80, CD86, CD83 and CD40 and despite being expressed, its MHC II molecules will be mainly sequestered in late endocytic compartments (Mellman & Steinman, 2001). Such is not the case of mature dendritic cells, since they will have great number of both MHC class I and II at the cell surface, used for antigen presentation and stimulation of both $CD8^+$ and $CD4^+$ lymphocytes. At the same time the expression of costimulatory and other molecules will greatly increase

so that the formation of the immunological synapse, *i.e*, the contact area between APCs and T cells, may happen. In the process of maturation, DCs will also suffer extended morphological changes with cytoskeleton reorganization, extending long "dendritic processes" that are actually membrane folds, whose biological function is believed to be the increase of the opportunity for T cell contact.

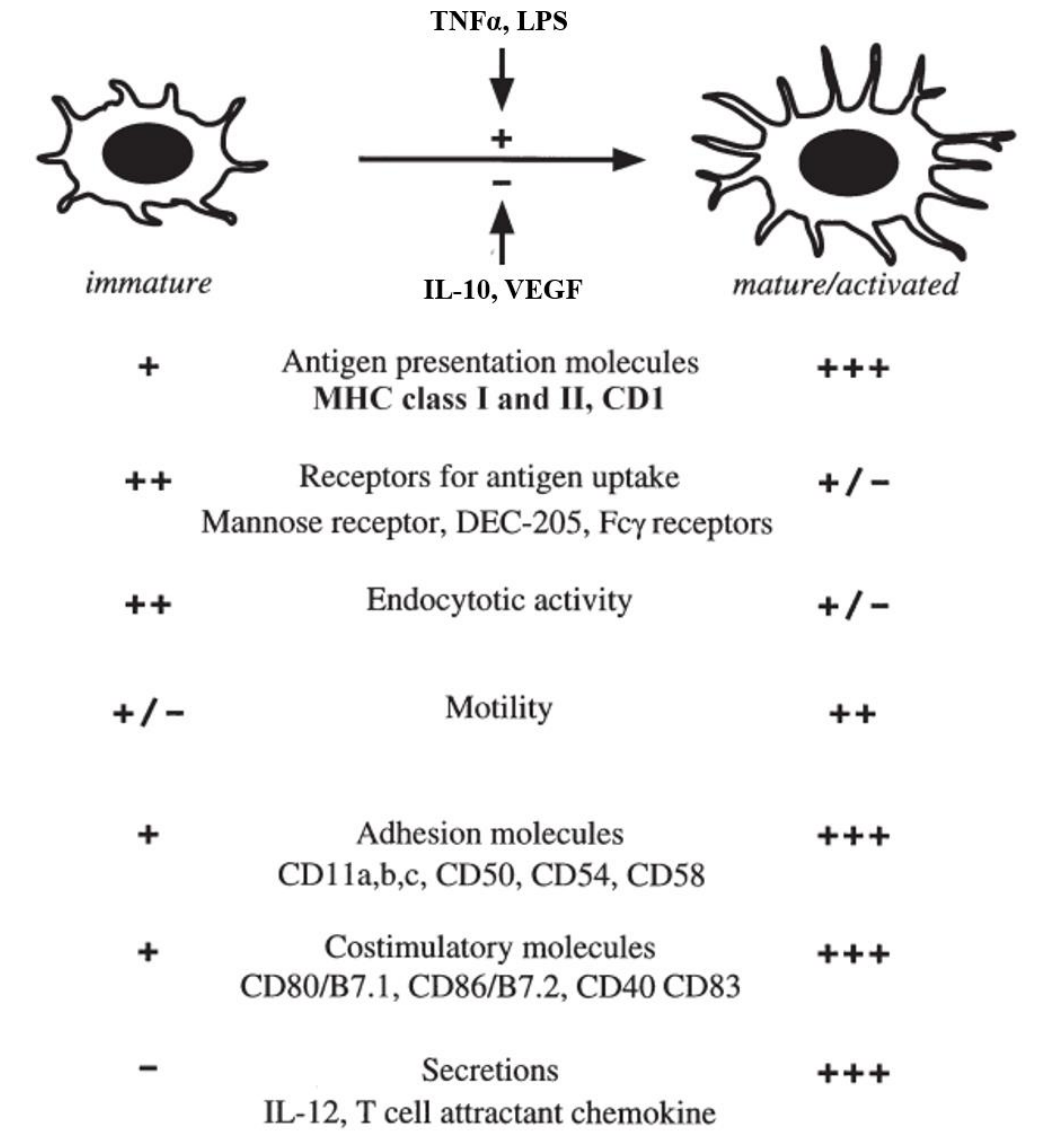

FIGURE 1.2 | Features of dendritic cells in their immature and mature (activated) states. Activation and functional maturation can be influenced by inflammatory stimuli, cytokines and growth factors. Properties as shown are generalized and not necessarily uniform for DCs derived from different sources. TNF, tumor necrosis factor; LPS, lipopolysaccharide; VEGF, vascular endothelial growth factor. Image was adapted from Timmerman & Levy, 1999, Image 2.

During maturation, DCs will also acquire mobility, necessary to reach T–cell-rich lymphatic nodes. In this, endocytosis capacity will decrease and cytokine and chemokine excretion profile will change depending on the nature of the immune response, but may well culminate in recruitment of specific T cells, monocytes and other DCs to the infection site (O'Neill *et al.*, 2004). Maturation is triggered by products of microbial pathogens (LPSs, CpG DNA, dsRNA), that can be understood with the help of Tool-like receptors and other PPR and also by some pro-inflammatory cytokines as TNFα and IL-1β (Banchereau & Steinman, 1998; Li *et al.*, 2012; Dudek *et al.*, 2013). Maturation can also be induced by a non-pathogenic-related stimuli (Pierre *et al.*, 1997).

Immature DCs can make use of three different endocytic processes: macropinocytosis, phagocytosis and clathrin-mediated endocytosis, with phagocytosis possibly being the most physiologically relevant, evolutionarily speaking (regarding the antigen uptake with its amoeba-like-processes). What mainly distinguishes these three different paths is the size of the particles to be uptaken and also the receptors needed for it to happen. This function is generally thought as part of the adaptive immune system because when extrinsic pathogenic antigens are endocyted, they will be processed and assembled on MHC II molecules to be transported to the cell membrane, posteriorly being presented to T cells that will generate a specific response to that new antigen. After endocytosis, the antigens will be subjected to processing (cleavage into smaller pieces, more fit to assemble the peptide binding cleft of MHC molecules – around 10 amino acids) and there are three known pathways by which small peptides may be presented to T cells. In the class II MHC pathway, after antigen internalization, the endosome will fuse with the lysosome and it will be degraded into smaller fragments that will bind to MHC II molecules, forming a complex that will be transported to the cell membrane. The conjugation between antigen and MHC II will allow interaction with T helper cells ($CD4^+$), some of them able to stimulate antibody production by B cells, when matured. Class I MHC pathway is more related to cells in abnormal conditions, producing altered proteins or infected by microorganisms. If those peptides are presented with MHC I it is a signal for cytotoxic T cells ($CD8^+$) that something is wrong and that the cell where it happened should be eliminated. Finally, there is a last and interesting "option" called cross-presentation, where antigens that come from outside the cell are endocyted but presented through MHC I, leading to the differentiation and activation of cytotoxic T lymphocytes (Abbas *et al.*, 2012).

1.4 | Umbilical cord blood.

During the embryonic development, there is a structure responsible for making the connection between the embryo and the placenta. Through it, all the nutrients, hormones, antibodies and all the important molecules will pass in this mother and child's bloodstream communication. It is referred to as umbilical cord and it contains two arteries and one vein. After birth, it is cut, which means that some blood will remain in it. This blood is no different from adults' peripheral blood, apart from the fact of

reportedly having an easy to access higher number of early and committed progenitor cells, similar to the ones found in the bone marrow (Cairo & Wagner, 1997). Because of this, it has become an accepted source of blood containing stem cells for bone marrow transplants, both in related as in unrelated patients. In 1974, Knudtzon understood the presence of colony-forming cells in cord blood and fourteen years later Gluckman performed the first transplant, in a patient with Fanconi anemia where the donor was his HLA-identical sister, as reported in New England Journal of Medicine's 1989's article (Gluckman *et al.*, 1989; Gluckman & Rocha, 2005; Rubinstein, 2006).

After this, banks where umbilical cord could be stored were created, giving a chance for unrelated transplants to happen more often and in a controlled way. The success of this kind of transplants is generally thought to be related to the number of hematopoietic stem cells (HSCs) and multipotential progenitors in cord blood and the HLA matching between donor and recipient, that, if too different, can lead to graft vs. host disease. That is why HLA pattern must be identified in cord blood banking in order to try to avoid it.

Significant effort has been put in expanding HSCs *in vitro* so that adults needing the transplant could have them in reasonable number, since most cord blood units will not have sufficient stem cells. (Chou *et al.*, 2010). This means that these cell progenitors' numbers could be augmented *in vitro* for a variety of goals, such as the production of dendritic cells. HSCs are often designated $CD34^+$, because they express CD34, a membrane protein with cellular adhesion properties that is thought to mediate attachment to the bone marrow. In order to obtain DCs from $CD34^+$ progenitors, different culture methods have been tried, and they can basically be resumed into the one or two-step culture systems (**FIGURE 1.3**).

The first system (1-step culture) tends to directly differentiate the cells into DCs and, for that, a set of cytokines is added to culture media. But this may constitute a problem, since it will limit the final number of DCs at the end of the process, plus the fact of possibly not getting a homogenous population, needed for clinical applications (Balan *et al.*, 2009). A combination of certain cytokines and one growth factor, FMS-like tyrosine kinase 3 ligand (Flt-3L) has been used: stem cell factor (SCF), GM-CSF, IL-4 and TNFα. The two-step culture system consists of/or involves, first, the induction of DC precursor proliferation (that can be accompanied by monocytic lineage commitment), followed by a second step of differentiation into DCs. The first step expands $CD34^+$ cells using early acting cytokines and growth factors which may include again SCF and Flt-3L and thrombopoietin (TPO), IL-3, IL-6 and granulocyte-colony stimulating factor (G-CSF). The presence of proteins like these in the culture may result in a 100 or higher fold increase of the initial number of cells (Montesoro *et al.*, 2006), reflecting the main advantage of the 2-step culture system: great augmentation of the number of DC precursors, comparing to the 1-step culture. Secondly, DC differentiation is induced by GM-CSF ad IL-4 (Balan *et al.*, 2009)

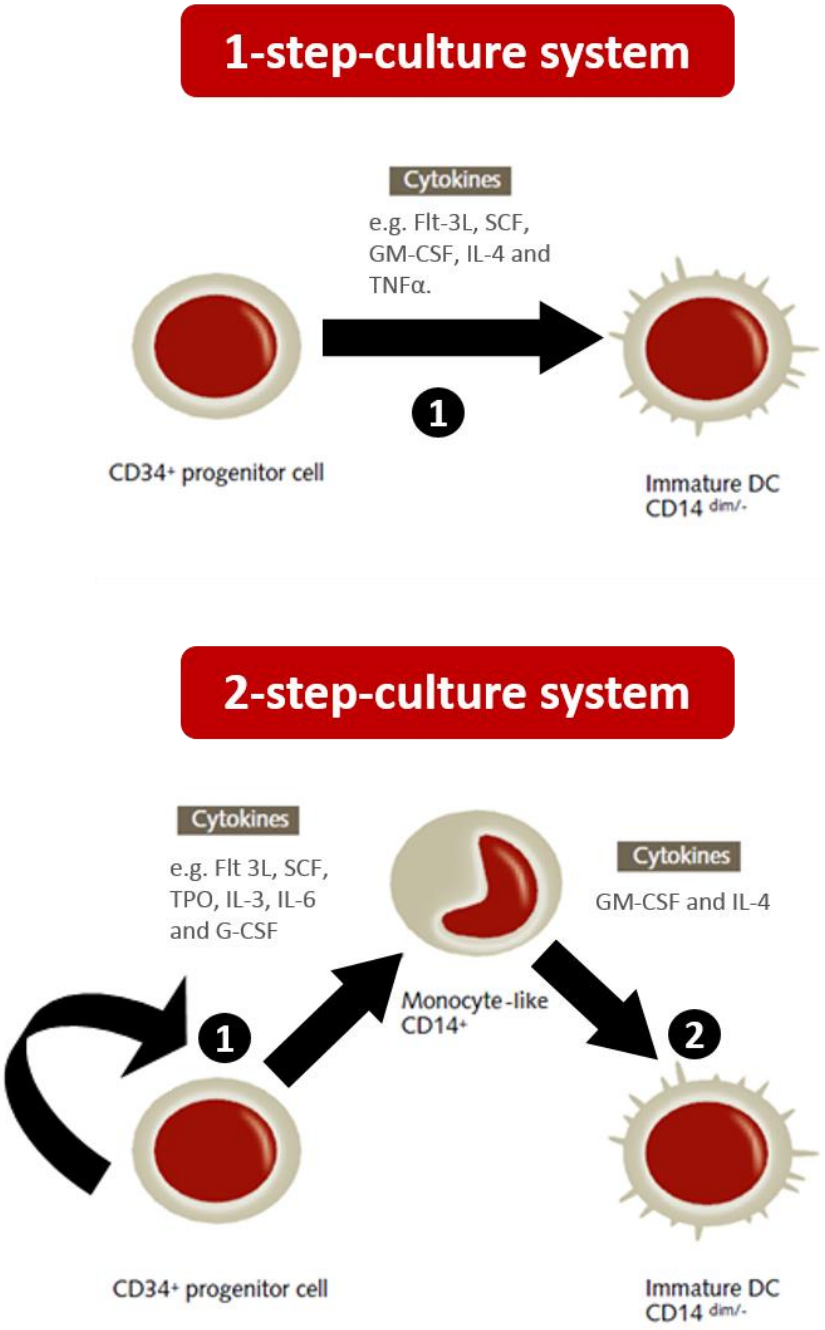

FIGURE 1.3 | Generation of dendritic cells using CD34$^+$ umbilical cord blood hematopoietic stem cells as a starting population. Two cell culture strategies are known to be used for obtaining DCs from HSCs on cord blood. The 1 and 2-step culture systems vary in the temporal separation of induction of proliferation in HSCs and posterior differentiation into DCs. The 1-step culture system focus only on differentiation (1). The 2-step culture system contemplates first proliferation and eventual lineage commitment (1) and then differentiation into immature DCs (2). Different sets of cytokines can be used *in vitro* for both strategies, as shown in the figure. Image was adapted from PromoCell's, "Generation of monocyte-derived Dendritic Cells (moDCs)" Figure 1, found in http://www.promocell.com/fileadmin/promocell/PDF/ Generation_of_monocyte-derived_Dendritic_Cells.pdf

1.5 | Cancer and its interactions with the immune system.

As one of the most nefarious diseases of the first world countries, cancer is one of the leading causes of death, in a list where cardiovascular diseases, dementia, chronic obstructive lung disease or diabetes are also included (WHO, 2014). People affected by cancer can increase their chance of survival by early identification and treatment of the disease (Sudhakar, 2009).

Cancer is a general name given to a collection of related diseases having in common the uncontrolled proliferation of cells and their consequent invasion of surrounding tissues. As malignant tumors refer to 'solid cancers', the different cancers starting on the bone marrow will be called leukemias and will not form solid tumors. Depending on the type of tissues and or/organ where it initially took place, cancer will acquire different names and characteristics, even if metastasis occur, and that is a complex process where the disease is transferred to another organ or tissue. Even though not always hereditary, we can say that cancer is a genetic disease, arising from mutations in the DNA that may come for a variety of reasons. These mutations mostly affect three types of genes – proto-oncogenes, tumor suppressor genes and DNA repair genes. The route that leads to these alterations is substantially environmental, with radiation, chemicals and viruses as inducing factors. Genetic predisposition is also involved in some cases. Most people who die of cancer will die because of metastatic disease, causing severe damage to the body functions (Blanpain, 2013; National Cancer Institute, 2015).

The understanding of cancer biology has undoubtedly brought new insights into the way new therapeutics could be engineered. Cell journal's most cited article (Hanahan & Weinberg, 2000) has proposed six biological capabilities adjacent to the development of tumors. Those are sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, activating invasion and metastasis. Eleven years later, the same authors proposed two more to add: reprogramming of energy metabolism and evading immune destruction. Other concept that has been having some light shed is the tumor microenvironment, and the recognition that tumors are way more than insular masses of proliferating cancer cells. Rather, 'normal' cells adjacent to the tumor and infiltrating immune cells do not act as passive bystanders, but are also known to contribute to the process of tumorigenesis (Hanahan & Weinberg, 2011)

Concerning cancer interactions with the immune system, the two most relevant cancer features may be its malignant and pre-malignant inflammatory state, driven by the immune system, and the avoidance of elimination by immune cells. The immune system will have two divergent roles here, denoted by the fact that it may serve to enhance tumor progression and/or to prevent it. When talking about inflammation, it is known that tumors can have infiltrating adaptive and innate immune cells in amounts that range from the subtle to the incredibly high (Pagès *et al.*, 2010). This has been seen as part of an attempt to eliminate the cancer cells, but paradoxical clues have also been pointing to another role,

the enhancement of tumorigenesis, leading to the acquisition of the cancer hallmark capabilities, mostly through innate cells (Hanahan & Weinberg, 2011). In this more tragic role, inflammation would supply the tumor microenvironment with growth, survival and pro-angiogenic factors, enzymes that could modify the extracellular matrix and reactive oxygen species that could promote malignancy by their mutagenic potency (Grivennikov *et al.*, 2011). As a matter of fact more and more cells are being proposed with tumor promoting actions, various macrophages, mast cells, neutrophils, B and T lymphocytes releasing EGF (epithelial growth factor), VEGF and FGF2 (vascular endothelial growth factor and fibroblast growth factor 2, promoting angiogenesis), extracellular matrix degrading enzymes and others that could promote tumor progression and amplify the inflammatory status (Hanahan & Weinberg, 2011).

Another subject is the evasion from immune destruction. The theory of immune surveillance states that the cells and tissues are always being monitored by the immune system, so that any alterations can be recognized and, in this way, cancer cells can posteriorly be eliminated. But if a tumor appears, it could mean that it avoided that recognition and so forth evaded eradication. Tumor elimination by the immune system is more obvious in some types of cancers that are caused by viruses, maybe due to the viral burden and the recognition of patterns in virus-infected cells. The immune system represents an important barrier to tumor formation even in non-virus induced cancers. In fact, in immune system deficient mice, tumors arise more frequently and grow more rapidly in comparison with the immunocompetent control. Those deficiencies were more dramatic when the functions of cytotoxic T cells, Th1 and NK cells were damaged. Other interesting results show that tumors transplanted from immunodeficient to immunocompetent mice are often inefficient in initiating secondary tumors. This last experiment gets more interesting because the results do not go both ways: if the transplantation route is switched (from immunocompetent to the immunodeficient), cancerous cells would proliferate as efficiently in immunodeficient, as in the immunocompetent mice where they came from. This leads to the interpretation that the more immunogenic cancer cells (those that are able to be successfully recognized by the immune system and further activate an immune response against themselves) are easily eliminated than the weakly immunogenic ones, one of the reasons why the last could grow (Teng *et al.*, 2008). In certain types of cancer, epidemiology has also brought some interesting facts: patients with colon and ovarian cancer that are highly infiltrated by cytotoxic T cells have a better prognosis than the less infiltrated (Jochems & Schlom, 2011). But never forgetting that these two types can be associated with viruses, and it might be taken as an argument against the importance of immune surveillance as a barrier to tumorigenesis and progression. Another perspective is that highly immunogenic cancer cells could also evade elimination by secretion of immunosuppressive cytokines as TGF-β, inciting lymphocytes to become dormant. Intermediaries have to be considered too, with immunosuppressive Tregs and myeloid derived suppressor cells (MDSCs) being recruited to counteract the action of cytotoxic T cells near the tumor site. Anti or pro-tumor immunity is something that must be further studied, since knowledge is still rudimentary (Hanahan & Weinberg, 2011).

DCs can capture tumor antigens that are released from either alive of dying tumor cells, cross-present these antigens to T cells in tumor-draining lymph nodes and posteriorly generate tumor-specific CTLs that contribute to tumor rejection (Fuertes *et al.*, 2011) When interacting with DCs and other phagocytes, there are a variety of signals from dying tumor cells that can lead to inhibition or enhancing of phagocytosis. Tumors can prevent the priming of tumor-specific T cells by DCs, switching the differentiation route of monocytes to macrophages instead of DCs (interplaying in the IL-6 and M-CSF signalization). They also interfere with DC maturation, promoting their anergy through the secretion of IL-10, or even direct their maturation to the generation of a Th2 response, where these T cells secrete IL-4 and IL-13, preventing tumor cell apoptosis and promoting their proliferation by stimulating macrophages to secrete epidermal growth factor (EGF) (Palucka & Banchereau, 2012). A recent study in lung cancer has again confirmed that the presence of myeloid antigen-presenting cells (mAPCs) near the tumor site may not correlate with an anti-tumor response, since increased levels of immunosuppressive cytokines were reported and those mAPCs understood to have decreased expression of co-stimulatory molecules CD80/CD86 and a tolerance-inducing cytokine profile (Bugalho *et al.*, 2015)

1.6 | Cancer therapies in brief.

In order to treat cancer there are several approaches physicians have been taking, but the need for new methods and strategies is still constant, so that the best (or better) treatment efficacies and an overall higher success rate is achieved. Therefore improving the number of lives saved, or at least, to a considerable prolonged survival from the moment of diagnosis.

Surgical removal of a tumor is helped from the 70's by techniques such as sonography, Computer tomography (CT) scans, magnetic resonance imaging and positron emission tomography (PET), which made a non-invasive diagnosis possible. Recently, cryosurgery and laser helped surgeries have also been in study. But it is obvious that the spreading of a tumor will impose some limitations here.

