NOVA SCHOOL OF SCIENCE & TECHNOLOGY

DEPARTAMENT OF LIFE SCIENCES

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BSc in Cell and Molecular Biology

EXPLORING THE EFFECTS OF ALTERED GLYCOSYLATION ON THE IMMUNE RESPONSE

MASTER IN MOLECULAR GENETICS AND BIOMEDICINE

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Exploring the effects of altered glycosylation on the immune response

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"Believe in yourself and all that you are. Know that there is something inside you that is greater than any obstacle."

Christian D. Larson

Abstract

Glycosylation is involved in a plethora of biological functions, playing a major role in human health and disease. In fact, diseases like cancer and Congenital Disorders of Glycosylation (CDG) are known to have altered glycosylation.

In cancer, aberrant glycosylation, especially hypersialylation, has been associated with tumour immunosuppressive environment, hindering the efficacy of immunotherapies like dendritic cell (DCs)-based vaccines. DCs are antigen-presenting cells essential to T lymphocyte activation and targeted cell death. DCs anti-tumour activity requires the expression of antigen-presenting and co-stimulatory molecules as well as cytokine secretion.

PMM2-CDG, the most frequent CDG-type, is characterized by defective *N*-glycosylation, leading to multi-systemic clinical manifestations, including immune defects. However, the underlying mechanisms and consequences of glycosylation changes on patients' immunity is poorly known.

Here we hypothesise that increased sialylation and *N*-glycosylation defects suppress the proper immune response. The main goal of this work was to understand the impact and consequences of these altered glycosylation features using DCs from healthy individuals and fibroblasts of PMM2-CDG patients. For that, two different approaches were followed: (1) comparison of maturation markers expression in cells with different sialylated content by using sialidase, sialidase inhibitors and the standard maturation cytokine cocktail; (2) characterisation of surface glycosylation and proinflammatory cytokines in PMM2-CDG patients' cells.

The data obtained indicated that sialidase treated DCs had higher expression of antigen-presenting and co-stimulatory molecules, such as MHC-I, MHC-II and CD40, and increased cytokine production, namely IL-12 and TNF-α, when compared to controls. Furthermore, decreased surface glycosylation and secretion of the proinflammatory cytokine IL-6 were detected in PMM2-CDG fibroblasts.

The results obtained here validate our hypothesis by highlighting how glycosylation affects the immune response. These findings may unravel new strategies that target glycans to potentiate the efficacy of existing cancer immunotherapies, like DC-based vaccines, and eventually explain immune-related manifestations in CDG patients.

Keywords: Glycosylation, dendritic cells, sialic acids, sialidase, Congenital Disorders of Glycosylation, PMM2-CDG

Resumo

A glicosilação está envolvida em diversas funções biológicas, desempenhando um papel preponderante na saúde humana. Doenças como o cancro e Doenças Congénitas da Glicosilação (CDG) são conhecidas por terem glicosilação alterada.

No cancro, a glicosilação aberrante, especialmente a hipersialilação, encontra-se associada ao seu ambiente imunossupressor, afetando a eficácia de imunoterapias como vacinas de células dendríticas (DCs). As DCs são células apresentadoras de antigénios essenciais para a ativação de linfócitos T e consequente morte celular dirigida. Para esta função anti-tumoral, é necessária a expressão de moléculas apresentadoras e co-estimulatórias, e secreção de citocinas pelas DCs.

PMM2-CDG, a CDG mais frequente, é caracterizada por *N*-glicosilação defetiva, levando a manifestações clínicas multissistémicas. Contudo, as consequências de alterações na glicosilação no sistema imune destes pacientes encontram-se pouco exploradas.

Aqui, partimos da hipótese de que aumento da sialilação, e *N*-glicosilação defetiva, inibem uma resposta imunológica apropriada. O objetivo principal foi entender o impacto destas mudanças na glicosilação usando DCs de indivíduos saudáveis e fibroblastos de pacientes PMM2-CDG. Assim, duas abordagens foram seguidas: (1) comparação da expressão de marcadores de maturação em células com diferente conteúdo sialilado, usando sialidase, inibidores de sialidase e citocinas de maturação; (2) caracterização da glicosilação e produção de citocinas pro-inflamatórias em fibroblastos de pacientes PMM2-CDG.

Os dados obtidos indicaram que DCs tratadas com sialidase apresentaram maior expressão de algumas moléculas apresentadoras e co-estimulatórias, como MHC-I, MHC-II e CD40, e aumento de citocinas, como IL-12 e TNF-α, quando comparadas aos controlos. Além disso, verificou-se menor glicosilação e secreção de IL-6 em fibroblastos de pacientes PMM2-CDG.

Os resultados obtidos validam a nossa hipótese, destacando alguns efeitos da glicosilação na resposta imune. Estes dados podem originar novas estratégias, potenciando a eficácia de imunoterapias contra o cancro, como vacinas de DCs, e eventualmente explicar manifestações clínicas relacionadas com o sistema imune em pacientes CDG.

