Universidade de Aveiro
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Departamento de Química

Cátia da Silva Mendes

Análise de Células Dendríticas Derivadas de Monócitos Obtidas por Protocolo de Diferenciação/Maturação Convencional *vs*. Rápido

Analysis of Monocyte-Derived Dendritic Cells Obtained Through Conventional *vs*. Fast Differentiation/Maturation Protocols

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Bioquímica, ramo de Clínica, realizada sob a orientação científica do Doutor Lúcio Lara Santos, Professor de Oncologia Cirúrgica e Responsável pelo grupo de Patologia e Terapêutica Experimental do Departamento de Oncologia Cirúrgica do Instituto Português de Oncologia do Porto, Francisco Gentil, e sob a co-orientação do Doutor Bruno Miguel Rodrigues das Neves, Professor Auxiliar do Departamento de Ciências Médicas da Universidade de Aveiro, e da Doutora Iola Melissa Fernandes Duarte, Investigadora Principal do CICECO-Instituto de Materiais de Aveiro, Departamento de Química da Universidade de Aveiro.

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Dedico este trabalho aos meus pais.

o júri

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Palavras-chave Células dendríticas derivadas de monócitos, Protocolo de diferenciação *ex vivo*, protocolo rápido *vs.* convencional, cocktails de maturação, metabolismo e metabolómica.

Resumo

As células dendríticas (CDs) são células especializadas na apresentação antigénica que desempenham um importante papel na ligação entre a imunidade inata e a imunidade adaptativa. Estas células têm a capacidade única de reconhecer, capturar, processar e apresentar antigénios a células T naïve, levando à polarização destas nas suas diferentes populações efetoras. Devido a esta capacidade, as CDs têm sido usadas nas últimas décadas no desenvolvimento de abordagens de imunoterapia contra o cancro. Neste tipo de imunoterapia celular é necessária a produção das CDs *ex vivo,* existindo para tal vários protocolos. O objetivo do presente trabalho prendeu-se com a comparação de dois protocolos de diferenciação, Rápido (1 dia) e Convencional (6 dias), sendo também testados quatro cocktails de maturação diferentes, tendo sido utilizadas amostras de 8 dadores saudáveis. Para esta comparação, foram analisados o perfil imunofenotípico, a capacidade de *uptake*, a produção de citocinas e a atividade metabólica. Os resultados obtidos demonstraram que apesar de algumas diferenças, as células diferenciadas pelo protocolo Rápido apresentaram um perfil fenotípico e capacidade de *uptake* bastante semelhante às CDs Convencionais. Relativamente à resposta às diferentes condições de maturação, tanto as CDs Rápidas como as Convencionais maturaram de forma mais efetiva quando estimuladas com o cocktail Alpha ou ligandos de TLR. A nível metabólico, as principais diferenças entre os protocolos prenderam-se com a maior atividade glicolítica e/ou glutaminolítica das células diferenciadas pelo método Convencional. Em termos de maturação, o cocktail Alpha pareceu estimular a glutaminólise, enquanto que a maturação com o cocktail Standard pareceu favorecer a glicólise.

Keywords Monocyte derived dendritic cells, *ex vivo* differentiation protocol, fast *vs*. conventional, maturation cocktails, metabolism and metabolomics.

Abstract Dendritic cells (DCs) are cells specialized in antigen presentation that play an important role in the connection between innate and adaptive immunity. These cells have the unique ability to recognize, capture, process and present antigens to naïve T cells, leading to their polarization in different effector populations. Because of this ability, DCs have been used in recent decades to develop cancer immunotherapy approaches. In this type of cellular immunotherapy, the production of *ex vivo* DCs is necessary, and there are several protocols for this purpose. The aim of the present work was to compare two differentiation protocols, Fast (1 day) and Conventional (6 days), while four different maturation cocktails were also tested, where samples from 8 healthy donors were used. For this comparison, the immunophenotypic profile, uptake capacity, cytokine production and metabolic activity were assessed. The results showed that, despite some differences, the cells differentiated by the Fast protocol had a phenotypic profile and uptake capacity very similar to Conventional DCs. Regarding the response to the different maturation conditions, both Fast and Conventional CDs matured more effectively when stimulated with the Alpha cocktail or TLR ligands. At the metabolic level, the main differences between protocols were related to the higher glycolytic and/or glutaminolytic activity of the cells differentiated by the Conventional method. In terms of maturation, the Alpha cocktail appeared to stimulate glutaminolysis, while maturation with the Standard cocktail appeared to favor glycolysis.

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Abbreviations and Acronyms

Ab - Antibody

AG - Antigen

APC - Antigen-presenting cell

CBA – Cytometric bead array

CCL - C-C motif ligand

CCR - C-C chemokine receptors

CD40L - CD40 ligand

cDC - Conventional DC

cGAS-STING - cyclic GMP-AMP synthase-stimulator of interferon genes

CLP - Common lymphoid precursor

CLR - C-type lectin receptors

CMD - Common myeloid precursor

COX-2 - Cyclooxygenase-2

CTL - Cytotoxic T lymphocyte

CTLA-4 - Cytotoxic T-lymphocyte-associated antigen 4

CXCL - C-X-C motif ligand

CXCR - C-X-C chemokine receptor

DAMP - Damage-associated molecular pattern

DCs - Dendritic cells

Dex - DC-derived exosomes

ER - Endoplasmic reticulum

FDA - Food and Drug Administration

FID - Free induction decay

Flt3L - Fms-like tyrosine kinase 3 ligand

Foxp3 - Fork head box p3

GM-CSF - Granulocyte-macrophage colony-stimulating factor

HSC - Hematopoietic stem cell

I.d. - Intradermal

I.n. - Intranodal

I.t. - Intertumoral

I.v. - Intravenous

iDC - Immature Dendritic cell

IFN - Interferon

IL - Interleukin

iTreg - induced T regulatory cell

LC - Langerhans cell

mAb - Monoclonal antibody

MDP - Macrophage and DC precursor

MDSC - Myeloid-derived suppressor cells

MFI - Mean Fluorescence Intensity

MHC - Major histocompatibility complex

MoDC - Monocyte-derived DC

mRNA - Messenger RNA

MVA - Multivariate analysis

NKT - NK T cells

NOD-like - Nucleotide-binding-oligomerization-domain

nTreg - Natural T regulatory cell

PAMP - Pathogen-associated molecular pattern

PBMCs - Peripheral blood mononuclear cells

PCA - Principal Component Analysis

PD-1 - Programmed death-1

pDC - Plasmacytoid DC

PGE_2 - Prostaglandin E_2

PKR - Active protein kinase

PLS-DA - Partial Least Squares-Discriminant Analysis

Poly I:C - Polyinosinic:polycytidylic acid

PRR - Pattern recognition receptor

PSA - Prostate-specific antigen

S.c. - Subcutaneous

SCF - stem cell factor

SSC - Side Scatter

TAP - Transporter associated with antigen processing

TCA - Tricarboxylic acid

TGF-β -Transforming growth factor beta

Th1 - T helper type 1

Tlr - Toll-like receptors

TNF-α - Tumor necrosis factor-α

TRAIL - TNF-α-related apoptosis-inducing ligand

Treg - T regulatory cell

αDC1 - Alpha type 1 DC

CHAPTER I - **Introduction**

I.1. Dendritic cells (DCs)

Dendritic cells (DCs) are an essential link between innate and adaptative immunity. These cells are professional antigen presenting cells (APCs) that can prime naïve T cells, polarizing them into their different effector subsets. DCs have therefore the exquisite capacity to modulate adaptive immune responses, namely to potentiate immunity against specific antigens[1]. This characteristic is a very appealing tool for immunotherapeutic approaches, namely, to enhance the immune system during cancer treatment. Several immunotherapeutic approaches using DCs have been developed, including DC differentiation *ex vivo*[2,3]. These immunotherapeutic approaches aim to generate an immune response against cancer by using DCs that, if properly maturated and loaded with tumor antigens will present these antigens to T cells, enhancing specific immunity against the tumor.

I.1.1 Ontogeny and differentiation of DC subtypes

In 1973, Steinman and Cohn identified in the spleen a new class of leukocytes with activating functions of the immune system, the DCs[4,5]. In subsequent years, DCs were shown to be potent APCs with a central role in the link between innate and adaptive immunity[1,6]. These cells consist of a small and heterogeneous population of leucocytes with powerful and unique immunoregulatory abilities. DCs act as sentinels of the innate immune system, seeking potential danger signals in peripheral tissues. When in the presence of such danger signals, that can be pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs), DCs initiate a series of functional and phenotypical alterations termed maturation[7]. These changes allow them to migrate from peripheral tissues to lymph nodes where they will interact with T cells. DCs are therefore able to recognize, capture, process and present antigens (AGs) to T naïve cells, causing their polarization into different effector or tolerogenic subpopulations[1].

Dendritic cells originate from $CD34^+$ hematopoietic stem cells (HSCs) in the bone marrow and circulate as precursors through the bloodstream to target tissues[7]. HSCs give rise to common lymphoid precursors (CLP) and common myeloid precursors (CMP), which

then originate intermediate progenitors, such as macrophages and DC precursors (MDPs)[8,9]. Further, MDPs differentiate into common DC precursors (CDPs) restricted to the generation of plasmacytoid DCs (pDCs) and conventional DCs (cDCs)[10,11]. Next, while pDCs terminally differentiate in the bone marrow, the pre-DCs exit the bone marrow and migrate through the blood to lymphoid and non-lymphoid organs, where they lastly differentiate into cDCs[12,13] (Figure 1). Independent of the precursor lineage, the differentiation, and expansion of specific DCs subtypes is controlled, under steady state, by different hematopoietic cytokines such as Fms-like tyrosine kinase 3 ligand (Flt3L), while during inflammation and infection, another cytokine, granulocyte-macrophage colony-stimulating factor (GM-CSF), mobilizes and stimulates the production of monocyte-derived DCs[14,15].

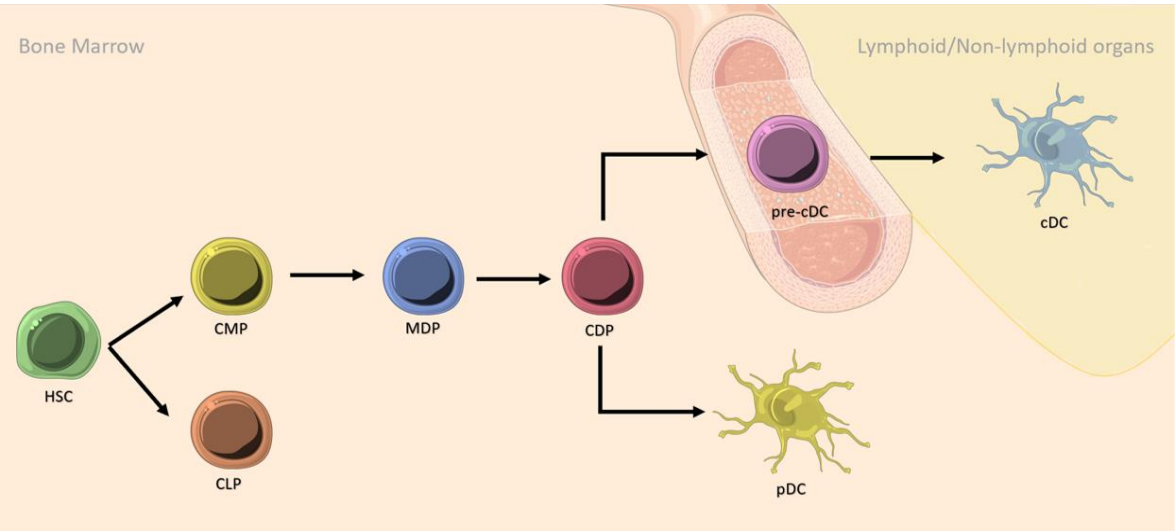


Figure 1. Ontogeny of DCs. HSC: hematopoietic stem cell; CLP: common lymphoid precursor; CMP: common myeloid precursor; MDP: macrophage and DC precursor; CDP: common DC precursor; pDC: plasmacytoid DCs; pre-cDC: pre-conventional DC; cDC: conventional DC.

As mentioned previously, DCs consist of a very heterogeneous group of leucocytes. Overall, they can be divided in pDCs and cDCs according to their origin and expression of specific markers. The well-known human pDC markers are CD123, CD45RA, CD303, CD304, CD85k, and CD85g, and more recently some antigens were added, like FcεR1, BTLA, DR6, and CD300A[16–18]. In addition, pDC also expresses a specific set of toll-like receptors (TLRs), mainly TLR7 and TLR9[19]. The role of pDCs in immunity is to sense and respond to viral infections by the rapid production of high quantities of type I and III interferons (IFN) and

secretion of cytokines[18,20,21]. However, conflicting roles for pDC have been reported in allergy[22,23], and tolerogenic pDCs under the influence of GM-CSF have also been associated with more aggressive breast cancer subtypes[24]. In turn, cDCs can be subdivided according to their localization: lymphoid organ-resident DCs; peripheral tissue-resident DCs, Langerhans cells (LCs); and circulating DCs. In the immune system, cDCs have the function of recognizing bacterial components and produce proinflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin 6 (IL-6), and IL-12p70 to activate proinflammatory T-cell subsets (T helper type 1(Th1) and Th17), and consequently recruit Cytotoxic T lymphocytes (CTLs)[7]. cDCs express a specific set of TLR, namely, TLR1, TLR2, TLR3, TLR4, and TLR8[19] and according to their expression levels of CD141 and CD1c, they have been subdivided into two subtypes[25]. It has been shown that the gene-expression profiles and functions of human $CD141^+$ DCs and $CD1c^+$ DCs resemble those of mouse cDCs1 and cDCs2, respectively[26]. Given this, it was proposed that human $CD141^+$ DCs would be referred to as cDC1s and $CD1c^+$ DCs referred as cDC2s[27]. In the blood and tissues, the frequency of human myeloid cDC1 is lower than cDC2, in steady-state[25,26]. In common, both cDC1 and cDC2 express CD13 and CD33[26]. The human cDC1 are found in blood and among resident DC of lymph nodes, tonsil, spleen and bone marrow, and non-lymphoid tissues such as skin, lung, intestine, and liver[26]. Phenotypically, cDC1s are characterized by expression of CLEC9A[28,29], Necl2[30], and the antigen BTLA[31], while XCR1[32] and intracellular detection of IRF8 are other markers that might also be considered to identify this lineage[33]. At the functional level, cDC1 are defined as having a high intrinsic capacity to cross-present antigens via major histocompatibility complex (MHC) class I to activate $CD8^+$ T cells and to promote Th1 and natural killer (NK) responses through production of IL-12[26]. About human cDC2, this population can be found in blood, tissues and lymphoid organs. These myeloid cDC express CD2, FcεR1, SIRPα and myeloid antigens CD11b and CD11c[31]. Recently, CD301a, VEGFA, and CD34A were added as cDC2 consistent markers[34]. To distinguish cDC2 from LC in the skin, the cDC2 have a higher CD11c and CD11b expression but lower CD1a, Langerin and Ep-CAM expression than LC[35]. cDC2 have the ability to respond well to lipopolysaccharide, flagellin, poly IC and R848, this capacity being due to expression of a wide range of lectins, TLRs,

nucleotide-binding-oligomerization-domain (NOD-like) receptors and RIG-I-like receptor [36]. Human cDC2s have a greater ability to produce IL-12 than cDC1, and can also present significant cross-presenting capacity [37]. They also secrete IL-23, IL-1, TNF-α, IL-8 and IL-10 but are consistently low in the secretion of type III interferon[38,39]. *In vitro*, human cDC2 have been shown to act as potent inducers of Th1, Th2, Th17 and $CD8^+$ T cells[38], which suggests that these cells might have the capacity to promote a wide range of immune responses *in vivo*.

I.1.2 Immunobiology of dendritic cells

I.1.2.1 Antigen capture, processing and presentation

Dendritic cells are usually present in peripheral tissues in an immature state. When encountering danger signals, immature DCs recognize them via pattern recognition receptors (PRRs), such as TLRs, NOD-like receptors, RIG-I-like helicases, C-type lectin receptors (CLRs) and active protein kinase (PKR)[40]. After recognition, antigen capture can occur by different mechanisms, that include receptor-mediated endocytosis, receptor-mediated phagocytosis and micropinocytosis (reviewed at [41]). The internalization of AGs by endocytosis and phagocytosis is mediated by receptors, namely, the receptor for Fc portion of the immunoglobulins, complement receptors, heat shock proteins, scavenger receptors[42] and C-type lectin receptor[43].

The antigen processing mechanism depends on the origin (endogenous or exogenous) and molecular nature of the antigen (protein or lipid). Therefore, there are three types of processing and presentation mechanisms, that are extensively reviewed at [44,45], and will be briefly described below. The presentation via MHC-II pathway occurs when the AGs are extracellular proteins. DCs capture these AGs and internalize them into endosomes that later fuse with lysosomes forming phagolysosomes. This results in AGs degradation into peptides that are loaded on MHC-II molecules. Once formed, the MHC-II-AG peptides cross the cytoplasm into exocytic vesicles, reaching the cell surface where they are presented to $CD4^+$ T cells. The MHC-I pathway is used when the AGs are proteins of intracellular origin. The endogenous proteins are degraded by the proteasome[46], and the resulting peptides are

directed, via the transporter associated with antigen processing (TAP), to the endoplasmic reticulum (ER), where they are coupled to MHC-I molecules[47]. This MHC-I-AG peptide complex is transported to the Golgi apparatus and then to the plasma membrane, being presented to $CD8^+$ T cells. Besides this mechanism, there is the cross-presentation pathway, where extracellular AGs can also be presented to $CD8^+$ T cells via MHC-I complexes. This type of presentation is exclusive of DCs and can originate either an immunogenic or a tolerogenic response (cross-tolerance)[48], being crucial for antitumor immunity. The last AG presentation pathway characteristic of DCs is the presentation of lipidic AGs, whether from endogenous or exogenous origin, via molecules of the CD1 family. The CD1 proteins can be classified into two groups: group I that englobes the CD1a, CD1b, CD1c proteins, and group II that includes the CD1d protein. These CD1 family proteins form complexes with lipidic AGs, and then the complex is presented to CD8+ T cells, γ/δ T cells or natural killer T cells (NKT)[49].

I.1.2.2 Maturation of DCs

The contact of immature DCs with a "danger signal" triggers a complex series of morphologic, functional and phenotypic modifications, referred to as maturation. The maturation process can be initiated by many stimuli, including pathogen-related molecules, such as lipopolysaccharides, bacterial DNA, double-stranded RNA, proinflammatory cytokines (TNF-α, IL-1β, IL-6), prostaglandins, and T cell-derived signals[50]. The process initiates with the loss of adhesive structures, acquisition of high cellular motility and rearrangement of the cytoskeleton[51]. There is also increased activity of proteolytic enzymes, which favors the processing of internalized AGs[52], and increased availability of MHC-II molecules[53]. Another functional change is the downregulation of phagocytosis and micropinocytosis, while the ability to capture AGs via receptor-mediated endocytosis is maintained in maturing DCs[54]. Maturation is accompanied by profound phenotypic modifications, namely the increased expression of costimulatory molecules such as CD40, CD54, CD80, CD83 and CD86[50] and the production of cytokines like TNF-α, IL-10, IL-1α/β, IL-12p70, IFN-γ, IL-8, IL-6, IL-23[55]. Additionally, there is also an alteration in chemokines

produced by mature DCs, with increases in the expression of C-C motif ligand (CCL) type 2 (CCL2), CCL3, CCL4, CCL5, CCL8 and C-X-C motif ligand (CXCL) type 8 (CXCL8). These chemokines are important for the recruitment of neutrophils and monocytes to the site of inflammation or infection. At a later stage, an increase in some lymphoid chemokines such as CCL17, CCL18, CCL19, CCL22, and CXCL10 is also observed, these molecules being important for the recruitment and interaction with T and B cells [56]. The expression profile of cytokines and chemokines is highly dependent on the type of DC and stimulus that triggers the maturation process, being of major relevance for the polarization of T cells into different subsets [57]. Relatively to chemokines receptors expression, immature DCs express C-C chemokine receptors (CCR) type 1 (CCR1), CCR2, CCR5, CCR6, C-X-C chemokine receptor (CXCR) type I (CXCR1), and CXCR2. During maturation, cells downregulate the expression of these receptors and increase the expression of the receptors CXCR4 and CCR7. The expression of CCR7 makes DCs responsive to lymphoid chemokines CCL19 and CCL21, which direct their migration to lymphoid organs[58]. All these phenotypical and functional modifications allow the egress of DCs from peripheral tissues to the marginal zones in the draining lymph nodes where they will present AGs to naïve T cells, thereby initiating a specific immune response.

I.1.2.3 Interaction with T cells and NK cells

Dendritic cells' immunomodulatory potential is intimately connected to their capacity to interact and polarize naïve T cells into their different effector or tolerogenic subsets. During antigen presentation, DCs provide 3 signals that drive the activation and polarization of T cells[59]. Signal 1 is initiated by the interaction of the T cell receptor on $CDT8^+$ and $CDT4^+$ T cells, respectively with the MHC-I or MHC-II-antigen complexes presented by the DCs. Signal 1 without any co-stimulation leads to the inactivation of the naïve T cells by anergy or deletion, which promotes tolerogenic responses. Signal 2 results from the interaction between the costimulatory molecules expressed by DCs with the respective ligands present on the surface of T cells. The synergy between signal 2 and signal 1 promotes the survival and proliferation of T cells and stabilizes their cytokine production[60]. For a long time, these

two signals were seen as a fundamental requirement for the transition from tolerogenic to immunogenic polarizing abilities[61]. However, numerous data show that a certain level of positive co-stimulation is also required for the establishment of tolerogenic responses. For example, the binding of the costimulatory molecules CD80 and CD86 to cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) act as a negative regulator of T cell activation. The interaction of CD80 and CD86 with CTLA-4 and CD28 on T regulatory cells (Treg) has shown to be essential for Treg development, homeostasis, and suppression activity[62]. On the other hand, the interaction between 4-1BB ligand and OX40, expressed on DCs, with their receptor in T cells can promote immunogenic[63] or tolerogenic[64] responses. So, the output from signal 2 results from an equilibrium between positive and negative costimulatory signals, leading to either immunogenic or tolerogenic responses.

Signal 3 is related to cytokines and chemokines secreted by DCs, which are important for $CD8^+$ T cells differentiation into CTLs[65] and for $CD4^+$ T cells polarization into their different effectors (Th1, Th2 and Th17)[66] or regulatory subpopulations (Treg, Tr1 and Th3)[67]. Relatively to the interaction of DCs with $CD8^+$ T cells, the presence of the cytokines IL-12p70, IL-15 and IFN-α/β during the presentation of MHC-I-antigen complexes leads to differentiation of $CD8^+$ T cells into CTLs and memory $CD8^+$ T cells[68], that are important in the establishment of immunity against tumors and intracellular infections[69,70]. For the $CD4^+$ T cells, the differentiation in the subpopulation Th1 is typically dependent on the pro-inflammatory chemokine IL-12[71,72]. This subpopulation is characterized for IFN-γ, TNF-α and IL-2 production, and for playing a critical role in anti-tumor immunity[73] and in the immune response against many virus infections[74–78]. By contrast, Th2 cells are differentiated by IL-4 through STAT6 and the transcription factor GATA-3[79,80]. Th2 cells secrete IL-4, IL-5, IL-9, IL-13 and IL-25 when activated by bacterial, helminth or parasitic pathogens[81]. Th17 cells secrete IL-17[82], and are important for resistance to extracellular bacteria and fungi, and also contribute to allergic[83] responses and autoimmune pathogenesis[83]. For induction and differentiation of human Th17 cells, IL-6 and IL-1β are essential[84,85]. The regulatory subpopulations of $CD4^+$ T cells comprise, among others, the Treg cells that have high suppressor activity and serve to prevent autoimmunity and immunopathology[86]. The

process of differentiation of these cells is mediated by the presence of IL-2 and TGF-β[62,87]. Treg cells can be classified in two main subsets according to their origin and suppressive activity. Natural $CD4^+$ Treg cells (nTregs) constitutively express fork head box p3 (Foxp3), and the activation marker CD25[88]. Other types of Treg cells are Tr1 and Th3, that are called "induced" Treg subpopulations (iTreg), and these subpopulations aren't associated with a high level of Foxp3 expression[89]. Tr1 cells also have suppressor activity via high production of IL-10 and TGF-β[90]. The differentiation of naïve $CD4^+$ T cells in Tr1 happens in the presence of IL-10[91]. Finally, the Th3 cells secrete high levels of TGF-β and play an important role in maintaining intestinal mucosal tolerance to ingested AGs[92]. Their differentiation is mediated by TGF-β and is co-adjuvated by the presence of retinoic acid[92].

Besides T cells, DCs also interact with NK cells, and this interaction results in a mutual regulation, as demonstrated in Figure 2. NK cells are crucial for controlling infections and immune surveillance through their production of cytokines and lysis of transformed cells[93]. Several studies showed that NK-DC interaction results in activation, maturation, survival or even death of DCs. First, DCs promote NK cell survival, activation and maturation through several mechanisms that involve direct cell-cell interactions and indirect cytokine-mediated interactions[94]. The cytokines secreted by DCs that are involved in the survival, activation and proliferation of NK cells are: IFN-α, IFN-β, IL-2, IL-12, IL-15 and IL-18[21,95–98]. The cytokine IL-12, secreted by mature DCs, has an important role in the immune response because it enhances NK cell-mediated cytotoxicity and IFN-γ production[99]. DCs might also activate NK cells indirectly, by the promotion of T cells expansion, which secretes IL-2 that, in turn, activates NK cells[100]. pDCs also have an important role here because they produce high levels of type I IFN[101]. Type I IFN could potentiate NK cell cytotoxicity by upregulating TNF-α-related apoptosis-inducing ligand (TRAIL), and, in addition, promotes NK survival[102]. On the other hand, NK also regulates DCs. Activated NK cells, by producing IFN-γ, promote maturation of DCs with Th1 polarizing abilities, which boost the ongoing adaptative response. DCs activated by NK cells also present increased CTL-polarizing capacities [103]. Alternatively, NK cells can induce the lysis of some DCs, namely, immature DCs (iDCs), because of their downregulated MHC-I expression. Mature DCs are resistant to NK-

mediated killing because of their high level of MCH expression. This phenomenon seems to depend on the NK:DC ratio, *in vitro,* and is regulated by the NK activating receptor NKp30[104–107]. Thus, activated NK cells eliminate iDCs from inflammation/infection site, favoring antigen presentation to T cells by mature DCs, which results in a more effective immune response. So, as we can see, DCs and NK cells regulate each other, and their interaction is important for a better immune response, as summarized in Figure 2.