World War II brought some hype in cancer treatment after the realization that the bone marrow and lymph nodes of troops accidentally spilled with sulfur mustards (the known mustard gas) were markedly depleted. The hype came from the people's excitement, that perhaps drugs could cure patients with cancer, but the reality is that the use sulfur mustards has had decadent success in treating lymphomas, something that was understood not long after (DeVita & Chu, 2008) And so the concept of chemotherapy became more important and the exploration of new drugs has taken a new emphasis, with many being used to treat a variety of types of cancers over the years. 'New' desirable improvements to chemotherapy include strategies to diminish the unspecificity of treatments and side effects that usual chemotherapeutic drugs have to other cells of the body besides cancer cells. Those are monoclonal

antibody conjugated drugs which can improve targeting (and diminish the side effects), using of liposomal conjugations, new combination of drugs, chemo-protective agents (so that, again, side effects could be better prevented), HSC transplantation and agents to overcome multidrug resistance. Hormonal therapy constitutes another class of drugs that have been used to treat breast and prostate cancer, making use of the knowledge of how hormones could influence the growth of cancer. Radiotherapy aims to damage the DNA in the treating area, normally using X-rays (Sudhakar, 2009). One relatively recent approach was not been mentioned, and will be further discussed in the next chapter – immunotherapy.

1.7 | Immunotherapy and cancer immunotherapies.

Immunotherapy represents a set of strategies that act by harnessing the immune system into a specific direction. For that, treatments can work in different ways, with some boosting the body's immune system in a very general way and others "teaching" immune system cells more specifically, for example in attacking cancer cells. And for that, immunotherapy agents can be represented by immune on non-immune molecules that will directly interfere with the patient's immune system. Or even, and more interestingly, using laboratory enhanced cells as the immunotherapy agent itself, as a cellular therapy (Mellman *et al.*, 2011)

To highlight the present importance of immunotherapy and due to the progress made in the last decades it has been considered the breakthrough of the year 2013 by *Science* journal and a big effort is being put in this approach for cancer therapy. One can say that immunotherapy started in the 19th century with the use of heat killed *S. pyogenes* and *S. marcescens* injections in patients with sarcoma by William Coley. Later, that came to be known as "Coley's toxin" (Lam *et al.*, 2015).

Up to July 2015 there are registries of at least 1084 clinical trials related to cancer immunotherapies, with at least 361 of which still on the go (numbers from ClinicalTrials.gov, advanced search term 'immunotherapy', condition 'cancer')

A significant amount of effort has been put on identifying tumor-specific or associated-antigens, leading to the discovery of MAGE-1 (melanoma), NY-ESO-1 (prostate cancer) and HER2 (breast cancer). These tumor specific antigens were then used as peptide vaccines on an attempt to induce tumor specific immunity, but this strategy brought only limited success in the past. The principle of action of this peptide vaccines consists on facilitating the antigen uptake and processing by APCs due to their reduced size, so that their presentation by MHC molecules could also be made easier and expectedly more efficient in generating a specific immune response (Novellino *et al.*, 2005). Newer peptide vaccines combine different tumor antigens capable of being better presented by MHC I, with adjuvants such as IFNs and IL-12 as cytokines and with tool like receptors (TLRs) ligands. Whole tumor cell vaccines have also been tried, with GVAX representing the most clinically relevant. This vaccine

consists of an allogenic prostate cancer cell line, genetically engineered to secrete GM-CSF, which promote a better interaction with DCs and other APCs (Simons & Sacks, 2006).

Tumor-specific monoclonal antibodies (mAbs) and specially the three of its top selling drugs (rituximab, trastuzumab, and bevacizumab) had major impact on clinical oncology, using the so called antibody-dependent cell-mediated cytotoxicity to impress the clinic. *In vitro* studies indicated that the interaction between these antibodies and the Fc Receptor (FcR) of immune cells led to a better elimination of cancer cells, something also denoted by some patients whose high affinity FcR polymorphism may have led them to better responses. Depending on the target having the antigen against which the antibody was made, the anti-tumor activity also comes from the alteration of the associated pathway. Complement-mediated cytotoxicity and enhancement of the adaptive immune response are also therapeutic effects that can come from the use of mAbs in the clinic (Vacchelli *et al.*, 2013)

Adoptive cell transfer (ACT) is another form of immunotherapy usually associated with the idea of lymphocytes being harvested from the patient and manipulated *in-vitro* so that an antitumor potency may be stimulated on the cells. These cells are to be injected back on the patient. Hematologic cancers are also treated with the use of bone marrow transplantation and, in this case, it can be considered ACT too. If T cells are to be used in ACT, they can be obtained from the peripheral blood, tumor draining lymph nodes or even from tumor infiltrating lymphocytes. $CD4^+$ and $CD8^+$ T cells can also be engineered to recognize specific tumor antigens such as gp100 or MART-1, something that is in clinical trial for melanoma (June, 2007) Within ACT, a new and promising state-of-the-art technology is the patients' T cells genetically engineered to express a chimeric antigen receptor (CAR). In this, a chimeric antibody-mimicking receptor (for targeting) fused with an intracellular CD3ζ molecule (part of the TCR complex, for signal transduction) is added to the T cell. Firstly, it was used in patients to treat leukemia and complete remissions were reported in some cases (Grupp *et al.*, 2013), with the clinical trial still going (ClinicalTrials.gov Identifier: NCT01593696). Their use in solid tumors is more recent and some unpublished success was stated in *American Society of Clinical Oncology 2015's Annual Meeting*.

Given the findings on the tolerogenic effect that cancer can have on immune cells, a new trend appeared to counteract possible mechanisms responsible for it. And so, the immune modulating antibodies came to existence. In the interaction between DCs and T cells there is an 'immune checkpoint', where before the activation of the T cell when presented to an antigen, the T cell must have an appropriate molecular pattern for it to be activated, and proliferate in response. Some molecules are needed for the costimulation (subject already covered a few chapters ago) and some of them exist to inhibit costimulation, leading to inhibitory signaling cascades. This is part of a natural strategy to control overstimulation of the immune system (note that these molecules contribute for controlling both activation and post-activation of T cells). CTLA-4 is one of those inhibitors and since it is known that tumors are able to induce tolerance, what better than to try to contrapose its mechanism of action? And so, two anti CTLA-4 blocking mAbs, ipilimumab and tremelimumab came to the clinic for patients with metastatic melanoma. Their success included some mixed regressions and long-term complete

remissions in a small percentage of patients. Some autoimmune effects were also reported. There are other antibodies against other members of CTLA-4 family already in the clinic, specifically PD-1 and PD-L1, going by the name MDX-1106, CT-011 and MK-3475 (Topalian *et al.*, 2011)

1.8 | Dendritic cell immunotherapy.

Cancer vaccines are another form of active immunotherapies and, maybe because of the tremendous success of early vaccines in the prophylaxis of innumerous infectious diseases in the past, regarded as one of the most interesting and hopeful approaches, despite cancer vaccines of today being more focused on treating existing cancers rather than to prevent the development of it (prophylaxis).

Because of dendritic cells outstanding capacity for antigen presentation and clues that they could be used *in vivo* to generate an immune response against tumors, their use in immunotherapy was proposed. In this, DCs from patients could be put in culture and loaded with specific tumor antigens or even whole tumor cell lysates. The final goal is to create a cellular therapy that directs the immune system into a cancer killing profile, for that using the body's natural resources to promote cancer cell targeting (Gilboa, 2007; Palucka & Banchereau, 2012).

Provenge (sipuleucel-T) was the first cellular immunotherapy that has ever received FDA's (Food and Drug Administration) approval, in 2010. It represents a dendritic cell vaccine used for hormone-refractory prostate cancer and reportedly able to extend survival in 4.1 months (median) and a 22.5% reduction in risk of death but without significant tumor burden reduction (probably because of lymphocyte infiltration after the vaccine). This vaccine is achieved in three steps, where patient's leukapharesis product is obtained and then incubated with a fusion protein, consisting on a serum tumor marker for prostate cancer, prostatic acid phosphatase (PAP), linked by its C-terminus to a functional GM-CSF protein by a Gly-Ser linker. The product is then re-infused into the patient so that an immune response against the cancer cells would be generated, since 95% of prostate cancer cells are said to be positive for the marker (Gardner *et al.*, 2012).

'The goal of therapeutic vaccination via DCs is to elicit tumour-specific $CD8^+$ T cell-mediated immune responses that will be sufficiently robust and long-lasting to generate durable tumour regression and/or eradication' (Palucka & Banchereau, 2012). This is the very straight forward main goal when talking about DCs for cancer therapy. Cytotoxic T lymphocytes (that are $CD8^+$) should easily recognize MHC I conjugated peptides being presented to them by tumor cells, so that elimination may take place. Not only antigen recognition, but also high levels of granzyme and perforin in these lymphocytes, the ability to enter the tumor microenvironment and to overcome its negative immunomodulatory regulation act as important factors**.** A tumor antigen specific naive T cell will recognize the antigen after being activated by an antigen-presenting DC. Activation means that that same cell will proliferate and

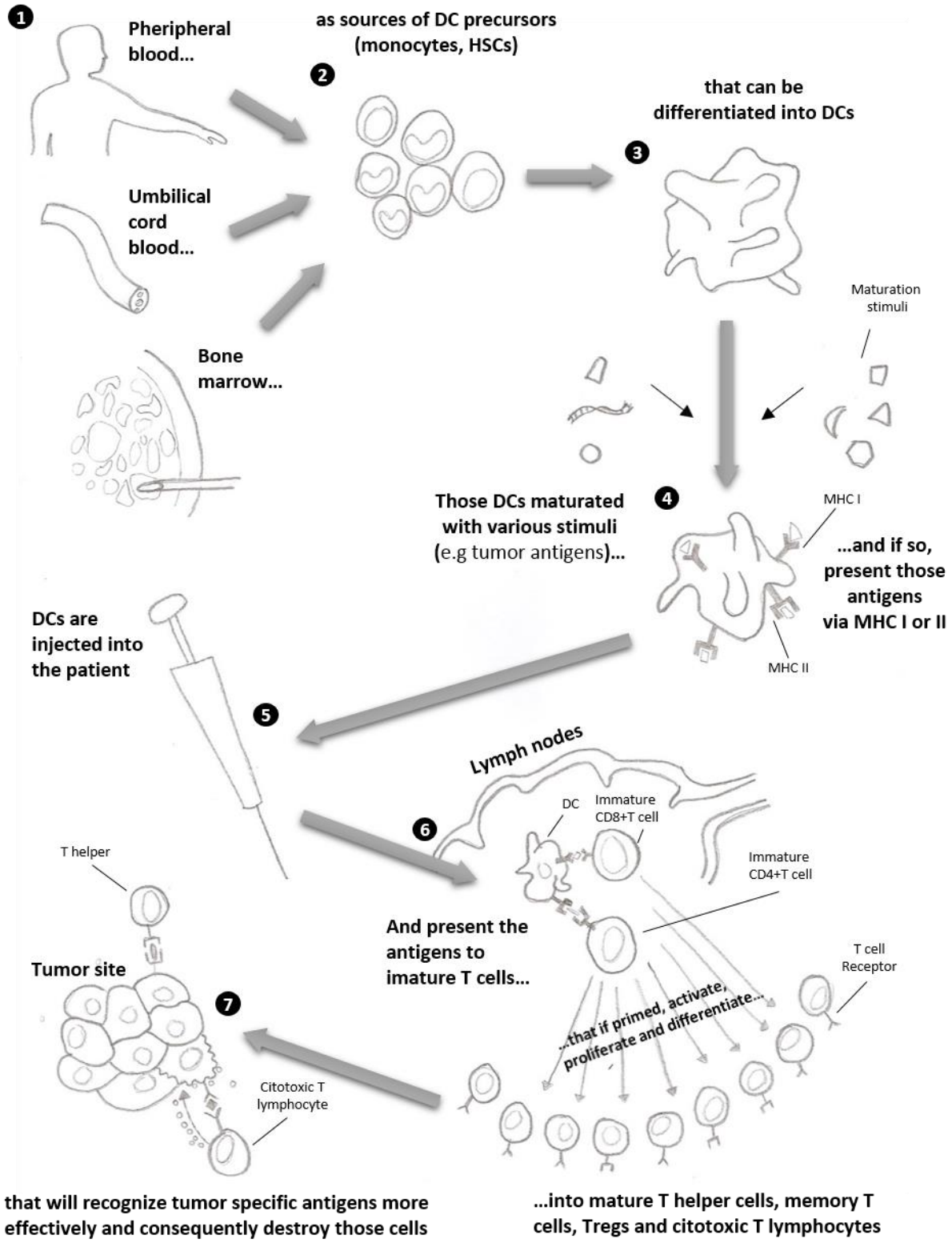

FIGURE 1.4 | Approach on the use of Dendritic Cell vaccines to treat cancer and possible *in vivo* mechanism of action. Precursors of DCs can be obtained from various biological sources and manipulated *in vitro* so that mature cancer-antigen-presenting-DCs can be generated, posteriorly infused into the patient and induce an immune response against the tumor. This image was achieved taking art pieces present at Nature Vol 504, No. 7480 Suppl, S2–S3 (2013) as a starting point.

differentiate into a tumor-specific cytotoxic T cell, with a "killing phenotype" by complex signals such as costimulation (e.g. CD80 and CD70) and cytokine pathways as IL-12 and IL-15. And these can be provided by specific antigen matured DCs, directing CTLs into specific directions. Not forgetting the other cells that can contribute to the goal: Th cells ($CD4^+$) given their capacity to influence CTLs and activate macrophages near the tumor, and long term memory $CD8^+$ cells also. A not so in favor biological function of also $CD4^+$ cells is Tregs inhibition of CTL differentiation by IL-10 and TGFβ and some of them will compete for IL-2, necessary for both Treg and CTL differentiation (Palucka & Banchereau, 2012).

In **FIGURE 1.4**, a simple representation of a hypothetic dendritic cell vaccine is represented, focusing on some important checkpoints that were already discussed up to this chapter.

The views on the potential role of DCs in cancer immunotherapy have expanded remarkably, moving from the early trials with ex vivo DC vaccines to a whole new array of therapeutic options that include joint efforts of other therapies such as radiotherapy and anti-tumor or T-cell immune checkpoint blockade antibodies. And 'just as immunotherapy is moving to the forefront of cancer therapy, DC-based therapy is moving to the forefront of cancer immunotherapy' (Palucka & Banchereau, 2012)

1.9 | Use of cord blood in medicine

Cord blood has been used in the treatment of more than 80 different diseases so far. The most common set of diseases has been the different kinds of leukemia, but also lymphomas, bone marrow failure syndromes, immune deficiencies and metabolic diseases (NCBP's NYBC, Q&A data, website consulted 24th July 2015). In the case of cancer, after chemotherapy new cells will be needed to repopulate the ones that were eliminated by the treatment. Umbilical cord blood can provide those cell progenitors.

Cord blood is a solution to a problem also answered by bone marrow transplantations and because of the last decades of cryopreservation through cord blood banking, it is now easier to find an HLA match, compatible for transplantation. Initially, it was thought that immune reconstitution after an umbilical cord blood transplantation was delayed in comparison to bone marrow and peripheral blood transplantations. Such thoughts were related to the fact of a lower number of transplanted cells being present in cord blood, plus the 'naïve' pattern of its immune cells. But the truth is that the pattern of immune recovery after a cord blood transplant was demonstrated to be remarkably similar to the other two HSC sources (Smith & Thomson, 2010)

In the clinic, in order to have a certified cord blood unit in correct conditions to be used it must pass through a series standardized, reliable and reproducible tests, HLA genotyping and then cryopreserved for banking and posterior use. Tests will include post processing enumeration of the nucleated, mononuclear, $CD45^+$, and $CD34^+$ cells; microbiological contamination; genotyping of ABO

and Rh erythrocyte markers; genotyping of *HLA-A* and *HLA-B* (both MHC I genes with highly variable alleles) and of *DRB1* (representative of MHC II) and others recommended; *in vitro* culture of CFUs (Colony forming units) and $CD34^+$ cell viability determination before transplantation; testing for hemoglobinopathies and viral agents screening as HIVs and hepatitis virus (and on mother's blood as well). The next critical step is the cryopreservation of cord blood, where few slightly different methods can be applied in order to achieve so. For logistic reasons there must be a volume reduction where most of the erythrocytes and plasma are removed so that a final uniformed volume of 20 mL can be obtained and 5 ml of cryoprotectant dimethyl sulfoxide (DMSO) added before freezing in liquid nitrogen. Nowadays, the volume reduction can be achieved through hydroxyethyl starch (HES) sedimentation which is a manual method or by two recent fully automated ways, Sepax (from Biosafe) or AXP (from Thermogenesis). Comparing the three methods, the total nucleated and $CD34^+$ cells recovery would range from about 81.8 to 97% (Rubinstein 2009).

If we are to compare normal research laboratory settings with those that we find in the clinic, we see the second to be based on stricter rules of asepsis and of less manipulation of human samples. And because governmental authorities controlling cord blood banking require these settings, the common laboratory practice of separating blood components (using ficoll density gradient centrifugation) cannot be applied, if a clinical-grade sample to be cryopreserved is the goal.

1.10 | Introduction to the aim of the dissertation.

The work developed throughout this one year work is part of a major project (aDVANCE, Desenvolvimento de novas vacinas anti-cancro, QREN-ADI 2013-2015) which aims the optimization of production of anti-cancer vaccines based on dendritic cells. Regarding this project, the specific objectives were the application of an innovative technology consisting of 'glycan-engineering' of DCs from various sources to produce cell based vaccines able to potentiate better anti-tumor response. In it, the greatest importance was given to moDCs, differentiated from human peripheral blood monocytes, since they are the most usual and easily accessible source of DC progenitors.

In the scope of this thesis, the main goal of the work was to understand the suitability of umbilical cord blood derived-DCs for applications in cancer treatment. For that, clinical-grade samples of cryopreserved umbilical cord blood (UCB) were used as a source of DC precursors. Those were provided by a Portuguese private cord blood bank, Crioestaminal, the only in Portugal credited by the FDA and AABB (American Association of Blood Banks).

The specific goals were:

❶ To optimize the thawing process of cryopreserved UCB for posterior immunomagnetic cell separation and culture;

❷ To optimize the isolation, culture and differentiation of DC precursors ($CD14^+$ monocytes and $CD34^+$ HSC) into DCs;

❸ To compare the phenotype and functionality of DCs obtained from different precursors;

❹ To evaluate the effect of the enzymatic removal of sialic acid from DCs' surface on the functionality of those cells;

❺ To establish cancer cell lines from patients' primary breast cancer cells.

2 | MATERIALS AND METHODS

2.1 | Adult human peripheral blood.

Part of the work developed for this dissertation required the use of adult human peripheral blood cells. These were isolated from leuco-platelet concentrates, "buffy coats", of healthy volunteers provided and ethically approved by Instituto Português do Sangue e da Transplantação (IPST) through an existing protocol between IPST and Prof. Paula Videira from NOVA Medical School (Faculdade de Ciências Médicas da Universidade Nova de Lisboa). In order to reduce inter-sample variability blood was requested from male donors aged 18 to 60 years old and has to be collected the day before. Approximately 450-500 mL of blood is collected into an adequate plastic bag containing 63 mL of citrate phosphate dextrose (CPD), a preserving and anticoagulant solution. A serological control is performed for the following: *T. pallidum*, HB, HC and HIV, all of which should be negative for blood to be suitable for use.

Each blood bag supplied for investigation purposes contains approximately 60 mL of blood cell concentrate obtained from a slow rate filtered, saline-adenine-glucose-mannitol (SAGMAN) diluted whole blood and contains most of the white blood cells present in the original 450-500 mL. It is then kept safe at room temperature until further use.

All blood collection, handling, testing and processing steps until we get the 60 mL "buffy coat" containing bag is performed entirely by IPST. Each bag is supplied with information such as the collection date, (negative confirmation) results for the serological control, blood type and age of the donor.

2.2 | Human cryopreserved umbilical cord blood.

After the birth of the newborn at the hospital, the nurses collect as much umbilical cord blood as possible. For that, a collection kit is provided by a Portuguese umbilical cord blood banking company, Crioestaminal, when requested by the parents. In other cases, informed consent UCB donations for the purpose of investigation may also happen. The blood is then immediately shipped to Crioestaminal's headquarters in Cantanhede (Coimbra) where it is further tested, processed, cataloged and cryopreserved. Tests will include bacteriological and viral contamination (including screenings for HIV and hepatitis), which are performed on mothers' blood also. Genotyping of ABO and Rh erythrocyte markers and *HLA-A*, *HLA-B* (MHC class I representative genes) and *DRB1* (representative of MHC II) is performed. Post processing enumeration of the nucleated, mononuclear, $CD45^{+}$, and CD34+ cells will is also registered.

Before cryopreservation process, there was a sample volume reduction to 25 mL so that it can easily be further stored and maintained in liquid nitrogen. Volume reduction is performed in a fully automated way, using SEPAX (Biosafe) technology. Briefly, it consists of a centrifugal device and disposable built to allow spinning the CB around a vertical axis. The disposable has a computer-controlled piston that aspirates the CB (containing 1% Hespan). After centrifugation, erythrocytes will settle next to the walls and leukocytes into a concentric layer that after elevating the piston forces the fluid stream to exit to a sterile distribution tubing. Acellular plasma, leukocyte suspension and erythrocyte mass exit sequentially and are individually collected, with the leucocyte concentrate bag containing 20 mL (Rubinstein, 2009). 5 mL of DMSO (final [c] 10% (v/v) are then slowly added to this bag and gently mixed with an orbital rotator. For cryopreservation, the samples are placed in a nitrogen container, where the temperature is dropped at of 1ºC/min until -60 and 5°C/min until -120ºC. After that, CB samples are stored in the vapor phase of the nitrogen freezer. Small aliquots for quality control of cryopreservation procedure, determination of post-thaw characteristics and the number of cells (white blood cells, lymphocytes, monocytes, total nucleated mononuclear and $CD34^+$) were extracted before and after freezing.