Palavras-chave: Glicosilação, células dendríticas, ácidos siálicos, sialidase, Doenças Congénitas da Glicosilação, PMM2-CDG

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List of Abbreviations

β2M	β2-microglobulin
APCs	Antigen-presenting cells
APC	Allophycocyanin
BCA	Bicinchoninic acid
CD	Cluster of differentiation
CD1	Cluster of differentiation 1
CDG	Congenital Disorders of Glycosylation
ConA	Concanavalin A
CTLA-4	Cytotoxic T lymphocyte-associated antigen 4
DANA	2,3-dihydro-2-deoxy-N-acetylneuraminic acid
DC(s)	Dendritic cell(s)
(c)DMEM	(complete) Dulbecco's Modified Eagle Medium
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
FITC	Fluorescein isothiocyanate
FSC	Forward scatter
GalNAc	*N*-acetylgalactosamine
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GlcNAc	*N*-acetylglucosamine
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GNL	*Galanthus nivalis* lectin
HRP	Horseradish Peroxidase
IFN	Interferon

IL	Interleukin
ITAMs	Immunoreceptor tyrosine-based activation motifs
ITIMs	Immunoreceptor tyrosine-based inhibitory motifs
MFI	Median fluorescence intensity
MHC	Major histocompatibility complex
MHC-I	Major histocompatibility complex class I
MHC-II	Major histocompatibility complex class II
moDCs	Monocyte-derived dendritic cells
PAMPs	Pathogen associated molecular patterns
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PD-1	Programmed cell death protein 1
PD-L1	Programmed death-ligand 1
PD-L2	Programmed death-ligand 2
PE	Phycoerythrin
PGE2	Prostaglandin E2
PMM2	Phosphomannomutase 2
PNA	Peanut Agglutinin
PRRs	Pattern recognition receptors
(c)RPMI	(complete) Roswell Park Memorial Institute
RT	Room temperature
RT-qPCR	Real-Time Quantitative Polymerase Chain Reaction
SAMPs	Self-associated molecular patterns
Siglecs	Sialic acid-binding immunoglobulin-type lectins
SNA	*Sambucus nigra* lectin

SSC	Side scatter
ST3Gal	β-galactoside 2,3 sialyltransferase
ST6Gal	β-galactoside α2,6 sialyltransferase
ST6GalNac	*N*-acetyl-galactosamine α2,6 sialyltransferase
ST8Gal	α2,8 sialyltransferase
TAP	Transporter associated with antigen processing
TCR	T cell receptor
TGF-β	Transforming growth factor β
Th1	T helper type 1
Th2	T helper type 2
TMB	3,3',5,5'-Tetramethylbenzidine
TNF-α	Tumour necrosis factor-alpha
Zanamivir	4-Guanidino-2,4-dideoxy-2,3-dihydro-N-acetylneuraminic acid

Chapter 1. Introduction

1.1. Immune System

The immune system is a complex set of components and structures that maintain the organism protected against foreign bodies[1]. Thus, to develop an appropriate response, it needs to perceive and differentiate the host molecules ("self") from foreign molecules ("non-self"). The immune response is often characterised by the recruitment of several molecules and cells, neutralisation or elimination of foreign bodies, and ultimately, the development of immunological memory.

The immune system comprises two different types of immunity: innate and adaptive immunity. Although defined by a broad and fast response, the former is not specific to antigens, whilst the latter displays a slower response with high antigen specificity and can develop immunological memory[2].

1.1.1. Innate immunity

The innate immunity, normally indicated as the first line of defence, plays a significant role in the host defence against pathogens and comprises different types of defence mechanisms, namely, anatomic, physiologic, inflammatory, and endocytic and phagocytic barriers[2].

The endocytic and phagocytic barrier is composed of different cells, including dendritic cells (DCs) and macrophages, that express pattern recognition receptors (PRRs), involved in the detection and response to a broad range of pathogens[2,3]. That can be achieved due to the recognition of structures and molecules that several pathogens share, designated as pathogen-associated molecular patterns (PAMPs)[2,3].

The recognition of PAMPs activates diverse signal transduction pathways, such as nuclear factor κB, culminating in the secretion of cytokines, like interferon (IFN), tumour necrosis factor-alpha (TNF-α) and interleukins (IL)[1,3]. Cytokines are multifunctional proteins that have a major role in cellular recruitment and activation, promoting, therefore, phagocytosis and neutralisation or elimination of pathogens[1]. These processes are decisive in the activation of the adaptive immunity, as explored in the next section.

1.1.2. Adaptive immunity

The adaptive immunity, as previously mentioned, develops after the innate immunity, and displays a high antigen specificity.