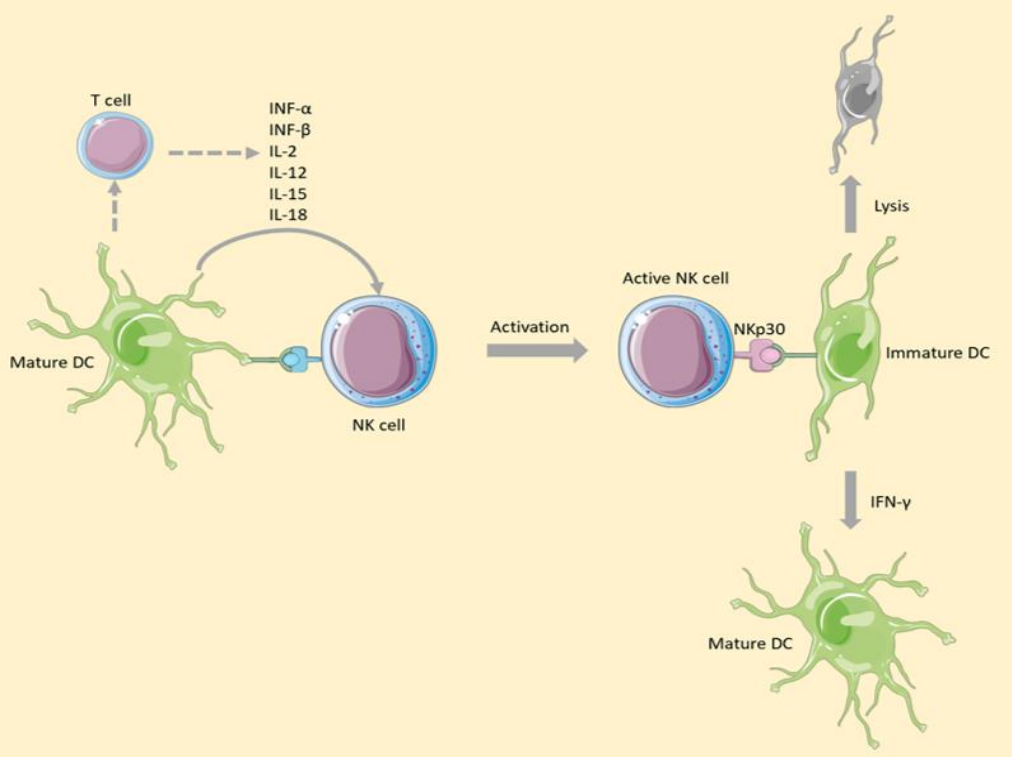


Figure 2. DC-NK cells interaction. Mature DCs make direct contact with NK cells, and together with cytokines secreted by mature DCs, NK cells are activated. DCs also activate NK cells, indirectly, through the IL-2 production by T cells. Activated NK cells interact with immature DCs, through the NKp30 receptor, resulting either in their lysis or their maturation caused by IFN-γ secreted by NK cells. DC: Dendritic cell; IL: Interleukin; IFN: Interferon; NK: Natural Killer.

I.2. Dendritic cells in the immunobiology of cancer

The relationship between the immune system and cancer has been a matter of debate for many decades because the immune system can mediate protection against cancer but at the same time promote cancer progression. The term “cancer immunoediting” appears to better describe the dual function of host-protecting and tumor sculpting of the immune

system. Cancer immunoediting is the result of three phases: elimination, equilibrium and escape[108]. The elimination phase, that represents the concept of immunosurveillance, corresponds to the attempt of the immune system to eradicate tumor cells. If successful, it represents a complete immunoediting process without evolution to the following phases. So, the initiation of the antitumor immune response is triggered when the innate immune system becomes alerted by the presence of a growing tumor. This occurs due to local tissue disruption occurring as a result of stromal remodeling, which is part of the solid tumor development[109]. This tissue disruption induces inflammatory signals leading to the recruitment of immune cells, such as NKT, NK, γδ T cells, macrophages and DCs, to the tumor site[108,110,111]. In a second stage, the release of IFN-γ by these cells induces the local production of angiostatic chemokines that block the formation of new blood vessels within the tumor, leading to its death. Then, necrotic tumor cells are engulfed by iDCs, which mature under pro-inflammatory milieu and migrate to tumor-draining lymph nodes (TDLNs)[108,110,111]. Subsequently, the products generated during remodeling of the extracellular matrix might induce tumor-infiltrating macrophages to produce low quantities of IL-12 that stimulate the recruited NK cells to produce low amounts of IFN-γ. In turn, IFN-γ activates macrophages in the tumor to produce more IL-12, leading to increased IFN-γ production by NK cells. This continuous positive feedback system potentiates tumor killing by activating cytotoxic mechanisms such as perforin, TRAIL, and formation of reactive oxygen species (ROS). In the TDLNs, the migrated DCs present tumor antigens (TAs) to naïve $CD4^+$ and $CD8^+$ T cells that then differentiate into TA-specific $CD4^+$ and CTLs, respectively[108,110,111]. Finally, TA-specific $CD4^+$ and CTLs home to the primary tumor site, where the CTLs eliminate the remaining TA-expressing tumor cells whose immunogenicity has been enhanced by exposure to locally produced IFN-γ[108,110,111]. If any tumor cell variant survived the elimination process, then it enters in the equilibrium phase. In this process, the immune system holds the tumor in a state of functional dormancy. Some tumor cells undergo genetic and epigenetic changes, due to lymphocytes and IFN-γ potent and persistent selection pressure, which leads to continuous sculpting of tumor cells. This phase culminates in the selection of tumor cells with reduced immunogenicity and that are

therefore resistant to destruction by immune effectors[108,110,111]. Furthermore, in the equilibrium phase, there is a balance between IL-12, that promotes elimination, and IL-13, that promotes persistence, maintaining tumors in equilibrium[112]. This phase is likely the longest and might occur over a period of several years to decades.

In the escape phase, tumor cells immunoescape can occur through many different mechanisms. These mechanisms have been discussed elsewhere[113–118] and are summarized here in three points that include: reduced immune recognition, increased resistance or survival, or development of an immunosuppressive tumor microenvironment. Reduced immune recognition can be due to the absence of strong TAs, loss of MHC class I, or costimulatory molecules. The increased resistance or survival can be related to the increased expression of STAT-3 or anti-apoptotic molecules such as Bcl2. On the other hand, the development of an immunosuppressive tumor microenvironment is due to the production of cytokines like vascular endothelial growth factor (VEGF) and transforming growth factor (TGF)-β, and immunoregulatory molecules, such as IDO, PD-1/PD-L1, Tim-3/galactin-9 and LAG-3[116,117].

As stated above, DCs have an important role in the process of cancer immunoediting, especially in the phase of elimination. In this phase, DCs present tumor antigens to T cells and polarize them into antigen-specific effectors that will eliminate tumor cells. However, sometimes tumors can escape this process by promoting an immunosuppressive microenvironment comprising VEGF[119], TGF-β[120], prostaglandin E_2 (PGE_2)[121], IL-10[122], IL-6[123], colony-stimulating factor 1 (CSF-1)[124] or prostate-specific antigen (PSA)[125]. These soluble mediators have been shown to inhibit or change maturation, differentiation and/or function of DCs. Another parameter that is important for DCs in the elimination phase is the immunogenicity of tumor cells. The major determinant that distinguishes an immunogenic from a non-immunogenic tumor is the frequency of neoantigens, as well as the relationship between mutational cargo and response to immune checkpoint blockade[126–129]. A second factor might be the degree of DC maturation, that differs depending on the type and extent of tumor cells death. A third factor is the level of local or systemic immune suppression caused by the tumor. The switch from immunogenic to immunosuppressive that occurs in

tumors during progression seems to correlate with a phenotypic change in DCs. DCs have been shown to suffer multiple transformations during tumor progression, losing their capacity to effectively present antigens and gaining the ability to actively suppress T cell responses by the promotion of Treg differentiation[130].

Due to this capacity of tumors to escape or subvert the immune system, numerous immunotherapeutic strategies have been investigated in the last years to overcome this problem. One of these strategies is to boost anti-tumor immune responses by the administration of tumor antigen loaded DCs. These so-called anti-tumor DC-based vaccines will be briefly described in the following sections.

I.3. Dendritic cell vaccines in antitumor immunotherapy

Due to their capacity for antigen presentation and ability to modulate immune responses, dendritic cells are a perfect tool for immunotherapeutic interventions. So, DC vaccines emerged as a potential way to boost immune responses against cancer cells. Numerous studies have been carried out in this field and some methods to produce DC vaccines have been developed in the last decades. Among the approaches explored, *ex vivo* differentiation and manipulation of DCs is by far the most common process, both at experimental and clinical levels[2]. Nevertheless, there are other methods that have been explored, such as *in vivo* DC-targeting, non-targeted AG based vaccines, GM-CSF-secreting tumor cell vaccines, implanted DC-recruiting and activating scaffolds, or the use of DC-derived exosomes (Dex).

I.3.1. *Ex vivo* manipulation of DCs

In *ex vivo* manipulation, DCs are differentiated from patients' blood precursors, loaded with tumor antigens, induced to mature, and reinjected into the patient in order to activate pre-existing tumor-specific T cells and induce *de novo* anti-tumor immune responses. Due to all these steps, the production of DC-based vaccines is time-consuming and expensive. Moreover, there is still no standardized procedure, which renders the comparison of studies quite difficult. The source of DCs, maturation cocktails, the nature and procedures for AG

loading, and the route of administration are therefore important variables in the production of DC-based vaccines[2] (Figure 3).

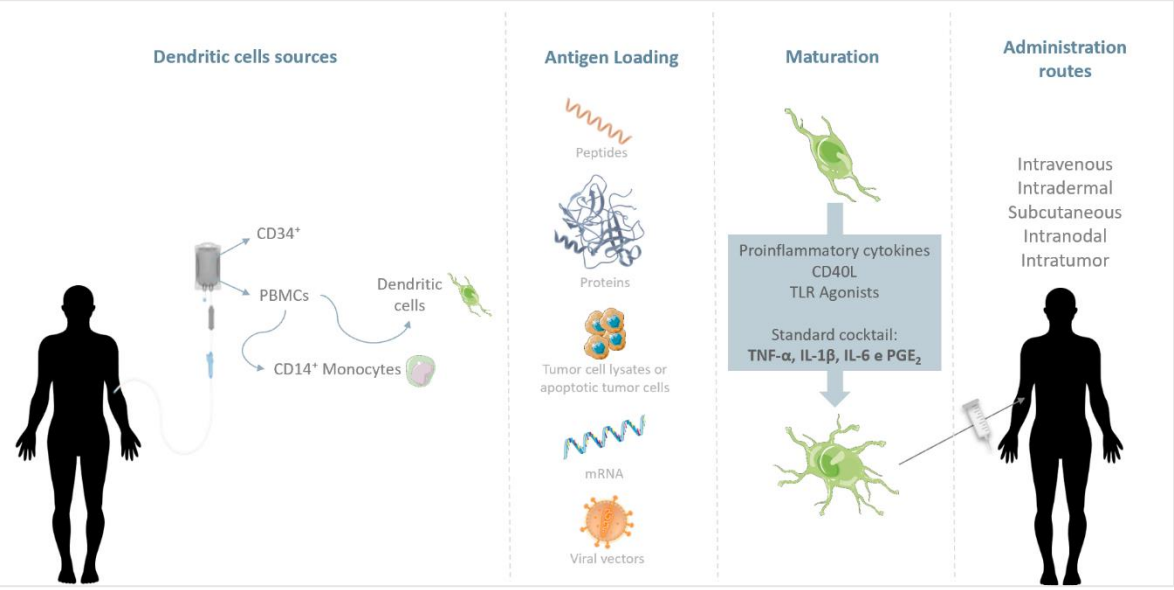


Figure 3. Schematic representation of the phases involved in *ex vivo* manipulation of DCs and the approaches currently available. The whole process includes: choosing a DC source, loading DCs with antigens of the tumour, maturating DCs, and finally administering the DCs loaded with tumour antigens and maturated. Adapted from[131]

I.3.1.1. DC Source

In the production of DCs there is an important aspect to keep in mind: the source of DCs that will be used. Natural DCs constitute only 1% of peripheral blood mononuclear cells (PBMCs) which is a major limitation to their clinical application. Although challenging, the use of natural DCs is still being addressed in several ongoing clinical trials using pDCs and cDCs for the treatment of different cancer types, including melanoma (NCT02574377, NCT number, or ClinicalTrials.gov identifier) and prostate cancer (NCT02692976) [132,133] .

Besides, another possibility for generating DCs involves the use of peripheral blood monocytic precursors or hematopoietic $CD34^+$ stem cells. The protocols for differentiation of DCs from these precursors will be addressed in detail in section 4 of the present introduction.

I.3.1.2. Strategies for Antigen Loading

The selection of tumor-associated antigens (TAA) and the loading procedures are important steps for DC-based vaccine production[134]. Of the existing TAAs, only a limited fraction (approximately 10%) appear to be immunogenic, and among these, just a limited number is associated with tumor rejection[135]. TAAs are normally unique mutated proteins, AG derived from oncogenic viruses, or shared non-mutated self-AG[136]. In current procedures, DCs have been pulsed with peptides, proteins, and tumor cell lysates, exposed to apoptotic tumor cells, loaded with genes via viral vectors, and transfected with messenger RNA (mRNA) coding for TAAs. Pulsing DCs with peptides, proteins, and apoptotic tumor cells is the most common approach. While peptides are loaded directly onto MHC-I and MHC-II molecules on the surface of DCs, proteins or tumor cells need to be internalized and processed by DCs before presentation to $CD4^+$ and $CD8^+$ T cells[137]. The major disadvantage of defined peptides is the limited number of characterized TAAs and the need to know the patient's haplotypes to ensure that peptides would bind to them. In the case of proteins and tumor cells, the main drawback is that the MHC-I pathway is not specifically targeted, however studies have demonstrated activation of $CD8^+$ T cells, which indicates the occurrence of cross-presentation[137–139].

Another strategy is the use of viral vectors that allow the insertion of genes encoding tumor AGs or whole proteins. Some vectors might also induce DC maturation, avoiding the need for a separate maturation procedure. Another advantage of this method is the possibility of adding genes encoding cytokines or costimulatory molecules. The disadvantage of viral vectors is that pre-existing immunity against the vector might reduce the ability to induce *in vivo* responses[138,140]. Finally, another promising approach is the loading of DCs with mRNA encoding for TAAs that were demonstrated to induce $CD4^+$ and $CD8^+$ T cell responses. mRNA has a short half-life, is not part of the host genome, and can be loaded on DCs without using vectors or requiring knowledge of patient's haplotypes[141]. Another advantage is the possibility to induce expression of multiple AG epitopes, maturation markers or cytokines. To introduce mRNA into DCs, electroporation, without the need for additional reagents, has been shown to be the most efficient method[138].

I.3.1.3. DC Maturation

The maturation of DCs is another important step in DC-based vaccines production. Studies using *in vitro* matured DCs have shown that these cells have a good expression of costimulatory molecules and an increased production of cytokines and chemokines, which is necessary for the efficient activation of T-cell responses[50]. Besides, immature DCs were shown to induce limited immunogenic antigen-specific responses[142] and to promote the differentiation of regulatory T cells[143,144]. The appropriate DC maturation stimulus remains a matter of discussion, and several protocols have been tested using different maturation cocktails. The most common approach in clinical trials is the use of cocktails comprising pro-inflammatory cytokines, CD40L, and TLR agonists. In the past, TNF-α, IL-1β, IL-6, and IFN-α were used to mature DCs, however this cocktail originated highly variable responses from donor to donor[145]. Therefore, another DC maturation cocktail was proposed, being presently the most used at clinical level, it includes TNF-α, IL-1β, IL-6 and PGE_2[145]. The use of PGE_2 is paradoxical because it improves DC migratory capacities by inducing CCR7 expression[146], but on the other hand reduces IL-12p70 production[147]. Despite the weak IL-12p70 production, the protocols that include PGE_2 result in a more uniform maturation regarding the upregulation of phenotypic markers[145]. To overcome the problem of low IL-12p70 production, protocols that combine the exposure to the TLR3 ligand polyinosinic:polycytidylic acid (Poly I:C) and PGE_2 were tested[148]. These efforts have originated the second most common DC maturation cocktail: TNF-α, IL-1β, IFN-α, IFN- γ and poly I:C. The exposure of cells to this cocktail gives rise to the so-called alpha type 1 DCs (αDC1s). These cells efficiently secrete IL-12p70 and have been shown to elicit more potent CTL responses than the standard matured DCs[149].

Simon, Fonteneau, and Grégoire[150] proposed, however, that these maturation approaches raised two important concerns: 1) maturation stimuli might be insufficient to generate fully functional mature DCs with efficient immunogenic properties and 2) the 48-h maturation period might be too long, resulting in DC exhaustion, a situation where DCs have already reached their optimal maturation status *in vitro* and are exhausted when reinjected into the patient. Moreover, *in vitro* studies have demonstrated that prolonged

periods of maturation lead to the development of DCs that already exhausted their IL-12 production capacity[151]. So, the period of exposure of DCs to maturation cocktails is an important point to be considered in clinical trials in order to avoid the use of exhausted DCs with reduced capacity to induce cellular immune responses[152].

I.3.1.4. Administration routes

The importance of the administration route is related to the requirement of the injected DCs to reach the lymph nodes and stimulate immune responses. Several administration routes have been tested, including: intravenous (i.v.), intradermal (i.d.), subcutaneous (s.c.), intranodal (i.n.) and intratumor (i.t.). All these routes allow anti-tumor immune responses, but each one has different characteristics and efficiency. I.v. administration of DCs leads to their transient lung accumulation before redistribution to the liver, spleen and bone marrow[153]. In i.d. and s.c. routes, the majority of DCs remain at the injection sites and are cleared by infiltrating macrophages. However, a small percentage of these cells migrate to peripheral lymph nodes, where they are sufficient to induce specific antitumor T cells[154,155]. S.c. and i.d. routes are the less technically exigent and the less expensive and are therefore the most used in trials for solid tumors tratments[2]. The i.n. route needs to be performed under ultrasound guidance by an experienced radiologist and presents great variability between patients[2]. Comparing i.n. to i.d. vaccination in treatment of melanoma patients, while in i.n. more DC reach the T cell areas in the lymph nodes, the antitumor T-cell responses are comparable or inferior to i.d. administration[154,156]. Another route of administration is the i.t., this approach is technically exigent and associated with a high rate of intervention-associated morbidity. This technique was already tested in several solid tumors, and the studies demonstrated that the procedure is safe and could elicit anti-tumor immunity in some patients[157–159]. Overall, data indicate that there is not a “perfect” route of administration. The tendency is to combine several administration strategies, the most frequently tested being the combinations of i.d. + s.c. and i.d. + i.v.[2].

I.3.2. Other DC-based approaches

Other DC-based approaches that have been analyzed are *in vivo* DC targeting, nontargeted AG based vaccines, GM-CSF-secreting tumor cell vaccines, implanted DC-recruiting and activating scaffolds, and DC-derived exosomes (Dex). *In vivo* DC targeting has the advantage of not requiring the production of DCs *ex vivo* and to take advantage of superior functional characteristics of endogenous DC populations [160]. This process involves the coupling of AGs to monoclonal antibodies (mAbs) that are specific for DC surface molecules. However, in the absence of adjuvants this process induces tolerance, and therefore, requires the co-administration of DC maturation agents[161,162]. Besides *ex vivo* manipulation and *in vivo* targeting, there are other approaches that explore the immunogenic potential of DCs against cancer. Non-targeted AG based vaccines can be composed by peptides, proteins or tumor nucleic acids, and, when injected, are captured and processed by DCs, leading to an antigen-specific immune response[163]. In GM-CSF-secreting tumor cell vaccines, irradiated autologous tumor cells or allogenic tumor cell lines are manipulated to secrete GM-CSF and are then injected into patients. These transformed tumor cells can strongly attract macrophages, granulocytes, T cells, and DCs, which potentiate tumor AG presentation[164]. Implanted DC-recruiting and activating scaffolds use the same concept as GM-CSF-secreting tumor cell vaccines. In implanted DC-recruiting scaffolds, biocompatible polymers incorporate and release a DC chemotactic agent, an adjuvant, and tumor AGs in a controlled way[165,166]. Finally, Dex are also used to prime specific CTLs[167,168]. All these methods have been tested and are still being tested, and clinical trials have arisen. However, besides all these different approaches, *ex vivo* differentiation and manipulation of DCs remains the most common process used at experimental and clinical levels.

I.4. Dendritic cells differentiation protocols

As stated in previous sections, *ex vivo* manipulation of DCs is by far the most used technique to produce DC-based vaccines against cancer. In this approach, the crucial phase is the differentiation of DCs from precursor cells collected from patients. Although the

protocols found in the literature share some features, it is clear from their analysis that a universal standardized procedure to obtain DCs is still not available.

There are basically two sources of precursors to produce DCs *ex vivo*: $CD34^+$ hematopoietic progenitors and $CD14^+$ monocytes isolated from PBMCs fraction. In the first approach, $CD34^+$ precursors are mobilized from the bone marrow by treatment of patients with GM-CSF before leukapheresis procedures[169]. $CD34^+$ cells are then cultured with GM-CSF, TNF-α, Flt3L, TGF- α, and stem cell factor (SCF) for 11–12 days. After culture, the resulting cell mixture includes monocyte-derived DCs (MoDCs), DCs that are phenotypically similar do LCs and myeloid cells at different stages of differentiation[138]. The limited number of $CD34^+$ that can be isolated and the extended time that these cells need to differentiate renders this approach difficult to implement, the differentiation from $CD14^+$ monocytes being frequently preferred [170]. The differentiation of DCs from $CD14^+$ monocytes (MoDCs) was established in the early '90s by Romani and collaborators in a work where they showed that incubation with GM-CSF and IL-4 for 5 to 7 days allowed the production of a large number of cells with characteristics of immature DCs[171]. GM-CSF is an essential growth factor that supports monocytes and DCs survival, while IL-4 acts by limiting monocyte differentiation towards macrophages [172].

As previously stated, mature DCs have a superior capacity to induce an efficient activation of T-cell responses[50,142]. So, a second step is required in order to mature DCs before their injection into the patient. The classical DC maturation cocktail includes TNF-α, IL-1β, IL-6 and PGE_2[145], and in most protocols this step takes 48 hours. As previously discussed, this 48h period is not consensual because it might lead to the development of DCs that already exhausted their IL-12 production capacity[151]. So, until now, the most used method to produce mature DCs takes 7 days (Conventional method): 5-6 days differentiation with GM-CSF and IL-4, plus 1-2 days with the maturation cocktail[173–175].

DCs differentiated by this standard protocol generate good results but have the big disadvantage of their production being time consuming. Therefore, new approaches have

been tested in order to overcome this problem. One of these approaches consists in the production of DCs in only 2 days: 24h treatment with GM-CSF and IL-4, plus 24h with the maturation cocktail. This approach was first tested by Dauer et al[175], in 2003, giving rise to the so-called FastDCs. In the referred study, the authors compared the standard 7-day protocol with their new 48h protocol, using the same maturation cocktail for both: TNF-α + IL-1β + IL-6 + PGE_2. The results showed that the cells undergo all phases of DC differentiation including: increased endocytic uptake of soluble dextran before they develop into mature DCs and classical mature phenotype ($CD83^+CD40^+CCR7^+CD14^-CD80^+CD86^{high}$MHC-$II^{high}$). Relatively to size and complexity, FastDCs are considerably smaller and less complex than 7-days DCs, but present elongated dendrites. Despite their reduced size and complexity, AG-loaded FastDCs not only stimulate immune responses to recall AGs but were also effective in initiating primary immune responses. Moreover, FastDCs showed to be efficient in stimulating a Th1-type immune response as AG-loaded. The authors also concluded that IL-6 is dispensable for the maturation step and that the cytokines can only induce maturation when used together because none of the stimulus individually induced maturation of FastDCs by itself. The flaw of the method is the low production of IL-12p70, something that was expected due to the use of PGE_2[175].

Further studies confirmed this seminal work [173,174,176]. For instance, the generation of DCs in 8, 5 and 2 days with the same maturation cocktail as the one used by Dauer was tested[173]. The results showed that immature FastDC efficiently endocytosed dextran, underwent DC maturation, upregulated the lymph node directing chemokine receptor CCR7 (the percentage of migrating cells increased with decreased time in culture), and acquired T cell stimulatory capacity comparable to the standard (8 days) DCs. Here, despite the presence of PGE_2 in the maturation cocktail, IL-12p70 production was triggered through TLR4 and TLR7/8 stimulation in all three types of DC. The strongest production was observed in the 8-day DCs, IL-10 secretion being concomitantly increased. The authors conclude that FastDC were equally capable of stimulating AG specific Th1 responses, however, they could also be more susceptible to immune suppressive tumor-derived factors[173]. In another work,

FastDCs were directly differentiated from PBMCs using GM-CSF, IL-4, and IFN-β, being then matured with standard maturation cocktail without IL-6. [174]. The authors did not find obvious differences between DCs differentiated from PBMC and from purified monocytes. Furthermore, matured 2-day-DC/PBMC showed to be potent stimulators of IFN-γ, but not IL-4 or IL-10 by allogeneic T cells. It is of interest that the levels of IFN-γ production were higher in $CD4^+$ T cells immunized with 2-day-DC/PBMC than those immunized with 7-day-DC/Mo[174]. Finally, another approach was the 2 days differentiation of αDC1 using poly I:C and IFN-γ instead of IL-6 and PGE_2[176]. The objective of producing this type of DC is to overcome the lack of IL-12p70 secretion by conventionally differentiated cells. In this study, 2-days αDC1s were compared to FastDCs, and in fact, the results showed that 2-days αDC1s could secrete higher levels of IL-12p70 while maintaining the same maturation phenotype (increased HLA-DR, CD80, CCR7, and CD83 expression and decreased CD209 and CD14 expression). The authors concluded that 2-days αDC1s induce specific responses and expansion of T cells, similarly to FastDCs, but with an increased Th1 cytokine production[176].

Overall, these studies provide some evidence that the differentiation of DCs in 2 days is feasible and that these cells are phenotypically and functionally similar to the ones obtained by the conventional 7 days protocol. More studies are however necessary to optimize and standardize these short protocols. Their implementation is of great importance for the extended use of anti-tumor DC-based vaccines, as they would allow the decrease in time and production costs, two major limiting factors nowadays.

Objectives

The main objective of this dissertation was to perform a comparative study between MoDCs differentiated by Fast and Conventional protocols, with a view to evaluate the future implementation of the former at the Immunology Service of IPO-Porto. *Ex-vivo* DC differentiation and maturation coupled to immunogenicity testing is expected to help predicting, for each patient, the success of treatment with DC vaccines.