Important to mention, all these steps were performed in Crioestaminal's headquarters. When needed, samples were shipped to Lisbon, to the Glycoimmunology laboratory at CEDOC, in dry ice and kept at -80ºC until further use.

2.3 | Thawing process on cryopreserved cord blood.

Each individual sample contains 25 mL of concentrated white blood cells inside an appropriate plastic bag which is divided into two smaller closed compartments, one containing approximately 20 mL and the other 5 mL.

When necessary, samples were thawed as follows: the bag was quickly submerged in a 37ºC water bath, and the thawing process was helped by massaging the bag. The goal is not to warm the sample for a long time in the presence of DMSO. With a pointy edge, the bag was pierced in the appropriate location and all the content of the bag poured into a 50 mL tube already containing 25 mL of room temperature DMEM 20% FBS (v/v). With the help of a plastic Pasteur pipette the interior of the bag was washed with this same medium and collected into the same tube. The total volume is now approximately 50 mL (1:2 dilution) and the sample was then incubated for 20 minutes at room temperature. After, the sample was centrifuged at 300xg for 15 min. The supernatant was carefully discarded until about 1 mL of pelleted cells remained. Since the pelleted cells always presented a red color, the step that followed was erythrocyte lysis. For that 50 mL of RBC lysis buffer (Biolegend) was added at the concentration recommended by the manufacturer, the cells were resuspended and divided into five 15 mL tubes and incubated for 5-10 min in the dark at room temperature. After, the tubes were

centrifuged at 300xg for 5 min and the pellets were resuspended in 5 mL of beads buffer (see chapter 2.19 for composition of buffer). Before further immunomagnetic isolation or cell culture, cells were homogenized, passed through a 40 µm pore strainer (Falcon) and an aliquot was taken for cell counting. Meanwhile, the cells were centrifuged at 300xg for 5 minutes and the respective supernatant discarded.

In an attempt to recover the highest amounts of living cells we tried other strategies: density gradient separation in erythrocyte lysed (and non lysed) cells for obtainment of cord blood mononuclear cells; panning method for immediate monocyte isolation, using cells right after the first wash of the thawing protocol mentioned in the beginning of the chapter – panning method is simply resuspending the cells in $RPMI_c$ medium (see chapter 2.19) and posterior incubation at 37ºC for 1h using a plastic petri dish – plastic adherent monocytes form a monolayer; panning method for erythrocyte lysed (and non lysed) cells, again following the protocol with which we started the chapter. None of these alternatives showed sufficient cell recovery apart from the next one. As the last and most successful alternative, we have chosen to include a DNase step on the thawing process. Freezing and thawing procedures may contribute extensively to cell clumping upon thawing, which have been something very common to almost all our samples. Clumping is promoted by cell debris arising from dead cells and so the presence of negatively charged stringed nucleic acids could also take part in it (García-Piñeres *et al.*, 2006). In some experiments, the thawing procedure described above was modified, as follows: the pellet originated from the first centrifugation was resuspended in RPMI medium containing 0.1 mg/mL of DNase solution (Stem Cell). After incubation at room temperature for 15 min, cells were abundantly washed with RPMI medium and passed through a 40 µm pore strainer (Falcon) before being centrifuged at 300xg for 5 min.

2.4 | Isolation of adult peripheral blood mononuclear cells (PBMCs).

Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation. The first step in this process consists in distributing the approximate 60 mL "buffy coat" into 50 ml tubes and diluting that volume in a phosphate buffered saline solution (PBS, see chapter 2.19) in a 3:2 ratio; then, centrifuged at 1100xg for 10 min without brake. At this point, the upper layer was aspirated so that the intermediate layer consisting mainly of leucocytes was easily accessible and removed to a new tube. Then, this leucocyte fraction was diluted with PBS until a final volume of 40 ml. After proper homogenization of this cell suspension, it was carefully layered on top of ficoll solution (1.077 g/ml) (Biochrom AG) in the proportion (5:3), using a plastic Pasteur pipette. This step is followed by a new centrifugation at 1100xg for 20 minutes (without brake). Ficoll is a hydrophilic polysaccharide polymer with a particular density, superior to PBMCs, but inferior to erythrocytes and granulocytes, allowing a specific separation of the different components, after centrifugation. Thus, at this point, blood components are separated by density gradient and four phases should be differentiable: erythrocytes and

granulocytes, present as pellets, followed by ficoll on top and then the PBMCs right beneath the most abundant layer, which corresponds to diluted plasma and the majority of the platelets. Plasma was removed and PBMCs were collected and transferred to a new 50 ml tube, diluted four times in PBS in order to remove the remaining cell debris and platelets and centrifuged at 250xg for 10 min. The supernatant was discarded and the pellet resuspended in 10 mL of PBS, of which an aliquot was taken so that the PBMCs number could be determined with the use of a hemacytometer (Bright-Line) and an optic microscope (Nikon). The cell suspension was centrifuged one last time at 120xg for 5 min to improve platelet removal (Silva *et al.*, 2011; Cabral *et al.*, 2013; Marques, 2014).

Once PBMCs are isolated, downstream applications may be performed, and one could be to further isolate more specific cell types, such as $CD14^+$ cells which will be covered in chapter 2.5.

2.5 | Immunomagnetic isolation of monocytes from fresh PBMCs and cryopreserved cord blood.

Peripheral blood monocytes ($CD14^+$ cells) were isolated using freshly isolated PBMCs as a starting population (chapter 2.4). Monocytes from cryopreserved cord blood were isolated using a processed total white blood cell pellet resulting from the thawing process of the cord blood (chapter 2.3). Regardless of that, a positive selective immunomagnetic selection kit was used: CD14 MicroBeads + MACS Column (Miltenyi Biotec). The procedures for $CD14^+$ cell isolation were generally as recommended by the manufacturer, except for the amount of beads. The amount of beads used with the Miltenyi´s kit had already been optimized in our laboratory and determined to be 1/3 of the recommended concentration.

Once the number of PBMCs (or white blood cells if referring to cord blood) had been determined and the resultant supernatant from the last centrifugation had been discarded, cell labeling was performed as described next. Cells were resuspended in 80 µl of beads buffer and 20 µl of CD14 Microbeads (Miltenyi Biotec) per 3 x 10^7 cells. The cell suspension was well mixed and incubated at 4ºC for 15 minutes. After, cells were washed by adding beads buffer up to a volume of 1 mL per 10^7 cells and centrifuged at 250xg for 10 min. Once the resulting supernatant had been discarded, the cell pellet was resuspended in 500 µL of beads buffer per 10^8 cells. If the number of cells exceeded 400 x 10^6 the suspension was strained through a 40 µm strainer (Falcon). Meanwhile, a LS column (Miltenyi Biotec) was placed in the magnetic field of a MidiMACS Separator (Miltenyi Biotec) and rinsed with 3 ml of beads buffer. Once the column reservoir had emptied, the cell suspension was applied onto it and the resulting effluent collected. The column was then washed 3 times, using 3 ml of beads buffer, never applying the next 3 ml before the column reservoir had emptied. At this point, the total effluent collected is composed only by unlabeled cells ($CD14^-$), representing the negative fraction of the isolation. In order to obtain the labeled cells (positive fraction), the column was transferred to a new 15 ml tube, where it

was loaded with 5 ml of beads buffer. Then, the plunger supplied with the column, was immediately applied and the eluted fraction $CD14^+$ fraction was collected. . Both the negative ($CD14^-$) and the positive ($CD14^+$) fractions were then counted in a hemacytometer (Reichert Bright-Line). Both fractions were centrifuged at 250xg for 10 min and the pellet subjected to downstream applications. Monocytes ($CD14^+$ cells) were then cultured for differentiation into DCs. The purity of the isolation was assessed by flow cytometry analysis.

2.6 | Immunomagnetic isolation of $CD34^+$ cells (HSCs) from cryopreserved cord blood.

Since by the time we performed experiments related with hematopoietic stem cell isolation we already had an optimized thawing procedure, we have then chosen to use a DNase step on the thawing protocol as well. The kit and procedures used for $CD34^+$ cell isolation were in all similar to the one used for monocyte isolation covered in chapter 2.5. For this type of cells, CD34 MicroBeads + MACS Column (Miltenyi Biotec) for immunomagnetic cell separation were used. This being said, the procedure is almost the same as in $CD14^+$ cell isolation, but with some differences: in the first step, when the pelleted cells are resuspended in beads buffer and beads, 100 µL of the first and 30 µL of the second (CD34 microbeads) are to be used for every 10^8 cells. Also and as importantly, 30 µL of FcR Blocking Reagent (from the kit) is to be added as well – this prevents nonspecific cell isolation related to microbeads binding to cell's Fc receptors. After resuspending the cells, they must be incubated for 30 min at 4ºC. The rest of the protocol is completely parallel to the one performed on monocyte isolation and can be consulted in chapter 2.5**.**

2.7 | Proliferation protocol for $CD34^+$ hematopoietic stem cells.

Stem cells present on cord blood are known to have proliferative and differentiation capacity. For our studies on proliferation an adapted method from Balan and colleagues (Balan *et al.*, 2009) was used. It is based on expansion cultures containing TPO, Fl3-3L and SCF as growth factors which will lead to proliferation of these stem cells.

For that, after $CD34^+$ immunomagnetic cell isolation from cryopreserved cord blood, cells were resuspended in warm Iscove's Modified Dulbecco's Medium (Gibco) containing 10% FBS (v/v), 100 U/mL of penicillin and 100 µg/mL of streptomycin (Gibco). To this medium, TPO (PeproTech) at 10 ng/mL, SCF (R&D Systems) at 20 ng/mL and FltT-3L (R&D Systems) at 25ng/mL was also added. Cells were resuspended at 10^5 cells/mL and distributed in a 24-well plate (Orange Scientific), 1 mL per well and were incubated for 3 weeks at a 37ºC, 5% CO_2 humidified atmosphere. The medium renewal

was performed at the 3rd, 7th and 14th days of culture, always supplemented with the growth factors mentioned above at the same concentration.

At chosen intervals, part of the cells was taken from culture in order to evaluate their surface phenotype, perform functional experiments and to be counted, so that the fold increase resulting from proliferation could be calculated.

2.8 | Generation of dendritic cells.

After the isolation of monocytes ($CD14^+$ cells) or expanded HSC ($CD34^+$ cells) was complete, they were resuspended at a concentration of 10^6 cells/ml in $RPMI_c$ (see chapter 2.19 for media composition) supplemented with 750 U/mL of interleukin-4 (IL-4) (R&D Systems) and 1000 U/mL of granulocyte macrophage colony-stimulating factor (GM-CSF) (Immunotools) to promote differentiation into DCs. The cell suspension was then distributed in a 6 well culture plate (Greiner Bio-one), 3 mL per well. Cells were transferred into a CO_2 incubator (Panasonic) and kept in culture at 37 ºC, in a humidified atmosphere with 5% CO_2. Every two days, 1/3 of the culture media was gently removed and replaced by 1/2 of an equal amount of fresh one, supplemented with IL-4 and GM-CSF at the same concentration, as previously referred. Cells were allowed to differentiate for 7 days (for moDCs) or 9 days (for CD34-derived DCs). Along culture time, the differentiation was monitored by staining with surface markers and analysis by flow cytometry. After this time, DCs were removed from culture in order to access the number of cells, the differentiation status, and/or to use them in further experiments such as maturation experiments, sialidase treatment and functional assays.

2.9 | Induction of DC and monocyte maturation.

In order to induce a matured status on the DCs from different origin as well as on monocytes, different approaches were taken, but all based upon the incubation of cells with specific cytokines or treatments.

Washed cells were resuspended in $RPMI_c$ at 10^6 cells/mL and stimulated with 5 µg/mL LPS (Sigma) and 1000 U/mL TNFα (Miltenyi Biotec); or MFC-7 cell line lysates at $2x10^5$ lysed cells/mL. Another type of stimulus was sialidase treatment and posterior culture, where cells were subjected to this enzymatic removal of surface sialic acids (see chapter 2.10). In these experiments, cells were placed in the incubator for 1, 24 or 48 hours, after which they were removed from culture and used for flow cytometry immunophenotyping (chapter 2.20) or other functional experiments (chapters 2.12, 2.13 and 2.14). Supernatants resulting from a 300xg for 7 min centrifugation were also kept at -20ºC to be analyzed by ELISA.

2.10 | Sialidase (neuraminidase) treatment.

Cells that have previously been in culture were removed from culture, their number determined, washed and centrifuged for 7 min at 200xg. The supernatant was completely discard and the cells resuspended in simple RPMI, so that a concentration of 50 x 10^6 cells/mL may was achieved. Sialidase enzyme from *C. perfigens* (Roche Diagnostics) was added in order to obtain a final concentration of 0.1 U/mL/50x10^6 cells and then incubated for 1h at 37 ºC, in a humidified atmosphere with 5% CO_2. After the incubation, warm RPMI containing FBS was added in order to stop sialidase enzymatic activity. Finally, cells were washed by centrifugation at 200xg for 7 min and put in culture after that, in maturation experiments. Another version of sialidase treatment used was simply to resuspend cells that were previously been in culture in 0.01 U/mL of sialidase and, without washing, place the cells back in culture.

2.11 | Generation of tumor cell lysates.

When needed, MCF-7/GFP (Cell Biolabs) cells were resuspended at 5 x 10^6 cells/mL in RPMI and subjected to freeze-thaw cycles so that cell's integrity could be compromised, generating antigens for maturation and endocytosis experiments. For that, cells were removed from culture, washed and once resuspended, stored at -80ºC. Cells were quickly thawed in a 37ºC water bath until the sample was in a liquid state, and then was again immediately frozen at -80ºC. This cycle is to be performed at least 4 times and after the last cycle, cells are centrifuged at 250xg for 5 min so that the supernatant containing the soluble antigens may be separated from the most dense cell debris. This supernatant was transferred into a new tube and stored at -80ºC.

2.12 | Endocytosis assays.

This assay will compare endocytosis capacity of cells subjected to different treatments using Fluorescein isothiocyanate (FITC) -conjugated ovalbumin (Molecular Probes) and two incubation temperatures, 37ºC and 4ºC. Incubation at 4ºC is used as a negative control, because at this temperature endocytosis is inhibited. After the incubation, OVA-FITC incorporation will be assessed by flow cytometry in which FITC fluorescence in target population is used to measure endocytosis. The values measured for the negative control (4ºC) are considered background fluorescence due to OVA molecules attached to the cells but not internalized and are discounted from the values measured at 37ºC.

For this assay, cells were resuspended in simple RPMI at a concentration of 5 x10^6/mL. Each individual eppendorf represents a different condition: one should have the exact same cells in different tubes both incubated at 4ºC and 37ºC and, within these settings, one tube must contain the agent to be

endocyted while the other does not. Each tube contained 100 µL of cell suspension and first, the cells were incubated at a 4ºC water bath and at a 37ºC incubator for 10 min as described by Cabral et al. 2010. After, OVA-FITC was quickly added at a final concentration of 0.1 mg/mL or 0.2 mg/mL and cells were incubated for 1h more at the same conditions. In some experiments, instead of OVA-FITC, cell lysates from MCF-7/GFP (Cell Biolabs) at a final concentration of 10^6 lysed cells/mL were used as endocytic agents. Then, 50 µL of trypan blue 0.4% (w/v) was added to every tube and incubated for 30 sec before washing the cells with PBS. Trypan blue will quench "green" fluorescence associated to eventual OVA-FITC clutching outside the cell membrane since it is known to actually absorb light in the range of FITC (Busetto *et al.*, 2004). In this way, data output will mainly represent only OVA-FITC endocyted molecules inside the cell. Back to the process, cells were abundantly washed with PBS twice, resuspended in 1 mL of PBS and analyzed by flow cytometry. The endocytosis capacity was evaluated in terms of median of fluorescence intensity (MFI), and the following ratio was used:

$$\text{Endocytosis capacity} = \frac{\textbf{with}\ \text{endocyted agent at } 37℃\ \text{MFI} - \textbf{without}\ \text{endocyted agent at } 37℃\ \text{MFI}}{\textbf{with}\ \text{endocyted agent at } 4℃\ \text{MFI} - \textbf{without}\ \text{endocyted agent at } 4℃\ \text{MFI}}$$

2.13 | Cytokine quantification by Sandwich ELISA (enzyme-linked immunosorbent assay) technique.

Four cytokines were evaluated in cell supernatants that resulted from maturation experiments: IL-6, IL-10, IL-12 and TNFα (all using kits from Immunotools).

The capture antibody corresponding to each cytokine was diluted 1:100 in PBS (Nzytech) and 100 µL of this working solution was distributed per each well to be used, in a 96-well high binding microplate (Costar) and incubated overnight at 4ºC. After, discarding the capture-antibody solution, 200 µL of blocking buffer (PBS 2% BSA [Sigma] 0.05% Tween20 [Sigma]) was added to each well and further incubated for 1h at room temperature. The blocking buffer was completely removed and 50 µL of cell supernatant was added to each well. At the same time, standard samples were also prepared, using serial dilutions of the lyophilized protein provided by the kit, in blocking buffer. Standard curves were prepared so that they could range values between 15 and 2000 ng/mL, and so 50 µL of each dilution was also added to the respective standard wells (in duplicate). Then, an incubation for 2h at room temperature followed. Samples were washed 5 times with 100 µL of washing buffer (PBS 0.05% Tween20 [v/v]) and after a biotinylated detector antibody was added. For that, detector antibody was diluted in a 1:100 ratio in blocking buffer and 100 µL of this solution was transferred to each well and incubated for 2h at room temperature. After that, wells were again washed 5 times with 200 µL of washing buffer. The next step was to add a biotin recognizing streptavidin, conjugated with Poly-HRP (Horseradish peroxidase).

1:1000 diluted in blocking buffer poly-HRP-streptavidin was added, 100 µL per well and then incubated for 20 min at room temperature. After washing 5x with washing buffer, 100 µL of 37ºC pre-warmed TMB (3,3',5,5'-tetramethylbenzidine) substrate (Sigma) was added to the wells. TMB is a chromogen that yields a blue color when oxidized, typically because of oxygen radicals produced by the hydrolysis of hydrogen peroxide by HRP. So, TMB is used to detect horseradish peroxidase activity, yielding a blue color (A_{max} = 370nm and 652nm) that changes to yellow (A_{max} = 450nm) upon addition of a sulfuric acid solution. Given this, after the adding of TMB solution, the plate was incubated at room temperature, protected from light, until the development of blue color. When so, the enzymatic color reaction was stopped by adding 50 µL of a 2M sulfuric acid solution. The microplate was read at 450 nm in an iMark microplate absorbance reader (Bio-Rad) and the absorbance values used for construction of the standard curve so that concentration of the different cytokines could be determined. Each condition was evaluated in duplicates.

2.14 | Allogenic mixed leucocyte reaction (MLR).

The question we wanted to address here was that if cryopreserved cord blood moDCs were able to induce (or not) a proliferative response when in contact with allogenic (*i.e* cells from a different donor) adult lymphocytes.

For that, stimulated CBmoDCs and non-stimulated CB moDCs were resuspended in $RPMI_c$ and distributed in a round bottom 96-well plate (Costar). The number of cells in each well was $4x10^4$, resuspended in 200 µL.

For the preparation of T cells, $CD14^-$ fraction resultant from immunomagnetic cell separation of adults PBMCs (chapter 2.5) was used. This fraction is enriched in lymphocytes and can further be used in allogenic MLR. In order to evaluate proliferation, cytometry methods were used and will be later explained. Output comes from fluorescence dilution upon cell division and for that lymphocytes need to be stained with carboxyfluorescein succinimidyl ester (CFSE) before being put in contact with CB moDCs. For that, a frozen aliquot of CD14- was thawed, washed and put in culture in the previous day. On the next day, approximately $50x10^6$ cells were resuspended in 1 mL of PBS and added to 1 more mL of PBS containing CFSE (Molecular Probes), so that a final concentration of 5 µM CFSE was obtained. The cells were immediately vortexed afterwards. And, after a 5 min incubation at room temperature, cells were washed 3 times with 20 mL of PBS 5% FBS (v/v), with centrifugations at 300xg for 5 min. Cells were counted and resuspended in $4x10^6$ cells/mL of $RPMI_c$, so that after adding 50 µL of this suspension to the CB moDCs containing wells, the ratio DC:T cells was 1:5. That is, 200,000 $CD14^-$ cells were put in contact with 40,000 DCs. Some of the wells were prepared for controls, such as wells with only T cells (stained and also not stained with CFSE) and wells with T cells (CFSE stained) that

were later stimulated with PMA (Phorbol 12-myristate 13-acetate) and ionomycin calcium salt as mitogens.

18 hours later, 10 µL of interleukin-2 (IL-2) (Miltenyi Biotec) was added to every well, to a final concentration of 0.1 µg/mL. At this time, control wells containing only T cells (CFSE stained and not stained) were harvested and centrifuged at 5000xg for 2 minutes. The supernatant was kept at -20ºC for eventual analysis and the cell pellet was resuspended in 300 µL of 2% paraformaldehyde (PFA) solution (Polysciences) and kept at 4ºC, protected from light. These cells will represent the parental T cell population for the cytometric analysis in the conditions to be studied.

Three days before the next harvesting of cells (that will happen in the fifth day of co-culture), some control T cells were subjected to stimulation with mitogens in 2 differential concentrations: the first at 100 nM of PMA (Sigma) and 10 µM of ionomycin (Sigma); the second at 10 nM of PMA and 1 µM of ionomycin. These mitogens were diluted in RPMI containing 10% FBS (v/v) and 20 µL of solution was added to the wells. PMA is small organic compound which will diffuse through the cell membrane into the cytoplasm, activating on protein kinase C (PKC) without needing the surface receptor stimulation while ionomycin is a calcium ionophore, triggering the release of NFAT (Nuclear factor of activated T-cells) signaling (Hashimoto *et al.*, 1991). After the 3 days of stimulation (5^{th} day of MLR), cells of every condition to be analyzed and also these controls were harvested and centrifuged at 5000xg for 2 minutes. Again, the supernatant was kept at -20ºC and the cells were resuspended with 300 µL of PFA solution and kept at 4ºC, protected from light. This was repeated at the 7^{th} day of MLR (except for PMA/ionomycin stimulated T cells, only analyzed at the 5^{th} MLR day.