The main cells involved in the adaptive response are B and T lymphocytes that express receptors capable of recognising specific antigens[2]. Furthermore, these cells allow the development of immunological memory, characterised by a faster and more efficient response when in contact with an antigen that the host has been previously exposed to[1].

Each B lymphocyte recognises a specific antigen, with surface receptors, in its native form, leading to its activation and differentiation into plasma cells. Plasma cells secrete antibodies with high antigen specificity, resulting in antigen neutralisation or elimination, a mechanism known as humoral immunity[2].

Cell-mediated immunity is carried out by T lymphocytes, that after activation, differentiate into effector cells, namely cytotoxic, helper and regulatory T cells[2].

T lymphocytes require the antigen to be presented through a specialised molecule, such as the major histocompatibility complex (MHC), expressed in antigen-presenting cells (APCs) like DCs[2].

The antigen recognition, however, is not sufficient on its own to result in the proper activation of T lymphocytes. Besides it, the presence of co-stimulatory molecules and cytokines are required (Figure 1.1.), as further explained in the next topics.

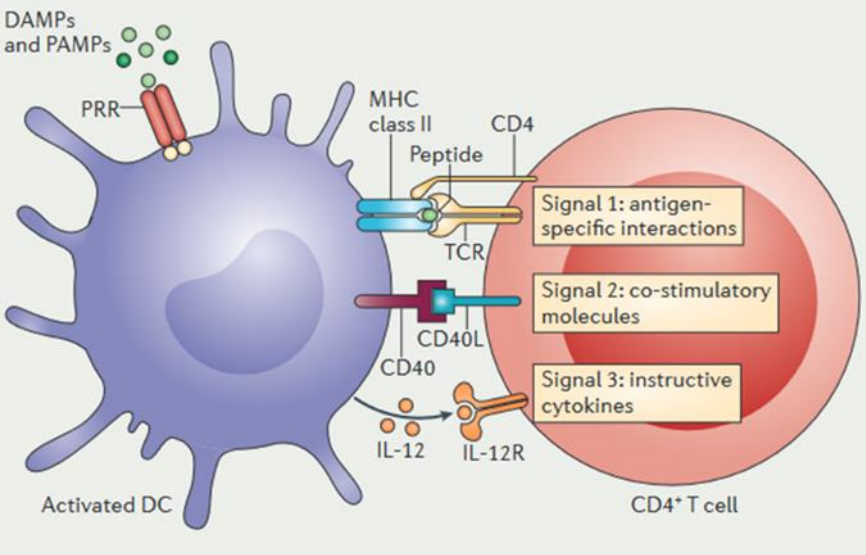

Figure 1.1. The three dendritic cell-derived signals necessary for T lymphocyte activation. From Kambayashi and Laufer[4]

1.1.3. Antigen presentation

As previously referred, for the antigen to be recognised by T lymphocytes, it must be presented through certain molecules, namely MHC and Cluster of Differentiation (CD) 1 (CD1). MHC

class I (MHC-I) and class II (MHC-II) are essential to peptide presentation to cytotoxic and helper T cells, respectively. MHC-I can be found in all nucleated cells, whilst MHC-II is only expressed in APCs[5].

There are two distinct pathways in which the peptides are processed to be available for presentation by MHC-I and MHC-II, the cytosolic and endocytic pathways, respectively[5].

The cytosolic pathway comprises proteolysis of cytosolic proteins mainly by the action of the proteasome[5]. The resulting peptides are transported to the endoplasmic reticulum (ER) by the transporter associated with antigen processing (TAP), where they are loaded onto MHC-I, a heterodimer formed by a heavy chain and β2-microglobulin (β2M)[5]. The MHC-I-peptide complex cycles through the ER and cell surface, where the peptide can be presented to cytotoxic lymphocytes, leading to its activation and cytotoxic activity. These T lymphocytes play an important role in targeted cell death, mainly in cancer and virus-infected cells, by death-inducing granules secretion, containing molecules such as perforin and granzymes[6].

The endocytic pathway includes antigen processing from internalised proteins via endocytosis, pinocytosis, and phagocytosis[5]. These proteins traffic to late endosomes, where they are processed and loaded to MHC-II[5]. The MHC-II-peptide complex is transported to the cell surface and recognised by helper T lymphocytes. These lymphocytes are essential in achieving an effective immune response, as they mediate the activation and proliferation of several immune cells by cytokine secretion[7].

Both MHC-I and MHC-II with loaded antigens are recognised by T cells through the T cell receptor (TCR) and CD3 complex[8]. The TCR interacts with the MHC-peptide complex, and CD3 molecules are responsible for activation signals transduction in T cells[8].