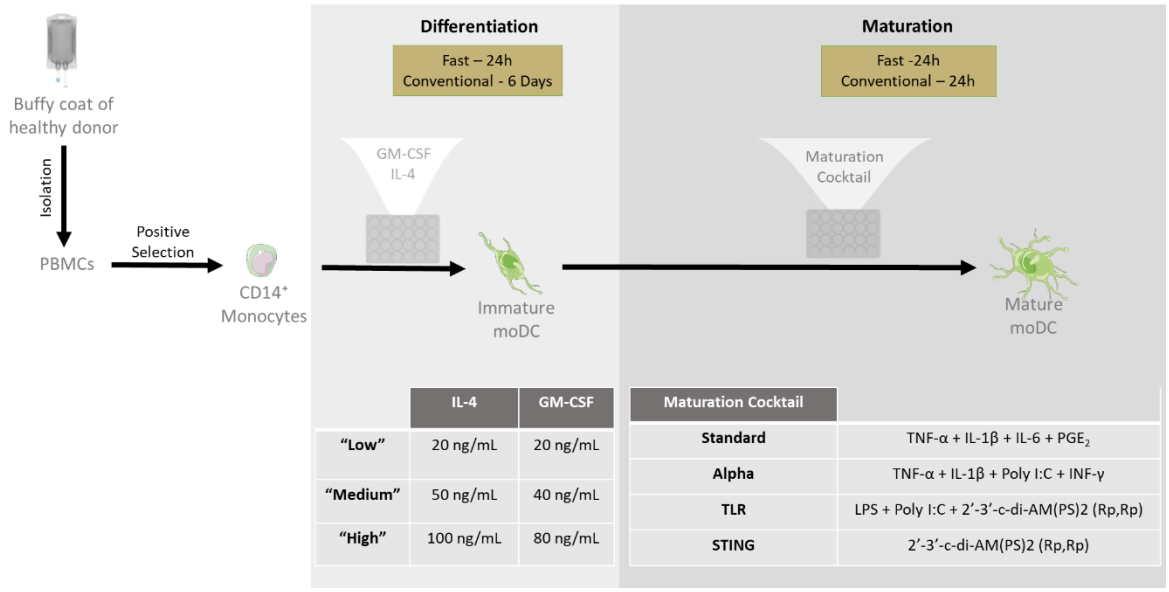
Specific aims were to:

- Assess the influence of differentiation factors´ concentration on MoDCs obtained through the Fast protocol.
- Evaluate the impact of different maturation cocktails, combined with either Fast or Conventional differentiation protocols, on MoDCs immunophenotype.
- Evaluate the uptake capacity of MoDCs resulting from Fast and Conventional differentiation, as a mean to predict the ability of MoDCs to take up tumor AGs.
- Compare the potential of different maturation cocktails to induce immunogenic or tolerogenic responses, based on cytokine quantification in the media of mature cells.
- Characterize the metabolic activity of MoDCs resulting from the different protocols tested, based on the analysis of changes in the cells exometabolome.

CHAPTER II - **Material and Methods**

II.1. Isolation of monocytes and DCs differentiation

PBMCs were isolated from buffy coats of 8 healthy donors by Histopaque-1077 (Sigma-Aldrich) gradient centrifugation. $CD14^+$ monocytes were purified by positive selection using MACS CD14 isolation kit (Miltenyi Biotech) and were then cultured in twelve-well plates (1.0 x 10^6 cells/mL) with RPMI Medium 1640 + GlutaMAX (Gibco) supplemented with antibiotics and 10% fetal calf serum (FCS). Additionally, IL-4 and GM-CSF (both from Peprotech) were added in the medium in three different concentrations. For Fast DCs, 20 ng/mL of IL-4 and GM-CSF were used as a "low" concentration condition, whereas for a "high" concentration, 100 ng/mL of IL-4 and 80 ng/mL GM-CSF were added. Conventional DCs were obtained with a standard cocktail (medium concentration) of 50 ng/mL and 40 ng/mL of IL-4 and GM-CSF, respectively. To differentiate the monocytes into Fast MoDCs, the cells were cultured 24h with IL-4 and GM-CSF, while in the case of Conventional MoDCs they were cultured for 6 days, at 37˚C in a 5% CO_2 atmosphere. In the Conventional method, the medium was changed on days 2 and 5. After differentiation, Fast and Conventional DCs were cultured for 24h with four different maturation cocktails, namely Standard Cocktail[177]: 50 ng/mL TNF-α (Peprotech), 25 ng/mL IL-1β (BioLegend), 1 µg/mL PGE_2 (Sigma-Aldrich) and 10 ng/mL IL-6 (BioLegend); Alpha Cocktail[176]: 20 µg/mL Poly I:C (Novus Biologicals), 100 ng/mL IFN-γ (Peprotech), 50 ng/mL TNF-α and 25 ng/mL IL-1β; TLR Cocktail: 1 µg/mL LPS (Sigma-Aldrich), 20 µg/mL Poly I:C and 1 µg/mL 2'-3'-c-di-AM(PS)2 (Rp, Rp) (InvivoGen); and STING activating stimulus: 1 µg/mL 2'-3'-c-di-AM(PS)2 (Rp, Rp). On days 1, 2, 6 and 7, the cells and supernatant were collected for characterization. Cell viability was evaluated by the trypan blue exclusion method. The experimental scheme is presented in Figure 4.



	IL-4	GM-CSF
"Low"	20 ng/mL	20 ng/mL
"Medium"	50 ng/mL	40 ng/mL
"High"	100 ng/mL	80 ng/mL

Maturation Cocktail	
Standard	TNF-α + IL-1β + IL-6 + PGE_2
Alpha	TNF-α + IL-1β + Poly I:C + INF-γ
TLR	LPS + Poly I:C + 2'-3'-c-di-AM(PS)2 (Rp,Rp)
STING	2'-3'-c-di-AM(PS)2 (Rp,Rp)

Figure 4. Schematic representation of cell isolation and culture. First, PBMCs were isolated from peripheral blood buffy coat by Histopaque-1077 gradient. Then, $CD14^+$ monocytes were separated through positive selection, using the MACS CD14 isolation kit. After that, $CD14^+$ monocytes were cultured with IL-4 and GM-CSF, in three different concentrations (Low, Medium, and High), for 1 day in the Fast Method, and 6 days in the Conventional method, where the monocytes differentiated to immature MoDCs. After differentiation, four maturation cocktails/stimulus (Standard, Alpha, TLR and STING) were added to the culture and incubated for 24h, before collection of mature MoDCs.

II.2. Immunophenotyping

For immunophenotyping, the cells from days 0, 1, 2, 6 and 7 were labeled with the following monoclonal antibodies (mAbs), as indicated in Table 1: anti-CD11c (BD Bioscience), anti-CD14 (BD Biosciences), anti-CD16+56+3 (Beckman Coulter), anti-CD19 (Beckman Coulter), anti-CD80 (BD Pharmigen), anti-CD86 (BD Biosciences), anti-HLA-DR (BioLegend/Beckman Coulter), anti-HLA-ABC (BD Horizon), anti-CD197 (CCR7)(BD Pharmigen). The cells were incubated 30 minutes with the mAbs, and then washed with a wash solution (BSA [Sigma-Aldrich] + EDTA [MERK] + Sodium Azide [Sigma-Aldrich] + FACSFlow solution [BD Bioscience]) followed by centrifugation. Lastly, the cells were resuspended with FACSFlow and then analyzed on FACSCanto II flow cytometer (BD Biosciences). Data was analyzed using Infinicyt software (Cytognos S.L.) and FlowJo software (Tree Star, version 10).

Table 1. mAbs panel and respective fluorochromes used

	Tube 1	Tube 2	Tube 3
PB	-	HLA-ABC	HLA-DR
PO	CD45	CD45	CD45
FITC	-	-	CD86
PE	CD16(FITC)+CD56(PE)+CD3(ECD)	-	CD197
PercPcys.5	-	-	-
PC7	CD19	HLA-DR	CD80
APC	-	CD11c	CD11c
APC-H7	CD14	CD14	CD14

II.3. Uptake Capacity

II.3.1. FITC-Dextran

The endocytic activity was measured in day 1 (Fast method) and 6 (Conventional method), when the differentiation phase ended, in all the 8 donors. The endocytic activity was assessed by incubating cells for 20 minutes with FITC-Dextran (100 µg/ml) (Thermo Fisher) at 37°C. Unspecific FITC-dextran binding to the cell surface was assessed by incubating cells at 4°C. When the incubation was complete, cells were washed extensively with PBS (Biochrom GmbH). After that, cells were labelled with anti-CD14 (APC-H7) and then incubated for 30 min. After incubation, cells were washed, centrifuged and resuspended with FACSFlow. Samples were run on the FACSCanto II flow cytometer and data were analyzed using Infinicyt and FlowJo software.

II.3.2. Internalization of apoptotic/necrotic cells

To test the capacity of differentiated cells to internalize apoptotic/necrotic cells , DCs from day 1 (Fast method) and day 6 (Conventional method) from 2 donors were tested with two different cell lines, namely with RKO cells, derived from colon carcinoma (CRL-2577), and colorectal adenocarcinoma HCT-15 cells (American Type Culture Collection), that were kindly provided by the Institute for Health Research and Innovation (i3S). Initially, the tumor

cell lines were labeled with 1 µM CFSE (BD Horizon) during 20 min at 37˚C in the dark. After that, cell death was induced by incubating the cells at 60˚C for 30 min. Apoptotic/necrotic cells were then layered on top of Fast / Conventional MoDCs for 2 hours at 37˚C. Unspecific CFSE binding to the cell surface was assessed by incubating the cell lines with MoDCs at 4°C. As a negative control, MoDCs were incubated with RPMI medium only, for 2 hours at 37°C. After the 2 hours incubation, cells were washed with PBS and labeled with mAbs: anti-HLA-DR (PB), anti-CD45 (PO), anti-CD11c (APC) and anti-CD-14 (APC-H7), to identify the MoDCs. The cells were incubated 30 minutes with the mAbs, washed, centrifuged and resuspended with FACSFlow. Data were acquired on the FACSCanto II flow cytometer and analyzed using Infinicyt software.

II.4. Cytokines quantification

The cell culture supernatants were collected after the maturation step, at days 2 and 7, for Fast or Conventional DCs, respectively. The medium with the cells went through a centrifugation to separate the supernatant from the cells, and then an aliquot of the supernatant was collected and stored at −80°C until analysis. Cytokine profiles of the MoDCs were checked using cytometric bead array (CBA) Flex Set (BD Biosciences), where IL-12p70 and IL-10 were measured, according to the manufacturer's instructions (BD Biosciences). The limit of detection of each cytokine was 9,14 - 2589,43 pg/mL (IL-12p70) and 9,08 - 2595,46 pg/mL (IL-10). The acquisition was performed with a FACSCanto II flow cytometer (BD Biosciences). Quantitative results were generated using FCAP Array v3 software (Soft Flow Inc.).

II.5. Metabolomics

II.5.1. Sample collection and preparation

At the end of differentiation (day 1 or 6) and maturation (day 2 or 7) phases, cells were collected, washed with PBS, resuspended with fresh RPMI medium supplemented with antibiotics and FCS, and incubated for additional 24h at 37˚C in a 5% CO_2 atmosphere.

After the 24 h incubation, supernatants were collected and stored at -80˚C (Figure 5). As a negative control, cell-free RPMI medium, supplemented with antibiotics and FCS, was also incubated for 24 h, at 37˚C in a 5% CO_2 atmosphere, collected and stored under the same conditions.

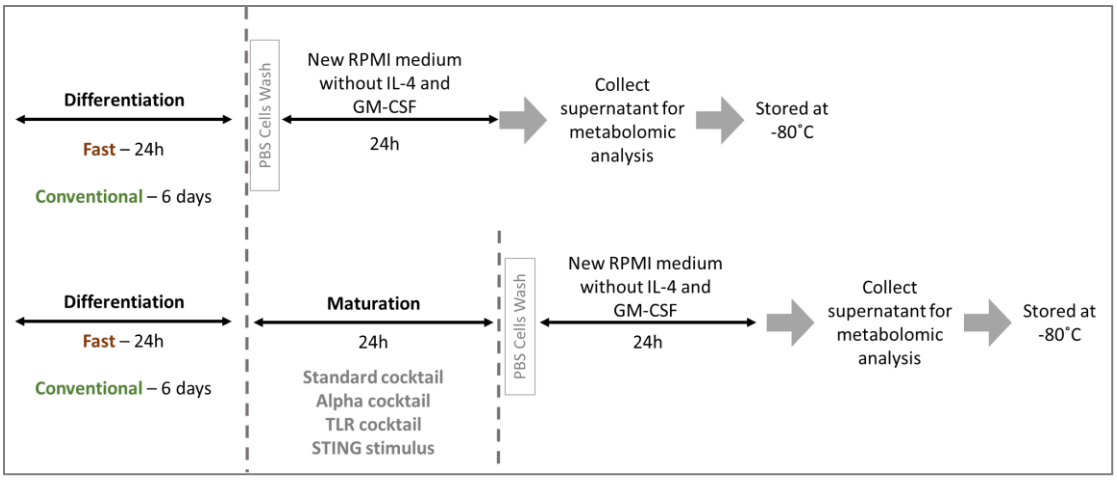


Figure 5. Schematic representation of samples collection for metabolic analysis. After differentiation or differentiation + maturation, cells were washed with PBS, resuspended in fresh RPMI medium and incubated for 24h at 37˚C in a 5 % CO_2 atmosphere. After that, the supernatant was collected and stored at -80˚C. Acellular medium incubated for 24h was used as control.

To remove interfering proteins, thawed supernatants were subjected to a protein-precipitation procedure[178]. First, 300 µL of cold methanol 100% (v/v) at -80˚C were added to 150 µL of supernatant (1:3 proportion). The aliquots were kept at -20˚C for 30 min, and subsequently centrifuged at 13000 x G for 20 min. The supernatant was then transferred to another vial, vacuum dried (SpeedVac, Eppendorf) and stored at -80˚C until NMR acquisition. At the time of analysis, the dried samples were resuspended in 600 µL of deuterated phosphate buffer (PBS 100 mM, pH 7) containing 0.1 mM 3-(trimethylsilyl) propanoic acid (TSP-d_4), and 550µL of each sample were then transferred to 5 mm NMR tubes.

II.5.2. ^{1}H-NMR Spectroscopy

Cell-conditioned and cell-free medium samples (prepared as described above) were analysed in a Bruker Avance III HD 500 NMR spectrometer (University of Aveiro, PT NMR

Network) operating at 500.13 MHz for ^{1}H observation, at 298 K. Standard 1D ^{1}H spectra with water presaturation (pulse program 'noesypr1d', Bruker library) were recorded with 32k points, 7002.801 Hz spectral width, a 2 s relaxation delay and 512 scans . After obtaining the NMR spectra, spectral processing was carried out using TopSpin 4.0.7 (Bruker Biospin), where each free induction decay (FID) was multiplied by a cosine function (with a ssb value of 2), zero filled to 64k data points and Fourier-transformed. The resulting spectra were then manually phased, baseline corrected and calibrated to the glucose signal (δ 5.236 ppm).

II.5.3. Multivariate analysis

After spectral processing, the spectra were visualized and prepared for multivariate analysis (MVA) using Amix-Viewer 3.9.15 (Bruker Biospin). Each spectrum was normalized by its total area, excluding the water-suppression region and residual contaminant signals. The normalized data were then organized into matrices, where signal intensities are displayed for each sample spectrum (rows) and chemical shift (columns). Data matrices were uploaded into SIMCA-P 11.5 (Umetrics) and subjected to Principal Component Analysis (PCA) and Partial Least Squares-Discriminant Analysis (PLS-DA). Different scaling types were tested and unit-variance scaling (UV), in which each column (containing the intensities at a particular chemical shift) is divided by its respective standard deviation, was chosen. This process gives equal variance to all variables, allowing for variations in less abundant metabolites to have the same weight in multivariate models as more intense signals. The results were then visualized through factorial coordinates ('scores') and factorial contributions ('loadings') coloured according to variable importance to projection (VIP). For PLS-DA models, Q^2 and R^2 values, respectively reflecting predictive capability and explained variance, were used to assess the robustness of class discrimination. Generally, model reliability increases as Q^2 approaches 1, so higher Q^2 are desirable.

II.5.4. Spectral integration

To provide a quantitative measurement of metabolic variations, spectral integration of selected signals was carried out in Amix-Viewer 3.9.15. Signals representative of each metabolite, which were found to be relatively free of overlap, were integrated and normalized by the total spectral area. For each metabolite, the percentage of variation in treated samples was calculated relative to respective controls, along with the effect size (ES)[179] and the statistical significance.

II.6. Statistical analysis

Data are expressed as mean (for the number of samples indicated) ± standard error of the mean (SEM). To assess differences between samples, the t-student test was applied using the softwares Microsoft Excel 2019 and GraphPad Prism 6. A P value < 0.05 was considered statistically significant (represented by asterisks in bar charts).

CHAPTER III - **Results and Discussion**

III.1. Isolation of monocytes and DCs differentiation

To isolate $CD14^+$ monocytes from PBMCs, positive selection was used, and through flow cytometry, using the "Tube 1" from Table 1, some cell populations were identified over the days. The populations that were identified were: classical monocytes ($CD14^+$; $CD16^{-/low}$; $CD3^-$; $CD19^-$), MoDCs ($CD14^{-/low}$; $CD16^{-/low}$; $CD3^-$; $CD19^-$), T cells ($CD3^+$; $CD19^-$; $CD16^-$; $CD56^-$; $CD14^-$), B cells ($CD19^+$; $CD3^-$; $CD16^-$; $CD56^-$; $CD14^-$) and NK cells ($CD16^+$; $CD56^+$; $CD3^-$; $CD19^-$; $CD14^-$). The percentage of variations of cell populations over the days are represented in Figure 6. On day 0, after isolation, 95.9% of the leucocytes were monocytes, 2.88% T cells, 0.59% B cells and 0.68% NK cells. It is therefore possible to conclude that the method used to isolate $CD14^+$ monocytes gave good results, as almost all the cells in culture were $CD14^+$ monocytes.

Throughout the days, there were some oscillations in the percentage of cell populations in culture. In the differentiation phase (days 1 and 6), the average of MoDCs ranged from

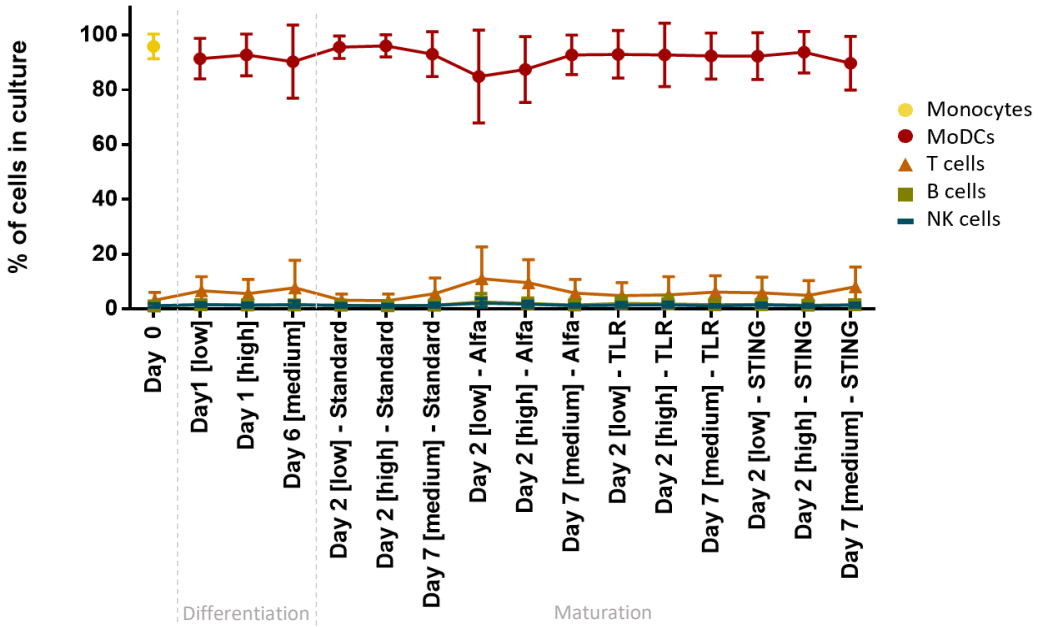


Figure 6. Percentage of cells of each population of leucocytes between the total of leucocytes, in the day 0, after the isolation, and over the days in culture. The populations that were measured on day 0 were: monocytes, T cells, B cells, and NK cells. From the day 1 to 7 the populations that were measured were: MoDCs, T cells, B cells, and NK cells.

90.3% (Day 6 [medium]) to 92.7% (Day 1 [high]). For T cells, the average was between 5.5% (Day 1 [high]) and 7.7% (Day 6 [medium]), while B cells oscillated between 0.94% (Day1 [high]) and 1.01% (Day 1 [low]) and NK cells between 0.88% (Day1 [high]) and 1.08% (Day 1 [low]). For all cell types, there was just a small difference in the percentage of cells in culture between 1 and 6 days of differentiation, and the concentration of GM-CSF and IL-4 didn't seem to have a big influence. If we compare the percentage of cells on day 0 and days 1/6, it is possible to observe a small increase in the percentage of T cells, B cells, and NK cells, this being probably due to nonspecific differentiation or death of some MoDCs. If we look separately to the maturation phase, the Alpha cocktail was the one in which the percentage of MoDCs was lower (84.8-92.8%) and the percentage of other leucocytes populations were higher, which could result from some MoDCs death. Among the other cocktails/stimulus there were almost no variations. From these results, it was possible to conclude that the concentration of IL-4 and GM-CSF didn't influence the percentage of each population of leucocytes in culture, and that the percentage of each population in culture didn't suffer great oscillations throughout the days.

The viability of cells was measured after the samples were collected, and the values oscillated between 80-93% throughout the days (Figure 7). There weren't big differences between [Low] and [High] concentrations in the Fast method, and the most influencing factors were the time in culture and the maturation cocktails/stimulus used. With the same maturation cocktail, Conventional MoDCs showed lower %viability, which was expected due to the longer culture time. Between maturation cocktails/stimulus, the TLR cocktail was the one producing lower cell viability (80-85%), followed by the Alpha cocktail, while the highest %viability was obtained with the Standard and STING stimuli. The cell viability along the differentiation process was above 80% in all conditions.

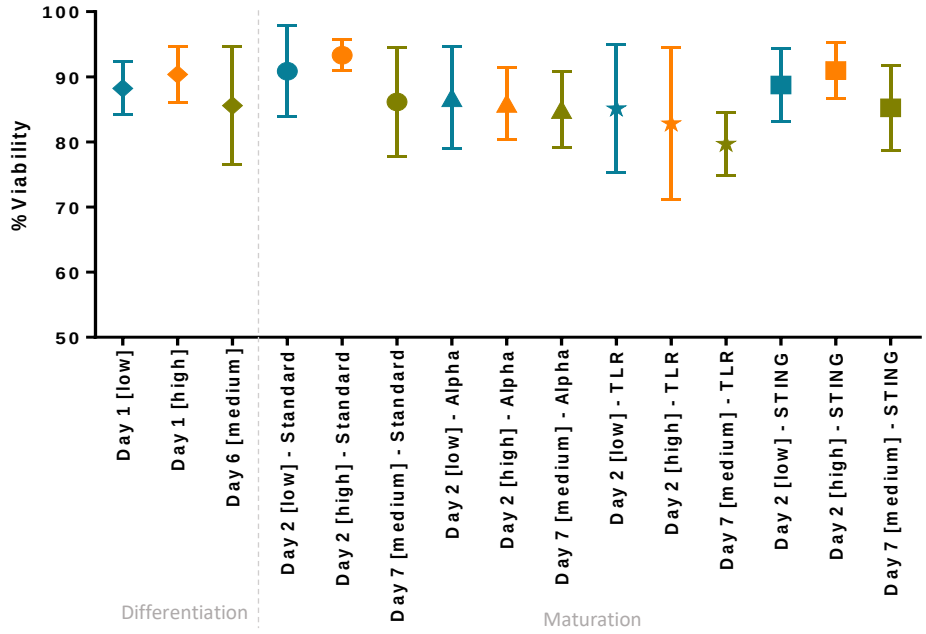


Figure 7. Percentage of viable cells throughout the days where samples were collected, in all conditions tested. Day 1 and 2 – Fast method; Day 6 and 7 – Conventional method.

III.2. Immunophenotype

The differentiation of monocytes into MoDCs involves several phenotypical modifications that allow the process to be followed and evaluated. The phenotypical changes analyzed in the current work are summarized in Figure 8. Apart from $CD14^{+}$, monocytes express $CD11c^{low}$, HLA-$DR^{low,}$ HLA-ABC^{low}, $CD80^{-}$, $CD86^{-}$ and CD197 $(CCR7)^{-}$. When in the presence of IL-4 and GM-CSF for a certain period, monocytes differentiate into immature MoDCs, and the expression of CD14 decreases while the levels of CD11c, HLA-DR (MHC-II) and HLA-ABC (MHC-I) increase. Therefore, MoDCs immunophenotype ca be resumed as follows: $CD14^{low/-}$, $CD11c^{high}$, HLA-DR^{high}, HLA-ABC^{high}, $CD80^{-}$, $CD86^{-}$ and CD197 $(CCR7)^{-}$. When immature MoDCs are exposed to maturation factors, the expression of CD80, CD86 and CD197 increases, resulting in the following immunophenotype: $CD14^{-}$, $CD11c^{+}$, HLA-DR^{high}, HLA-ABC^{high}, $CD80^{+}$, $CD86^{high}$ and CD197 $(CCR7)^{+}$. Besides all these changes, the complexity is expected to increase along the process, mature MoDC being much more complex then immature MoDC or monocytes. In the next subchapters, the results obtained

for each marker on day 0 (monocytes), days 1 and 6 (immature MoDCs) and days 2 and 7 (mature MoDCs) will be analyzed individually. The Conventional method was used as reference for how the markers should behave while, in the maturation phase, the reference was the Standard cocktail.