In flow cytometry analysis, the percentage of proliferative $CD14^-$ fraction was assessed as the cells presenting lower degree fluorescence than that of the parental population (18h CFSE stained CD14- fraction).

2.15 | Breast cancer biopsy samples acquisition, processing and culture.

Following Dr. Sofia Braga's patients normal breast cancer monitoring in CUF Descobertas Hospital and with the appropriate informed consent, biopsies were removed from the patients and sent to the pathologic anatomy department for cancer biomarker evaluation. There, immunohistochemistry, Fluorescent in situ hybridization (FISH), Silver in situ hybridization (SISH) and Chromogenic in situ hybridization (CISH) techniques are performed so that cancer biomarkers profile may be clinically determined. Part of the biopsy was placed in a 15 mL tube containing DMEM medium (Gibco) with 20% heat inactivated FBS (Gibco) and kept at -4ºC until transport to our laboratory, which would happen on the same day.

Once in our laboratory, all the content of the tube was placed on a glass petri dish and the tissue was dilacerated using tweezers and a scalpel. Once the tissue was sufficiently fragmented, all its content

was transferred into a glass tube and centrifuged at 200xg for 4 min. The sometimes greasy (maybe due to tumor closeness to adipose tissue) supernatant was removed and the solid pellet was resuspended in 5 mL of $DMEM_c$ (see chapter 2.19 for composition). Collagenase type IV from *C. histolyticum* (Sigma-Aldrich) was added to a final concentration of 1 mg/mL and incubated overnight at a 37ºC, 5% CO_2, 100% relative humidity cell culture incubator. In the next day, the cell suspension was carefully resuspended after adding 5 more mL of $DMEM_c$ and centrifuged at 200xg for 7 minutes. After discarding the supernatant, cells were diluted in 6 mL of fresh warm $DMEM_c$ and put in culture inside a T-25 tissue culture flask (Sarstedt) so that the cells could adhere to the plastic surface. 48 hours later we proceeded to the culture medium renewal and all the medium was removed, so that only the adherent cells remained in culture. The old medium was not discarded but centrifuged (5000xg for 2 min) to save cancer antigens for future experiments. Every four days the medium was renewed (only 3 mL of it) and when plastic surface confluence reached 80-90% the cells were subcultured in a 1:2 ratio, into two new T-25 flasks. For that, the medium was removed, cells were washed two times with 3 mL of PBS, given 1 mL of trypsin-EDTA solution (Gibco) and incubated at 37ºC for 5-7 min so that cells could detach. Tripsinization process was stopped by adding 6 mL of DMEM 10% FBS, centrifuged at 300xg for 7 min, resuspended in new medium and finally subcultured. On the first passages, pre-passage medium was always checked for antigens, given that sometimes there was debris in suspension. After the 5^{th} passage one could start using larger T-75 flasks (Sarstedt).

The objective was to have enough number of cells so that we could freeze and keep them at -80ºC for further experiments, 10^7 cell per vial in DMEM 10% DMSO (Sigma-Aldrich), and so cells were kept in culture with that goal. We had breast cancer biopsy samples coming from two patients, and because of some interesting characteristics one of it presented, we decided both analyzing several cell markers using flow cytometry and also to immortalize it into a cell line.

2.16 | Immortalization of a primary culture derived breast cancer cell line.

Because of the interest concerning one of the breast cancer samples we had, given that the patient went thought four different types of chemotherapy with the tumor showing low regression, plus the fact of it not showing three usual breast cancer markers, we decided to immortalize it so that we had a stable cell line for future applications. For that, a lentiviral construction containing *hTERT* gene was used in the process. *hTERT* is a gene coding for a major telomerase subunit, telomerase being the enzymatic complex responsible for telomere maintenance. Telomere shortening is known to be a phenomenon associated with cellular senescence (Stewart & Weinberg, 2002) and an over-function of telomerase activity would theoretically promote overlapping of Hayflick's limit, that is, the number of times normal cells would enter mitosis when cultured *in vitro*. What will be used in the process of immortalization is a cell line supernatant containing 10^7 viral particles/mL, a kind gift from Prof. José Ramalho, CEDOC's

Molecular Mechanisms of Disease Laboratory. Briefly, lentivirus production was achieved through a successful integration of three different plasmids into HEK293 cell line: two of them coding for viral capsid and packaging proteins, integrase and reverse transcriptase, and the other plasmid having an inserted cloned *hTERT* gene being expressed through a CMV promotor. This last plasmid would also contain a gene responsible for resistance to blasticidin S, an antibiotic that inhibits protein synthesis. If integration of the three plasmids is successful, our gene of interest will be packed inside a lentiviral particle that will exit the cell line by exocytosis, into the cell culture medium. This lentivirus containing medium will then be used to infect the primary culture derived breast cancer cell line, so that immortalization could be achieved.

The number of cells that would occupy approximately 30% of a 12-well plate single well area (1.14 cm^2) was determined. That amount of cells was resuspended in 2 mL of DMEM 20% FBS (v/v), added 250 µL of lentiviral supernatant (final concentration of approximately 10^6 viral particles/mL) and 6 mg/mL of polybrene (Sigma), a cationic polymer used to increase infection efficiency in cell culture that acts by neutralizing repulsion between virions and cell surface sialic acids (Davis et al. 2002). Cells were then incubated in a 37ºC 5% CO_2 atmosphere. A control well with the same number of cells but without virus or polybrene was also prepared. 24h later 100 µL more of viral supernatant was added to the well containing the cells to be immortalized. When a the middle part of the wells shown itself to be confluent, we subcultured the cells in a 1:2 ratio and also added blasticidin (Sigma-Aldrich) at 3,2 µg/mL since a successful transfection would promote cell survival in the presence of this antibiotic, trough *BSR* gene, also present in the viral construction. Once the control cells were all dead (not adherent) and the immortalized cells confluent, that should mean a successful transduction, where triple negative breast cancer cells not only integrated *hTERT* gene but also the reporter gene, conferring resistance to blasticidin which represents cell division capacity in the presence of the compound. Then, immortalized cells, from that moment named TNBC1 (Triple negative Breast Cancer 1), were again subcultured (1:2 ratio) to two new wells, always using fresh medium. From the moment these wells got confluent, cell culture was performed on T-25 flasks. In this case, successful immortalization represents cells with similar cell division time when compared to the pre-immortalized cells.

2.17 | Immortalized breast cancer cell line (TNBC1) immunophenotyping by flow cytometry.

The protocol for understanding of cancer biomarkers using flow cytometry was somehow different than that used for immunophenotyping of moDCs, monocytes and other blood circulating cells (that protocol is covered in chapter 2.20). When needed, other breast cancer cell lines were also phenotyped. Working volume of cell suspension upon antibody incubation is reduced to 50 µL.

For intracellular staining cells had to be permeabilized before, for that using a permeabilization solution, BD Cytofix/Cytoperm kit (BD Biosciences), following manufacturer's recommendations. Then, cells were incubated with anti-human HER2/ErbB2 (29D8) rabbit mAb (Cell Signaling) for 30 min at 4ºC. After, cells were washed with the kit's washing buffer and centrifuged for 2 min at 5000xg. Cells were again resuspended in 50 µL of PBS and incubated with the goat anti-rabbit IgG-FITC secondary antibody (clone F1262-1ML, Sigma) in the same conditions. For cytokeratin staining, mouse anti-human cytokeratin mAbs (Clones AE1/AE3, Dako) were used as primary antibodies and polyclonal goat anti-mouse Igs-FITC (Lot: 00058107, Dako). Finally cells were resuspended in 1 mL of PBS and analyzed by flow cytometry. Unstained controls as well as controls with only the secondary antibody were also performed for every sample.

Surface marker staining, with anti-human MUC1 mouse mAbs, MUC5 (both from Santa Cruz Biotechnology) and CEA (CB30 clone, Immunotools) were also done (on non-permeabilized cells), with a 30 min at 4ºC incubation period. After washing with PBS and a centrifugation for 2 min at 5000xg, the pellet was resuspended and incubated in the presence of a goat anti-mouse IgG–FITC secondary antibody (Lot: 20005617, Dako). Incubation, washing and resuspension conditions were as described for the permeabilized cells. For ER and PR staining, anti-human ERα and PR rabbit polyclonal IgGs were used (clones MC-20 and C-20, respectively, Santa Cruz Biotechnology), with the respective goat anti-rabbit IgG-FITC secondary antibody (Lot: F1262-1ML, Sigma). Direct detection of MHCI molecules was also done with an anti-human HLA-ABC FITC conjugated antibody (Lot: mab-m38, Immunotools).

2.18 | Cell freezing and thawing protocol.

For cell cryopreservation, after primary cells were isolated or after cell lines were expanded, cells were washed and resuspended in RPMI medium containing 20% FBS (v/v). An equal volume of medium containing 20% FBS and 20% cryoprotectant DMSO (Sigma-Aldrich) is then added at a drop by drop rate. In this, the final concentration of DMSO is 10% (v/v). Each 1.8 mL cryovial will contain a maximum of 50 x 10^6 cells that are transferred to Mr. Frosty (Nalgene) and kept at -80ºC. This will promote a 1ºC/min temperature decrease rate. 50 x 10^6 cells per cryovial makes sense when freezing PBMCs or $CD14^-$ fraction of monocyte isolation. When freezing MCF-7/GFP breast cancer cell line the amount of cells in a confluent T-25 flask in enough. Breast cancer patients' primary culture derived cell lines were frozen at 10 x 10^6 cells/vial, and instead of using RPMI, DMEM (Gibco) was used for all cancer cell lines.

For thawing, place the vial in a pre-warmed 37ºC water bath and gently swirl the vial until there is only a small bit of ice left. Dilute the content of the cryovial in medium containing FBS and centrifuge

it for 5 min at 200xg. Remove the supernatant and carefully resuspend the cells in appropriate pre-warmed medium if cell culture is desired.

2.19 | Cell culture media and other reagents used.

Primary cultures of cryopreserved cord blood and fresh peripheral blood monocytes were done in complete RPMI medium ($RPMI_c$) - RPMI medium supplemented with 10% (v/v) FBS, 2 mM L-glutamine, 100 U/mL of penicillin and 100 µg/mL of streptomycin, 1 mM sodium pyruvate and 1% MEM non-essential amino acids; Primary cultures of cryopreserved cord blood hematopoietic stem cells ($CD34^+$ cells) were performed in complete IMDM ($IMDM_c$) – IMDM medium supplemented with 10% (v/v) FBS, 100 U/mL of penicillin and 100 µg/mL of streptomycin; Cell lines derived from breast cancer primary cultures were cultured in complete DMEM medium ($DMEM_c$) – which is basically DMEM medium, supplemented with 20% (v/v) FBS, 2 mM L-glutamine and 100 U/mL of penicillin and 100 µg/mL of streptomycin. MCF-7/GFP (Cell Biolabs) commercial breast cancer cell line was cultured in DMEM supplemented with 10% (v/v) FBS, 2 mM L-glutamine and 100 U/mL of penicillin and 100 µg/mL of streptomycin. All products were bought from Gibco.

Phosphate buffer solution (PBS) for both cell culture procedures and flow cytometry analysis is a solution with 1.47 mM KH_2PO_4, 4.29 mM $NaHPO_4$.7H2O, 137 mM NaCl and 2.68 mM KCL in distilled water (pH=7.3).

Beads buffer, used for immunomagnetic cell separation is constituted by PBS containing 0.5% (w/v) BSA (Sigma-Aldrich) and 2 mM EDTA (Sigma-Aldrich).

2.20 | Flow cytometry.

Most of this work was done with the use of a flow cytometer, Attune Acoustic Focusing Cytometer (Applied Biosystems). Flow cytometry is a gold-standard technique in immunology.. Basically a single cell suspension is used as a sample and the fluidic system of the cytometer will drive the cells and present them to a laser beam, which in the case of this cytometer is composed by two different ones, Blue (488 nm) and Red (638 nm). Light will then be refracted, and the photons with the lower angle of refraction will be detected by what we call a Forward Scatter (FSC) detector. The cytometer will then transform this light signal into electronic data that gives information on cell size. A Side Scatter (SSC) detector will capture light that has been refracted with 90º angle and this gives information about the complexity of the cell, or granularity. Now, if a cell has an antibody (linked to a fluorochrome) attached to itself, the lasers will excite the fluorochrome, and depending on the fluorochrome specific emission spectrum, light will be generated and detected on specific detectors. BL1, BL2, BL3 and BL4 channels will detect light that comes from blue laser excited fluorochromes.

515–545 nm, 561–587 nm, 665–715 nm and 750–810 nm are the filter ranges for each of the detectors, respectively. Red laser will excite others that should be read in RL1 (650–670 nm) and RL2 (750–810 nm) detectors. Apart from FSC all other detectors need a complex set of dichroic mirrors set in order for the light to reach the detector. Once it does, the signal from the cell (or particle) is detected in the form of a voltage pulse defined by its area (A), height (H) and width (W). W refers to the 'duration of the signal', H to the intensity of it. A = W x H, and in all our experiments this is the one voltage pulse input that we choose to evaluate.

Describing the sample preparation for cytometry, cells were washed and centrifuged at 1000xg for 2 minutes. The pellet was resuspended in PBS and distributed in eppendorfs so that in each one there was 20,000 to 100,000 cells suspended in 100 µL. In order to proceed with immunophenotyping, antibodies, lectins or other fluorophore conjugated markers (**TABLE 2.1**) were added to cell suspension and incubated for 15 min at room temperature. The table only shows markers used for blood derived cells (antibodies and protocol for primary breast cancer cell immunophenotyping on chapter 2.17). After this time, cells were washed with 900 µL of PBS and centrifuged for 2 min at 1000xg. The pellet was then resuspended in up to 1 mL of PBS and soon after read on the cytometer. Most of the work on this dissertation was performed in this way. If the samples were to be analyzed later at the flow cytometer, cell pellet was resuspended in 300 µL of 2% paraformaldehyde fixation buffer (Polysciences) and is kept at 4ºC, protected from light. Upon flow cytometric acquisition, the strategy we adopted to evaluate the cells in terms of their markers was as described on **FIGURE 2.1**

TABLE 2.1 | List of antibodies and other cell markers used in flow cytometric assays. *n.a. is non-applicable.

Marker	Fluorochrome	Manufacturer	Clone/Lot
Anti-human CD1a	FITC	BD Pharmigen	79742
Anti-human CD1c	PE	Biolegend	L161
Anti-human CD3	APC	Immunotools	620368
Anti-human CD4	FITC	Immunotools	620305
Anti-human CD8	APC	Immunotools	620390
Anti-human CD14	FITC	Immunotools	620263
Anti-human CD14	PE	Immunotools	620307
Anti-human CD16	PE	Immunotools	274121
Anti-human CD20	PE	Immunotools	mab-m54
Anti-human CD34	PE	Miltenyi Biotec	5150225320
Anti-human CD40	PE	Immunotools	81241
Anti-human CD45	PerCP	Biolegend	H130
Anti-human CD80	PE	Immunotools	274354
Anti-human CD86	FITC	Immunotools	480118
Anti-human HLA-ABC	FITC	Immnunotools	mab-m38
Anti-human HLA-DR	APC	Immunostep	GRB-1
7AAD	n.a*	Sigma	044M4013V

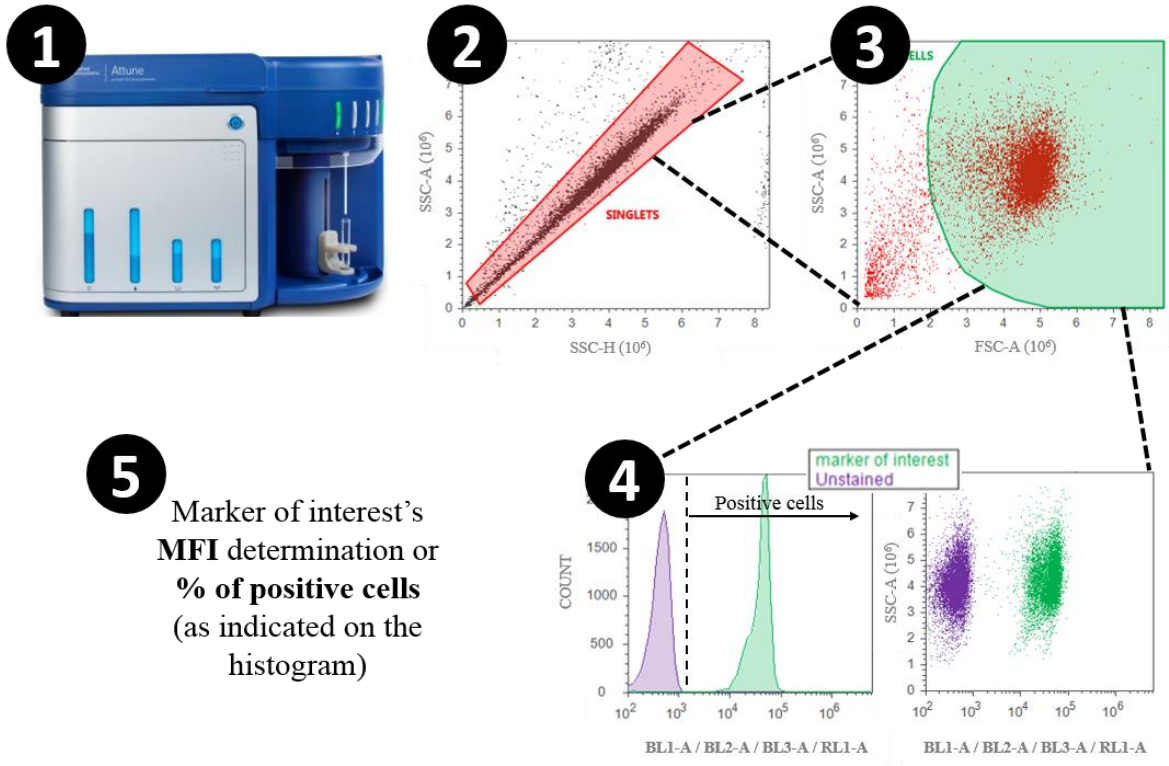

FIGURE 2.1 | **Gating strategy for flow cytometry assays.** The scheme represents all the steps between placing a cell suspension on the flow cytometer and data acquisition. Once the cells were properly incubated with the antibodies, washed and resuspended in PBS, the sample was placed in contact with the cytometer's needle in order for it to be aspirated (**1**); the sample is run and autofluorescence is manually lowered to basal levels. A gate covering the singlets (in red) is created on a SSC-H *vs* SSC-A dot blot (**2**). This gate is used to open a FSC-A *vs* SSC-A dot plot, were a gate covering 10,000 events, far from debris, is created (in green) (**3**). That gate is used to open a histogram (fluorescence channel *vs* cell count) or a SSC-A *vs* fluorescence channel dot blot (**4**), from where the marker of interest's MFI (Median Fluorescence Intensity) or % of positive cells may be extrapolated (**5**). In (**4**) it is represented the fluorescence threshold after which the cells are considered positive for the marker. Autofluorescence of unstained cells are used as the threshold. Flow cytometry channels used in this thesis were BL1-A, BL2-A, BL3-A, RL1-A.

2.21 | Statistical analysis and data acquisition software.

Experimental data was analyzed using GraphPad Prism 6 (GraphPad Software, Inc). Statistical differences were determined using Student's t-test, considering a p-value <0.05 as statistically significant. Both acquisition and cytometric data processing was done with the use of Attune Cytometric Software v2.1.

3 | RESULTS AND DISCUSSION

3.1 | Protocol optimization for thawing and cell recovery from cryopreserved cord blood units.

Thawing procedures for cryopreserved cord blood units are well documented and are routinely performed before cord blood transplantation, in the context of clinical settings. If for research purposes, most frequently, mononuclear cells are isolated from fresh umbilical cord blood using density gradient separation before being cryopreserved. However, for this thesis, UCB cell concentrates prepared by SEPAX technology were aimed to be used as starting material for monocyte isolation followed by cell culture and differentiation. So, due to this need of subsequent cell isolation after thawing, extra manipulation steps were required. Moreover, there is a subsequent prolonged *in vitro* cell culture and differentiation step, where preservation of cell viability and function is a must.

In this first part of results section we will describe our efforts to optimize the thawing and monocyte-isolation procedures with the aim of getting the highest cell recovery rates as well as the highest cell viabilities. Since our starting material was UCB cell concentrates prepared by SEPAX technology, which include not only mononuclear cells but also other nucleated cells and large amounts of contaminating RBCs that were not removed in the SEPAX concentration procedure. Therefore, our study is useful and relevant for these particular type of samples and especially for the purposes of cell isolation and subsequent culture.

The project initially started with the goal of isolating monocytes from cryopreserved cord blood. As for the lack of hands-on experience with these kind of samples in our laboratory, we followed Crioestaminal's standardized procedure for thawing. The overall protocol can be found on Materials and Methods section and, basically consisted in thawing, washing and erythrocyte lysis. When using this protocol we observed that large cell clumps were formed. Cell clumping is a problem when immunomagnetic separation is envisaged because it prevents cell passage through the column, limiting cell separation efficiency. It can also affect cell recovery, because live cells may be trapped by DNA (García-Piñeres *et al*., 2006).

As an attempt to overcome the problems mentioned above, we have decided to try other thawing and isolation variants, as reported in **TABLE 3.1.** In this table, some parameters are compared: the cell aggregation during the different processes, the monocyte recovery and the overall sample handling time.

TABLE 3.1 | Summary of the results obtained using different methods for thawing of cryopreserved UCB units and posterior monocyte isolation. "Cell aggregation" was evaluated by visual observation of cell aggregates during all process; "Monocyte recovery" was calculated as the percentage of isolated $CD14^+$ cells, taking the number of pre-thaw monocytes as reference. Data is presented as mean % ± SD; "Approximate duration" refers to the time taken for handling a sample, comprises thawing and isolation of monocytes.