Besides peptide presentation via MHC molecules, lipid presentation through CD1 molecules has been described. CD1 molecules, in humans, are separated into two different groups by sequence homology[9]. Group 1 contains CD1a, CD1b and CD1c, whilst group 2 consists of CD1d[9]. CD1-restricted T cells is a certain subset of T lymphocytes that do not bind with MHC-peptide complexes but can interact with CD1-lipid complexes[9,10]. Studies about group 1 CD1-restricted T cells have only been recently emerging, and its mechanism is not yet well understood[9,10].

1.1.4. Co-stimulatory and co-inhibition molecules

As stated before, antigen presentation is not, on its own, sufficient for a proper T cell activation, as the absence of co-stimulatory molecules leads to T lymphocytes anergy[11].

CD80 and CD86, both members of the B7 family, are two of the most important co-stimulatory molecules expressed on APCs as they interact with CD28, expressed on T cells, promoting the activation of these lymphocytes[11,12]. This association proves crucial as it leads to increased production of IL-2 by T cells, a cytokine that favours T lymphocyte survival and proliferation[12,13].

However, CD80 and CD86 do not only promote T cell activation, as they also bind, and with higher affinity, to CD152, a molecule that is upregulated in T cells after activation[12,14]. CD152, also known as cytotoxic T lymphocyte-associated antigen 4 (CTLA-4), is a negative regulator of T cell activation, having an active role in preventing an exacerbated immune response[12,14].

Another inhibitory molecule expressed in T cells, mainly in the activated state, is programmed cell death protein 1 (PD-1) that interacts with programmed death-ligand 1 (PD-L1) and 2 (PD-L2), expressed in a wide range of hematopoietic, non-hematopoietic and even in cancer cells[14]. This binding culminates in reduced T lymphocyte survival and proliferation, as well as reduced cytokine expression[14].

The APCs-T cell crosstalk involves other molecules, namely CD40, expressed in APCs, that interacts with CD154 in T cells, known as CD40 ligand. This binding is crucial as it promotes T cell activation and differentiation[12,14]. Furthermore, this interaction also leads to an increased expression of antigen-presenting and co-stimulatory molecules and cytokine production, such as IL-12, in DCs[12].

1.1.5. Cytokine secretion

T lymphocytes, besides antigen presentation and co-stimulatory molecules, also require the presence of cytokines to fully develop a functional immune response[15,16]. Cytokines are not only essential to prevent cell death and immunological tolerance but also play an active part in the differentiation pathways of T cells[15].

Cytokines secreted by APCs, like DCs, have been shown to modulate helper T lymphocytes differentiation into different subsets, namely T helper type 1 (Th1) and 2 (Th2)[17,18]. Th1 mainly secrete IFN-γ and are associated with the host defence against intracellular pathogens, whilst Th2 are especially important against extracellular parasites by producing several cytokines

like IL-4, IL-5, and IL-13[17,18]. Additionally, the secreted cytokines by these two subsets are also relevant for the induction of their own differentiation, creating a positive feedback loop[18].
Nonetheless, APCs are also modulated by the helper T cells cytokines, as IFN-γ is crucial for IL-12 production by DCs[12]. The latter is considered necessary for cytotoxic T lymphocytes cytolytic activity and responsiveness[16].
In fact, other cells, like fibroblasts, present in all tissues, also mediate immune response by cytokine secretion[19]. After recognising PAMPs, they release chemokines, modulating the recruitment of immune cells, and diverse cytokines, namely IFN-γ, IL-6 and TNF-α, that promote a pro-inflammatory environment and pathogen elimination[19].
All the examples above demonstrate an interplay between different types of cells within our immune system for a proper and effective response against pathogens.

1.1.6. Dendritic cells

As described above, DCs have a major role in establishing the interaction between innate and adaptive immunity.
DCs arise more frequently from common myeloid progenitors, although they can also be originated from common lymphoid progenitors[20]. DCs are widely distributed but typically are found in places with a higher chance of encountering pathogens, such as skin and gastrointestinal and respiratory systems[21].
In our tissues, DCs are normally in an immature state, where they possess a great antigen capturing capacity, mainly through receptor-mediated endocytosis and pinocytosis. Despite that, immature DCs have low co-stimulatory molecules expression and do not secrete immunostimulatory cytokines[22]. After capturing an antigen, they undergo maturation as they migrate to secondary lymphoid organs where they present the antigens to T lymphocytes[22]. The maturation process is accompanied by a decrease in the endocytic capacity, but a higher expression of MHC and co-stimulatory molecules, as well as cytokine secretion, that, as explained before, are all necessary for a proper T cell activation[22].

1.2. Glycosylation

Glycosylation is the process in which saccharides are enzymatically linked to proteins, lipids, or other saccharides, through glycosidic linkages, being one of the most relevant post-

translational modifications in eukaryotes[23]. Glycosylation occurs in the ER and Golgi apparatus and is orchestrated by the action of glycosyltransferases and glycosidases[24].

When considering protein glycosylation, the most common types of this modification are *N*- and *O*-glycosylation.