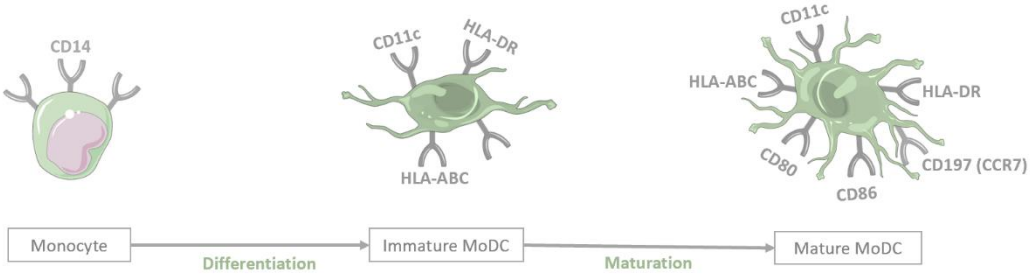


Figure 8. Representation of changes that occur on the cell markers, from monocyte to a mature MoDC. In monocyte the main marker is the CD14, and after differentiation into an immature MoDC that marker downregulate, and other markers like CD11c, HLA-DR, and HLA-ABC, upregulate. After maturation the MoDC gain CD80, CD86 and CD197 (CCR7) becoming a mature MoDC.

III.2.1. CD14

CD14 is a marker for $CD14^+$ monocytes and its expression is expected to be downregulated when the cells differentiate into immature MoDCs. As shown in Figure 9A, there was a loss of CD14 in both methods. On day 1 [low] the loss was of 52%, on day 1 [high] 53% and on day 6 [medium] 60%, when compared with day 0. Thus, we may conclude that, for the differentiation phase, the results of the Fast method were very similar to the Conventional method, and that the different concentrations didn't have a relevant influence on the percentage of CD14 loss.

After differentiation, different maturations cocktails/stimulus were added to immature MoDCs, and modulation of CD14 expression relative to immature cells was analyzed (Figure 9B). These results showed that with the STING activating stimulus the cells lost fewer CD14

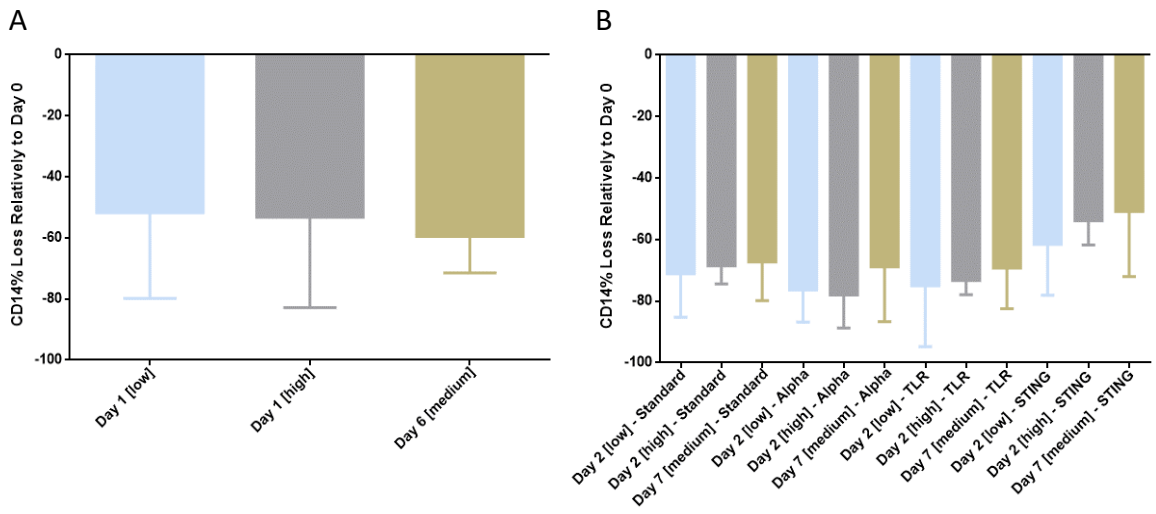


Figure 9. **A -** Percentage of CD14 MFI (mean fluorescence intensity) loss, relatively to day 0, of the cells after differentiation, in both Fast (day 1) and Conventional (day 6) method. **B -** Percentage of CD14 MFI loss, relatively to day 0, of the cells after maturation, in both Fast (day 2) and Conventional (day 7) method, with Standard, Alpha, TLR cocktails and STING stimulus.

(51-62%) than with the other cocktails/stimulus, in both concentrations and methods, the minimum loss being observed for the Conventional method. On the other hand, the maximum loss of CD14 was observed in the Fast method with the Alpha Cocktail, with 78% of loss with [High] and 76% with [Low]. The TLR cocktail also gave good results when using the Fast method, with 75% of loss with [Low] and 73% of loss with [High]. Comparing the Fast and Conventional methods, the former produced higher CD14 loss than the latter, for all maturation conditions. The concentration of differentiation factors (IL-4 and GM-CSF) in Fast MoDCs didn't have a large influence on the results. Overall, it is possible to conclude that the Fast and Conventional methods gave similar results, both in the differentiation and maturation phases.

The loss of CD14 marker is an important parameter to guarantee that monocytes are differentiating into MoDCs. However, other parameters need to be analyzed to ensure the identity and functionality of MoDCs.

III.2.2. CD11c

CD11c is expressed by monocytes at low levels but strongly increases with differentiation into immature MoDCs, making it a good marker for these cells. Figure 10A shows the percentage of CD11c MFI gain by differentiated cells, compared with monocytes (day 0). As expected, an increase of CD11c expression relative to day 0 was observed in both methods. In the Fast method, there was basically no difference between the [Low] (338%) and the [High] (332%). However, CD11c expression increased more than twice in the Conventional method (893%) compared with the Fast method. So, in our experimental setting, the increase of CD11c expression appeared to be related with the time in culture and not with the concentration of the differentiation factors.

In mature cells, the expression of CD11c varied with both differentiation and maturation conditions, as can be seen in Figure 10B. The tendency for higher CD11c gain in the Conventional method remained in mature cells. However, gains after maturation (793-868%) were lower than in the differentiation phase, an occurrence observed with all maturation cocktails/stimulus. Focusing on the Fast method, the TLR cocktail presented the

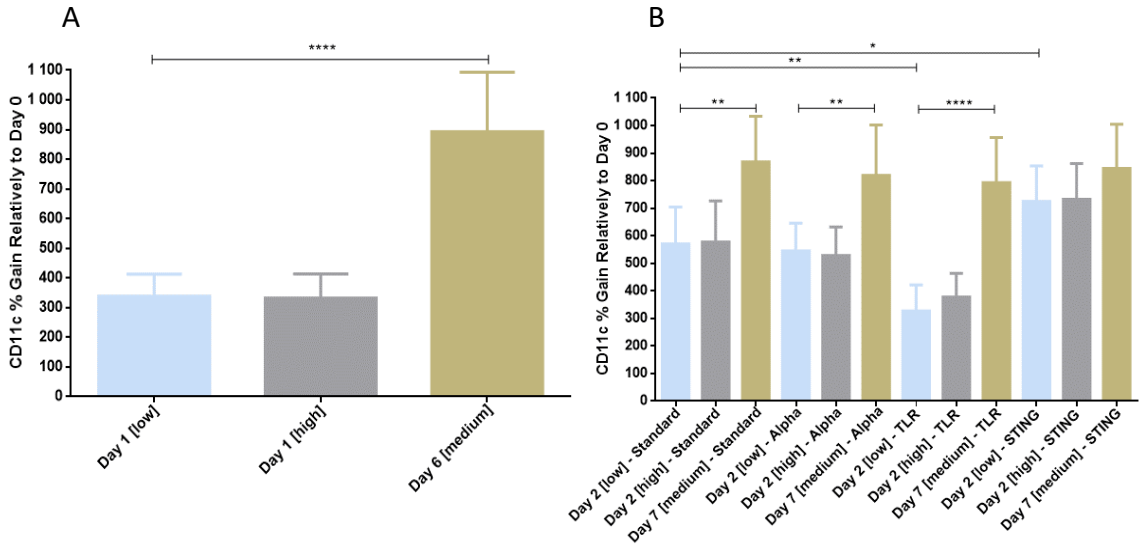


Figure 10. **A** - Percentage of CD11c MFI gain, relatively to day 0, of the cells after differentiation, in both Fast (day 1) and Conventional (day 6) method. **B -** Percentage of CD11c MFI gain, relatively to day 0, of the cells after maturation, in both Fast (day 2) and Conventional (day 7) method, with Standard, Alpha, TLR cocktails and STING stimulus.

lowest CD11c gain (327-377%), while the STING activating stimulus gave the highest values (725-733%). Standard (570-577%) and Alpha cocktails (529-545%) produced similar results. There were no significant differences between [Low] and [High]. Overall, regarding CD11c expression in Fast MoDCs, it is possible to conclude that STING was the activating stimulus causing the closes result to that obtained for mature Conventional DCs. We hypothesize that LPS and/or Poly I:C (present in Alpha and TLR maturation cocktails) might be responsible for downregulating the expression of CD11c, with marked effects in Fast MoDCs. Accordingly it was already reported that these two TLR agonists influence the expression of CD11c in DCs[180].

III.2.3. MHC-II (HLA-DR) and MHC-I (HLA-ABC)

III.2.3.1. HLA-DR

The HLA-DR protein belongs to the MHC-II complex, and is found on the surface of certain cells like DCs, B lymphocytes, and macrophages[181]. In DCs, extracellular proteins are presented via MHC-II to $CD4^+$ T cells. This marker is useful to identify MoDCs because it is highly expressed by these cells when compared with monocytes. In present work, the results obtained for the differentiation phase showed that the expression of HLA-DR increased compared with day 0. This increase was similar in both concentrations of the Fast method and was higher for the Fast method compared with the Conventional method, although the difference was not significant (Figure 11A).

In the maturation phase, there were differences in HLA-DR expression between the tested differentiation concentrations and methods, as can be seen in Figure 11B. The Standard, Alpha and TLR cocktails in Day 2 [Low] and Day 7 [Medium] gave similar results for %HLA-DR gain. However, when looking at Day 2 [High] the results were more heterogeneous, mainly between the Alpha and TLR cocktails, where the difference was significant. Finally, the STING activating stimulus presented the most marked difference between Fast and Conventional methods, producing the highest %HLA-DR gain in the Fast method (405 and 446%), but the lowest %HLA-DR gain in the Conventional method (170%). This difference between Fast and Conventional MoDCs when the STING activating stimulus

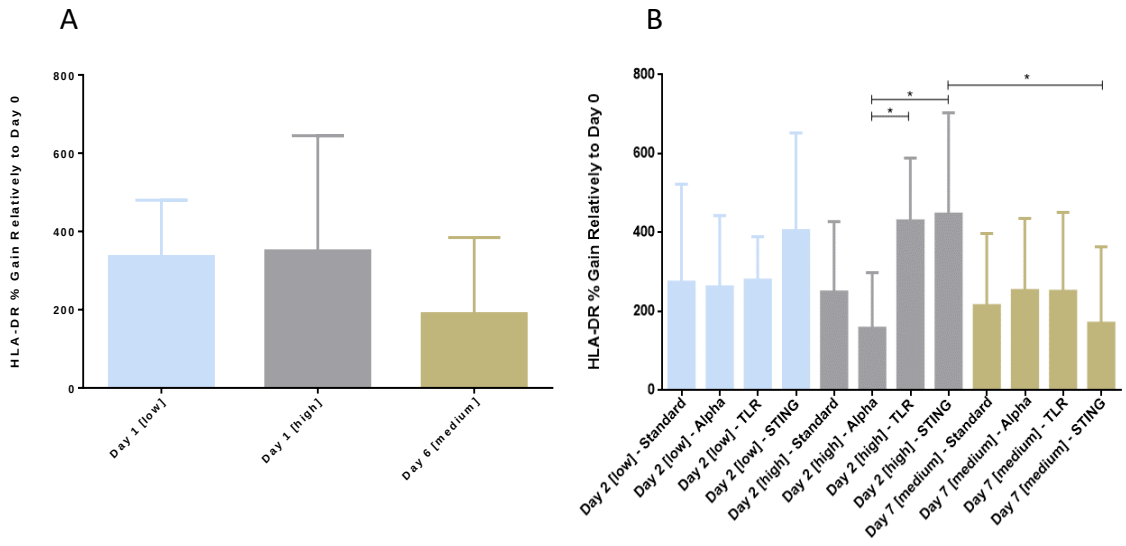


Figure 11. **A** - Percentage of HLA-DR MFI gain, relatively to day 0, of the cells after differentiation, in both Fast (day 1) and Conventional (day 6) method. **B -** Percentage of HLA-DR MFI gain, relatively to day 0, of the cells after maturation, in both Fast (day 2) and Conventional (day 7) method, with Standard, Alpha, TLR cocktails and STING stimulus.

was used could probably relate to the fact that in the Fast method the cells were more similar to monocytes than the ones differentiated by the Conventional protocol. The machinery that responds to 2'-3'-c-di-AM(PS)2 (Rp, Rp), the activator of the cyclic GMP-AMP synthase-stimulator of interferon genes (cGAS-STING) may be more expressed/effective in monocytes. Overall, all the cocktails/stimulus led to the upregulation of the MHC-II that will present the AGs to $CD4^+$ T cells that in turn will polarize into their different effectors (Th1, Th2 and Th17)[66] or regulatory subpopulations (Treg, Tr1 and Th3)[67], depending on the cytokines present on the environment .

III.2.3.2. HLA-ABC

MHC-I (HLA-ABC) is a complex present on all nucleated cells, including DCs[181]. On DCs the MHC-I pathway is used to load and present AGs resulting from intracellular proteins to $CD8^+$ T cells. Besides this canonical pathway , DCs present the exquisite capacity to cross-present extracellular AGs to $CD8^+$ T cells via MHC-I complexes[46,47]. The results of the percentage of HLA-ABC gain, compared with day 0, are displayed in Figure 12. Regarding the differentiation phase (days 1 and 6), an increase in HLA-ABC expression was observed

when compared with day 0 monocytes (Figure 12A). The greater increase was observed in the Conventional method (128%), followed by the Fast method with [Low] (117%), and the Fast method with [High] (78%). However, these differences were not significant. Overall, in terms of differentiation, the expression of HLA-ABC in cells obtained by the Fast method was very similar to that obtained by the Conventional protocol.

Regarding the effect of maturation on HLA-ABC expression, the values oscillate a lot and no statistically significant differences were found (Figure 12B). Still, a trend is observed for increased HLA-ABC expression in cells obtained through the Conventional method with the Alpha cocktail, or through the Fast method with the TRL cocktail. The upregulation of this marker is important for the immunogenic response of DCs, because it is through MHC-I that AGs are presented to $CD8^+$ T cells. The presentation of AGs by DCs to $CD8^+$ T cells in the presence of the cytokines IL-12p70, IL-15 and IFN-α/β leads to their differentiation into antigen-specific CTLs and memory $CD8^+$ T cells, that are crucial in the establishment of antitumor immunity [68–70]. So, for the purpose of immunity against tumors, based on HLA-ABC expression, the best conditions to produce MoDCs appear to be the Conventional method combined with the Alpha cocktail, or the Fast method combined with the TRL

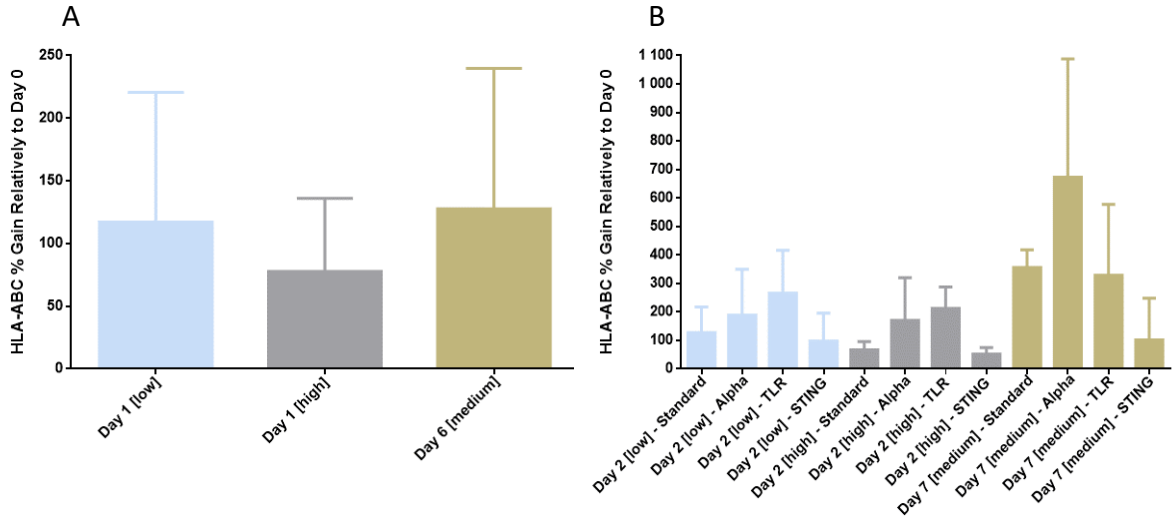


Figure 12. **A** - Percentage of HLA-ABC MFI gain, relatively to day 0, of the cells after differentiation, in both Fast (day 1) and Conventional (day 6) methods. **B -** Percentage of HLA-ABC MFI gain, relatively to day 0, of the cells after maturation, in both Fast (day 2) and Conventional (day 7) method, with Standard, Alpha, TLR cocktails and STING stimulus.

cocktail. However, this analysis needs to be complemented with the quantification of the cytokines, to be addressed in Chapter 4.

III.2.4. CD80 and CD86

The maturation of DCs involves functional changes and is accompanied by profound phenotypic modifications, namely increased expression of costimulatory molecules such as CD40, CD54, CD80, CD83, and CD86[50]. So, these markers can be used to address the effectiveness of the maturation stimuli. In present work, the markers chosen to evaluate DC maturation were CD80 and CD86.

Figure 13 shows the percentage of CD80 gain relatively to day 0. It is possible to observe that the results obtained for the Fast method were very similar between [Low] and [High], with no significant difference between them. Also, the increase of CD80 was much higher in Conventional MoDCs than in Fast MoDCs. Among maturation conditions, the highest expression was obtained with the TLR cocktail (≈44000% in both concentrations), followed by the Alpha (≈27000% and ≈26000%) and the Standard cocktail (≈24000% in both concentrations), with the STING activating stimulus presenting the lowest values. To confirm that there were significant variations upon maturation induction, CD80 expression on days 1/6 was compared with the results obtained for days 2/7. Differences were found for all maturation conditions, except for the STING activating stimulus. A non-significant trend for CD80 increase was observed from day 1 to day 2 MoDCs incubated with STING and almost no difference was seen between day 6 and day 7 MoDCs, suggesting that this stimulus was not very effective in MoDCs maturation. Concluding, when using the Fast method, the TLR cocktail led to the most “effective” maturation status, as seen by higher upregulation of CD80, with expression levels closest to the Conventional method using the Standard cocktail (reference for mature MoDCs). In the Conventional method the more “effective” maturation cocktails were Alpha and TLR agonists.

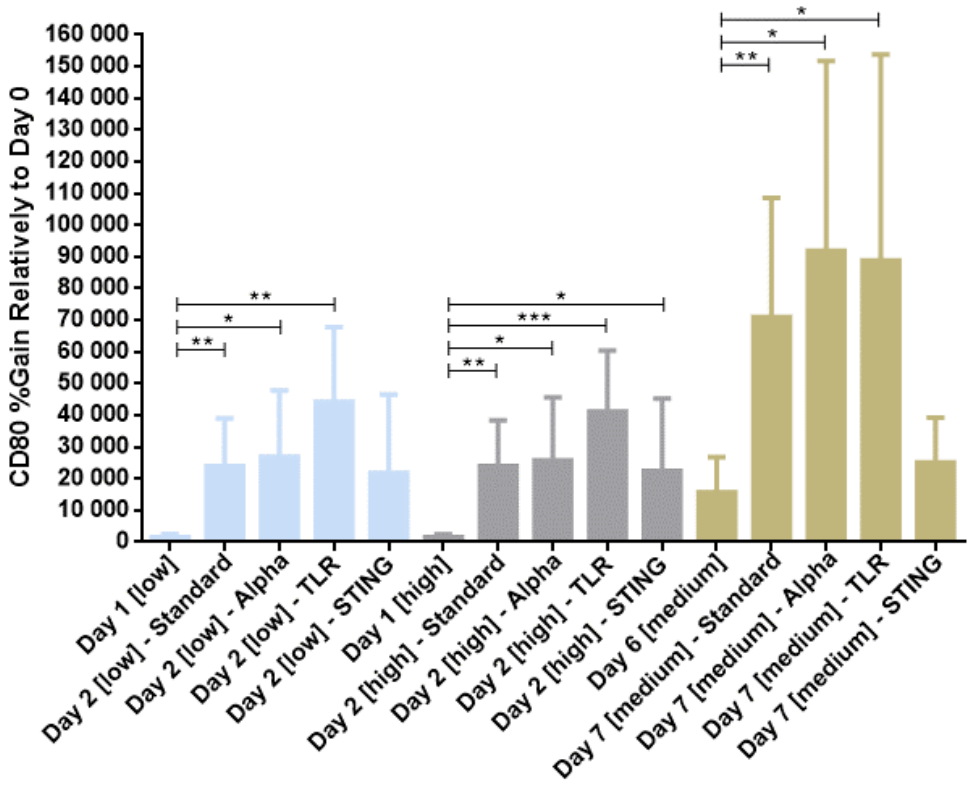


Figure 13. Percentage of CD80 MFI gain, relatively to day 0, after differentiation (day 1 and 6) and after the different maturation cocktails/stimulus (day 2 and 7): Standard, Alpha, TLR and STING, in the Fast and Conventional method.

CD86 is another marker that can be used to verify the maturation status of MoDCs. The results obtained for this marker are depicted in Figure 14. As expected, CD86 expression generally increased with maturation. Within Fast MoDCs, the results were similar for [Low] and [High], and between maturation cocktails/stimulus. Within Conventional MoDCs, the STING stimulus did not induce CD86 expression.

Taking in account the results from the two maturation markers described above, we can conclude that the STING activating stimulus is not effective in inducing maturation of Conventional MoDCs. The Alpha and TLR cocktails, on the other side, were the ones inducing the highest upregulation of CD80 and CD86 markers, in both Fast and Conventional MoDCs, so they are good candidates for an efficient maturation of MoDCs. However, it should also be noticed that CD80 and CD86 levels were lower in mature Fast MoDCs than in Conventional MoDCs (taking Day 7 [Medium] – Standard as reference), thus indicating

that these DCs may be limited in providing co-stimulatory stimulus during AG presentation to T cells.

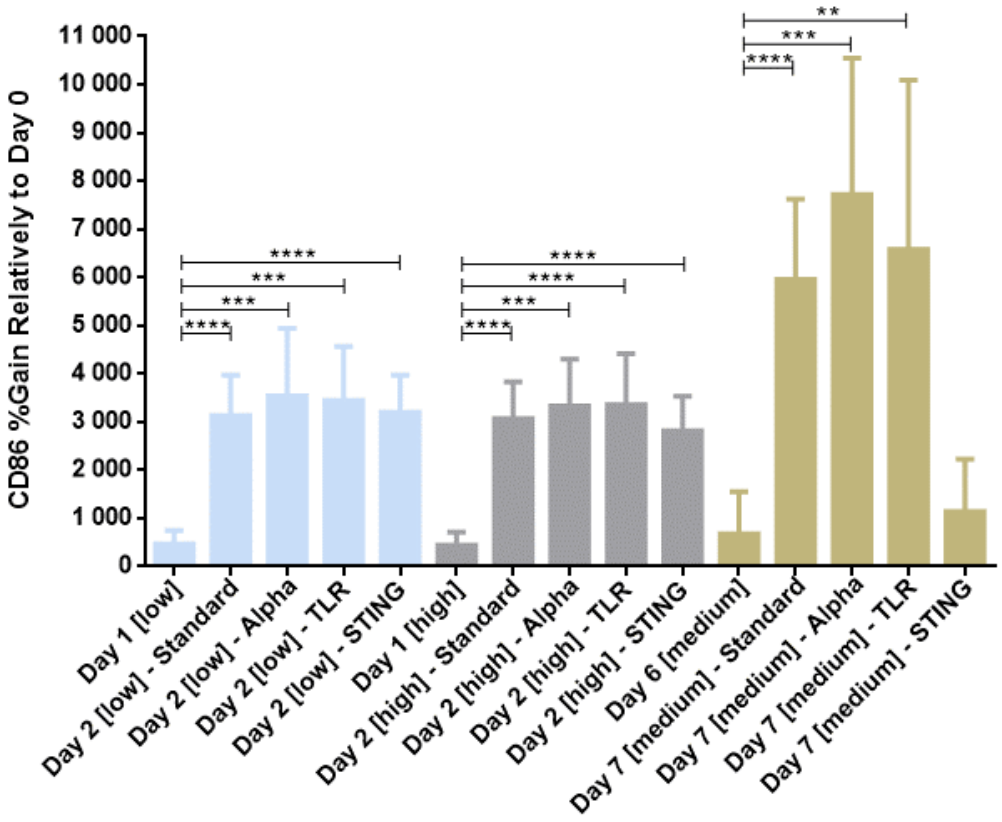


Figure 14. Percentage of CD86 MFI gain, relatively to day 0, after differentiation (day 1 and 6) and after the different maturation cocktails/stimulus (day 2 and 7): Standard, Alpha, TLR and STING, in the Fast and Conventional method.

III.2.5. CD197(CCR7)

During maturation, cells increase the expression of the chemokine receptors CXCR4 and CCR7. The expression of CCR7 makes DCs responsive to lymphoid chemokines CCL19 and CCL21, which allows them to migrate to lymphoid organs and to interact with T cells[58].The gain of CCR7 , also known as CD197, relatively to day 0 is represented in Figure 15. In the Fast method, the Standard and Alpha cocktails gave similar results between them and between [Low] and [High]. The TLR cocktail caused the highest increase in CD197, in both concentrations. The STING activating stimulus deserves special attention, because this

stimulus alone led to values closer to the ones obtained with the TLR cocktail. Once the STING activating stimulus (2'-3'-c-di-AM(PS)2 (Rp, Rp)) was part of the TLR cocktail, it might be the main responsible for the high values observed with this cocktail. Looking at the results obtained for the Conventional method, CD197 expression was higher than in the Fast method. It should however be noted that, contrarily to the observed in Fast MoDCs, CD197 expression was already high in immature Conventional MoDCs and did not increase upon maturation. Moreover, there were no significant differences between maturation conditions, although a trend for higher expression was noted for the STING stimulus.