Reference	Method of isolation	n	Cell aggregation	Monocyte recovery	Approximate duration
A	Thawing + Washing + $CD14^+$ immunomagnetic cell separation	2	+	17.6 ± 1.4 %	2.5 h
B	Thawing + Washing + Erythrocyte lysis step + $CD14^+$ immunomagnetic cell separation	7	+++	30.3 ± 13.4 %	3 – 3.5 h
C	Thawing + Washing + Panning method for adherent cell isolation	1	+	0 %	2 h
D	Thawing + Washing + Erythrocyte lysis step + Panning method for adherent cell isolation	1	+++	0 %	2.5 h
E	Thawing + Washing + Erythrocyte lysis step + Density gradient mononuclear cell isolation + $CD14^+$ immunomagnetic cell separation	2	++	1.4 ± 1.8 %	4 h
F	Thawing + Washing + Density gradient mononuclear cell separation + $CD14^+$ immunomagnetic cell isolation	1	++	0.1 %	1.5 h
G	Thawing + Washing + DNase incubation step + $CD14^+$ immunomagnetic cell separation (with FcR Blocker)	2	+	34.6 ± 23.4 %	3 h

As we perceived that extensive manipulation led to successive cell clumping, which occurred every time cells were washed by centrifugation, we decided to use a longtime established method for monocyte isolation (Johnson *et al.*, 1977), panning - or cell adherence (**method C and D, TABLE 3.1**). Results show that even without the possibly rough step of erythrocyte lysis (**method C**), cells have not adhered to the plastic surface, as observed under the microscope, making us recognize this method as the least efficient of all, since the reported capacity of monocyte adherence was not present in this case.

The presence of charged stringed nucleic acids arising from dead cells and the cryopreservation process in itself may be preventing the optimal condition for the binding of cell surface´s adherence proteins. This would block the way for the chemical interactions between them and the charged polystyrene surface that are needed for adherence to happen. Our results show this as "no-go" protocol for monocyte isolation.

Another step that is constantly used by scientists in fresh blood experiments is the isolation of mononuclear cells by density gradient centrifugation, based on cell separation depending on their density. For that, a layer of ficoll, a polymer with 1.077g/mL, is used in a protocol that, after centrifugation, will generate a less dense layer of cells, where mononuclear cells – lymphocytes and monocytes are the most predominant population. Erythrocytes and polymorphonuclear cells (granulocytes – neutrophils, eosinophils and basophiles) will remain in the ficoll layer since they are denser (Bøyum, 1976). With this in mind, and again with or without a previous erythrocyte lysis step, monocyte isolation was tried (**methods E and F**, respectively). As shown in **TABLE 3.1**, a very much reduced percentage of monocytes was recovered. The layer of mononuclear cells was not formed above ficoll polymer. We believe that cell aggregation that resulted from thawing may have prevented the distribution of cells according to their density. Instead, mononuclear cells would certainly be scattered in the ficoll and/or at the bottom erythrocyte layer and therefore, most of monocytes were lost in this step.

The above experiments were performed as attempts to increase the percentage of isolated monocytes, something that our most used protocol (n=7) (**method B**) was able to do, showing a cell recovery of 30.3 ± 13.4 %. This protocol was also tried without the erythrocyte lysis step (**method A**) but resulted in a lower recovery (17.6 ± 1.4 %). As can be observed in the table, whenever a erythrocyte lysis step was not applied, less cell aggregation is reported but it does not translate in higher cell recovery so erythrocyte lysis acted positively.

Last but not least, we report our most successful protocol for monocyte isolation, in terms of cell recovery, with a mean of 34.6 ± 23.4 % (**method G**). The idea for using this last protocol appeared because of another demand for the thesis project: the isolation of $CD34^+$ HSCs from these samples. These are known to exist in the blood in very small numbers, and so, this was contemplated in the $CD34^+$ immunomagnetic isolation of these cells in the form of an additional step - DNase treatment. That is, DNase should prevent cell clumping because it breaks long DNA molecules that trap cells and promote clump formation, and given the reduced amount of HSC cells this step would be a must. We firstly encountered a reference, where Spanholtz and colleagues (Spanholtz *et al.*, 2011) used Pulmozyme, a rhDNase containing solution used in cystic fibrosis treatment, for improving cell recovery in thawed umbilical cord units. Given that, we decided to add a DNase incubation step in $CD34^+$ cell isolation. So, we also applied this to monocyte, $CD14^+$, isolation. We decided to add one more variant too, which was the presence of FcR blocker upon $CD14^+$ immunomagnetic bead incubation. Results from that

demonstrate a 4.3 % higher monocyte recovery in comparison with our most used protocol, not forgetting a large decrease in cell clumping also.

Commenting on what would be the preferential method for monocyte isolation and strictly based in our experience, having higher cell numbers, less cell aggregates and within a reasonable protocol duration (and with the least amount of manipulation), we would suggest this last protocol (**method G**) as the most fit. And the incorporation of a DNase step has already been reported as safe for immunologic assays, regarding cell function and viability (García-Piñeres *et al.*, 2006). Most of our experience in isolating cells present in cryopreserved cord blood units comes mainly from monocyte isolation and that is why data regarding only monocytes is presented. Throughout this year's work there was a need for isolating hematopoietic stem cells, $CD34^+$. For $CD34^+$ cell isolation the protocol used was always the one including FcR blocker (recovery of two $CD34^+$ cell isolation experiments = 44 ± 19.2% [mean ± SD]; the inclusion of the DNase step was only performed in one experiment, providing a higher increase of recovery – 57.6 %). Although all these different protocols were used for the isolation of one single type of cells, we propose that the conclusions that come from them may be useful when applied to the isolation of other cell types from cryopreserved blood samples.

3.2 | Immunophenotype characterization of Cord Blood CD14-derived moDCs.

Monocytes are known to have the ability of *in vivo* differentiation into both functional dendritic cells as well as macrophages, something that will greatly depend on the microenvironment. *In vitro*, dendritic cell differentiation can be achieved by culturing these cells for 5 to 8 days in the presence of two cytokines, IL-4 and GM-CSF. This procedure is a routine procedure in our laboratory for the

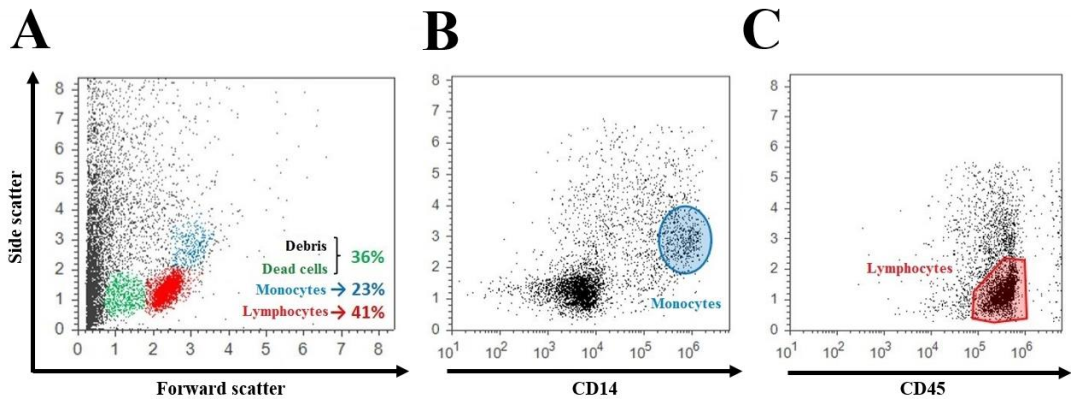

FIGURE 3.1 | Immunophenotype of a concentrated cord blood sample after thawing and erythrocyte lysis. Sample was analyzed by flow cytometry. **A -** FSC *vs*. SSC profile and identification of the dead cells ($7\text{-}AAD^+$) (green), monocyte ($CD14^+$) (blue) and lymphocyte populations ($CD45^{++}$) (red). The percentage of each of these populations is presented and relates to the total number of events; **B** and **C** represent the cell marker staining used to identify the populations in **A.**

differentiation of adult peripheral monocytes into DCs (Videira *et al*., 2008; Crespo *et al*., 2009; Silva *et al.*, 2011; Cabral *et al.*, 2013).

After thawing of UCB, small aliquots were taken for flow cytometry analysis, where cells populations were characterized and viability was accessed with 7AAD staining. The results of 4 independent experiments show that $CD14^+$ cells represent 19.5% ± 11.45 of all cells and that 75.25% ± 8.85 of all cells are viable. In **FIGURE 3.1,** dot plots from one representative sample are shown.

After isolation from a cryopreserved umbilical cord blood unit, monocytes were then cultured at 10^6 cells/mL with 1000 U/mL of GM-CSF and 750 U/mL of IL-4 for 8 days. Immunophenotyping of cells was performed every two days so that we could follow differentiation (**FIGURE 3.2**). When analyzed by flow cytometry and comparing forward and side scatter of the cells at different days there is no significant alteration in cell size or complexity. Microscope observation of cells shows that they appear to maintain a monocyte round shape over time. (**FIGURE 3.2**, 2nd and 1st columns, respectively).

On the three chosen markers of differentiation, we report a drastic loss of CD14 right after the cells have been put in culture, dropping from a mean of 92.2% of the cells presenting the marker at the cell surface to ≤ 0.4% on the next days. The percentage of cells expressing CD1c is also decreased to half after 2 days, and decreases again between the 4th and 6th day of culture (**see FIGURE 3.2**). Most of the monocytes driven into differentiation have shown high HLA-DR levels during all culture, oscillating from 88.3% maximum on day 4 to 78.9 % of the cells presenting the marker on day 8. In **FIGURE 3.2** it should be noted that there is an increase in the cell's autofluorescence after culture. During sample analysis cytometer settings were not optimized to compensate for this increment in autofluorescence, so it is possible that percentages of CD1c and HLA-DR positive cells are slightly underestimated for days 2, 4, 6, and 8.

Experience from our laboratory (with peripheral blood monocytes) and the literature show that during monocyte differentiation into DCs there is down-regulation of the expression of CD14 marker, but it takes 5-6 days until it is completely lost, which is typically used to prove for efficient monocyte differentiation. However, with these monocytes, this seems to be a very fast event. We went further to analyze the kinetics of CD14 loss and observed that after culture for 6h, only to 12% were $CD14^+$ and after 18h, there was a drop to 2% (results not shown). CD14 is known to be a receptor of a variety of ligands, specifically of gram-negative bacteria lipopolysaccharide (LPS). This receptor is anchored to the outer membrane in close association with LBP (lipopolysaccharide binding protein) and Tool-like receptors, but it also exists in a shed soluble form, facilitating LPS signaling for other cells (Jersmann, 2005). When we followed differentiation of monocytes isolated from fresh CB, the phenotypic profile of moDCs was very similar to adult moDCs except that the differentiation time, that is the loss of CD14, was longer - it took 7-9 days instead of 5-6 days (results not shown). So, it is probable that the quick loss of CD14 we are seeing is, in fact, CD14 shedding being facilitated by the thawing and manipulation

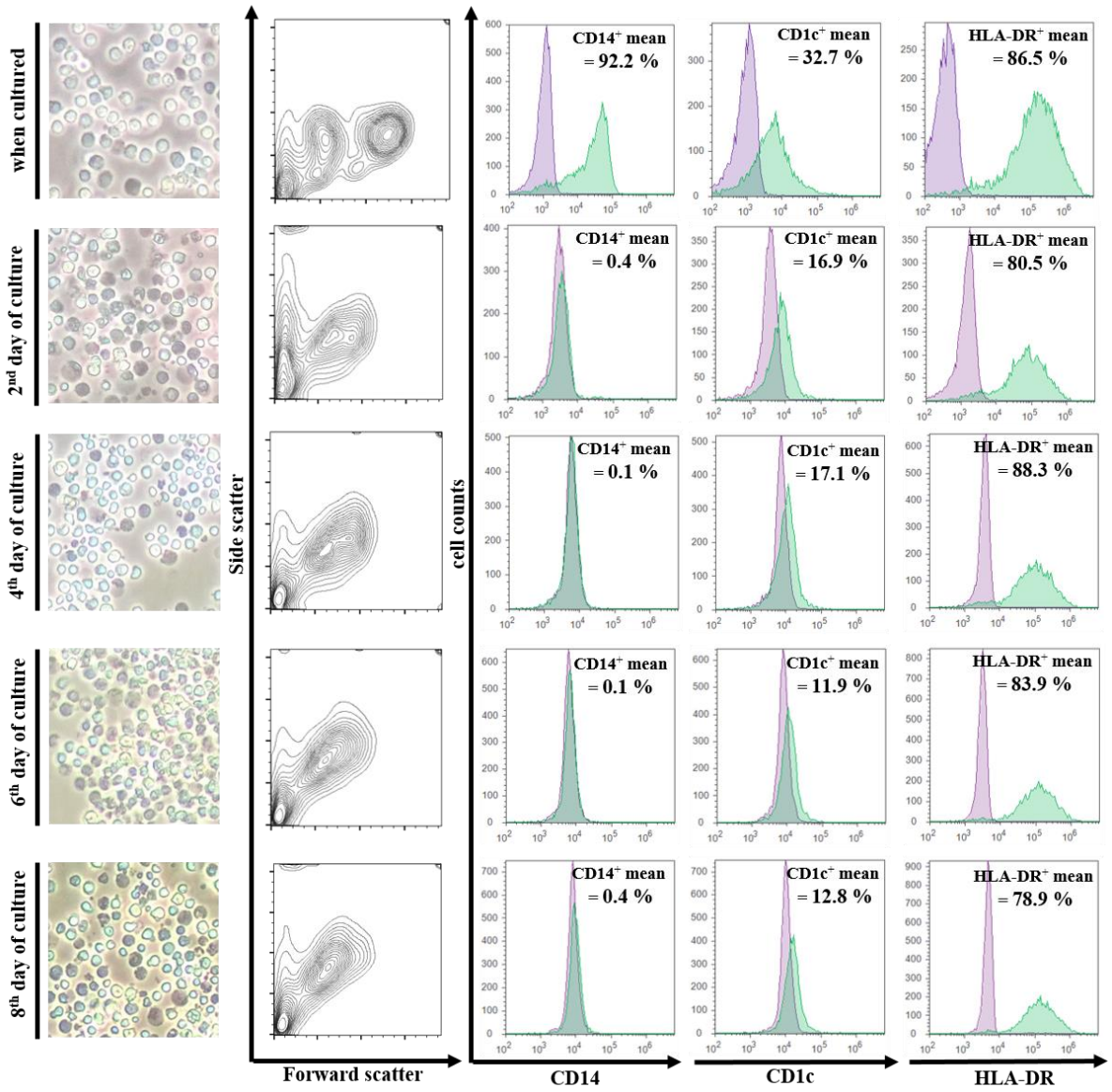

FIGURE 3.2 | Differentiation of monocytes obtained from cryopreserved UCB into dendritic cells. CB monocytes were immunomagnetically isolated from thawed CB units and cultured in the presence of GM-CSF and IL-4. Each row shows data obtained every 2 days**.** In the 1st column are pictures from monocytes induced into differentiation, taken with a regular smartphone camera (Microsoft Lumia 920) placed above the ocular of an inverted microscope (Nikon Eclipse TE2000) – for that reason, interpretation should only consider the appearance of the cells and not their size. The rest of the data regards flow cytometry analysis, as described under Materials and Methods. The second column represents the FSC/SSC profile. Last three columns show histograms for differentiation markers - CD14, CD1c, HLA-DR (MHC II molecule). In every histogram, the y-axis is the cell count and x-axis is the staining of specific cell surface marker. The cell's autofluorescence is represented in purple and the differentiation markers in green. In every graph the mean percentage of positive staining cells for the marker is also represented. Data is the result of 4 independent experiments. Representative graphs from one experiment were chosen.

process performed in cryopreserved samples.

Monocyte differentiation into immature DCs is regarded as a synonym of appearance of a typical dendritic morphology, high expression of major histocompatibility complex (MHC) molecules and other antigen presenting molecules such as CD1 family and Fc receptors, with the lacking of CD14 protein (Sallusto & Lanzavecchia, 1994). Our results show that the expression of HLA-DR (MHC II molecule) at cell's surface is abundant and unchanged since the first day of culture, pointing to the fact that the cells may be somehow stimulated to antigen presentation between collection and cryopreservation process of the cord blood. CD1c molecule (also known as BDCA-1) is structurally related to MHC proteins, with the difference that the first one mediates the presentation of lipid and glycolipid antigens of self or microbial origin to T cells (Sköld & Behar, 2005). In contrast to HLA-DR expression, for CD1c we actually report a decreased expression during induction of differentiation. The unusual phenotype and the round monocyte-like appearance of the cells during the course of differentiation, make us question ourselves about the functionality of DCs obtained in this way. Therefore, we proceed with functional experiments. Two very well established assays to assess function of immature dendritic cells are the ability to maturate in response to stimuli and the endocytic capacity. With this in mind, and in order to compare results from functional experiments with DCs of different origins, these cryopreserved monocytes that have been differentiated for at least 7 days will be referred to as CB CD14-derived moDCs (Cord blood monocyte-derived dendritic cells) or simply CB moDCs. Those results are presented in chapter 3.4).

During our "monocyte to dendritic cell experiments" we also tried one easier and less manipulative approach, which consisted in replicating the protocol for monocyte cell culture (for DC generation) but instead of using isolated monocytes, using all cell populations. For that, the most positive expectation was that either $CD14^+$ or $CD34^+$ cells or both would respond to the presence of IL-4 and GM-CSF and differentiate into DCs. **TABLE 3.2** represents the results of culturing all the cells, in order to understand if DCs could be obtained with this methodology. Detailed information on cell culture may be found under Materials and Methods.

Although we only tried this strategy twice (n=2) in the presence or absence of differentiation cytokines (**TABLE 3.2**) it became clear that no dramatic change in the cell differentiation markers was seen. Results in TABLE 3.2 show us that CD14 loss is verified both in cells incubated with and without cytokines; seemingly negative to our goal is the fact that the percentage of cells positively staining for CD1c and HLA-DR (MHC class II) were always similar to when no cytokines were added. A very high percentage of cells stain with 7AAD, which means that a lot of cells die in culture and that may be also negatively interfering with the cells that would respond to the cytokines. With this last experiment we conclude that the methodology that was utilized is inefficient when it comes to DC generation.

TABLE 3.2 | Culture of all cell populations with GM-CSF and IL-4. For the follow- up of the differentiation of all cell populations we have used CD14, CD1c and HLA-DR as differentiation markers. Cell death was also evaluated by 7AAD staining. As a control for the effect of the cytokines, cells were also cultured in the absence of the DC differentiation cytokine cocktail, IL-4 and GM-CSF. The mean percentage of positive cells for each marker, assessed by flow cytometry, is shown. The % of cells for each marker appear derives from the full gated population. Cells were kept in culture until the 12th day and analyzed during this interval. Data is the result of two experiments.

	CD14$^+$ cells			**CD1c$^+$ cells**		**HLA-DR$^+$ cells**			**7AAD$^+$ cells (death)**	
	When cultured	6th day culture	12th day culture	8th day culture	12th day culture	When cultured	8th day culture	12th day culture	When cultured	6th day culture
- IL-4 and GM-CSF	12%	1%	0%	14%	12%	17%	29%	25%	25 %	88%
+ IL-4 and GM-CSF		1.5%	0%	11%	7%		17%	21%		78%

3.3 | Cord Blood CD34-derived DCs show a different phenotype from CD14-derived moDCs.

Cord blood application in medicine takes advantage of the presence of hematopoietic stem cells (CD34$^+$) with clonogenic capacity, when the goal is to reconstitute a damaged bone marrow with new and healthy cells, as is the procedure in some leukemia treatments. Together with the clonogenic capacity, these CD34$^+$ cells are able to differentiate into all the other hematopoietic cell families. CD34 is known to mediate cell adhesion to bone marrow stromal cells and is also important on lymphocyte migration to the lymph nodes (being expressed in its endothelia). Given that it is recognized as a marker of HSC for more than 30 years now (Nielsen & McNagny, 2008), it can be used to isolated HSC present on cord blood samples.

With regard to our work, and given the existing reports on using HSC to generate DCs we decided to try this alternative DC precursor source. The average number of CD34$^+$ cells per UCB sample before freezing was only 2.63 ± 1.26 x 10^6 (mean ± SD) (n=20, data provided by Crioestaminal). As stated before, CD34$^+$ cells have proliferating capacity and therefore can be expanded by proper cytokines. In this way, immunomagnetically isolated CD34$^+$ cells were cultured for 3 weeks in the presence of a cytokine cocktail composed by SCF, TPO and Flt-3L as reported by Balan *et al*., 2009. After immunomagnetic isolation, HSC cell recovery was 30.5% (considering the number of CD34$^+$ present in the sample before cryopreservation) and isolation purity of 91.4%, for this specific sample.

We followed the proliferation for 3 weeks and the first visual hint of proliferation appeared in the form of cell colonies that only started to become obvious around the 8th day of culture (**FIGURE 3.3 A**) and their presence being clear in the following weeks. Between the 6th and the 8th days there was an increase in cell size, which occurred only for some cells. At the end of the 3rd week in culture, cell yield was 5.5 fold the number at the time of culture. In the 2nd and 3rd weak, viability was ≥ 98% (7AAD$^-$).