N-glycosylation starts in the ER and is characterised by the attachment of *N*-acetylglucosamine (GlcNAc) to the amide group of an asparagine side chain[23,25]. Meanwhile, *O*-glycosylation is initiated in the Golgi apparatus by the addition of *N*-acetylgalactosamine (GalNAc) to the hydroxyl group of either a serine or a threonine side chain[23,25]. Both modifications described (Figure 1.2.) above can be further modified by the attachment of other different saccharides, such as sialic acid, resulting in a great variety of glycoconjugates.

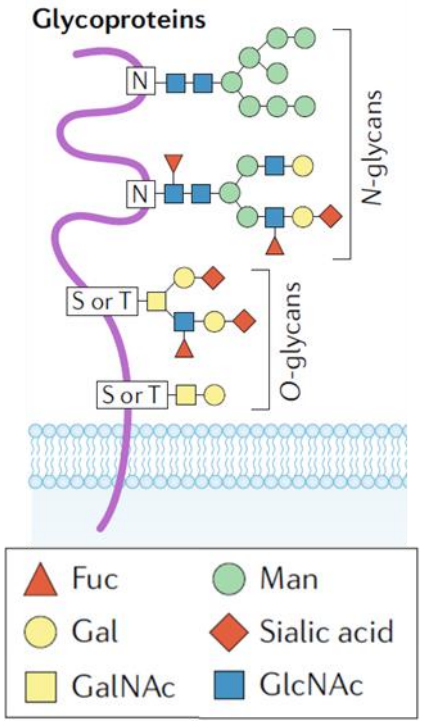

Figure 1.2. Schematic representation of *N*-glycans and *O*-glycans. *N*-glycans are attached to the nitrogen (N) atom of asparagine, whilst *O*-glycans are linked to the hydroxyl group of serine (S) or threonine (T). Fucose (Fuc), mannose (Man), galactose (Gal), *N*-acetylgalactosamine (GalNAc), *N*-acetylglucosamine (GlcNAc). Adapted from Reily *et al*.[25]

Glycosylation has been shown to be involved in the regulation of several physiological functions, like molecular trafficking and cellular interactions, being the reason why changes in its' regulation have been associated with diverse diseases, such as cancer, Congenital Disorders of Glycosylation and autoimmune diseases[23,25,26].

Given these functions, glycosylation also has a major role in the immune system, especially because most molecules involved in the immune response are glycoproteins[27]. Besides that, the binding of glycan-binding proteins or lectins, namely galectins, and sialic acid-binding immunoglobulin-type lectins (Siglecs), modulates a great part of innate and adaptive immune response[28,29], as will be addressed in the next topics.

1.2.1. Sialic Acids

Sialic acids are a group of 9-carbon backbone monosaccharides, derived from neuraminic acid, that are attached to the outermost ends of glycoconjugates in a process designated sialylation[30,31]. This modification is catalysed by sialyltransferases in the Golgi apparatus that transfer sialic acid residues from a donor, cytidine-5'-monophospho-N-acetylneuraminic acid, to glycans[31].

Considering the terminal position in which they are located, as well as its' molecular characteristics, such as their negative charge and hydrophilicity, sialic acids have a preponderant role in cellular interactions and immune system[30,32].

For instance, given its' negative charge, it has an important role in repulsion and attraction forces, modulating the interaction between cells. Furthermore, the presence of sialic acids acts like a coat, preventing the recognition of underlying glycans or other molecules, such as galactose residues, preventing the binding by galectins[32]. Galectins are a group of β-galactoside-binding lectins that regulate several cellular functions like adhesion, proliferation, and apoptosis[33].

Nonetheless, sialic acids are, in some cases, the binding target of certain molecules, like factor H and Siglecs[32]. The former is a complement regulator, and its' binding to sialic acids negatively regulates the activation of complement alternative pathway[31,34]. Although the recognition of sialic acids by Factor H as a "self" molecule is important to regulate complement activation, this mechanism is also used by certain bacteria that use sialic acids to evade complement response[31,34,35].

The binding of Siglecs to sialic acids has also been reported to regulate diverse signalling functions and cellular interactions, as will be further explored in the next section.

1.2.2. Sialic acid-binding immunoglobulin-type lectins (Siglecs)

Siglecs are glycan-binding proteins or lectins that bind specifically to sialic acids residues. These lectins are divided into two different subsets according to their evolutionary conservation and sequence similarity[34,36]. The first group comprises sialoadhesin (Siglecs-1), CD22 (Siglec-2), myelin-associated glycoprotein (Siglec-4) and Siglec-15, all having orthologs in diverse mammalian species but with little sequence similarity. The second group is composed of CD33 (Siglec-3) and numerous CD33-related Siglecs that share high sequence similarity but went fast evolutionary changes, namely by gene duplication[34,36].