From this data, we conclude that, using the Fast differentiation method, the TLR cocktail provided the best results, probably due to the influence of 2'-3'-c-di-AM(PS)2 (Rp, Rp), which seems to positively upregulate the CCR7 receptor. This upregulation may be due to the production of type I IFN by the cells matured with the STING stimulus[182], which acting

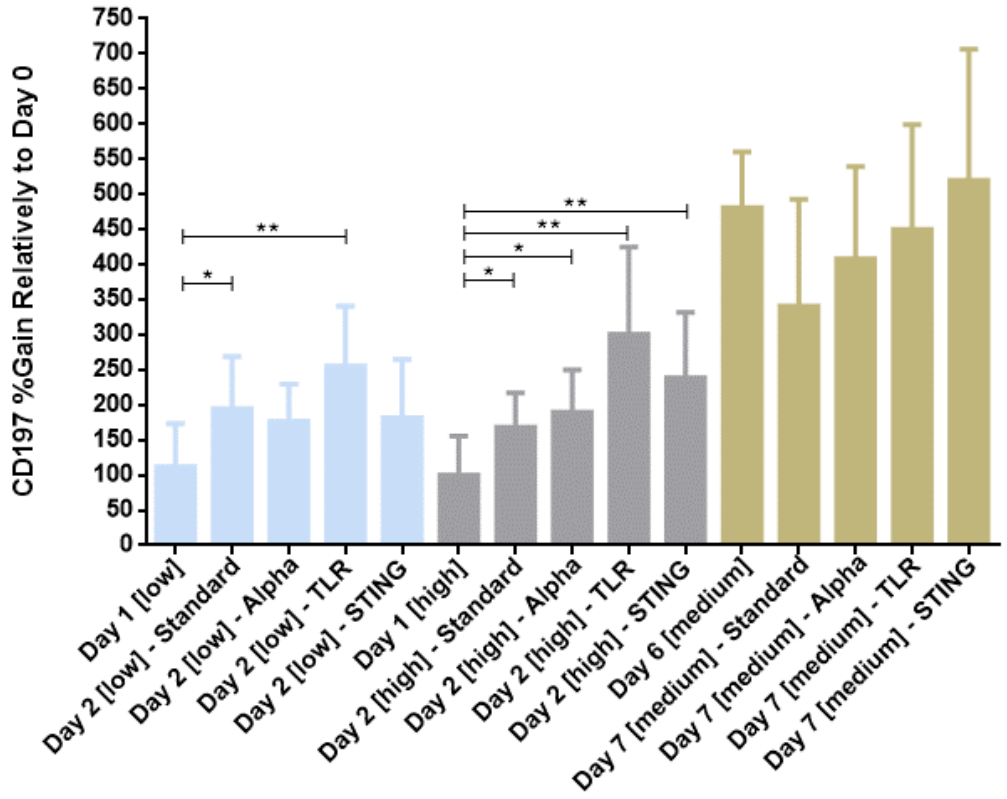


*Figure 15. P*ercentage of CD197 MFI gain, relatively to day 0, of the cells after maturation, in both Fast (day 2) and Conventional (day 7) method, with Standard, Alpha, TLR cocktails and STING stimulus.

autocrinally upregulates CCR7. This hypothesis is supported by previous works showing that the type I IFN leads to the increase expression of CCR7 on DCs[183].

Taking in account all the immunophenotypic data described in the previous sections, several conclusions can be drawn. First, the concentration of IL-4 and GM-CSF didn´t substantially affect the results of the Fast method, since [Low] and [High] produced similar levels of almost all markers, with exception for CD197, where [High] caused slightly higher levels than [Low]. However, [High] was disadvantageous for the HLA-ABC marker. Hence, given that [Low] gave similar or better results for almost all markers, compared to [High], only [Low] was analyzed for cytokine quantification and metabolomic analysis. Another important consideration is that with the Fast method it was possible to obtain an immature ($CD14^{low/-}$, $CD11c^{high}$, $HLA\text{-}DR^{high}$, $HLA\text{-}ABC^{high}$, $CD80^{-}$, $CD86^{-}$ and $CD197(CCR7)^{-}$) and a mature MoDC immunophenotype ($CD14^{-}$, $CD11c^{high}$, $HLA\text{-}DR^{high}$, $HLA\text{-}ABC^{high}$, $CD80^{+}$, $CD86^{+}$ and $CD197(CCR7)^{+}$). Comparing the data from the differentiation processes, we can conclude that, while there were quantitative differences between cells obtained with the Fast and Conventional protocols, notably, 1 day in culture appears to be sufficient to induce an immature DC phenotype. Regarding the maturation process, the STING activating stimulus appears to have a limited potential to mature Conventional MoDCs, downregulating the HLA-ABC marker while increasing CD11c and CD197 markers. In general, for the Fast method, the TLR cocktail was the one that worked better, while in the Conventional method it was the Alpha cocktail. However, all these immunophenotype results need to be complemented with data on cytokine production and functional assays such as uptake capacity.

III.2.6. Cellular complexity

Differentiation of monocytes into DCs is expected to be accompanied by an increase in internal complexity. This information can be obtained through the side scatter (SSC) parameter acquired by flow cytometry. All MoDCs in this work, both in the immature state and after maturation, showed higher complexity than the monocytes of day 0 (Figure 16). The results from the Fast method were very similar between [Low] and [High], and didn't

fluctuate much between the different cocktails/stimulus, with the highest value of MFI occurring in the STING activating stimulus (63587 and 67726) and the lowest in the Standard cocktail (55926 and 56597). In the Conventional method, the cells obtained were more complex than in the Fast method, both in differentiation and maturation phases. In the maturation phase, the Alpha cocktail gave the highest MFI (144314), while the STING

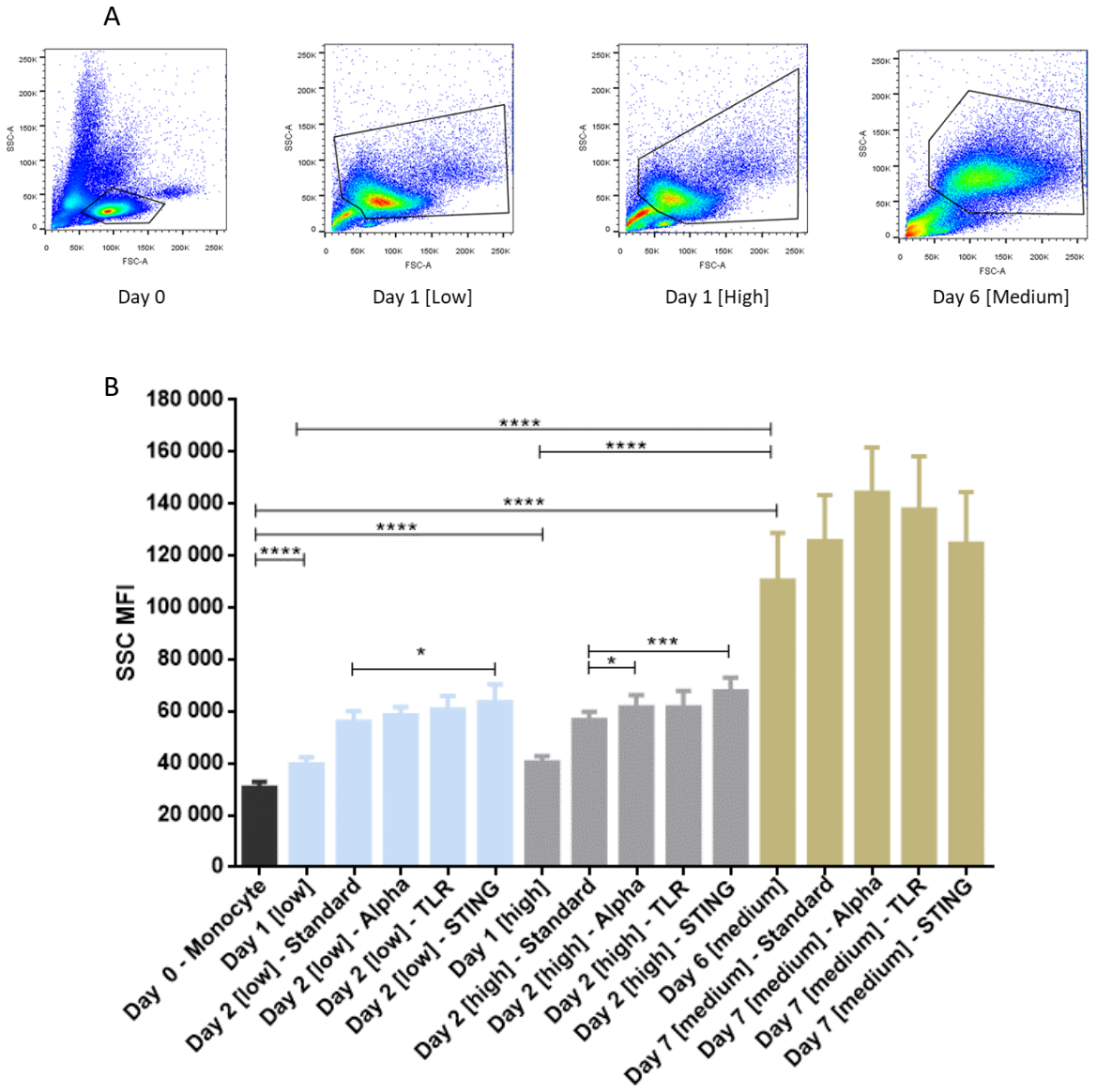


Figure 16. **A -** Dot Plot of Side Scatter (SSC) MFI through the differentiation days, from day 0 to day 6, and **B** - Graphic representation of SSC MFI through the days, from day 0 to day 7. On day 0 is the SSC of the monocytes, on day 1 and 6 of immature MoDCs, and on day 2 and 7 of mature DCs. The SSC the parameter that gives information about the complexity of the cells, in flow cytometry, and the parameter is directly proportional to cell complexity.

activating stimulus was the one with the lowest (124625). So, the Conventional method led to cells with a higher internal complexity, probably resulting from higher abundance of lysosomes, endosomes and/or organelles such as mitochondria.

III.3. Uptake Capacity

The capacity to take up and process AGs for presentation on MHC molecules is a hallmark of immature DCs that is rapidly lost upon maturation[54]. To assess the uptake capacity of MoDCs, immature cells differentiated from 8 donors by the Fast or Conventional method (1 and 6 days, respectively), were incubated with FITC-conjugated Dextran, and then analyzed by flow cytometry. The capacity of differentiated DCs to engulf apoptotic/necrotic cells was also addressed using two tumor cell lines RKO and HCT. These cell lines were marked with CFSE, and then heated at 60˚C for 30 minutes to induce necrosis. After that, each cell line was incubated with immature MoDCs, from the Fast and Conventional method, and the uptake capacity was assessed through flow cytometry. The results of those experiments are described in the next subchapters.

III.3.1. Dextran uptake

Immature MoDCs were incubated with the FITC-Dextran at 37˚C, and to assess the unspecific FITC-Dextran binding to the cell surface, the cells were also incubated at 4°C. Trough flow cytometry, SSC and CD14 were used to identify MoDCs, and then the FITC-Dextran MFI of that gate was acquired, at both 37˚C and 4˚C. The MFI value obtained at 4˚C was subtracted from the value obtained at 37˚C, to exclude unspecific binding, and these results are presented in Figure 17. The results showed that cells differentiated by both methods have endocytic capacity, and hence are expected to have the ability to take up antigens. Comparing the two methods, the Conventional MoDCs presented a higher uptake capacity, with an MFI 84% higher than Day 1 [Low] and 79% higher than Day 1 [High]. These results also showed a small (5%) difference between [Low] and [High], corroborating the immunophenotype results.

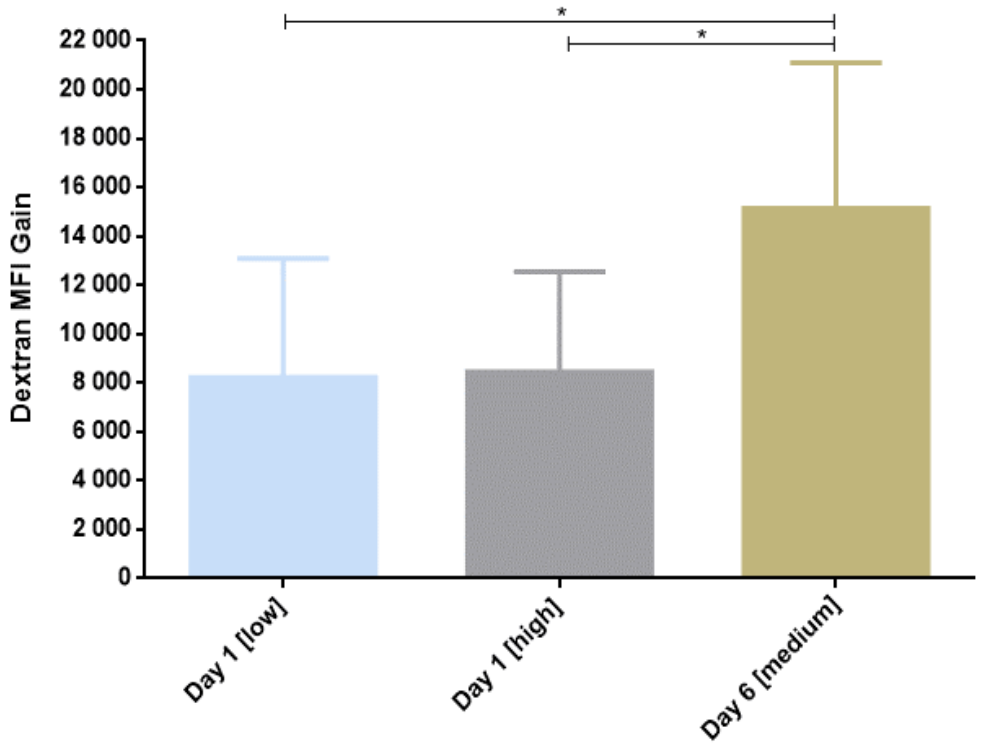


Figure 17. Capacity of MoDCs to capture FITC-Dextran particles

III.3.2. Internalization of apoptotic/necrotic cells

The capacity of MoDCs to engulf apoptotic/necrotic cells was also addressed. For that, RKO and HCT tumor cells were first stained with CFSE and then cell death was induced by heating at 60˚C. Dead cells were then incubated with immature MoDCs, from both methods, at 37˚C. After incubation the cells were labeled with anti-HLA-DR, anti-CD45, anti-CD11c and anti-CD14 that helped in the identification of MoDCs, by flow cytometry. To exclude unspecific binding, the MoDCs were also incubated with dead cells at 4˚C, and the value of CFSE MFI resulting from this unspecific binding was subtracted from the results obtained at 37˚C. As it can be observed in Figure 18, there wasn't a significative difference between [Low] and [High] used in the Fast method. However, Conventional MoDCs had a higher uptake capacity than Fast MoDCs, which was particularly noticeable for the engulfment of HCT cells. Although RKO and HCT were originated from colorectal carcinoma, they harbor different genetic backgrounds and cell surface molecules which can lead to

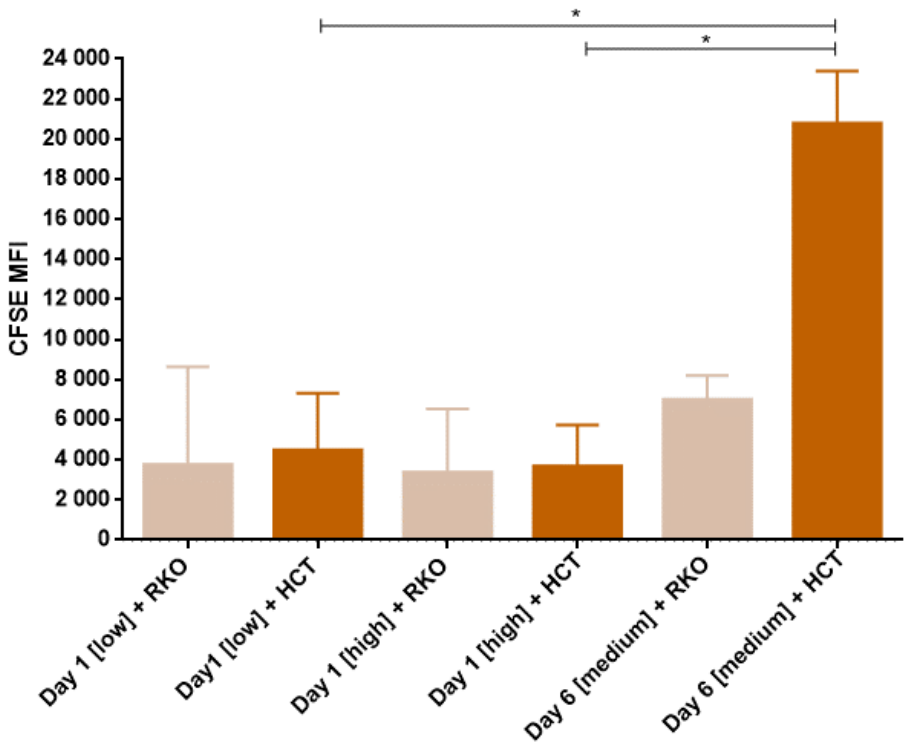


Figure 18. Capacity of MoDCs to capture RKO and HCT cell lines, stained with CFSE and subjected to cell death.

differences in their uptake by MoDCs[184]. Given that these results were obtained from only two samples, more experiments are needed to confirm the difference in the uptake of the two cells lines.

Overall, both differentiation methods produced cells with capacity to take up Dextran and apoptotic/necrotic cells. However, Conventional MoDCs presented a higher uptake capacity than Fast DCs.

III.4. Cytokines Quantification – IL-10 and IL-12p70

Cytokines play an important role in the modulation of the immune system. The cytokines expression profile is highly dependent on the type of DC and on the stimulus that triggers the maturation process, being of major relevance for the polarization of T cells into different subsets. The cytokines IL-12p70 and IL-10 are important to predict which type of immune response will be produced by the immune system. IL-10 is markedly anti-

inflammatory and involved in immunosuppressive responses, being associated with tumors escape[90,122]. On the other hand, IL-12p70 is proinflammatory and associated with the activation of T-cell subsets that are important in the establishment of immunity against tumors[68–70]. These cytokines were quantified to assess the kind of immune response that MoDCs obtained through different methods would induce. Quantification in all the 8 samples was performed using the cytometric bead array (CBA) technique. Since [Low] and [High] from Fast method had similar results in the previous experiments, cytokines were only quantified for the [Low] Fast method, in comparison with the Conventional method. For a better analysis, each maturation cocktail will be analyzed individually in the next subchapters.

III.4.1. Standard Cocktail

The Standard cocktail is constituted by TNF-α, IL-1β, PGE_2 and IL-6, and it is the cocktail most frequently used for MoDCs maturation. However, it is already known that the use of PGE_2 in this cocktail is paradoxical, because it improves DC migratory capacities by inducing CCR7 expression, but on the other hand, it reduces IL-12p70 production [146,147]. Therefore, as expected, the concentration of IL-12p70 was low (0.17 and 5.76 pg/mL) in both methods (Figure 19). The values for IL-12p70 were inferior to IL-10 (14.87 and 84.33 pg/mL) which could indicate that these DCs will have limited immunostimulatory abilities.

III.4.2. Alpha Cocktail

The Alpha cocktail is similar to the Standard cocktail, but instead of PGE_2 and IL-6, reported by some authors to have no effect on DCs maturation, this cocktail has Poly I:C and IFN-γ[176]. The objective of using this cocktail was to overcome the lack of IL-12p70 secretion by conventionally differentiated cells while maintaining the same maturation phenotype. The results presented in Figure 20 show that the production of IL-12p70 (107.28 and 747.07 pg/mL) with the Alpha cocktail was indeed much higher than with the Standard cocktail. Comparing the Fast with the Conventional method, the average of IL-12p70 was higher in the Conventional method, but the results were also more heterogeneous. IL-10

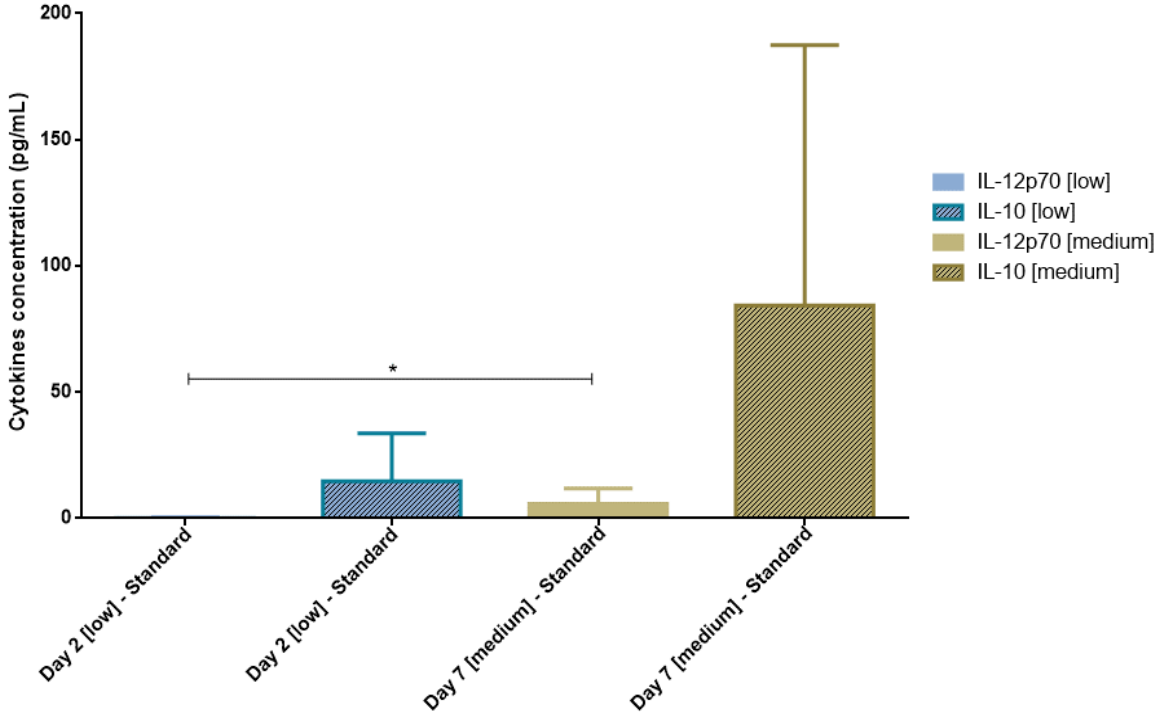


Figure 19. Concentration of IL-12p70 and IL-10 (pg/mL) obtained using the Standard cocktail, in both Fast and Conventional method.

levels were very consistent between samples and much lower (21.86 and 20.14 pg/mL) than IL-12p70, in both methods. The ratio between IL-12p70 and IL-10 let to anticipate that the DCs maturated by this cocktail will produce an immunogenic response, in both methods, as intended.

III.4.3. TLR Cocktail

The TLR cocktail consists of two TLRs agonists, Poly I:C (TLR3) and LPS (TLR4), and of an activator of the cGAS-STING pathway (2'-3'-c-di-AM(PS)2(Rp, Rp)). To our knowledge, this cocktail hasn´t been tested before to mature MoDCs, and the motivation to use it in this work was to assess its potential to induce MoDCs maturation and an immunogenic response. Poly I:C was used because it showed good results in previous maturation cocktails, by having a similar immunophenotype to Standard DCs and a cytokine profile associated with an immunogenic response[176,185]. LPS was used because it showed to be important for cytokine production and allogenic activation of T cells when using the

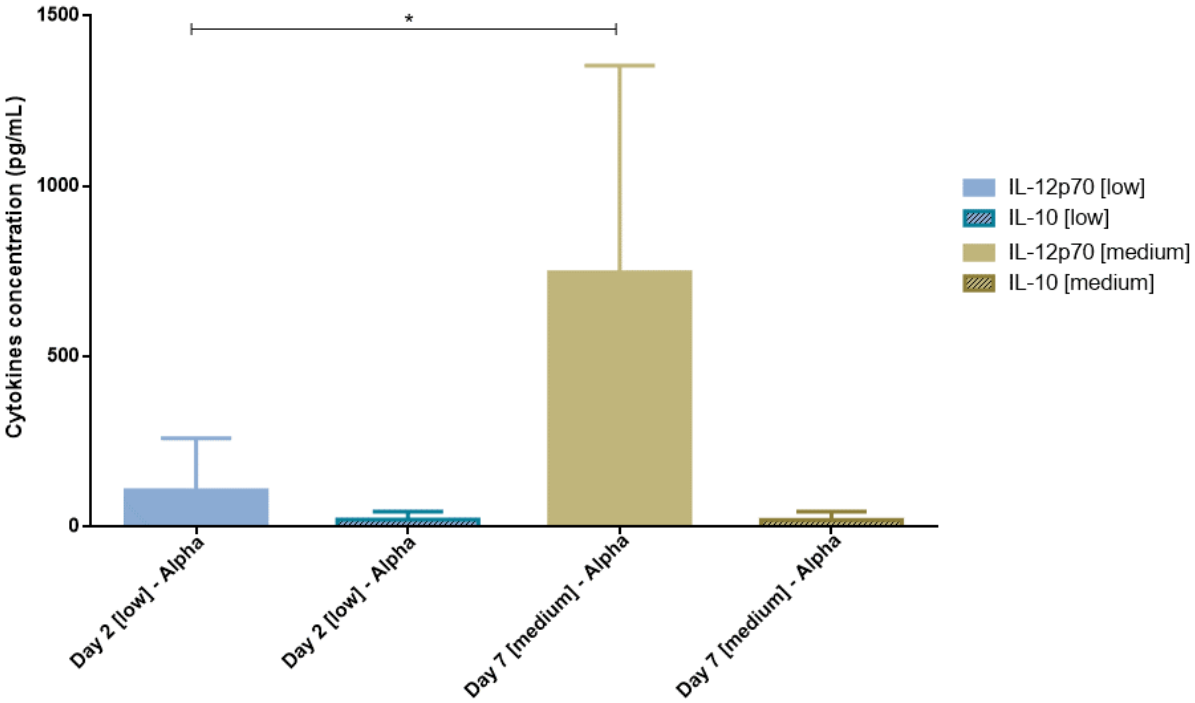


Figure 20. Concentration of IL-12p70 and IL-10 (pg/mL) obtained using the Alpha cocktail, in both Fast and Conventional method.