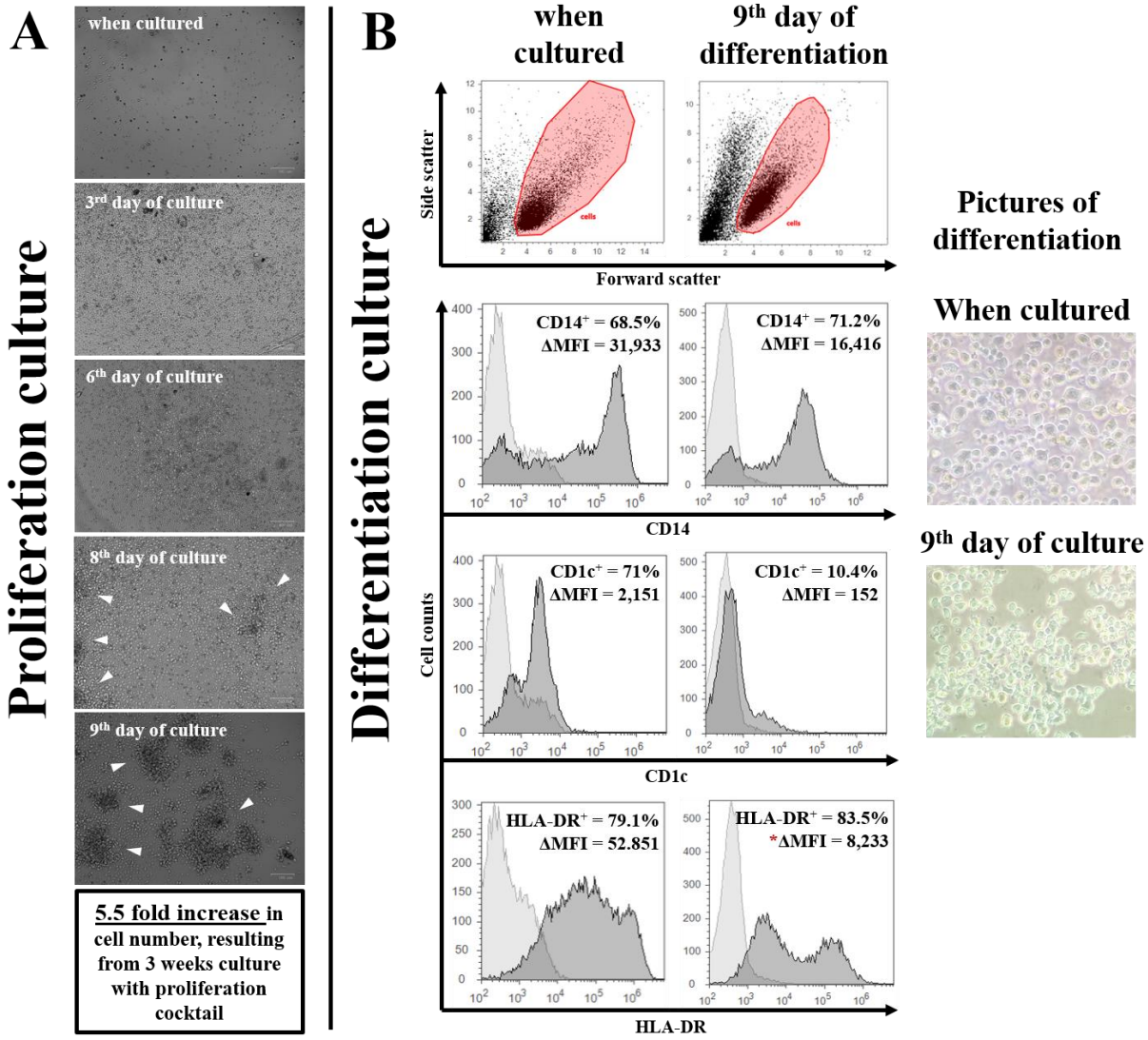

FIGURE 3.3 | Evaluation of cryopreserved UCB stem cells ($CD34^+$) proliferation and posterior differentiation into dendritic cells. A – Proliferation culture. Cells were immunomagnetically isolated from a thawed CB unit and cultured in the presence of a stem cell proliferation cytokine cocktail, SCF, FLT-3l and TPO, for 3 weeks. Pictures until the 9th day of culture were taken with the bright field setting of a fluorescence microscope (ZOE Fluorescent Cell Imager, Bio-Rad). Arrows point the visible proliferative aggregates. Scale bar = 100 µm; **B – Differentiation into DCs culture.** The graphs represent flow cytometry analysis of the 9-day induction into DC-differentiation (cell culture w/ GM-CSF and IL-4) on the 3 week expanded cells, as described under Materials and Methods. Analysis was performed on day 0 and on the 9th. The first row graphs compare FSC-A and SSC-A. Last three rows are the histograms representing the three chosen differentiation markers, CD14, CD1c and HLA-DR. The cell's autofluorescence is represented in grey and the differentiation markers in black. In every graph the mean percentage of cells staining positive for the marker and the ΔMFI (Median fluorescence intensity of the marker minus autofluorescence MFI) is also represented. Last column contains pictures from differentiating $CD34^+$ cells, taken with a regular smartphone camera (Microsoft Lumia 920) positioned above the ocular of an inverted microscope (Nikon Eclipse TE2000). Data is the result of a single experiment.

Previous studies report protocols for *ex vivo* expansion of HSC, where those which use fresh (not frozen) cord blood samples for expansion of HSCs usually have fold increases of 100-1000x or even higher within a few weeks of culture in liquid media (Flores-Guzman *et al*,. 2013). Other studies, in which clinical grade cryopreserved cord blood samples were used, show that lower numbers may be expected, with a fold increase of 30-40x after expansion of $CD34^+$ cells for 6 days (Querol *et al*,. 2000). At present, divergent approaches aiming to improve the amplification of HSCs are still under development, which include variations in the cytokine cocktails to be used and on the culture systems (Pineault & Abu-Khader, 2015).

We also tried another simpler cell culture approach in order to induce proliferation of $CD34^+$ cells. It consisted in culturing an aliquot of all cell populations obtained from cryopreserved cord blood after thawing. For that, using all the conditions that were used for culturing isolated $CD34^+$ cells with the exception of the number of cells per mL of medium, that in the case of this experiment (n=1) was $2x10^6$ cells/mL opposing to the 10^5 cells/mL used for the isolated cells, meaning a proportion of 20x more cells to the same amount of cytokines. Also, that in this experiment we only changed the media (supplemented with cytokines) strictly on a weekly basis (this is because in the $CD34^+$ isolated cells we additionally changed the media at day 3). **TABLE 3.3** shows this method as very unsuccessful way to proliferate HSCs since after two weeks of culture both cells in the presence or absence of SCF, FLT-3L and TPO show the same percentage (both 1.5%) of CD34 positive cells. Moreover, if almost no cells show this marker after this time of culture and no proliferation was seen until then, it is likely that no proliferation will come afterwards. If no proliferation occurred and no commitment to the myelo-monocytic lineage happened (as denoted by the almost close to 0 frequency of CD14) it is less likely

TABLE 3.3 | Proliferating HSCs having the "all cell populations" as a starting point. Instead of with the isolated $CD34^+$ cells (HSC), this table represents the results of culturing all the cells contained in the pellet that was used for HSC isolation, in order to understand if HSC could be led into proliferation with this methodology (n=1). Detailed information on cell culture may be found under Materials and Methods. For the follow- up of proliferation we have used CD34 (stem cell marker), CD45 (a common pan-leukocyte antigen) and CD14 (mainly monocyte lineage marker). Cell culture comparison was achieved by culturing the cells either in the presence or the absence of CD34+ cell proliferation cytokine cocktail, SCF, FLT-3l and TPO. The percentage of positive cells for each marker was accessed by flow cytometry. Cells were kept in culture for 5 weeks and analyzed during this interval (*consider that between the 2nd and the 5th week the culture medium was not renewed).

	$CD34^+$ cells		**$CD14^+$ cells**		**$CD45^+$ cells**	
	2 weeks culture	* 5 weeks culture	2 weeks culture	* 5 weeks culture	2 weeks culture	* 5 weeks culture
- SCF, FLT-3L and TPO	1.5%	0.24%	0.5%	0%	74%	56%
+ SCF, FLT-3L and TPO	1.5%	0.17%	0.36%	0%	61%	59%

During expansion of the immunomagnetically isolated HSCs (**FIGURE 3.3 A**), cells were checked for the presence of CD34 marker at week 1, 2 and 3, and since after the 3rd week 73% and 86% of the cells were positive for CD14 and HLA-DR (respectively) we proceeded to the induction of their differentiation into DCs with the regular protocol also used for CB $CD14^+$-derived moDCs. For that, cells were removed from culture, counted and plated in the presence of GM-CSF and IL-4 as previously described for monocytes. On that day, cells were immunophenotyped so that we could follow the same differentiation markers as performed for CB $CD14^+$-derived moDC. These $CD14^+$ DC precursors were cultured for 9 days and after this time were immunophenotyped again, as **FIGURE 3.3 B** shows. If we start by observing the aspect of the cells that can be seen in the pictures, one can comment about an alteration in their shape during the 9 day period, going from mostly round to an apparent membrane-projection-phenotype in some cells at the 9th day. Opposing to the CD14-derived moDCs, where all the cells remained non-adherent during the full time, cell went from loosely adherent to mostly non-adherent over the differentiation culture. This is more what to expect of a DCs differentiation culture (Chapuis *et al*., 1997). For the rest of differentiation parameters we can see a clear difference if we compare with CD14-derived moDCs. First, there is an apparent divergence in the cell size and granularity that CD14-derived DCs did not show. CD34-derived moDCs show a less uniform population, regarding these parameters. This may be explained, if a non-totally homogeneous set of DC precursors emerged from the expansion of HSCs. It has been claimed in the past that culturing cord blood CD34-derived DC precursors with a set of cytokines may lead to the differentiation of two independent DC pathways, one expressing CD1a and the other CD14 (Caux *et al.*, 1997), although this study did not use the same cytokines we did. Also, it is necessary to mention that in spite of it not being represented in the figure, CD1a has not been detected by flow cytometry in any of the days. This non-homogeneous population discussion is enhanced by the fact that no positive selection was performed on these expanded cells before DC differentiation induction. In **FIGURE 3.3 B** we do not report a complete loss of CD14 in these CD34-derived DCs (as was the case of CD14 derived moDCs) but nevertheless a considerable loss is seen, with a ΔMFI of 31,933 on day 0 and 16,416 on day 9 (in spite of the positive % of cells being close). CD1c has shown a ΔMFI starting on 2,151 drop to 152 after 9 days (accompanied by 71 to 10.4% positive cells) and HLA-DR mostly positive in all the cells during the process (ΔMFI on both days can't be compared because the detection antibody that was used was different, on account of our usual antibody being unavailable at the time). Ryu and colleagues have used the same procedure that our group also employed for the expansion of cord blood immunomagnetically isolated $CD34^+$ cells with the early acting cytokines SCF, TPO and Flt-3L. After 5 days of differentiation with GM-CSF and IL-4, DCs were obtained with a considerable 44.5% of $CD14^+$ cells too (Ryu *et al.*, 2004), which is concomitant with our results with regard to this marker.

3.4 | Cord Blood CD14-derived moDCs seem to show lower functionality when compared to CB CD34-derived DCs and adult moDCs.

DCs are cells with the ability to uptake, process and present antigens to lymphocytes, regulating immune responses. Antigen uptake as well as inflammatory stimuli will drive DCs into maturation, which in turn will enable DC interaction with T lymphocytes and initiate adaptive immune responses (Banchereau & Steinman, 1998). Maturation of DCs is characterized by phenotypic and physiological changes, *i.e*. the antigen-uptake machinery is down-regulated and the expression of surface MHC I, MHC II costimulatory molecules, such as CD80 and CD86, is up-regulated (Inaba *et al.*, 2000). Synthesis of DC-specific cytokines is also changed. CD40 is a co-stimulatory molecule that upon encounter with its ligand on a T cell membrane, CD40L, will up-regulate the expression of various genes, including of adhesion molecules that will participate in the DC:T cell interaction (Chatzigeorgiou *et al.*, 2009). CD80 and CD86 on the DC membrane will prime CD28 or CTLA-4 in the T cell's membrane, providing co-stimulatory signaling for enhancing or attenuation of an immune response, respectively (Vasilevko *et al.*, 2002).

DCs need to upregulate the expression of specific markers before the contact with T cells. The first test we performed to ascertain if immature CB moDCs had an intrinsic capacity to respond to maturation stimuli was to evaluate different maturation markers behavior upon different maturation stimuli, of which the enzymatic removal of surface sialic acids is included. In a general way, these are membrane proteins that in one way or another will interact with other proteins of a T cell's membrane. So, after maturation stimulus, these should be up or down-regulated (most of the chosen markers are known to up-regulate). Having that in mind, results were presented as "maturation ratio" where the marker's MFI in stimulated cells was divided by the same marker's MFI in non-stimulated control cells. This strategy will present a number, that being ≤ 1 shows the marker to be down-regulated, if ≈ 1, unresponsive, and above 1 up-regulated. But since our chosen markers should all be up-regulated in a successful maturation induction, one can assume that if most of them remain close to 1 that a mature status was not achieved by the cells.

FIGURE 3.4 A compares 4 different types of cells in terms of maturation status. CB moDC show a low response to LPS + TNFα even when the stimulus is prolonged for 48h. With regard to this, CD40 ratio is 0.86 ± 0.15 at 24h and 0.78 at the end of 48h. In the same page we have CD80 (0.7±0.16 at 24h and 1.029 at 48h), CD86 (1.02±0.01 at 24h and 1.09 at 48h), and HLA-DR with a ratio of 0.97±0.1 at 24h and 0.97 at 48h). For HLA-DR we also tried the 48h LPS+TNFα maturation stimulus in 12 days differentiated CB moDCs but the result (1.03) was very close. We also tried other stimulus in order to access HLA-DR expression, as sialidase treatment and posterior culture for 24h (1.3), sialidase in the culture medium for 24h (1.33) and sialidase treated and posterior culture for 24h in the presence of LPS + TNFα (1.16). This is the data on CB moDCs. Then, one question was raised, and that was if the culture

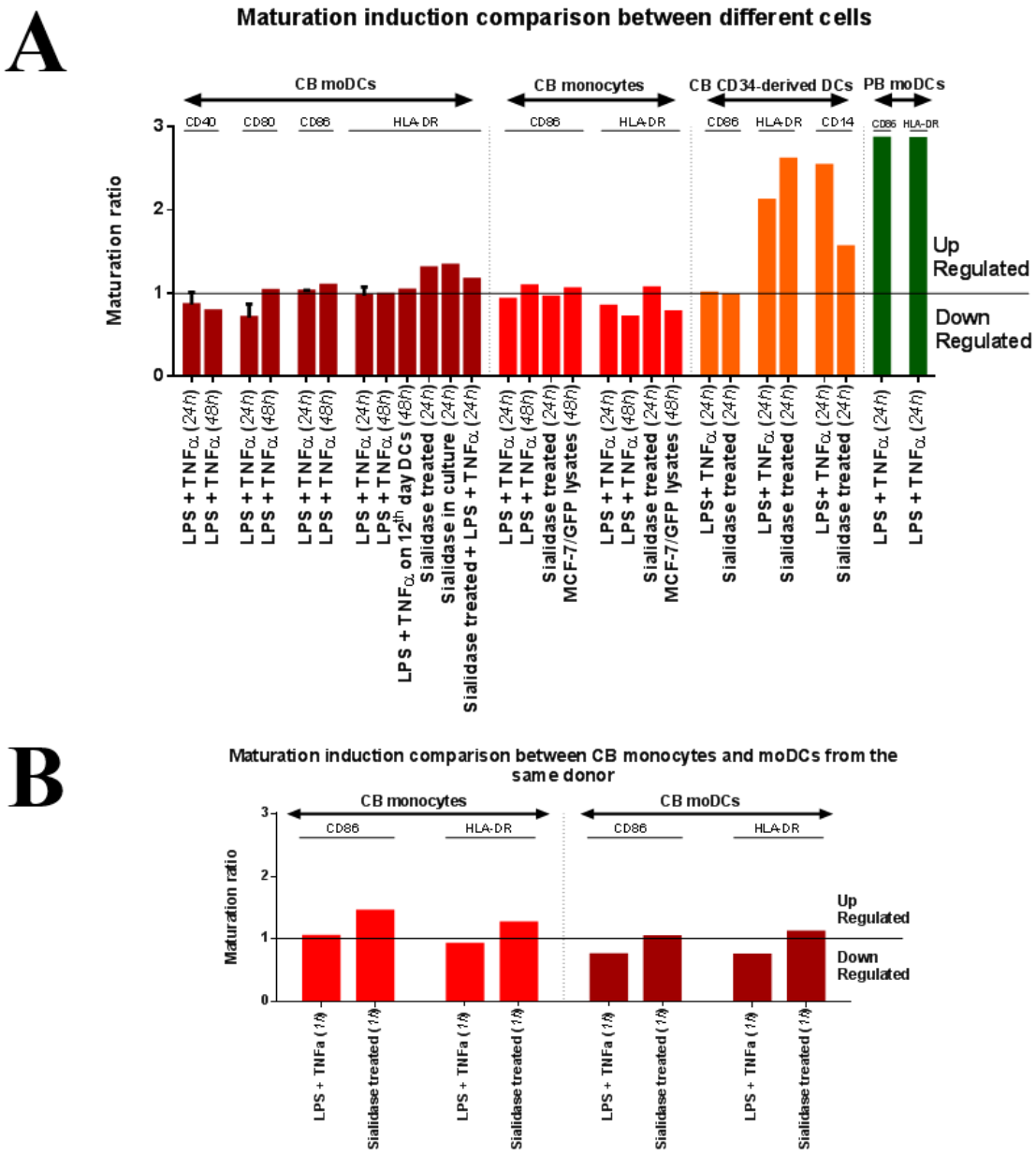

FIGURE 3.4 | Maturation markers comparison when cells of different origin are subjected to various maturation stimuli. Maturation stimuli comprises a 24 or 48h cell culture stimuli, with LPS+TNFα, LPS+TNFα after sialidase treatment of cells, only culture after sialidase treatment, sialidase in the culture medium (0.01 U/ml) or culture with MCF-7/GFP cell lysates. CD40, CD80, CD86, HLA-DR and CD14 markers were assessed by flow cytometry on cord blood (CB) moDCs, monocytes and CD34-derived DCs and on peripheral blood (PB) moDCs. Maturation ratio is obtained by dividing the specific maturation marker ΔMFI (Fluorochrome stained marker minus cell's autofluorescence) after induction of maturation by the unstimulated control cells' ΔMFI. Numbers above 1 may be considered "up-regulation" since it is the translation of an increased marker expression, and below 1, "down-regulation". When experiments were performed more than once, values represent the mean ratio (with error bars as SD) or a single value when the experiment was performed only once (without error bars)**. A - Maturation induction comparison between cells of different origin.** CB moDCs maturation ratio for different markers comes in dark red, CB monocytes in red, CB CD34^{+}-derived DCs in orange and PBmoDCs in green; **B – Maturation induction comparison between CB monocytes and moDCs from the same donor.** Color code is the same as in "A". In this specific experiment, cells were only stimulated for 1h hour before flow cytometry analysis.

of monocytes for differentiation into DC could be somehow affecting these cells' ability to be inducted into a mature status. So the next step was to try to mature CB monocytes, what can be understood as a 'negative' control. CD86 showed not much difference, with LPS + TNFα ratio as 0.92 (24h) and 1.08 (48h) and 0.95 in sialidase treated and 24h cultured cells. When ultimately exposed to cancer cell lysates for 48h the results were practically the same, with a ratio of 1.05. HLA-DR marker was also followed, with LPS + TNFα (24 and 48h), sialidase treated and cancer cell lysates exposed cells with 0.84 and 0.71 (24 vs 48h), 1.06 and 0.77 ratios, respectively. The experiment we performed for CB CD34-derived DCs obtainment was also able to provide us with information on the maturation of these cells. With regard to these DCs, CD86 did not show to be up-regulated either in LPS + TNFα stimulated cells (0.99) as in sialidase treated ones (0.97). The same can't definitely be said about HLA-DR, where in comparison with moDCs these cells showed way higher up-regulation of the marker, with a ratio of 2.12 in LPS + TNFα stimulated cells and 2.6 if sialidase treated. Given that the immunophenotyping experiment of the differentiating CB CD34-derived DCs reported the presence of CD14 after 9 days of differentiation, this marker was also followed and showed increased up-regulation after incubation with LPS + TNFα (2.53 ratio) and after culture of the sialidase treated cells (1.56). As already said, most of our laboratory's experience comes from the use of peripheral blood moDCs, and when incubated for 24h with LPS + TNFα, reached the higher ratios of CD86 and HLA-DR registered in all the different cells and maturation conditions, with 2.86 both.

In another experiment (**FIGURE 3.4 B**) we also compared the maturation of monocytes and moDCs coming from the same cryopreserved cord blood sample. In this one experiment, and starting with LPS + TNFα stimulus (for 1h) monocytes showed higher CD86 (1.04) than moDCs (0.75) and higher HLA-DR also (0.91 vs 0.75). The other stimulus we tested was sialidase treatment on the cells, and after it monocytes presented a ratio of 1.49 up-regulation of CD86 and in moDCs of 1.04; HLA-DR ratio was 1.26 on monocytes and 1.11 on moDCs.

Taking all these results into account it is easily understandable that PB moDCs and CB CD34-derived DCs showed an increased ability to be stimulated into a matured status, when compared to both CB monocytes and CB moDCs. Given our results with cryopreserved CB monocytes and DCs, we can only assume a most likely ineffectiveness if participating in the induction of a specific immune response, once they have already shown themselves with very close to 1 (static status of maturation markers) maturation stratus. This is true when LPS and TNFα are used, and also true when a MCF-7/GFP cancer cell lysate was used as a maturation stimulus on monocytes. On the other hand, CB CD34-derived DCs were shown better able to reach a mature status, at least considering the expression of MHC II (since CD86 was not up-regulated).