Siglecs are expressed mainly on the cell surface of immune cells, like DCs, macrophages, monocytes, and B lymphocytes[34]. Siglecs are not only capable of binding sialic acid residues on other cells (in *trans*) but also on the same cell (in *cis*)[33].

The majority of Siglecs send inhibitory signals via immunoreceptor tyrosine-based inhibitory motifs (ITIMs), having a preponderant role in the regulation of activation pathways[31,36,37]. Not all Siglecs have ITIMs or ITIM-like motifs, such as Siglec-14 and Siglec-15. However, these Siglecs can interact with a transmembrane protein, DAP12, that contains immunoreceptor tyrosine-based activating motifs (ITAMs), resulting in the transduction of activation signals[36,37,38]. Furthermore, there are Siglecs that do not interact with either ITIMs or ITAMs[37]. Such is the case of Siglec-1, being associated with cellular interactions and phagocytosis of sialylated antigens[37].

Inhibitory Siglecs are especially important regulators of the immune response since sialic acids are considered self-associated-molecular patterns (SAMPs), thus playing an active part in the prevention of an inadequate immune response against our cells[37].

However, the presence of these Siglecs is also a mechanism used by cancer cells to evade immune responses, as increased sialylation in cancer cells has been described. For instance, sialic acid recognition in cancer cells by Siglec-7 and Siglec-9, expressed in DCs, lead to regulatory T cell induction and immunosuppressive environment[31,39,40].

For the reasons stated above, modulation of sialic acid content has been a novel strategy to combat several diseases, and hence, the importance of sialyltransferases and sialidases, detailed in the next topics.

1.2.3. Sialyltransferases

Sialyltransferases are a group of glycosyltransferases responsible for the addition of sialic acids to other glycans, as stated before.

There are 20 human sialyltransferases currently described, although it is thought that the human genome is likely to encode more than those[41,42,43]. They have been divided into four different families according to the substrate that acts as the acceptor of sialic acids and to the glycosidic linkage these enzymes form[41,42]. β-galactoside α2,3 sialyltransferase (ST3Gal) family comprises sialyltransferases that act on galactose residues present on glycoconjugates forming an α2,3 glycosidic bond with sialic acids[43,44]. β-galactoside α2,6 sialyltransferase (ST6Gal) family attaches sialic acids to galactose in *N*-glycans, through an α2,6 linkage[41,42]. *N*-acetyl-galactosamine α2,6 sialyltransferase (ST6GalNac) family links sialic acids to GalNac residues in *O*-glycans and glycolipids, in an α2,6 linkage[43]. Finally, α2,8 sialyltransferase (ST8Sia) family attaches sialic acids to other sialic acid residues (polysialic acid) via α2,8 glycosidic bond[33,36].

As referred before, sialic acid plays an important role in our immune system. Therefore, alteration in the expression of sialyltransferases is also expected to modulate our immune response. In fact, an increased expression in sialyltransferases has been reported in different cancer cells, improving their survival and immune evasion, as explored in section 1.4.1.

1.2.4. Sialidases

Sialidases, also known as neuraminidases, are glycosidases that catalyse the removal of sialic acid residues from glycoconjugates. Although a great part of sialylation regulation is attributed to sialyltransferases, sialidases have also been considered to modulate this complex process[45].

Up to this date, four different human neuraminidases have already been identified and characterised, Neu1, Neu2, Neu3 and Neu4, and they vary in their substrate specificity and cellular location[45,46].

Considering their location, Neu1 can be either found in lysosomes associated with several proteins or at the cell surface of different cells, whilst Neu2 is a cytosolic sialidase[45,46]. Neu3 has been described to be a plasma membrane-associated protein, although it can also be in early endosomes, and Neu4 has been associated with lysosomes, mitochondria, and ER[45,46].

In terms of substrate, Neu1 acts on glycoproteins and glycolipids, being crucial to several adhesion and signalling functions, given its' location on the cell surface[45,46]. Neu2 desialylates both glycoproteins and gangliosides, being reported to have an active role in neural and myoblast differentiation[45,46]. Neu3 desialylates gangliosides preferentially and is commonly associated with modulation of adhesion, apoptosis, and neuronal differentiation[45,46]. Neu4 is not yet well described, but some studies suggest its' activity on glycoproteins and gangliosides, having an impact on apoptosis and neuronal differentiation[46].

Besides cellular location and substrate, human neuraminidases also act preferably depending on the glycosidic linkage. Neu1 and Neu2 hydrolyse faster α2-3 linkages, compared to α2-6 and α2-8 sialyl linkages whilst Neu3 acts equally on α2-3 and α2-8 linkages and to less extent on α2-6 linkages[46].

As pathogens make use of sialic acid to evade the immune system or to elicit their pathogenicity, understanding the functions of sialyltransferases and sialidases, as well as developing inhibitors to both, is essential to unravel potential therapeutics[47].