Conventional method[185]. The 2'-3'-c-di-AM(PS)2 (Rp, Rp) was never used to mature DCs and the aim was to see if the cGAS-STING pathway has any impact on MoDCs maturation when used with TLRs agonists (Poly I:C and LPS). The results demonstrated that this cocktail had different cytokine profiles depending on the method used, as shown in Figure 21. In the Fast method, the concentration of IL-10 (895.45 pg/mL) was higher than IL-12p70 (335.50 pg/mL), leading to an immunosuppressive profile. On the other hand, in the Conventional method, the concentration of IL-12p70 (1170.80 pg/mL) was higher than IL-10 (345.20 pg/mL), leading to an immunogenic profile. In the Alpha cocktail, where Poly I:C was also used, this different profile between Fast and Conventional method was not seen, so this effect could be due to the lack of IFN-γ (used in the Alpha cocktail but not in the TLR cocktail), or due to the use of LPS. Promising results were previously reported for LPS in MoDCs differentiated over 48h or more, and in some cases used in conjunction with IFN-γ[185,186]. But, in our case, where the cells were differentiated for 24 hours only (Fast method), that didn't occur. This might be because cells could still be more similar to monocytes than

to DCs, and in the case of monocytes, incubation with LPS leads to high levels of IL-10[187]. The contribution of 2'-3'-c-di-AM(PS)2(Rp, Rp) to the production of IL-12p70 and IL-10 appears to be very low (Figure 22). In the Conventional method, where IL-12p70 concentration was higher than IL-10, LPS had a different effect. After 6 days of differentiation, most cells in culture are for sure MoDCs, and, in combination with the Poly I:C, LPS leads to a much higher production of IL-12p70 than the other maturation cocktails. The IL-10 concentration resulting from this method was lower than the IL-12p70, but still had a high concentration when compared with other maturation cocktails, which might be the effect of LPS, since that didn't happen using the Poly I:C (in the Alpha cocktail), nor with the STING activating stimulus. So, LPS had this dual effect of increasing IL-12p70 but also IL-10 production. However, it is important to keep in mind that LPS could be problematic when used in vaccines, since remaining LPS in the DC vaccine might induce septic shock. So, some authors stated that to use LPS in vaccines, it is essential that the vaccine is thoroughly

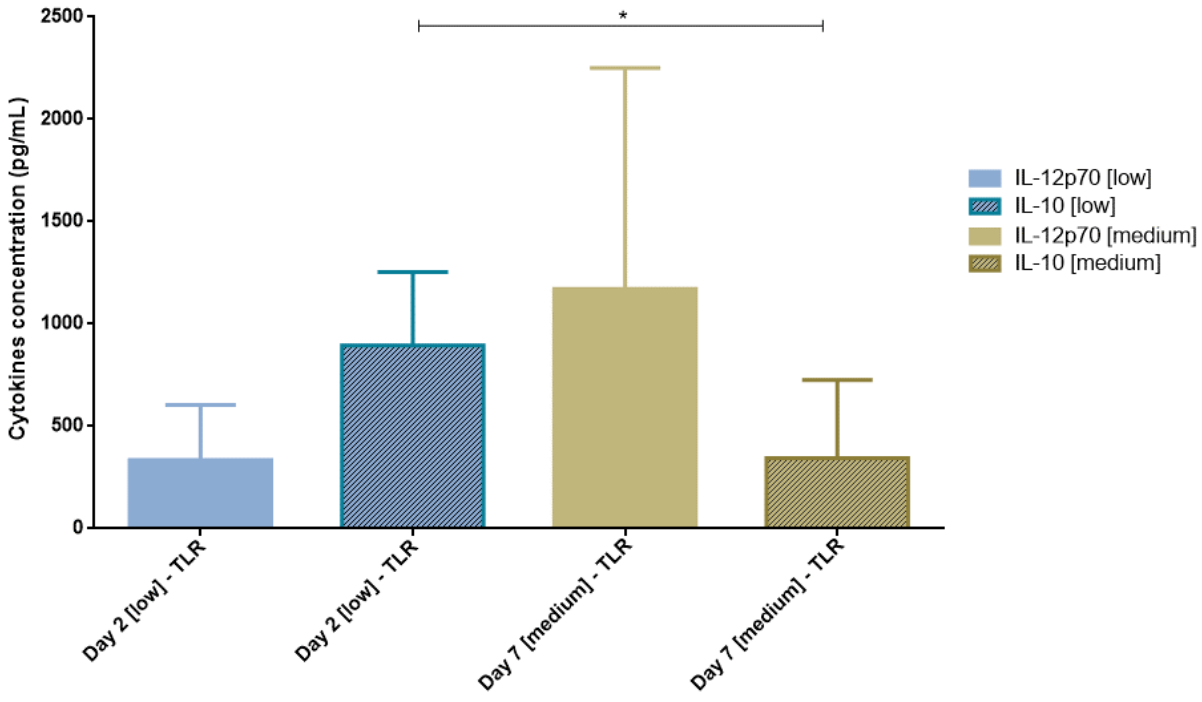


Figure 21. Concentration of IL-12p70 and IL-10 (pg/mL) obtained using the TLR cocktail, in both Fast and Conventional method.

washed and proven to be free of endotoxins by appropriate tests before administration to patients[185].

III.4.4. STING activating stimulus

The STING activating stimulus, as previously mentioned, consists of 2'-3'-c-di-AM(PS)2(Rp, Rp), an activator of the cGAS-STING pathway. The cGAS-STING pathway is known to play an important role in anti-cancer immunity, and it is generally acknowledged that the downstream signals of cGAS-STING, especially type I IFN, bridge innate and adaptive immunity. Type I IFN, induced by the cGAS-STING pathway, promotes the maturation and migration of DCs, enhancing cytotoxic T lymphocyte or NK cell-mediated cytotoxicity effect, and protect effector cells from apoptosis[182]. Due to these characteristics, besides testing its effects in conjunction with Poly I:C and LPS, it was important to test the effect of 2'-3'-c-di-AM(PS)2(Rp, Rp) alone. Briefly, the concentration of the cytokines IL-12p70 and IL-10 in both methods were very low, with a maximum of 8,07 pg/mL of IL-10 in

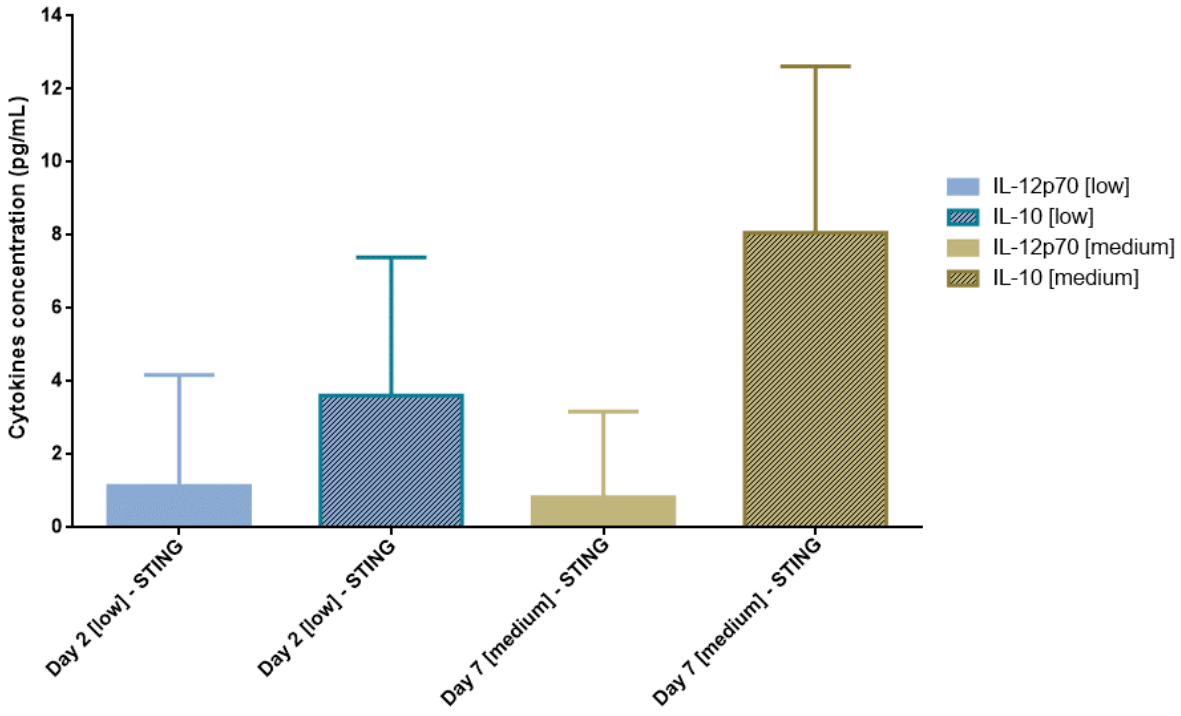


Figure 22. Concentration of IL-12p70 and IL-10 (pg/mL) obtained using the STING activating stimulus, in both Fast and Conventional method.

the Conventional method, as shown in Figure 22. In both methods, the concentration of IL-10 was higher than IL-12p70, but in the Conventional method that difference was more pronounced. These results showed that, even though the concentration of the cytokines was low, the profile of cytokines was immunosuppressive in both methods.

In summary (Figure 23), the Standard cocktail showed limited immunostimulatory abilities, while the Alpha cocktail is expected to induce an immunogenic response, regardless of the differentiation method used. The TLR cocktail had surprising results that were different for the Fast and Conventional methods. When using this cocktail after Fast differentiation, cells remained closer to monocytes than to DCs. This influences the response to LPS and helps explaining why in the Fast method the TLR cocktail led to an immunosuppressive response and in the Conventional method to an immunogenic response. The STING activating stimulus was shown to have a small impact on cytokines production since the concentration of both cytokines was very low, however the profile of

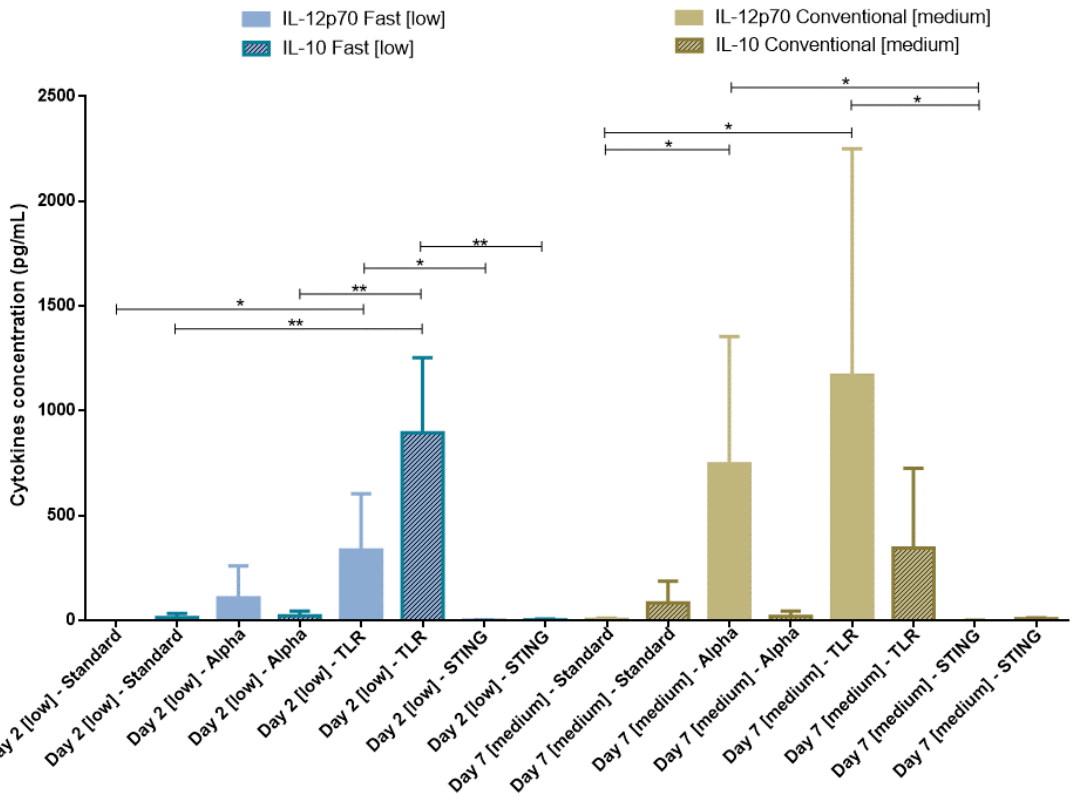


Figure 23. IL-12p70 and IL-10 concentration (pg/mL) in both Fast (day2) and Conventional (day7) method, in the Standard, Alpha, TLR cocktails and STING stimulus.

cytokines produced indicated a possible immunosuppressive response. Of notice, the Conventional method showed more heterogenous results than the Fast method, for all maturation conditions. As a main conclusion from these results, it can be stated that, among the methods tested, those with greater ability to produce immunogenic responses are the Fast method combined with the Alpha cocktail, and the Conventional method used with the Alpha or the TLR cocktails.

III.5. Metabolic profiling of medium samples

In this work, the metabolic activity of MoDCs obtained using multiple differentiation and/or maturation protocols was assessed through ^{1}H NMR analysis of culture medium supernatants (exometabolome). By comparing the NMR-derived metabolic composition of cells-conditioned medium with that of cells-free medium, it was possible to determine the metabolites consumed and excreted by cells, in a 24h incubation period, following either differentiation alone or differentiation and maturation (experimental scheme in Figure 5).

The most abundant metabolites detected in the media spectra were glucose, lactate, formate, acetate and several amino acids, such as valine, leucine and isoleucine (BCAA), glutamine, glutamate, alanine, lysine, asparagine and aspartate (Figure 24). All the metabolites identified were present in all samples, but some of them in different quantities, depending on the time of differentiation and on the maturation cocktail used, as described in the following sections.

III.5.1. Exometabolome of immature MoDCs obtained by Fast *vs.* Conventional differentiation

The effect of differentiation time on the cells exometabolome (extracellular metabolic composition) was assessed by multivariate analysis of ^{1}H-NMR spectra, followed by spectral integration. The PCA and PLS-DA scores scatter plots obtained for the comparison between cells differentiated by Fast (1 day) and Conventional (6 days) methods are presented in Figure 25, left and middle, respectively. The two sample groups showed reasonable separation in the PCA scores scatter plot (Figure 25, left). PLS-DA confirmed such

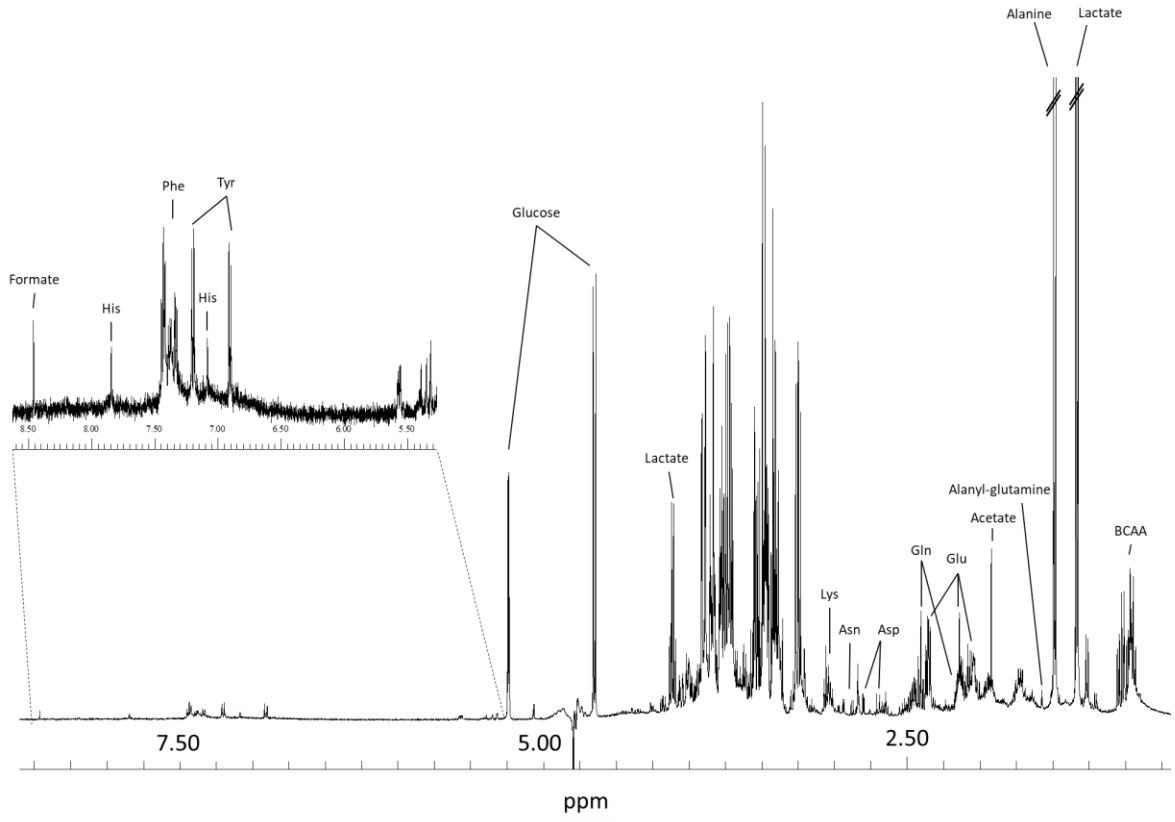


Figure 24. 500 MHz ^{1}H-NMR spectrum of medium supernatant extract after MoDCs maturation, with some metabolites assigned. Tree-letter codes used for amino acids: Asn - Asparagine, Asp - Aspartate, BCAA - Branched chain amino acids, Gln - Glutamine, Glu - Glutamate, His - Histidine, Lys - Lysine, Phe - Phenylalanine, Tyr – Tyrosine.

discrimination, with a Q^2 above 0.50 (Figure 26, middle). Examination of the PLS-DA loadings (Figure 25, right), colored according to variable importance to the projection (VIP), enabled the main metabolites responsible for sample discrimination to be identified. As the Conventional samples were mainly grouped in the positive side of the LV1 axis, positive loadings correspond to metabolites increased in the supernatant of Conventional MoDCs, while negative loadings correspond to metabolites elevated in the supernatant of Fast MoDCs. Hence, based on the loadings profile, the main metabolites suggested to be increased in the exometabolome of Conventional MoDCs were lactate, alanine, acetate, glutamate, aspartate and lysine, while the exometabolome of Fast MoDCs appeared to contain higher glutamine levels.

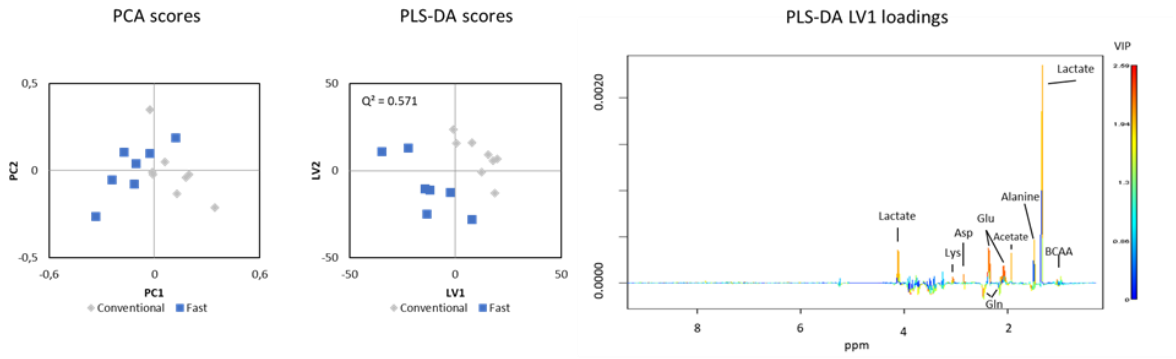


Figure 25. Multivariate analysis of ^{1}H-NMR spectra from the supernatants of MoDCs, which were differentiated through the Fast or Conventional methods, and subsequently incubated for 24h in fresh culture medium. PCA and PLS-DA scores scatter plots (left and middle, respectively) and LV1 loadings (right), coloured according to variable importance to projection (VIP).

For a more detailed analysis, spectral integration was carried out, and the magnitude and statistical significance of each metabolite variation, highlighted in the PLS-DA loadings, were analyzed. The results are presented in Figure 26, for metabolites showing variations above 5% (and smaller relative error) in cell-conditioned media compared with acellular culture media. The main substrate consumed by MoDCs (largest negative variation) was alanyl-glutamine, a cell culture medium supplement used for its higher stability compared to free glutamine[188]. While glutamine may degrade in acellular medium and originate products that are toxic to cells, the alanyl-glutamine peptide is only hydrolyzed in the presence of cell peptidases. The amino acids released (glutamine and alanine) may then enter cells and be used intracellularly. Accordingly, the levels of free glutamine in the cells-conditioned media didn't vary much compared to acellular medium, likely due to its rapid consumption by cells after alanyl-glutamine hydrolysis. Aspartate was also consumed, but to a smaller extent. On the other hand, MoDCs excreted mainly glutamate, formate, lactate, and acetate. Increased extracellular alanine levels could indicate either increased excretion or that alanine was being released from alanyl-glutamate at a higher rate than it was being consumed by cells.

When comparing Fast and Conventional MoDCs (Figure 26, blue and grey bars, respectively), the latter showed a trend for higher decrease in alanyl-glutamine, although the difference did not reach statistical significance. Glutamine also showed a distinct

variation trend, which suggested reduced utilization rate in Fast MoDCs compared to Conventional MoDCs. Moreover, the increases in glutamate, lactate, acetate and alanine, relatively to acellular medium, were significantly higher in the medium of Conventional

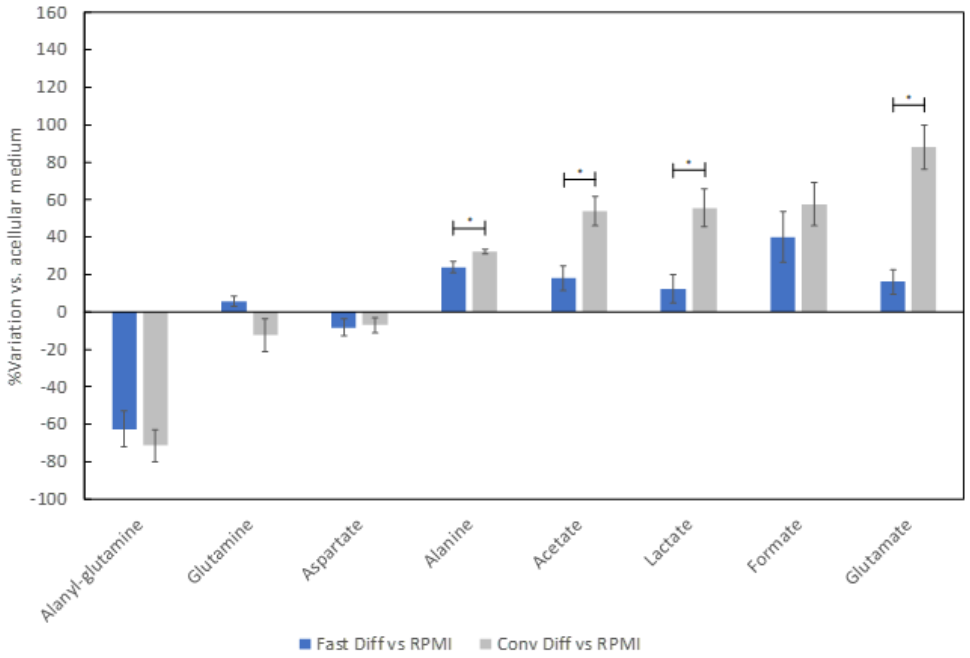


Figure 26. Variations in consumption (negative bars) and excretion (positive bars) of several metabolites in the cell culture supernatant of MoDCs which were differentiated through the Fast or Conventional methods, and subsequently incubated for 24h in fresh culture medium. * $p < 0.05$

MoDCs than in Fast MoDCs-conditioned medium. These results indicate higher excretion of glutamate, lactate and acetate by Conventional MoDCs (during the 24h incubation), while, as explained above, the variation in alanine is not as straightforward to interpret.

III.5.2. Exometabolome of mature MoDCs obtained by maturation after Fast *vs.* Conventional differentiation

The impact of Fast and Conventional differentiation methods on the metabolic activity of MoDCs was also evaluated after their 24h incubation with different maturation cocktails/stimulus, followed by 24h incubation in fresh medium (without maturation stimulus). A similar approach to that described in the previous section (multivariate analysis and spectral integration) was used to compare the different conditions. The PCA and PLS-

DA results are presented in Figure 27. The Fast and Conventional sample groups undergoing Alpha maturation displayed the best separation in PCA scores (Figure 27 b), left), while for the other maturation cocktails, there was some overlap between sample groups. Still, PLS-DA (Figure 27, middle) was able to discriminate between mature MoDCs obtained by Fast

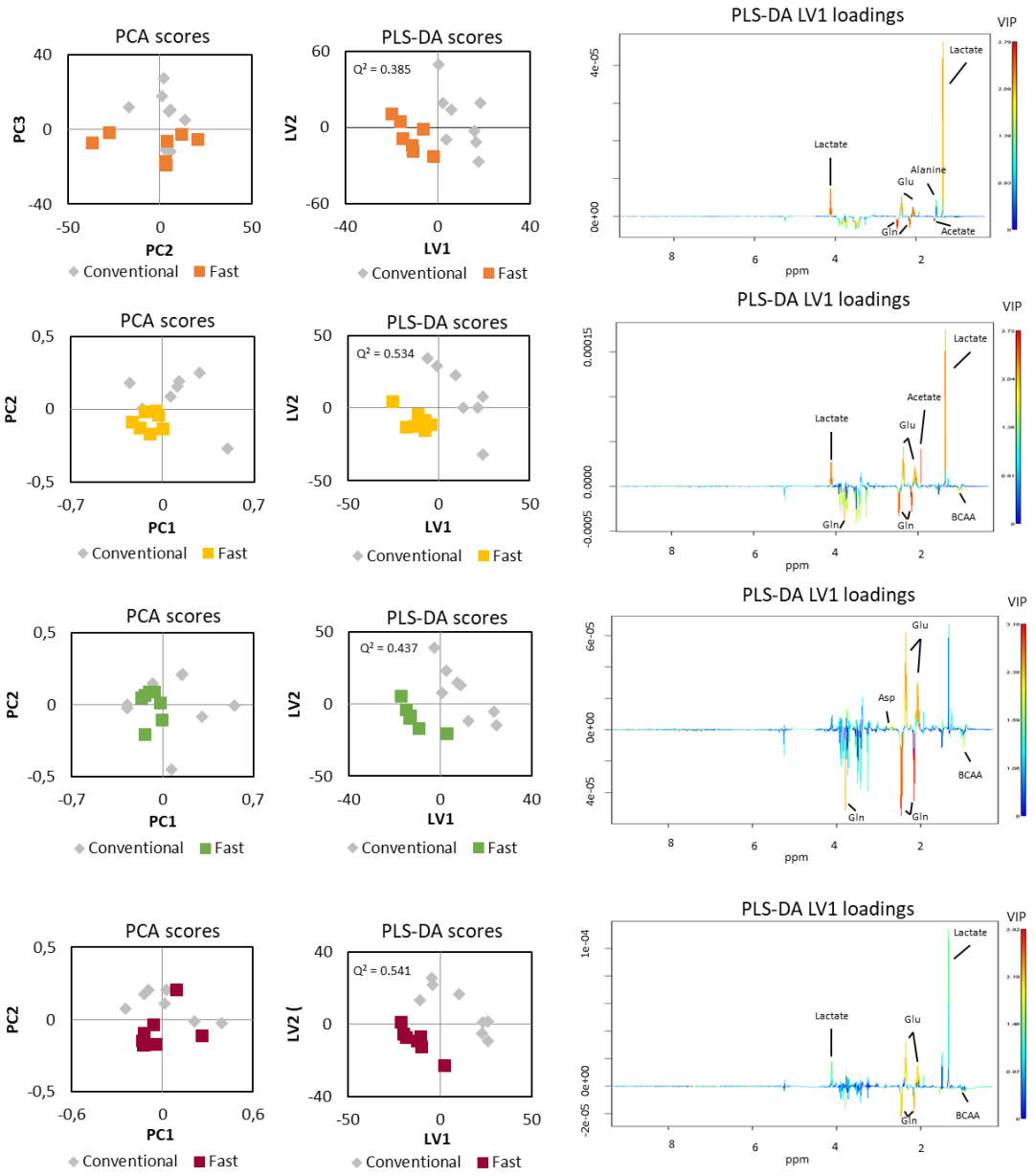


Figure 27. Multivariate analysis of ^{1}H-NMR spectra from the supernatants of MoDCs, which were maturated with a) Standard cocktail, b) Alpha cocktail, c) TLR cocktail and d) STING stimulus, after Fast or Conventional differentiation, and subsequently incubated for 24h in fresh culture medium. PCA and PLS-DA scores scatter plots (left and middle, respectively) and LV1 loadings (right), coloured according to variable importance to projection (VIP).

and Conventional differentiation with reasonable predictive power, especially in the case of Alpha and STING maturations ($Q^2 > 0.5$). The respective loadings (Figure 27, right) highlight the main features responsible for group discrimination, which were then analyzed in more detail through spectral integration.