For the comparison between the maturation of CB monocytes vs. moDCs from the same donor, the strategy we employed in the experiment may require additional interpretational effort because the maturation stimuli was only given for 1h and analyzed right after. Our results show that monocytes have better intrinsic maturation capacity than moDCs from the same donor (with a higher maturation ratio,

despite being both very close to 1, what means unresponsiveness). Sialidase treatment removes non-specifically α-2,3-linked and α-2,6-linked sialic acids from all cell surface sialylated constituents, and existing abundantly in glycosylated proteins (Crespo *et al.*, 2009). This means that the cell periphery will be less abundant in glycosidic structures, which may contribute to a facilitated access to the proteins that will be targeted by flow cytometry antibodies. This may constitute an interpretational bias, arising from the fact the cells were analyzed right after being treated. Either way, monocytes showed to be more prone to up-regulate CD86 and HLA-DR markers when treated with sialidase, when compared to their moDCs counterpart.

The next functional assessment we performed on cryopreserved CB moDCs, CD34-derived DCs and CB monocytes was the endocytosis capacity. We also wanted to understand the effects of cell periphery glycan loss upon endocytosis. Results are expressed in the form of MFI fold increase between cells incubated with Ovalbumin-FITC at 37ºC and 4ºC (negative control for endocytosis) which indicates that the cells with higher endocytic performance will also have an higher MFI fold increase (**FIGURE 3.5**). **FIGURE 3.5 A** shows PB moDCs to be the most capable, with mean MFI fold increase values of 71.92 ± 2.09 when incubated with 0.1 mg/mL of OVA-FITC and 81.64 if OVA-FITC is at 0.2 mg/mL concentration. CB moDCs endocyte less, with only 8.78 ± 2.06 fold increase when OVA-FITC is at 0.2 mg/mL and 4.68 ± 0.77 when at 0.1 mg/mL. This difference between the endocytosis of 0.1 mg/mL OVA-FITC of PB moDCs and CB moDC has also shown to be statistically significant ($p=0.0077$). CB CD34-derived DCs showed a value 8.97 (with OVA-FITC at 0.1 mg/mL), which is higher than the performance of CB moDCs and is in accordance with the also higher maturation capacity reported by our previous results. Not only we used OVA-FITC but also fluorescent MCF-7/GFP cell lysates as antigens to be endocyted but, the MFI fold increase was only 2.47 with PB moDC and 0.82 with CB moDCs. One could argue that lysate preparation may have affected the fluorescence of GFP molecules. The mechanism of endocytosis for lysates should be different from OVA with the first probably being endocyted by phagocytosis and the uptake of the second by a combination of mannose receptor-mediated and fluid-phase macropinocytosis (Garrett *et al.*, 2000; Platt *et al.*, 2010).

After comparison of dendritic cells obtained from different precursors we wanted to access sialidase treatment effects on endocytosis, given that the removal of glycans in the cell periphery has been suggested as a maturation stimulus in the past. Mature DCs should present a lower endocytosis capacity than immature DCs, advocated by the redirection of their primary function of antigen capturing and processing (as immature DCs) to an antigen presentation status with reduced endocytosis (as mature DCs). Our results show that when PB moDCs face sialic acid removal, OVA-FITC endocytosis drops from 17.07 fold increase value to 10.83 (**FIGURE 3.5 B**) and of fluorescent cell lysates from 1.25 to 0. **FIGURE 3.5 C** shows the same experiment performed with monocytes and moDCs from the same CB donor: monocytes keep a low OVA-FITC endocytosis before and after sialidase treatment (2.08 and 1.86) and the correspondent monocyte-derived DCs of 8.1 fold increase before treatment and 5.1 after.

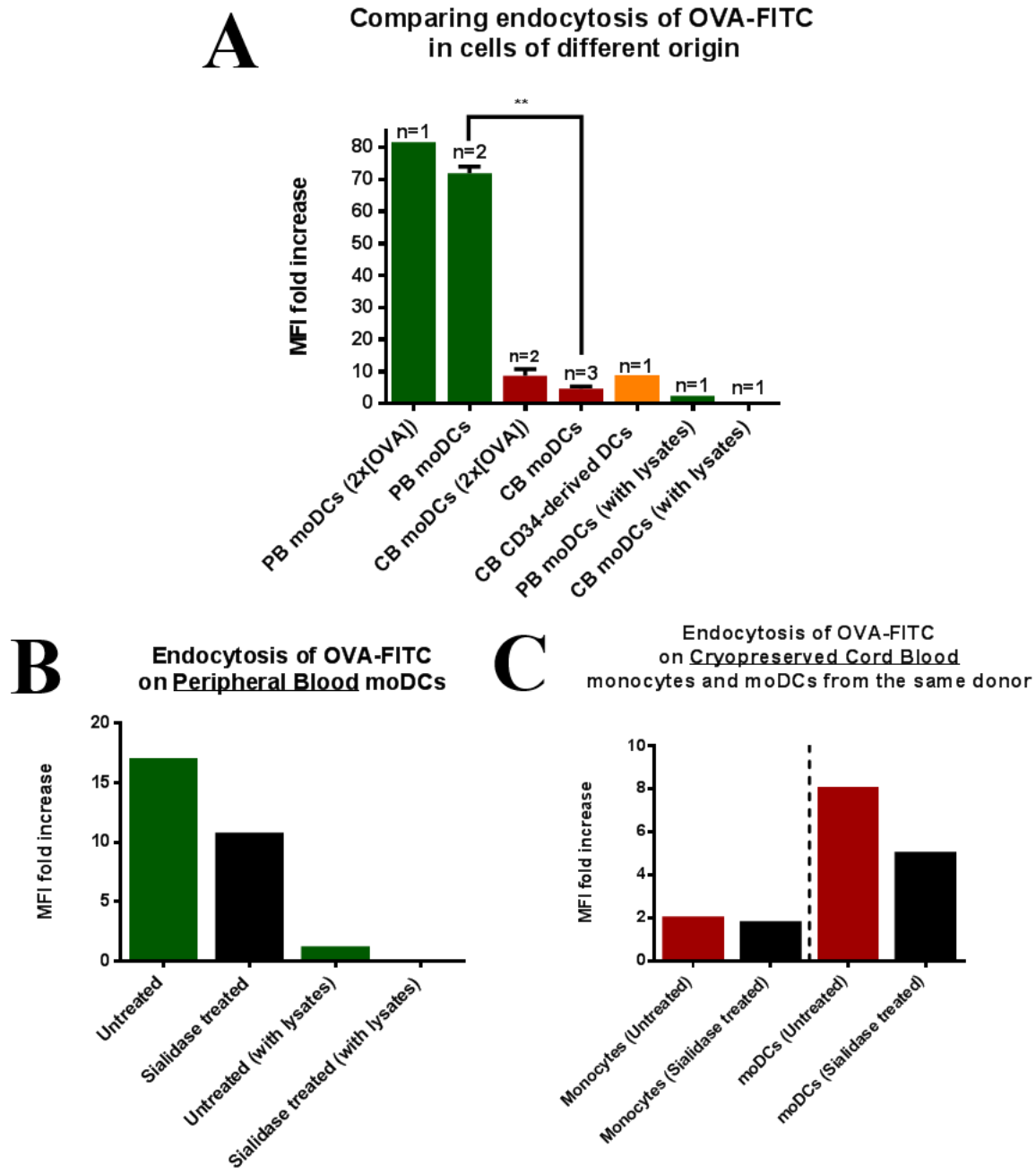

FIGURE 3.5 | **Endocytosis assays in cells of different origin.** Values represent the mean MFI fold increase ± SD (coming from the uptake of fluorochrome-conjugated molecules) when experiments were performed more than once, or a single value when n=1. **A – Comparing endocytosis of OVA-FITC in cells of different origin.** Endocytosis of cord blood (CB) moDCs and CD34-derived DCs and on peripheral blood (PB) moDCs was assessed. MFI (Median of fluorescence intensity) fold increase represents a fold increase in the uptake of the endocyted agent, obtained by the division of the 37ºC incubated cells' MFI by 4ºC control (detailed description in Materials and Methods). In the last two bars MCF-7/GFP cell lysates were also tried as an endocyted agent instead. PBmoDCs in green, CBmoDCs in red, CB CD34^{+}-derived DCs in orange. Statistical significance (**P < 0.01 unpaired Student's t-test) refers to the difference between PB moDCs and CBmoDCs in OVA-FITC endocytosis; **B – Endocytosis of OVA-FITC on PB moDCs**. Sialidase treated and untreated cells' endocytosis of OVA-FITC was compared. MCF-7/GFP cell lysates were also tried as an endocyted agent. Results are from a single experiment; **C - Endocytosis of OVA-FITC on cryopreserved cord blood monocytes and moDCs from the same donor.** Sialidase treated and untreated cells' endocytosis of OVA-FITC was compared. Results are from a single experiment.

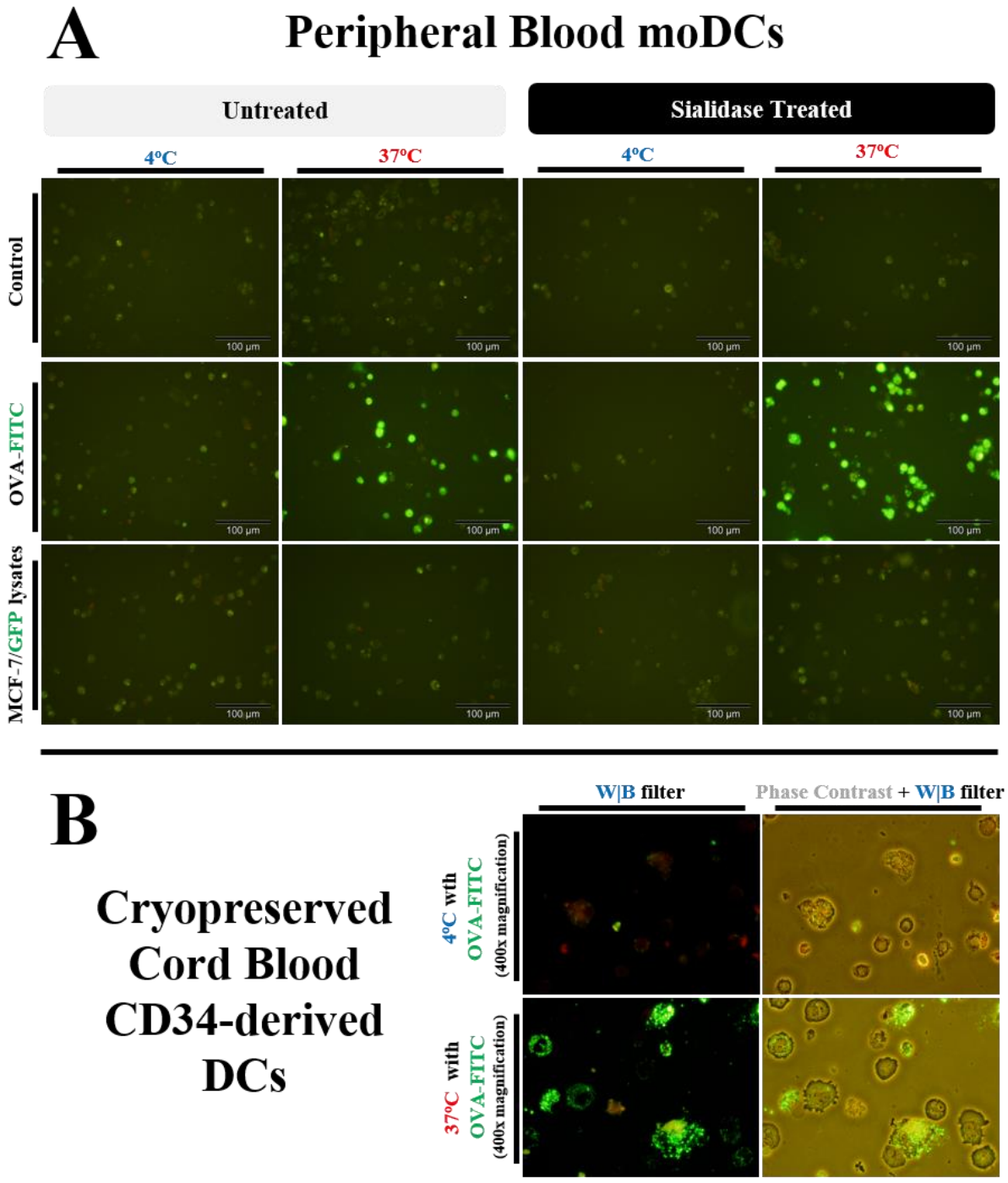

FIGURE 3.6 | Endocytosis functional assays in cells of different origin, comparing different stimuli and treatments (Fluorescence microscopy). Pictures were obtained with the use of a OLYMPUS DP72 camera coupled to a fluorescence microscope (OLYMPUS IX51) of which the W/B filter was used. Cells remaining from flow cytometry endocytosis analysis were centrifuged at 1000xg, resuspended in 20 µL of PBS and placed between a microscope slide and cover slip. **A – Evaluation of OVA-FITC and MCF-7/GFP endocytosis in sialidase treated and untreated peripheral blood moDCs.** 4ºC incubated cells represent a negative control for the experiment (also a control used in our flow cytometry analysis) and 37ºC is an incubation temperature at which endocytosis is promoted. The 'control' refers to cells incubated without an endocytic agent. Scale bar = 100 µm. **B – Evaluation of OVA-FITC endocytosis in cryopreserved cord blood CD34-derived DCs.** Analysis was different than image A only in the fact that pictures were also taken using a 'manual merge', where both full spectrum light and filtered blue light (using W/B filter) sources were used at the same time.

A simple fluorescence microscopy confirmation of the PB moDCs endocytosis experiment presented on **FIGURE 3.5 B** can be found on **FIGURE 3.6 A.** The pictures were taken solely for successful endocytosis confirmation and not with the aim of extensive data analysis. The same was also performed for CB CD34-derived DCs and is shown in **FIGURE 3.6 B**. The cells that were used in these last endocytosis pictures are the same which MFI fold increase is depicted in **FIGURE 3.5 A**. Microscopy has confirmed a higher amount of fluorescence in the cells incubated with OVA-FITC at 37ºC when compared to the controls (4ºC), and this is true for both PB moDCs and for CB CD34-derived DCs.

Previous work reported by our laboratory, using PB moDCs has shown that α2,6-sialic acid deficiency (promoted by sialidase treatment) leads to an increase in the phagocytosis of various pathogenic *E. coli* isolates (Cabral *et al.*, 2013). On the contrary, when OVA-FITC is used (that represent another endocytosis mechanisms – mannose receptor-mediated and macropinocytosis) we have demonstrated that removing surface sialic acid from immature human moDCs by sialidase treatment significantly decreased its endocytic capacity (Videira *et al.*, 2008). In agreement, bone marrow-derived DCs from ST3Gal.I$^{-/-}$ and ST6Gal.I$^{-/-}$ knock-out mice show lower OVA endocytosis capacity (Crespo *et al.*, 2009). So the endocytosis results presented in this thesis are in accordance to what our group has already published: we again confirm a lower endocytic capacity of sialidase treated PB moDCs and bring forth the same conclusions for when cryopreserved CB moDCs are used (having in mind that these specific sialidase experiments were performed only once (n=1) (**FIGURE 3.5 B and C**).

In parallel, the cytokine secretion profile of cryopreserved CB moDCs exposed to different maturation stimuli was also assessed. The supernatants of these cultures were checked for the presence of the following cytokines, by ELISA technique (**FIGURE 3.7 A**). Starting on IL-6, unstimulated cells produced 1.4 pg of the cytokine per mL of culture medium and sialidase treated moDCs 4.8 ± 6.8 pg/mL. For all the other conditions tested there was no production of IL-6. IL-10 was of 9.3 ± 13.1 pg/mL in unstimulated cells, 17 ± 17.1 pg/mL in the cells that were treated with sialidase, cells that had sialidase continuously in the culture medium for 24h registered 10 pg/mL, LPS + TNFα stimulated cells produced 0 pg/mL and sialidase treated + LPS + TNFα 3.3 ± 4.6 pg/mL. IL-12 secretion was not seen in any condition besides 'sialidase in culture medium for 24h' stimulated cells, that showed a value of 2.7 pg/mL. TNFα has shown to be the cytokine with the highest concentration values in all the conditions, with 41.9 ± 59.2 pg/mL in the unstimulated, 45.7 ± 64.6 pg/mL in the sialidase treated, 106.7 pg/mL if cultured with sialidase in the medium, 986.2 ± 797.5 pg/mL when stimulated with LPS + TNFα and 1766.8 ± 1664.7 if sialidase treated + LPS + TNFα.

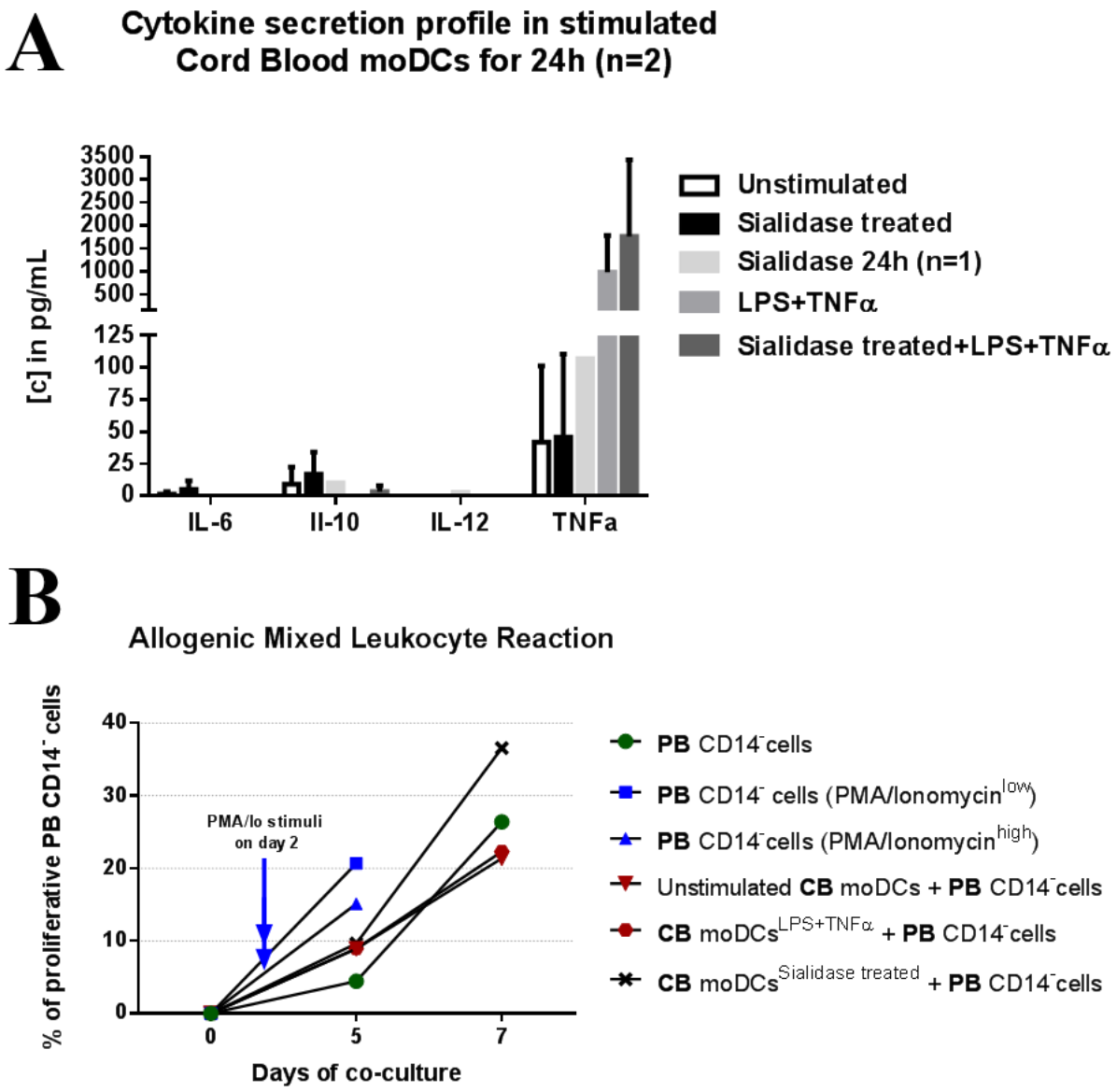

FIGURE 3.7 | Other functional essays performed on cryopreserved cord blood moDCs. A - Cytokine secretion profile in 24h stimulated CB moDCs. ELISA detection of cytokines secreted by CB moDCs after being treated with different maturation stimuli (unstimulated, sialidase treated and cultured, 0.01U/mL sialidase in culture media, sialidase treated and presence of LPS + TNFα or simply LPS + TNFα maturation stimulus. This stimulation occurred for 24h, after which cells have been centrifuged and the complete supernatant collected for ELISA (and the cells used for flow cytometry experiments). Levels of IL-6, IL-10, IL-12 and TNFα were measured. The values represent the mean ± SD from 2 independent experiments (exception for Sialidase in culture medium for 24h condition, only performed once) **B – Allogenic Mixed Leucocyte Reaction using stimulated CB moDCs as stimulator cells and PB CD14$^-$ cells as responders.** Results are the *in vitro* alloresponse of a thawed PB CD14$^-$ cell fraction (resulting from an immunomagnetic isolation) when cultured with CB moDCs. This cell fraction was stained with CFSE and cultured alone, or stimulated for 3 days with high or low [c] of PMA/Ionomycin, or cultured in a 5:1 ratio of unstimulated or LPS/TNFα stimulated or sialidase treated CB moDCs. The % of proliferative PB CD14$^-$ was assessed by flow cytometry on day 5 and 7, as the % of cells with less amount of green fluorescence (CFSE-derived) than the 18h cultured, CFSE stained, parental population. All samples were fixed in 2% Paraformaldehyde before flow cytometry. Values represent the mean % of proliferation from cells in duplicate wells.