An example of bacteria that use sialidases for pathogenicity is *Clostridium perfringens*, being one of the most common causes of food poisoning. It is able to adhere to intestinal cells where it secretes its toxins, after the removal of sialic acids, by reducing the negative charges[47].

Nonetheless, these sialidases have also become a powerful tool to understand the impact of sialylation in current studies, being one of *C. perfringens* sialidases used in this work, acting preferentially on α2,3 linkages and equally with less extent on α2,6 and α2,8 linkages.

Several sialidase inhibitors have been developed, such as 2,3-dihydro-2-deoxy-N-acetylneuraminic acid (DANA), a potent Neu1 and Neu3 inhibitor, or 4-Guanidino-2,4-dideoxy-2,3-dihydro-N-acetylneuraminic acid (Zanamivir), a strong Neu3 inhibitor, to help combat a great variety of diseases[45]. For instance, Zanamivir has been used as an anti-influenza drug to help prevent its pathogenicity.

Influenza virus has two different glycoproteins important for the infectious cycle: hemagglutinin and neuraminidase. Hemagglutinin is responsible for the attachment of the virus to cells through binding with sialic acids on glycoconjugates, initiating the infectious cycle. Later, neuraminidase is responsible for sialic acid cleavage, detaching and allowing progeny virions to spread to nearby cells[48,49]. When in the presence of a sialidase inhibitor, like Zanamivir, progeny virions aggregate at the infected cell surface, hindering the infection of other cells[49].

Furthermore, since hypersialylation has also been associated with cancer (section 1.4.1), the use of different sialidases has been considered in certain therapeutics.

In fact, several patents have been granted to technologies using sialidases in the treatment of diseases in which glycosylation takes part, especially cancer, as can be seen in Appendix 1.

1.3. Congenital Disorders of Glycosylation

Congenital Disorders of Glycosylation (CDG) are a group of genetic diseases that result from hyper- or hypo-glycosylation of glycoconjugates, namely glycoproteins and glycolipids[50]. These alterations on glycosylation patterns owe to different mutations in genes related to the glycan modification pathway, in which each mutation originates a different CDG type[51]. More than 150 CDG types have already been described, in which most are related to defective *N*-glycosylation (Figure 1.3.)[52]. Generally, CDG patients have multi-systemic manifestations, mainly on numerous functions of the nervous, gastrointestinal, hepatic, and immune systems[51,53]. The most common screening techniques for *N*-glycosylation related CDG detection are mass spectrometry or isoelectric focusing of serum transferrin[51,52]. Exome sequencing can also be performed to identify the mutation in CDG-associated genes[52]. As of today, most CDG treatment is symptomatic, being monosaccharide supplementation one of the most used approaches[52].

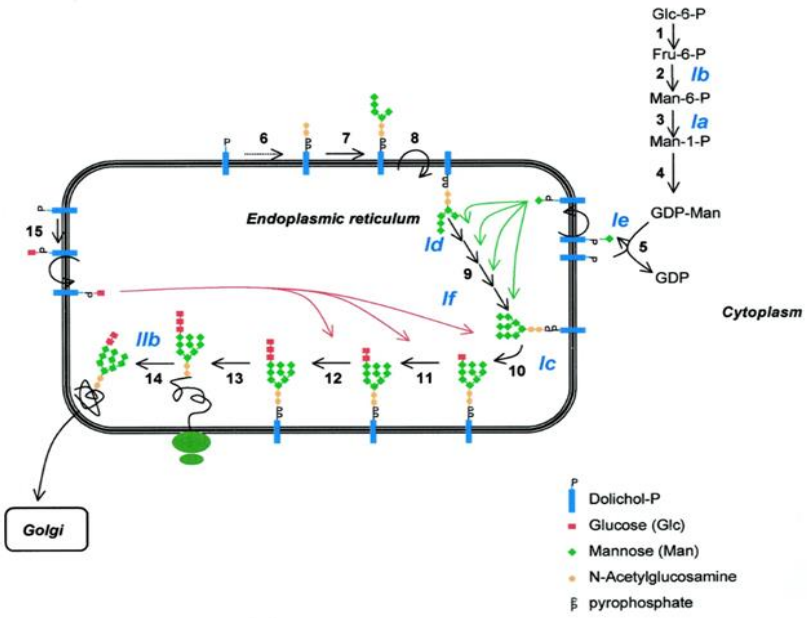

Figure 1.3. Schematic representation of the *N*-glycosylation pathway and the steps affected by different Congenital Disorders of Glycosylation (CDG). Here each CDG (represented in blue letters) is described using the old nomenclature. From Grünewald *et al.*[54]

1.3.1. PMM2-CDG

Phosphomannomutase 2-Congenital Disorder of Glycosylation (PMM2-CDG), previously known as CDG-Ia, is the most prevalent CDG (1:100 000) with more than 1000 patients diagnosed worldwide[52,55]. This CDG results from mutations in the *PMM2* gene that lead to a deficiency in phosphomannomutase 2 (PMM2). This enzyme catalyses the conversion of mannose-6-phosphate to mannose-1-phosphate, which is essential to the biosynthesis of *N*-glycans[56]. The decrease in the production of mannose-1-phosphate hinders the synthesis of guanosine 5'-diphospho-D-mannose (GDP-man), necessary for protein oligosaccharide chains[56].