The metabolite variations greater than 5% in cells-conditioned medium relatively to acellular culture medium are shown in Figure 28. Each graph displays the comparison between Fast and Conventional mature MoDCs for a specific maturation cocktail/stimulus. The trends for higher alanyl-glutamine hydrolysis and/or glutamine consumption, observed in Conventional *vs*. Fast immature cells are maintained upon cell maturation with all the cocktails/stimulus employed. Cells obtained through Standard or TLR maturation also maintained aspartate consumption, especially in the case of Fast differentiation. Additionally, mature cells were seen to consume leucine (all maturation cocktails except STING stimulus) and glucose (Standard maturation only). These additional consumptions were absent or less marked in mature cells obtained after Fast differentiation than in mature cells obtained through Conventional differentiation. Like for immature cells, acetate, formate, lactate and glutamate were the main metabolites excreted by mature MoDCs (positive bars in the graphs of Figure 28). Regarding the comparison between Fast and Conventional MoDCs, after maturation, the excretion of glutamate remained markedly higher in Conventional MoDCs than in Fast MoDCs, while the difference in lactate excretion was found to be significant only for the Standard and Alpha cocktails. Interestingly, in cells that underwent Standard maturation, the lactate increase was twice as much the one observed in immature cells, both after Conventional and Fast differentiation. Indeed, the percentages of variation in extracellular lactate levels, in cells-conditioned medium relative to acellular medium, were 12.4±7.4/55.5±10.1 in Fast/Conventional immature MoDCs and 29.7±4.8/110.9±15.2 in Fast/Conventional mature MoDCs, obtained using the Standard maturation protocol (Supplementary Table S1). As for acetate and formate, the difference between Fast and Conventional mature MoDCs was only significant when the Alpha maturation cocktail was used.

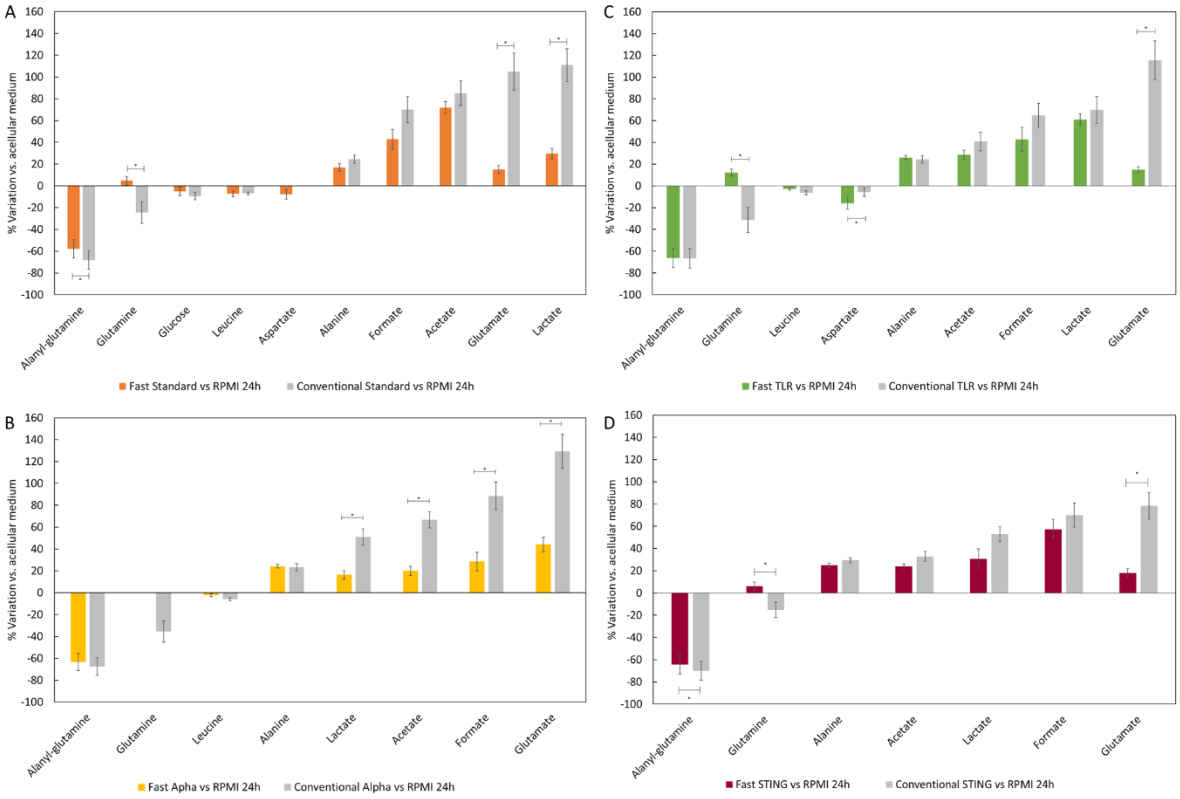


Figure 28. Variations in consumption (negative bars) and excretion (positive bars) of several metabolites in the cell culture supernatants of MoDCs, which were maturated with A) Standard cocktail, B) Alpha cocktail, C) TLR cocktail and d) STING stimulus, after Fast or Conventional differentiation, and subsequently incubated for 24h in fresh culture medium. * $p < 0.05$

III.5.3. Effect of different maturation cocktails/stimulus in the exometabolome of Fast and Conventional MoDCs

The variations induced by the four maturation cocktails/stimulus in the exometabolome of MoDCs were also compared. The results are presented in Figure 29A and 29B for Fast and Conventional MoDCs, respectively.

In the case of mature MoDCs obtained through Fast differentiation (Figure 29A), the main metabolic differences between maturation conditions were: i) Trend for higher hydrolysis of the alanyl-glutamine peptide in cells treated with the TLR cocktail, although the difference was statistical significant only in comparison with the Standard maturation; ii) Higher consumption of aspartate in TLR-treated cells; iii) Slight accumulation of glutamine in the medium of all mature cells, except for those treated with the Alpha cocktail, where

no change reflects fast glutamine uptake after its release in the medium from alanyl-glutamine; iv) Significantly higher lactate excretion in TLR-treated cells; v) Significantly higher glutamate excretion in MoDCs treated with the Alpha cocktail; vi) Higher excretion of formate in cells activated with STING (significant only in comparison to Alpha maturation); vii) Significantly higher excretion of acetate in cells resulting from Standard maturation.

As for cells differentiated through the Conventional method and subsequently treated with the different maturation cocktails/stimulus (Figure 29B), the main differences in their

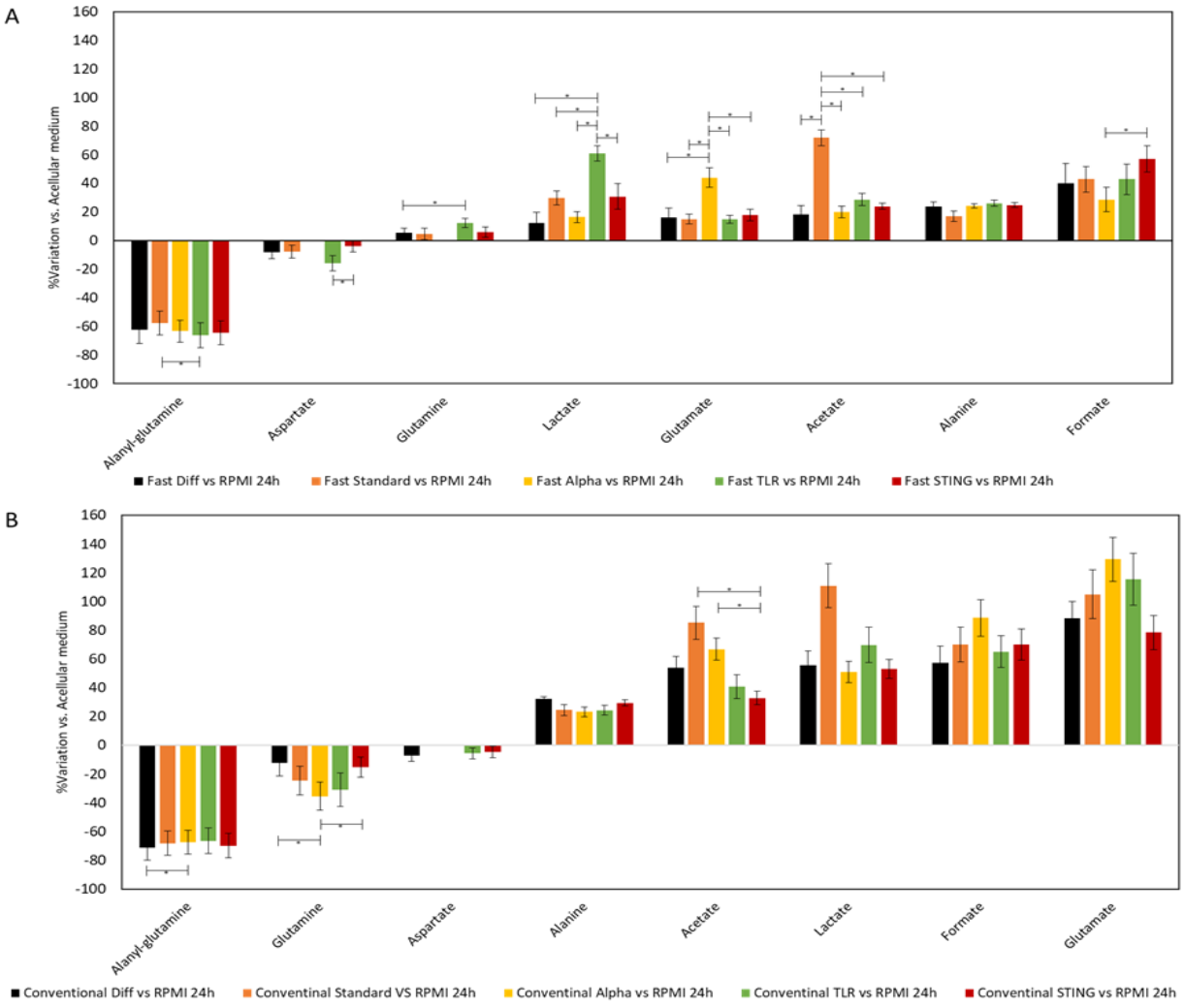


Figure 29. Variations in consumption (negative bars) and excretion (positive bars) of several metabolites in the cell culture supernatants of immature and mature MoDCs, which were maturated with different maturation cocktails/stimulus, and subsequently incubated for 24h in fresh culture medium, for the case of Fast (A) and Conventional (B) differentiation protocols. * $p < 0.05$.

consumption/excretion patterns were: i) Slightly higher glutamine consumption and glutamate excretion by MoDCs treated with Alpha and TLR cocktails; ii) Higher excretion of acetate and lactate by cells obtained through Standard maturation than by cells treated with the other cocktails/stimulus.

Overall, based on extracellular changes, the results suggest that the differences between maturation cocktails/stimulus were attenuated in cells that had been differentiated through the longer, conventional method.

III.5.4. Interpretation of metabolic changes

Untargeted analysis of the exometabolome enabled subtle differences in consumption and excretion patterns to be detected between cells obtained through Fast *vs.* Conventional differentiation and upon maturation with different stimuli. The metabolic differences observed are summarized in Figure 30 and briefly discussed below.

The high decrease of alanyl-glutamine levels in cell-conditioned medium reflects the hydrolysis of this peptide by cell peptidases, which releases glutamine and alanine, that can subsequently enter cells for intracellular use (Figure 30). In the medium of MoDCs obtained through Fast differentiation, there was a small net increase in glutamine levels, indicating that this amino acid was being consumed at a lower rate than it was being released from alanyl-glutamine hydrolysis. On the other hand, the medium of Conventional MoDCs showed depletion of glutamine, indicating its fast uptake and intense use by cells. This effect was even more pronounced in mature MoDCs, especially when the Alpha cocktail was used. Glutamine, processed by cells through glutaminolysis[188], has been reported to be an important substrate for immune cells proliferation, intracellular pathways and cytokines production[189].

Glutamate was excreted by MoDCs obtained in both differentiation methods, although at much higher levels by Conventional MoDCs. Glutamate may arise from glutamine (through the action of glutaminase) and be converted into α-ketoglutarate (by glutamate

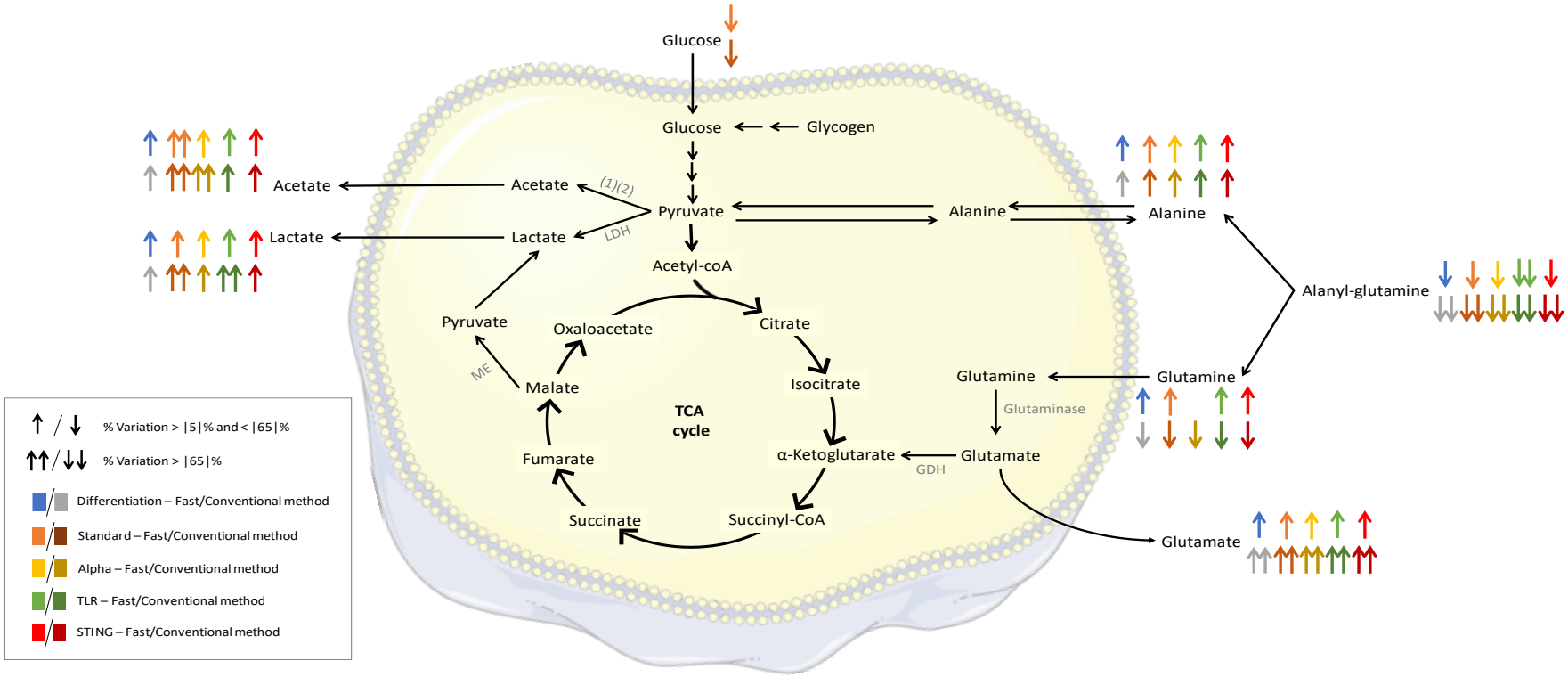


Figure 30. Schematic diagram of main metabolic differences between the MoDCs obtained with Fast and Conventional method. (1) via reactive oxygen species (ROS); (2) via ketoacid dehydrogenases (kDHs)196; GDH - glutamate dehydrogenase; LDH - Lactate dehydrogenase; ME – Malic enzymes.

dehydrogenase) to fuel the TCA cycle (Figure 30). Its excretion may reflect an excess of intracellular levels, beyond the capacity of processing by the TCA cycle. Interestingly, glutamate has been highlighted as an immunomediator in the cross-talk between DC and T cells. A study showed that DC-released glutamate acts during the interaction DC-T cell, impairing IL-6 production and consequently leading to T cell proliferation[190]. After T cell activation, glutamate led to enhancement of Th1 response and proinflammatory cytokine secretion[190]. Hence, higher glutaminolysis and glutamate excretion observed in MoDCs differentiated through the Conventional method and subjected to maturation (especially using the Alpha cocktail) could be linked to a better immunogenic response, when compared with the Fast method.

Glutamate excretion could also be related with the glutamate-cystine antiporter, that participates in the regulation of the *de novo* synthesis of glutathione (GSH), a metabolite with an important role in maintaining DC redox homeostasis and protecting DCs from oxidative stress. The glutamate-cystine antiporter has already been shown to play an important role in regulating DC differentiation from monocyte precursors, and to play a central role in exogenous antigen presentation to both class I and class II MHC[191]. In the future, analysis of intracellular levels of reactive oxygen species (ROS) and glutathione could help further assessing the role of glutamate excretion in MoDCs differentiation and maturation.

In this work, immature MoDCs incubated for 24h in fresh medium did not consume extracellular glucose. As for mature cells, slight extracellular glucose consumption was noticed only when the Standard maturation cocktail was used. This is consistent with a previous study showing that, in the early effector functions of TLR-activated DCs, stored glycogen is used as the main fuel, instead of extracellular glucose[192]. Hence, the observed small variations in medium glucose levels do not necessarily imply low glycolytic activity (conversion of glucose to pyruvate), but that the main glucose source could be glycogen-derived glucose rather than extracellular glucose.

Lactate was another main metabolite excreted by MoDCs. Lactate arises from pyruvate reduction by lactate dehydrogenase (LDH) and its excretion may reflect both glycolysis and

glutaminolysis. The secretion of lactate by immune cells is typical of inflammatory activation[193] and has been reported to occur upon DC activation[194]. In a previous study, the accumulation of lactate in the medium of dense MoDCs cultures was associated with promotion of IL-10 production instead of IL-12, in response to TLR stimuli[195]. Nevertheless, it was recently reported that lactate has an important role as carbon source for $CD8^+$ T cells. Lactate demonstrated to be used as a fuel during $CD8^+$ T cells activation. Moreover, during short term exposure (3 days after activation), lactate promoted the effector phenotype and conditioned the cells to proliferate more extensively[196]. So, while lactate accumulation in the medium during MoDCs maturation could be disadvantageous, leading to IL-10 promotion instead of IL-12, this metabolite is important during the DC-$CD8^+$ T cell interaction. In this work, Conventional MoDCs showed higher lactate excretion than Fast MoDCs, reflecting higher glycolytic and/or glutaminolytic activity. Upon maturation, this difference was maintained, except when the TLR cocktail was used. On the other hand, MoDCs incubated with the Standard cocktail showed a very large increase in lactate excretion, accompanied by consumption of extracellular glucose. These data suggest that Standard mature MoDCs are the most glycolytic among the different cells assessed. Besides that, the higher excretion of lactate by the Standard MoDCs (Conventional method) and by TLR MoDCs (Fast method) might have influenced the cytokine profile presented by these MoDCs, discussed in section III.4.

The excretion of acetate was also found to be more prominent in MoDCs subjected to Standard maturation. Recently, acetate has been described to result from glycolytic pyruvate, especially in conditions of metabolic overflow (when supply of nutrients overtakes metabolic demands)[197]. This is consistent with the high glycolytic/glutaminolytic activity described above. Interestingly, acetate has been reported to be required for mounting an optimal memory CD8 T cell response *in vitro* and in vivo[198].

Another metabolite that was highly excreted by MoDCs was formate. This metabolite may also reflect metabolism overflow, in this case related to the catabolism of serine, when the resulting one-carbon units exceed the cells biosynthetic demands and are excreted as formate, in a process coupled to energy production[199].

Overall, NMR metabolomics has revealed changes in cells consumption and excretion patterns, which reflect the close link between DC metabolism and their differentiation/maturation. The main findings suggested a preferential use of glutamine and, possibly, of glycogen-derived glucose, as well as a significant upregulation of glutaminolysis and/or glycolysis upon longer monocyte to DC differentiation (Conventional method). Additionally, maturation with the Alpha cocktail appeared to stimulate glutaminolysis, while maturation with the Standard cocktail appeared to favor glycolysis.

CHAPTER IV - **Conclusions and Prospects**

In present work, MoDCs obtained through different differentiation protocols and maturation stimuli were analyzed in respect to their immunophenotype, cytokine production, phagocytic capacity and metabolic activity.

Regarding the process of DC differentiation from monocytes, we found that the concentration of IL-4 and GM-CSF did not have a noticeable impact on the Fast differentiation protocol. Moreover, both Conventional (6 days) and Fast (1 day) methods allowed immature DCs ($CD14^{low/-}$, $CD11c^{high}$, $HLA\text{-}DR^{high}$, $HLA\text{-}ABC^{high}$, $CD80^{-}$, $CD86^{-}$ and $CD197(CCR7)^{-}$) to be obtained. After stimulation, these cells acquired a mature immunophenotype ($CD14^{-}$, $CD11c^{high}$, $HLA\text{-}DR^{high}$, $HLA\text{-}ABC^{high}$, $CD80^{+}$, $CD86^{+}$ and $CD197(CCR7)^{+}$). Although the cells differentiated with the Fast protocol presented a lower upregulation of phenotypic markers, the values were not very discrepant, allowing us to conclude that MoDCs could be obtained with only 1 day of differentiation. Regarding the maturation cocktails/stimulus compared, based on the immunophenotype results, the TLR cocktail appeared to work better with the Fast differentiation method, while for Conventional MoDCs it was the Alpha cocktail.

In terms of uptake capacity, cells from both methods demonstrated to effectively phagocyte Dextran particles and apoptotic/necrotic cells, with higher values obtained for Conventional MoDCs.

Regarding the production of IL-12p70 and IL-10, the Standard cocktail showed limited immunostimulatory abilities, while the Alpha cocktail was associated with an immunogenic response, regardless of the differentiation method used. The TLR cocktail produced different results between Fast and Conventional methods. With this maturation cocktail, the cells from Fast differentiation were still closer to monocytes than to DCs, which influences their capacity to respond to LPS. The STING activating stimulus had a marginal impact on maturation and cytokines production, for either Fast or Conventional differentiation. It should also be noticed that, for all maturation cocktails/stimulus, the Conventional method showed more heterogenous results than the Fast method. As the main conclusion from these results, it can be stated that, in order to produce the strongest immunogenic responses, the best maturation cocktail when using the Fast differentiation

method would be the Alpha cocktail, while for Conventional MoDCs the Alpha and TLR cocktails seemed to work better.

The modulation of the cell's metabolic activity upon Fast *vs*. Conventional differentiation and different maturation conditions was also assessed through ^{1}H NMR analysis of medium supernatants. Immature moDCs, collected after differentiation and cultured for additional 24h in fresh medium, consumed mainly glutamine (resulting from alanyl-glutamine hydrolysis) and aspartate, while excreting glutamate, formate, lactate and acetate, as determined by comparison with acellular medium (also incubated for 24h). Interestingly, Conventional moDCs showed intensified glutamine uptake and higher excretion of metabolic products, compared to Fast MoDCs, likely reflecting upregulated glycolysis and glutaminolysis. After maturation, the higher rate of glutamine consumption and of glutamate, formate, lactate and acetate excretion by Conventional MoDCs was maintained. Concerning the influence of the different maturation stimuli on the metabolic activity of mature MoDCs incubated for 24h in fresh medium, the results showed: i) higher rate of glutamine consumption and glutamate excretion for the Alpha cocktail (both differentiation methods); ii) Higher excretion of acetate by cells that had been matured by Standard cocktail (both methods); iii) Consumption of extracellular glucose only by cells that had been maturated by the Standard cocktail (both methods); iv) higher excretion of lactate by Fast TLR-treated cells and by Conventional Standard-treated cells. In general, the metabolic differences between maturation conditions were less pronounced with the Conventional method.

Overall, this work showed that the time of differentiation is more important than the concentration of the differentiation factors, and that MoDCs differentiated by a Fast protocol share multiple features with the ones obtained by the Conventional method. Within the maturation cocktails/stimulus tested, the Alpha and TLR cocktails showed the most promising results in terms of the ability to produce MoDCs with an immunogenic profile. In the future, it would be of interest to increase the period of differentiation from 1 to 2 or 3 days. Finally, it would be also very important to study other functional abilities of Fast MoDCs such as their capacity to activate and polarize T cells.