DCs produce cytokines and are susceptible to cytokine-mediated activation and, when activated, a cascade of pro-inflammatory cytokines is triggered, skewing T cell responses. Other cytokines, for example TNF, are able to trigger DC maturation via autocrine pathways. TNFα is a pleiotropic cytokine that induces many cell types (including DCs) to secrete pro-inflammatory cytokines, being part of both innate and adaptive immunity (Blanco *et al.*, 2008). IL-6 can be produced by many cell types and induces inflammatory and antigen-specific immune responses as proliferation of T cells, differentiation of immature $CD8^+$ T cells into cytotoxic T lymphocytes and of B cells into plasma cells (Kishimoto *et al.*, 1995). IL-12 shares a subunit with IL-23 and can mediate inflammatory disease. It is a cytokine mainly produced by myeloid DCs, differentiating and expanding Th1 cells) (Kastelein *et al.*, 2007). Contrary to these three cytokines, IL-10 is more associated with tolerogenicity instead of immunogenicity: autocrine IL-10 prevents spontaneous maturation of DCs *in vitro*, and the release of IL-10 will block maturation process by interfering with up-regulation of costimulatory molecules and production of IL-12, subsequently limiting the ability of DCs to initiate a Th1 response (Corinti *et al.*, 2001) Stimulated pDCs have also been reported to induce IL-10-secreting Tregs (Ito *et al.*, 2007). Relating our results to what has just been stated, what we see with our CB moDCs is a very low secretion of IL-6 and IL-12 inflammatory cytokines, allowing the suggestion that maybe no-antigen specific response would come from these cells. And this is observed for all the maturation conditions that we tested. In a general way IL-10 seems to be in the same page, despite showing (very slightly) higher concentrations, so this would mean that almost no anti-inflammatory properties would also come from the use of these cells. Not surprisingly, TNFα shows the highest concentrations in the cells where it was added to induce maturation. One could expect [TNFα] in LPS + TNFα condition to be closer to Sialidase + LPS + TNFα, but since it is not, two answers may come: positive feedback in TNFα production in this last condition, arising from sialidase treatment, or experimental error (considering that only 2 experiments were performed and that one of them had closer concentrations than the other).

The last functional assay performed on CB moDCs was allogenic Mixed Leucocyte Reaction (MLR). For our purposes, this assay is intended to evaluate the functionality of CB moDCs, namely their ability to activate T cells. This assay has been used for many years, so that a patient's individual response to a transplanted tissue or organ could be predicted. This assay measures the proliferative response of leucocytes from one individual to leucocytes from another individual. Those will be termed responder and stimulator cells, respectively.

Differently treated CB moDCs (stimulators) were co-cultured with PB lymphocyte rich $CD14^-$ cells (responders) from an adult donor, for 7 days. Given that the responder cells were homogeneously stained with CFSE prior to co-culture, it was possible to distinguish not proliferating from proliferating cells, the last ones having less fluorescence than the parental stained population. By flow cytometry assessment of the green fluorescence of cells, the percentage of proliferating cells can then be determined (**FIGURE 3.7 B**). By day 5 of co-culture, PB $CD14^-$ cells cultured alone had 4.45% of its population

proliferated. As a control, responder cells were stimulated for 3 days with two concentrations of mitogens PMA/ionomycin. PMA/ionomycin^{low} and PMA/ionomycin^{high} (10x higher than the lower [c]) resulted in 20.65% and 15.1% proliferation, respectively. Unstimulated CB moDCs co-cultured with PB CD14$^-$ cells induced 8.9% proliferation, CB moDCs maturated with LPS + TNFα induced 8.95% and sialidase treated CB moDCs 9.65%. At day 7 of culture, PB CD14$^-$ cells cultured alone had 26.4% of its population proliferated, higher than the same cells stimulated by LPS+TNFα-maturated CB moDCs (22.3%) and by not-stimulated CB moDCs (21.35%). Notably, it seems that sialidase treated stimulator cells gave rise to a higher proliferation of PB CD14$^-$ population (36.55%).

With this maybe oversimplified experience of ours, we only wanted to address the overall consequences in terms of cell division that would come from the presence of CB moDCs. We did not want to know what specific type of cells were dividing, but if we did, one would to stain specific cell markers (from either B cells, CD4$^+$ or CD8$^+$ T cells) in order for the response to be interpreted 'individualy', as usually found in the literature.. If we are to take conclusions by what is seen on day 5, exposed and not exposed to maturation stimulus CB moDCs induced ≈ 9% of cell division in the responder population, that after subtracting auto-stimulation (4.45%) would tell us that 4.55% of PB CD14$^-$ cells are dividing because of CBmoDCs influence. And that the maturation status of CB moDCs does not seem to affect these results until day 5. If conclusions are to be taken by day 7 observations, one may suggest that unstimulated and LPS/TNFα stimulated CB moDCs can promote a certain inhibition of PB lymphocyte proliferation since basal proliferation (26.4%) was higher than when in the presence of CB moDC (21.35 and 22.3%). That was not true for the glycan deficient sialidase treated CBmoDCs, that demonstrated to be responsible for 10.15% of CD14- cell proliferation (36.55 minus 26.4%). Could this mean a better physical MHC-TCR reconnaissance/interaction, between DCs and T-cells, promoted by the removal obstructing carbohydrates? Anyhow, this question could be different if we knew more about the histocompatibility between these two donors, which is unknown (we have the genotype of MHC class I's HLA-A locus of CB moDCs, provided by the CB bank, but not on PB cells). The relevance of this last observation lays within the fact that the more histocompatible the donors are, the less evident proliferation response is supposed to be. Ultimately, it seems unwise to take serious conclusions from this very simple experience, that was only performed once, but the clues are here. It is of our best interest to repeat it in the future, given that we have already proven in the past that the removal of surface sialic acids in the periphery of (PB) moDCs is able to maturate and promote DC functionality (Videira *et al.*, 2008; Crespo *et al.*, 2009; Cabral *et al.*, 2013; Crespo *et al.*, 2013).

3.5 | Culture, immortalization and tumor marker profiling in a Triple Negative Breast Cancer primary culture-derived cell line.

Following one of the initial goal of the work performed in this thesis, blood and tumor biopsy samples were to be taken from diverse breast cancer patients. DCs could then be obtained from the peripheral blood monocytes, enzymatically treated with sialidase and stimulated with tumor antigens deriving directly from the patient's own tumor cells. In this way, specific proliferative responses could be induced in patients' own T cells and cytotoxicity experiments involving these DC-stimulated-T cells and patient's primary breast cancer cells could ultimately be performed. This has proven to be quite demanding in terms of logistics for both our group and the other parts involved, but nevertheless an approach in immunotherapy that still remains to be tried in the future. Despite all that, our group still had the chance to work with two biopsies obtained from two breast cancer patients.

One of the tumor samples came from a 64-year-old patient that has never been treated with chemotherapy, whose clinical diagnosis pointed to an ER (Estrogen Receptor)$^+$ breast cancer. The other, from a 39-year-old patient with a recurrent highly proliferative triple negative breast cancer. Triple negative means that the tumor lacks three common breast cancer biomarkers – ER, progesterone receptor (PR) and lack HER2 (Human epidermal growth factor receptor type 2) overexpression (Foulkes *et al.*, 2010). This last patient has been treated with 4 different chemotherapy drugs, which included anthracyclines, taxanes and platinum salts and even after those treatments cancer remained its highly proliferative status. For these reasons, our laboratory decided to better understand these tumor biomarkers (by flow cytometry) and to immortalize the cells that derived from its primary culture (**FIGURE 3.8**). We previously had clinical indications on the status of some markers (or else we would not know from the start that this was the case of a triple negative breast cancer), and so PR and ER status was confirmed by our immunophenotyping analysis as depicted in **FIGURE 3.8 A**. Our first HER-2 analysis was not parallel to the clinical diagnosis, given that the cells were staining positive for it. But since HER2 association with breast cancer is overexpression, one should at least expect a basal level of the protein, as we noted. In the clinic, the threshold for one to consider a tumor to be HER2$^+$ is a 3$^+$ immunohistochemistry score, or only 2$^+$ if positive after in situ hybridization techniques (Foulkes *et al.*, 2010), so this is in syntony with our "flow cytometry positive status". But, in order to definitely set this straight ,we decided to compare the staining profile of HER2 with two other (not primary) breast cancer cell lines, reportedly clinically negative for HER2 (**FIGURE 3.8 B**). And because of the almost overlapping HER2 detection histograms between the three cell lines, flow cytometry technique confirmed the clinical status.

CEA is a glycoprotein involved in cell adhesion which is commonly more associated with colorectal cancer (Duffy, 2001), and was also not detected. It also stained negative for the tested mucins (MUC1 and 5). MUCI is associated with epithelial cancers and is sometimes overexpressed in breast

A

Tumor and other markers	ER		PR		HER2		CEA	MUC1	MUC5	Cytokeratin	HLA-ABC
	clinically	by flow cytometry	clinically	by flow cytometry	clinically	by flow cytometry					
TNBC1 status	-	-	-	-	-	+	-	-	-	+	+

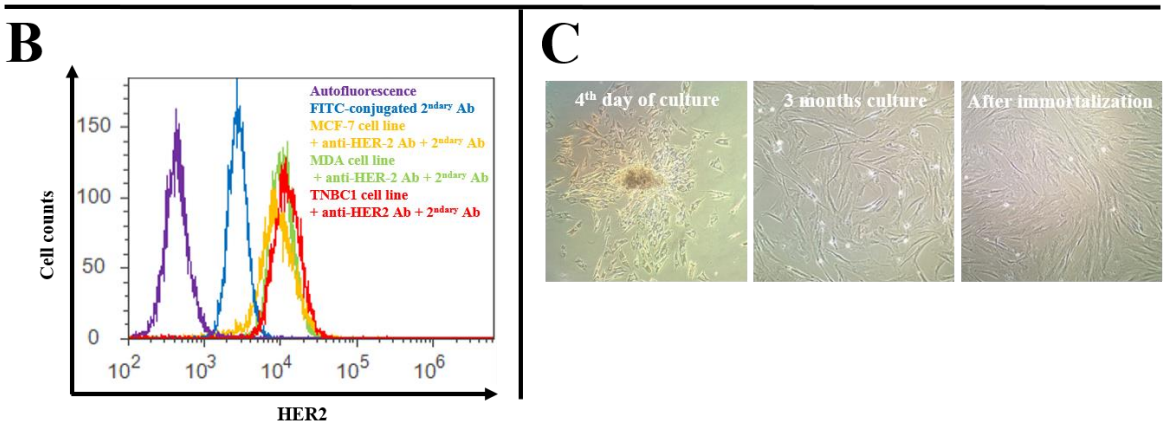

FIGURE 3.8 | Flow cytometry analysis of an immortalized Triple Negative Breast Cancer primary culture-derived cell line (TNBC1). A- TNBC1 cell line tumor biomarkers immunophenotyping by flow cytometry (and other non-tumor markers also). ER is the abbreviation of Estrogen Receptor, PR of Progesterone Receptor and CEA of Carcinoembryonic Antigen. HER2, MUC1 and 5, cytokeratin and HLA-ABC were also assessed. Positive (+) or negative (-) coining of the tumor marker comes from the positive detection of the marker in a cell count vs fluorescence flow cytometry histogram, having the secondary antibody single fluorescence (if one is used) as the negative threshold that needs to be surpassed in order for the marker to be stated as (+). Detailed information on the markers detection and staining under Materials and Methods. A regular marker histogram is described in "B"; **B –HER-2 positiveness vs cell count histogram.** X-axis represents the relative fluorescence intensity of the stained cells, for that, using flow cytometer BL1-A channel since the secondary antibody is FITC-conjugated. Y-axis is the cell count. HER-2 staining was performed on TNBC1 (red) and also on two other breast cancer cell lines available at the time (MDA-MB-231 [green] and MCF-7 [orange]), because of a higher complexity of this specific marker's evaluation. Cell's autofluorescence is in purple and the fluorescence of the secondary antibody (anti-rabbit FITC) is in blue. **C – Pictures from cells in culture at different times.** Pictures were taken with a regular smartphone camera (Nokia Lumia 920) above the ocular of an inverted microscope (Nikon Eclipse TE2000).

cancer (Gendler, 2001). The cell line showed itself to be recognizable by $CD8^+$ cytotoxic T; lymphocytes once it is positive for MHC class I. Cytokeratins are intermediate filament forming proteins that provide mechanical support and fulfill a variety of additional functions in epithelial cells and different cytokeratin may be used to separate different epithelial malignancies (Barak *et al.*, 2004). But our goal with the positive detection of cytokeratin was only to exclude possible contamination with fibroblasts which are not epithelial cells (**FIGURE 3.8 A,** 2nd last column). Regarding the follow-up of the immortalization process, what can be said is that cells maintained their overall morphology. Cells were

maintained in culture after immortalization, and the division rate was evaluated over-time in immortalized and non-immortalized counterparts. Both had decreased division rate over-time and we could not conclude about the effectiveness of immortalization procedure. To evaluate if *hTERT* (the gene that was inserted to prevent telomere shortening) is functional or not, sequencing or expression profiling of the inserted gene would be useful but were not yet tested.

Breast cancer is a heterogeneous disease that is classified into different immunohistological and gene-expression profile derived-molecular subclasses, presenting distinct clinical prognoses. Some of the generally accepted divisions are luminal breast tumors type, which derive from the mammary gland luminal cells and this may explain their ER and PR positiveness, representing around 75% of all breast cancers; $HER2^+$ tumors account for 15-20% of breast cancer subtypes and are characterized by overexpression of this gene or even others of the same signaling pathway; the mammary gland has another cell lineage that helps to expel the secretions from the lumen, called myoepithelial cells, which may be progenitors of the basal-like type of breast cancers: rarely express ER, PR and have very low prevalence of HER2 overexpression, but expressing specific myoepithelial cytoqueratins. $HER2^+$ and basal-like type are the most aggressive , linking to the shortest survival time in breast cancer patients (Blanpain, 2013; Yersal & Barutca, 2014). Triple negative breast cancer is considered to be a cousin of the basal-like breast and represents about 12 to 17% of woman with breast cancer, and, as its 'cousin', has a relatively poor outcome and cannot be treated with endocrine therapy or other targeted therapies (Foulkes *et al.*, 2010), and exploring new approaches for its treatment is crucial, given that the median survival of woman with metastatic triple negative breast cancer is of 12 months, which is the same to say that all women will die of the disease despite systemic treatment (Abramson *et al.*, 2015).

3.6 | General discussion and future directions.

The field of cancer immunotherapy is currently at its most promising phase. The immunotherapy based strategies are less damaging to the patients' general health because they will harness the individual's own immune system to better fight the progression of a cancer or even to prevent its emergence or re-emergence. Strategies in cancer immunotherapy still have lots of room to go forward, and are helped everyday by new discoveries provided by research in basic cancer biology. One of these cancer immunotherapies is what has been called of cancer vaccines, which consist of prophylactic therapy (as the case of vaccines against cancer-causing virus – human papillomavirus and hepatitis B virus) or treatment vaccines that are administered during the current time of the disease. One of these strategies consists on using DCs, taking advantage of their sublime capacity on antigen presentation. When antigens are successfully presented to naïve $CD8^+$ or $CD4^+$ T cells, the last will be induced into antigen-specific activation, proliferation and differentiation into function CTLs and T helpers. Simply put, a highly specific adaptive immune response could be harnessed against cancer cells displaying

specific antigens. Unfortunately, clinical trials with DCs in cancer immunotherapy have shown little success, aided by new discoveries showing that cancer is able to "escape" or even "put a break" on the immune system. This opens a path for research on how to avoid this "break" to be done. Cancer vaccines may also come in other forms, and for what regards the DCs in these approaches, much research still needs to be done in fundamental, applied and clinical settings.

Some of the research that has been developed in our laboratory fits into a project which aims to harness a better activation of T cells for recognition of cancer antigens and consequent killing of cancer cells. That, by fundamentally using our group's expertise on DCs biology and engineering of these cells' glycans, leading them into a more mature status. The work presented in this thesis is a part of the work that was gathered for a patent application. One of the main drawbacks that prevented us to go even further on our research was the limited time of the project. Initially, and also to the regard of this thesis, breast cancer patient cells were to be used in cytotoxic assays with their own (and differentiated in our laboratory) moDC-stimulated T cells, something that we did not have time and resources to accomplish in such limited time. My part of the work, and what we report here is the use of human cryopreserved cord blood samples provided by a cord blood Bank, Crioestaminal, to explore new sources of DC's precursors. These UCB samples are clinical-grade, SEPAX method volume reduced, cryopreserved cord blood samples, the same that are used in cord blood transplants. Initially, the purpose of these samples was to provide us monocytes, which would be differentiated into DCs, maturated and functionally tested. This aim was extended to the use of another DC's precursor– the Hematopoietic Stem Cells.

As to what our experiments allowed us to understand, the method that was employed for dendritic cell differentiation from CB monocytes seemed not to be successful in originating functional moDCs. After being tested for their capability for maturation after facing different maturation stimuli, it seems that no tested maturation markers showed to be up-regulated. These markers are supposed to be subjected to up-regulation before contact with T-cells for antigen presentation, so that this interaction may be promoted and stable. When assessed for endocytosis capacity, CB moDCs showed to be almost 10-fold inferior to that of peripheral blood moDCs. The final two tests on cryopreserved CB moDC seem to have closed the door for the use of these cells to generate a specific response against an antigen: secretion of both inflammatory and anti-inflammatory cytokines after maturation induction showed to be very low; when cultured with cells from another individual, they also seemed not to promote proliferation. Very important to mention, is that the methodology and protocol decisions taken on these cells must match those that have been taken for PB moDCs, for the sake of the project to what this thesis relates. Although no cancer cell cytotoxic assessment was made with CB moDC-stimulated T cells, all the clues until now point to the most likely incapacity to do so. Altogether, the results that we obtained with CB moDCs allowed us to make a comparison with adult PB moDCs (unpublished data) and to conclude that the first differentiate into DCs with a much more immature phenotype, having also a lower endocytic capacity than PB moDCs.

We could question the reasons for this anergy in CB moDCs that seems to be happening because of the cryopreservation process. The cell freezing has already been reported as having some influence in their function (Meijerink *et al.*, 2011; Silveira *et al.*, 2013). Anyway, studies have already proven that it is possible to generate DCs from fresh CB monocytes (Liu *et al.*, 2001), but these same studies have also given some clues about a lower capacity of cord blood moDCs (comparing to PB moDCs) to generate a strong immune response, and even said to have a 'more immature phenotype' (Bracho *et al.*, 2003; Nupponen *et al.*, 2013). We propose that investigating the IL-4 receptor and downstream signaling in the monocytes would be a good starting point, something we could not perform. This would give some clues as to what a true differentiation is happening or not, since the question still remains.

Given the inefficiency of CB moDCs to be modulated in order to perform the tasks necessary to our goal, the second and last option was to use the other source of DCs precursors available in cord blood – hematopoietic $CD34^+$ stem cells. After a 2 step culture that basically consisted in proliferating the rare $CD34^+$ cells present in cord blood before differentiating them in DCs. Despite the humbleness in the total numbers after proliferation culture, the fact is that after differentiation culture these cells demonstrated to be more prone to maturation than moDCs. Since obtaining DCs from HSCs takes more time and resources than from monocytes, we only had the possibility to work with the CD34-derived DCs from one cord blood unit. More units were also used before, so that some necessary optimizations could be done. So we addressed all the questions we wanted to a single unit of CB by the time this dissertation was written. So it is easy to say that future work will certainly take these CD34-derived DCs into use, performing with them all the experiments up until now only performed on monocyte-derived DCs. By then, we will understand if this source of DC precursors will be more of use in cancer immunotherapy than cryopreserved moDCs.

As was already said, the tumor cells from two breast cancer patients that we had the chance to receive were not used, to our regret, as originally intended. Working with all the cells deriving from the original patient would still be an interesting approach, though not possible in a one-year-period, the time of the project. Notwithstanding, cells were expanded in culture and properly frozen, so that they will be of use in the future. Moreover, and because of some interesting features of one of the breast cancers biopsies we got (also because of state of the patient), these cell's triple negative breast cancer phenotype was confirmed by flow cytometry and were immortalized for future studies.

Regardless of its goal, any project that would use cryopreserved cord blood monocytes for differentiation into DCs can take the results reported in this dissertation as a starting point. This specific part of our work is to be taken in consideration, since the number of experiments for which we have used CB moDCs was considerably higher than any other type of experiments reported here. Of course some of the results must be confirmed with a higher n but, in all the experiments, results have shown to

be consistent. Having said this, maybe this dissertation was aiming cancer immunotherapy but this doesn't mean that conclusions taken from it can't be applied to other set of diseases. There are certainly disorders that would benefit from the modulation of the immune system, as auto-immune diseases. Taking in mind our (one) result with allogenic MLR, where the presence of CB moDCs seemed to have inhibited a higher degree of basal-stimulation. Without wanting to seem naïve and reducing this commentary to the simplicity of our *in vitro* experiments, if this one result proves to be consistent in the future, maybe this means that these cells will not promote GvHD in un-related cord blood transplants.

The modulation of glycans that decorate the cell surface of DCs is of major interest to our research group, given that their terminal sialic acid removal by extrinsic enzymatic ways or intrinsic sialic acid absence caused by sialyltransferases deficiencies has already shown to be able to mature these cells in some way (reviewed in Crespo *et al.*, 2013). Something that we again confirmed with PB moDCs, on decreasing endocytosis capacity. However, CB moDCs showed low capability to be used for their original aim, and sialic acid removal (as a type of maturation stimulus) remains to be attempted in the future in CB CD34-derived DCs. Notably, in the one allogenic MLR experiment performed with CB moDCs, the sialidase treated cells were able to induce a higher allogenic response, even if unspecific. With these clues, we affirm this as a type of enzymatic treatment with great potential.

Most of the assays with cryopreserved CB developed throughout this year were not repeated in a sufficient number of times so that a high level of confidence in the results could be reached. Even though, the clues can't be denied and they will prove useful if the experiments are to be repeated again, for posterior publication.

We think that most of the relevance and originality related to this specific work holds to the fact that clinical grade cryopreserved CB units are being used for DC generation in 'translational' research settings. This is important not only because these are the exact 'products' that are used in clinical practice but also because the protocol optimization performed here to circumvent possible cell damage caused by cryopreservation and thawing processes may be very useful for future studies using this type of samples.

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