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Supplementary Information

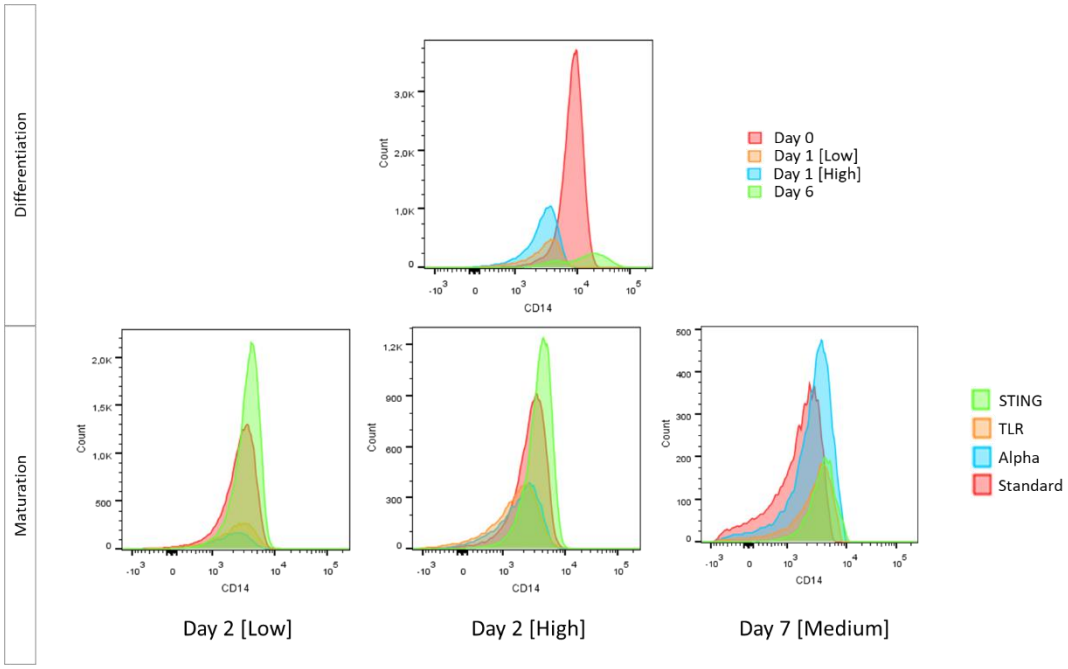

Figure S1. Graphic representation of the CD14 intensity in the samples analysed on day 0, after differentiation (day 1 and 6) and after maturation (day 2 and 7) with the different maturation cocktails/stimulus.

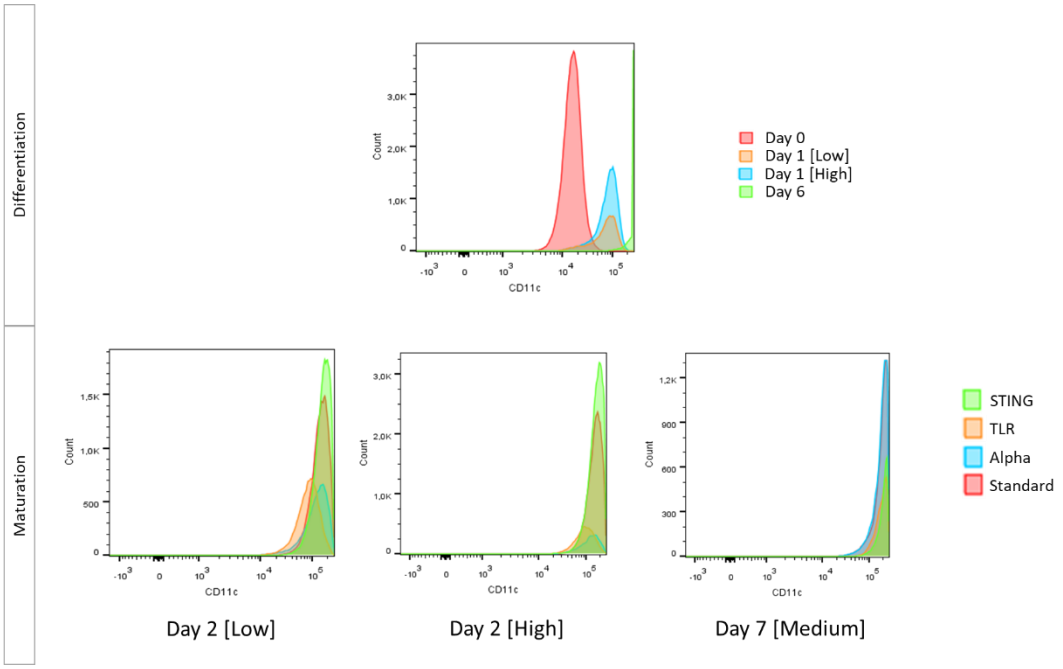

Figure S2. Graphic representation of the CD11c intensity in the samples analysed on day 0, after differentiation (day 1 and 6) and after maturation (day 2 and 7) with the different maturation cocktails/stimulus

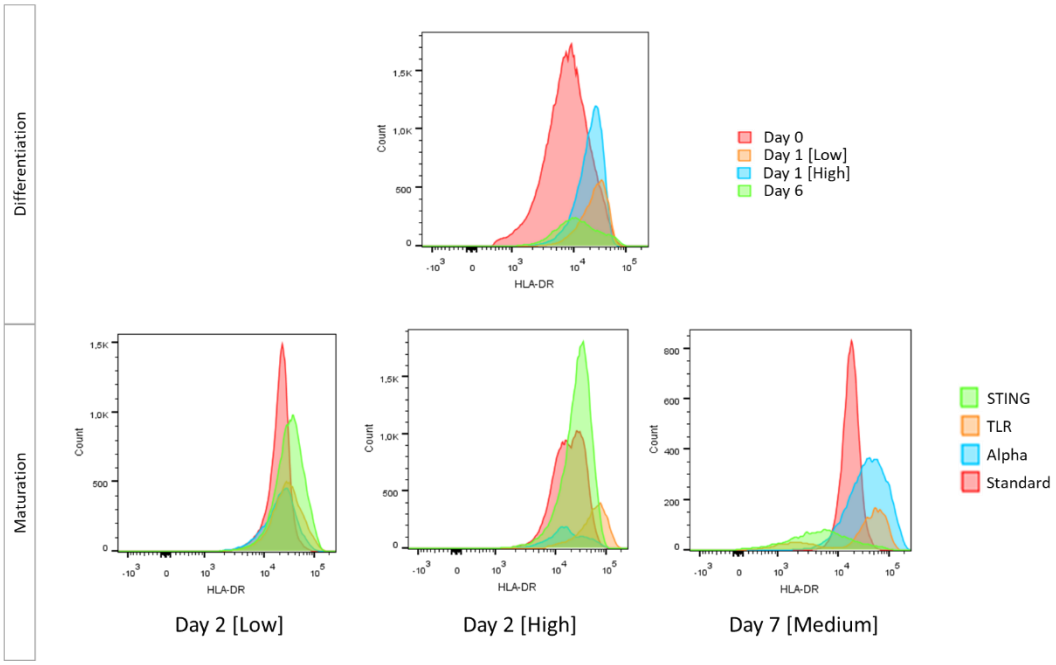

Figure S3. Graphic representation of the HLA-DR intensity in the samples analysed on day 0, after differentiation (day 1 and 6) and after maturation (day 2 and 7) with the different maturation cocktails/stimulus.

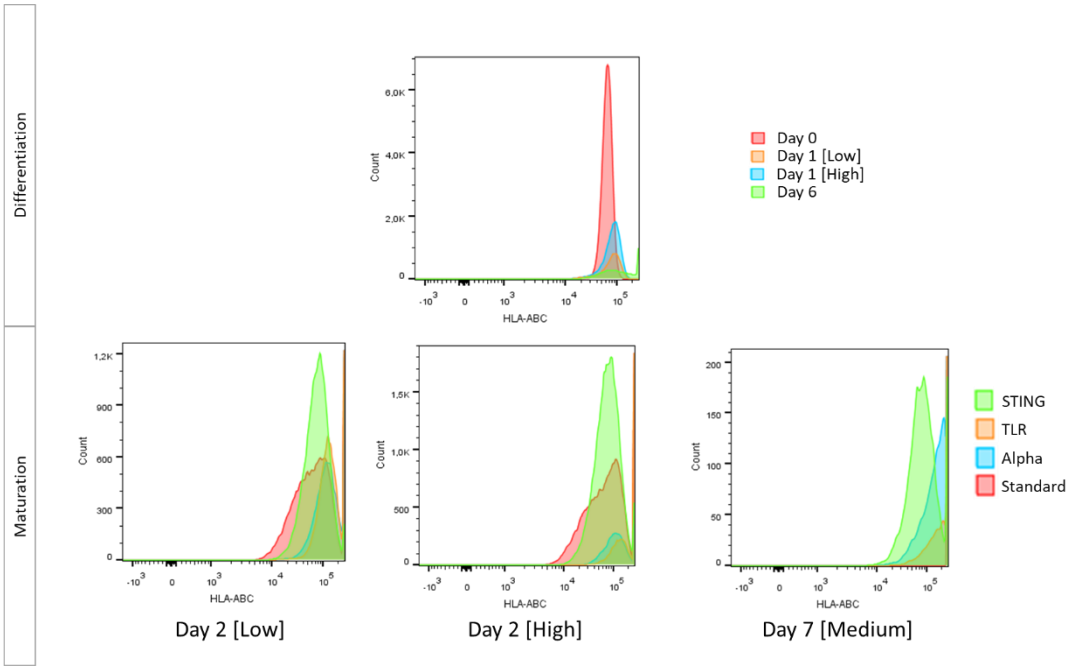

Figure S4. Graphic representation of the HLA-ABC intensity in the samples analysed on day 0, after differentiation (day 1 and 6) and after maturation (day 2 and 7) with the different maturation cocktails/stimulus.

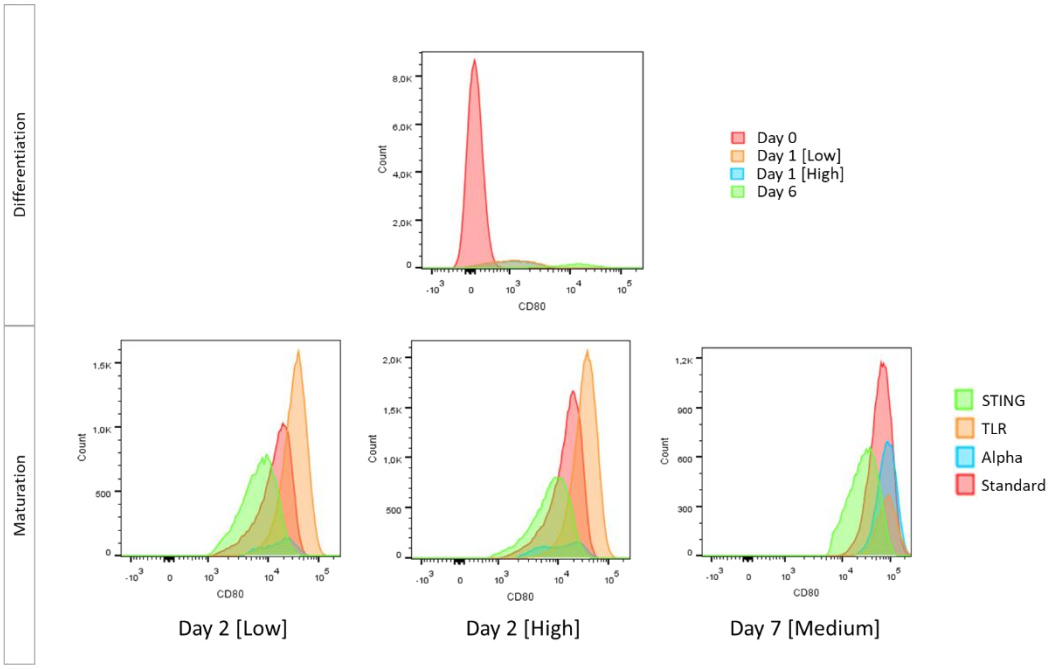

Figure S5. Graphic representation of the CD80 intensity in the samples analysed on day 0, after differentiation (day 1 and 6) and after maturation (day 2 and 7) with the different maturation cocktails/stimulus

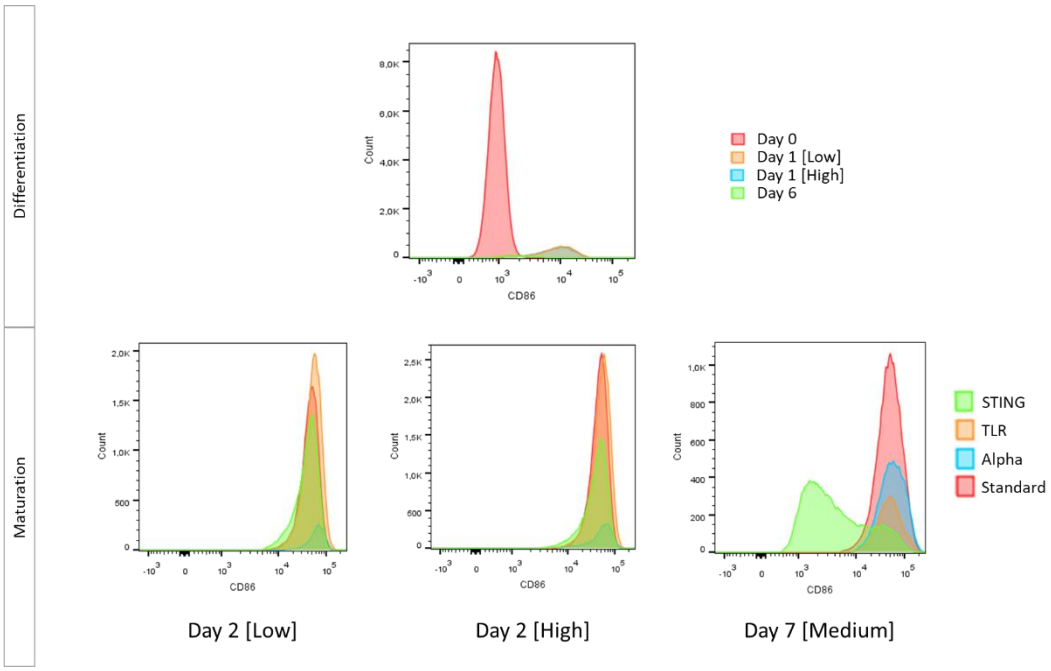

*Figure S6***.** Graphic representation of the CD86 intensity in the samples analysed on day 0, after differentiation (day 1 and 6) and after maturation (day 2 and 7) with the different maturation cocktails/stimulus.

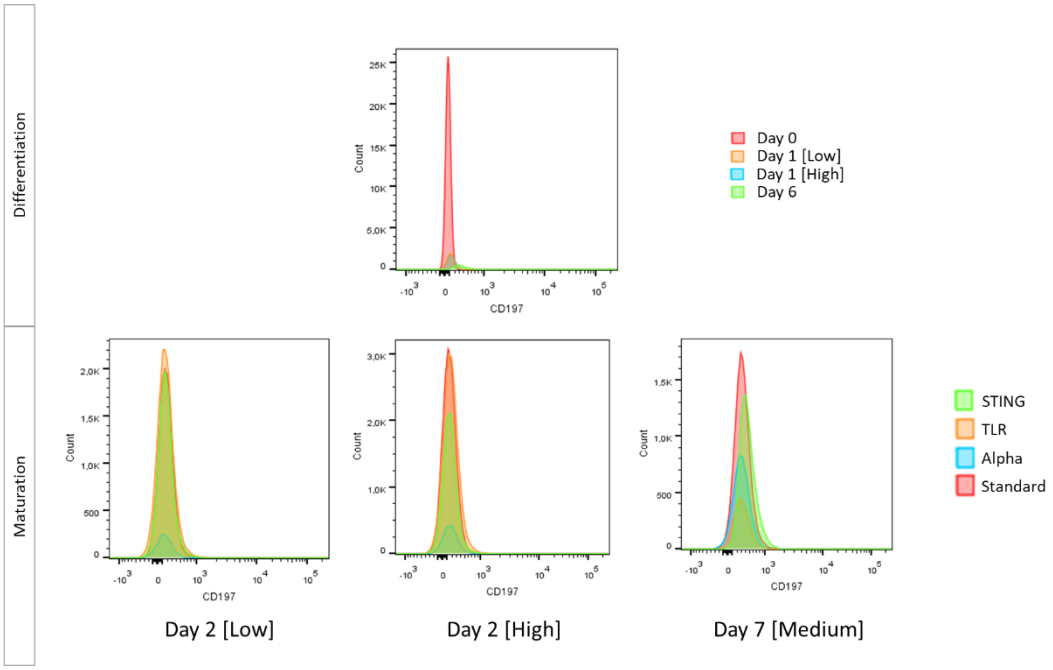

Figure S7. Graphic representation of the CD197 intensity in the samples analysed on day 0, after differentiation (day 1 and 6) and after maturation (day 2 and 7) with the different maturation cocktails/stimulus.

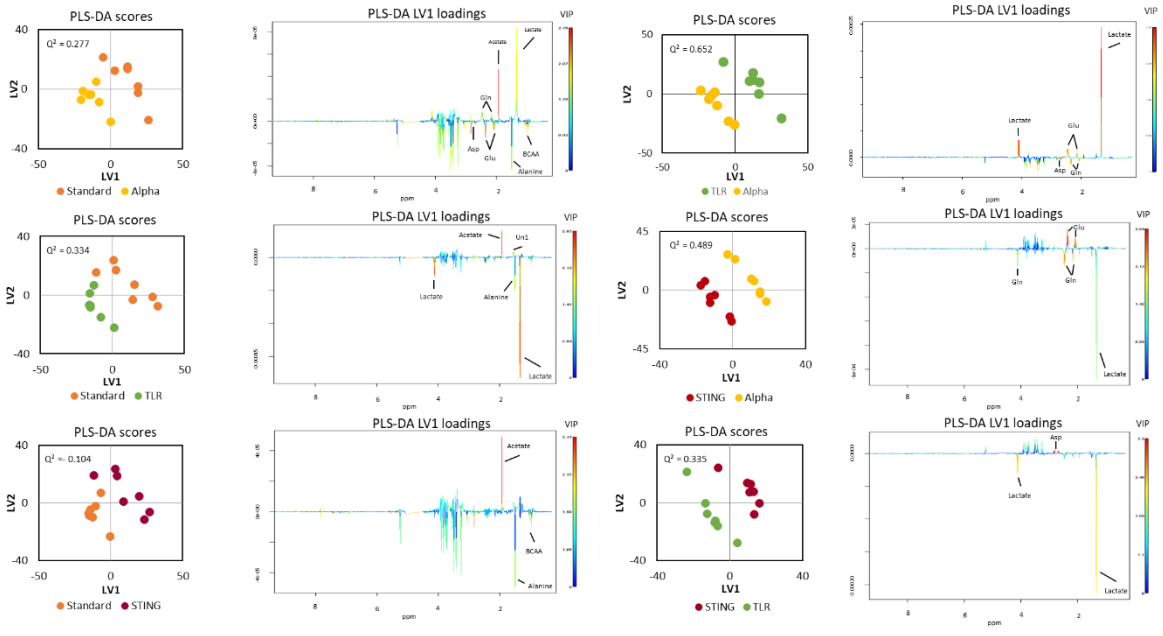

Figure S8. MVA of ^{1}H-NMR spectra from the supernatants of Fast MoDCs after maturation with a) Standard cocktail, b) Alpha cocktail, c) TLR cocktail and d) STING stimulus, incubated for 24h in RPMI medium. PLS-DA scores scatter plots and LV1 loadings, coloured according to variable importance to projection (VIP).

Table S1. %Variation of the metabolites, and respective relative error, of the cells-conditioned medium vs. cells-free medium of immature and mature MoDCs, which were differentiated by a Fast or Conventional protocol, and maturated with different maturation cocktails/stimulus, and subsequently incubated for 24h in fresh culture medium.

% Variation vs acellular medium

	Fast				
	Differentiation	**Standard**	**Alpha**	**TLR**	**STING**
Acetate	18.12 ± 6.49	71.85 ± 5.71	19.98 ± 4.05	28.68 ± 4.14	24.00 ± 2.06
Alanine	23.99 ± 2.84	16.90 ± 3.60	24.28 ± 1.65	26.07 ± 2.04	24.82 ± 1.83
Alanyl-glutamine	-62.52 ± 9.48	-57.61 ± 8.42	-63.44 ± 7.82	-66.15 ± 8.63	-64.43 ± 8.36
Aspartate	-8.19 ± 4.36	-7.72 ± 4.54	0.00 ± 3.87	-15.87 ± 5.31	-4.08 ± 3.75
Formate	40.00 ± 13.70	42.86 ± 9.03	28.57 ± 8.55	42.86 ± 10.81	57.14 ± 9.01
Glucose	0.00 ± 2.86	-5.05 ± 4.19	3.40 ± 2.03	0.00 ± 1.95	0.00 ± 2.62
Glutamate	16.16 ± 6.54	14.98 ± 3.49	43.98 ± 6.78	14.87 ± 2.74	17.87 ± 4.04
Glutamine	5.68 ± 2.89	4.79 ± 4.07	0.00 ± 4.48	12.33 ± 3.05	5.92 ± 3.79
Lactate	12.38 ± 7.40	29.73 ± 4.86	16.49 ± 3.86	60.85 ± 5.41	30.72 ± 8.97
Leucine	-2.26 ± 1.67	-7.25 ± 2.50	-2.15 ± 1.40	-2.90 ± 0.99	-2.79 ± 1.10
	Conventional				
	Differentiation	**Standard**	**Alpha**	**TLR**	**STING**
Acetate	54.04 ± 7.73	85.15 ± 11.30	66.81 ± 7.53	40.82 ± 8.42	32.90 ± 4.61
Alanine	32.41 ± 1.27	24.55 ± 3.75	23.25 ± 3.32	24.42 ± 3.26	29.51 ± 2.16
Alanyl-glutamine	-71.29 ± 8.54	-68.17 ± 8.52	-67.50 ± 8.14	-66.53 ± 8.97	-69.93 ± 8.55
Aspartate	-6.99 ± 4.05	0.00 ± 5.08	0.00 ± 4.31	-5.68 ± 3.96	-4.71 ± 3.98
Formate	57.50 ± 11.63	70.00 ± 12.00	88.57 ± 12.59	65.00 ± 11.00	70.00 ± 10.83
Glucose	4.09 ± 2.22	-9.41 ± 3.53	0.00 ± 3.87	-5.92 ± 4.93	0.00 ± 3.01
Glutamate	88.11 ± 11.89	105.01 ± 17.02	129.32 ± 15.44	115.45 ± 18.08	78.46 ± 11.83
Glutamine	-12.45 ± 8.88	-24.52 ± 9.96	-35.45 ± 9.68	-31.06 ± 11.65	-15.14 ± 6.95
Lactate	55.46 ± 10.06	110.92 ± 15.17	51.04 ± 7.42	69.84 ± 12.36	53.00 ± 6.53
Leucine	0.00 ± 1.14	-6.79 ± 1.30	-6.04 ± 1.22	-6.25 ± 1.77	-3.96 ± 0.89

Table S2. p value associated to % variation of each metabolite between immature and mature MoDCs, which were differentiated by a Fast or Conventional protocol, and maturated with different maturation cocktails/stimulus, and between mature MoDCs which were differentiated by a Fast or Conventional protocol and maturated with different maturation cocktails/stimulus.

p value

		Fast						Conventional			
		Differentiation	Standard	Alpha	TLR			Differentiation	Standard	Alpha	TLR
Alanyl-glutamine	Standard	0.3140	-	-	-	Alanyl-glutamine	Standard	0.0711	-	-	-
	Alpha	0.8194	0.1132	-	-		Alpha	**0.0366**	0.7404	-	-
	TLR	0.4153	**0.0437**	0.3434	-		TLR	0.0820	0.5553	0.7249	-
	STING	0.6597	0.0907	0.7120	0.5960		STING	0.3210	0.3384	0.1940	0.2116
Aspartate	Standard	0.8828	-	-	-	Aspartate	Standard	0.4376	-	-	-
	Alpha	**0.0129**	**0.0327**	-	-		Alpha	**0.0148**	0.2261	-	-
	TLR	0.0670	0.0631	**0.0022**	-		TLR	0.5311	0.6573	**0.0311**	-
	STING	0.0960	0.1871	0.0628	**0.0090**		STING	0.3026	0.8543	0.0584	0.6138
Glutamine	Standard	0.8219	-	-	-	Glutamine	Standard	0.2963	-	-	-
	Alpha	**0.0371**	0.0960	-	-		Alpha	**0.0476**	0.3240	-	-
	TLR	**0.0355**	0.0918	**0.0030**	-		TLR	0.1556	0.5927	0.7064	-
	STING	0.9458	0.8095	0.0550	0.1180		STING	0.7749	0.3571	**0.0458**	0.1589
Glutamate	Standard	0.8855	-	-	-	Glutamate	Standard	0.6329	-	-	-
	Alpha	**0.0285**	**0.0142**	-	-		Alpha	0.2492	0.5569	-	-
	TLR	0.8698	0.9814	**0.0139**	-		TLR	0.4556	0.8121	0.7497	-
	STING	0.8405	0.6155	**0.0240**	0.5674		STING	0.8005	0.4509	0.1572	0.3349
Alanine	Standard	0.1847	-	-	-	Alanine	Standard	0.1158	-	-	-
	Alpha	0.9357	0.1204	-	-		Alpha	0.0509	0.8212	-	-
	TLR	0.6005	0.0698	0.5377	-		TLR	0.0736	0.9813	0.8265	-
	STING	0.8270	0.1033	0.8403	0.6791		STING	0.3248	0.3361	0.1884	0.2729
Lactate	Standard	0.0844	-	-	-	Lactate	Standard	0.0885	-	-	-
	Alpha	0.6309	0.0553	-	-		Alpha	0.7933	0.0612	-	-
	TLR	**0.0005**	**0.0043**	**0.0003**	-		TLR	0.4823	0.2198	0.3744	-
	STING	0.1810	0.9339	0.2326	**0.0360**		STING	0.9907	0.0671	0.8796	0.4151
Formate	Standard	0.8712	-	-	-	Formate	Standard	0.5586	-	-	-
	Alpha	0.5022	0.1822	-	-		Alpha	0.1958	0.4488	-	-
	TLR	0.8816	1.0000	0.2842	-		TLR	0.5037	0.8120	0.3149	-
	STING	0.3556	0.2659	**0.0255**	0.3408		STING	0.3659	1.0000	0.4260	0.7981
Acetate	Standard	**0.0004**	-	-	-	Acetate	Standard	0.1581	-	-	-
	Alpha	0.8318	**0.0003**	-	-		Alpha	0.4103	0.3940	-	-
	TLR	0.2521	**0.0013**	0.2156	-		TLR	0.4433	0.0543	0.1099	-
	STING	0.4631	**0.0009**	0.4505	0.4088		STING	0.1570	**0.0217**	**0.0202**	0.5257