PMM2-CDG has a broad range of phenotypic manifestations, with emphasis on the nervous system. Affected hepatic and gastrointestinal systems, and immunological involvement, have also been associated with this CDG type[56,57]. The symptoms can range from mild to severe, with reported neonatal death[56]. There is still no cure for PMM2-CDG, with only symptomatic treatment available[56].

1.4. Cancer

Cancer is one of the most prevalent causes of mortality worldwide, being, inclusively, the leading cause in some countries[58]. Thus, early diagnosis, as well as effective therapeutics, are crucial.

Cancer arises from successive mutations caused by a variety of carcinogens, such as physical, chemical, and biological agents[59]. These mutations alter gene expression leading to increased proliferation and survival. This can be achieved, for instance, by the inhibition of proliferation regulation, the production of growth-promoting signals by cancer cells and the ability to resist cell death[60]. Other relevant characteristics that allow an increased proliferation are angiogenesis promotion, with the production of proteins such as vascular endothelial growth factors, as well as higher capacity for invasion and metastasis, due to altered regulation of proteins involved with cell-cell and cell-matrix interactions[60].

Although the immune system has a preponderant role in cancer elimination, cancer cells also have mechanisms to evade detection by immune cells[60,61]. Some of them are the recruitment of regulatory T lymphocytes to cancer site, hindering the function of other immune cells; secretion of inhibitory cytokines, namely transforming growth factor β (TGF- β) and IL-10; and negatively affect antigen presentation and T cell activation, either by affecting the antigen

processing pathways or by modulating the expression of co-stimulatory/co-inhibitory molecules[61]. Another way to escape immune detection is the deregulation of glycosylation, which will be explained in further detail in the next topic.

1.4.1. Glycosylation and cancer

It has already been reported changes in glycosylation patterns when comparing healthy and cancer cells, however, the consequences and its underlying mechanisms are not yet fully understood.

One of the most determinant glycosylation modifications in the malignant process is aberrant sialylation, resulting in increased cancer-associated sialylated glycans, also referred as sialoglycans[62,63]. Generally, this is caused by altered regulation of sialyltransferases and sialidases and is associated with an increase in tumour growth and metastasis capacity[64]. Although there is a great variety of affected sialyltransferases related to cancer, one of the most predominant is ST6GAL1, which catalyses the attachment of α2,6-linked sialic acids to *N*-glycans[64].

Upregulation of this enzyme has been correlated with higher survivability and ability to invade and metastasise[64,65]. For instance, hypersialylation hinders the binding of receptors and ligands associated with apoptotic pathways, such as galectins and Fas-Fas ligand[64,65].

Furthermore, overall changes in sialylation also affect interactions with Siglecs. This can be seen as these sialylated structures are recognised by Siglecs expressed in APCs, like DCs and macrophages, impairing their function and leading to immunosuppression, as explained before[63].

The examples above are only a few in the plethora of immune affectations resulting from aberrant glycosylation in cancer, and with such impact that it is now starting to be recognised as a new hallmark of cancer[64].

1.4.2. Immunotherapies

As mentioned, the development of effective therapeutics against cancer has been an emerging concern. Immunotherapy describes a treatment in which the immune system of the host is used to fight the disease[66].

Diverse immunotherapies have been arising in recent years, focusing on diverse aspects of the immune system, such as blockage of co-inhibitory molecules, use of cytokine cocktails, cell therapies, and dendritic cell-based vaccines[66,67,68,69].

Dendritic cell-based vaccines (Figure 1.4.) frequently make use of *ex-vivo* derived DCs loaded with tumour-associated antigens to elicit an immune response by activating T lymphocytes[70,71,72]. DCs are normally obtained by isolating monocytes from the patients' blood and differentiating them into monocyte-derived dendritic cells (moDCs)[71]. This differentiation step can be achieved by using cytokines like IL-4 and granulocyte-macrophage colony-stimulating factor (GM-CSF)[71,73]. After differentiation, moDCs are stimulated to promote their maturation using, for instance, cytokine cocktails with IL-1β, IL-6, prostaglandin E2 (PGE2) and TNF-α[70,72]. Then, moDCs are loaded with tumour-associated antigens and administered to the patient[70,72].

However, there are still certain obstacles related to the tumour immunosuppressive environment. Thus, in recent years, there has been a collective effort to understand the underlying mechanisms of the cancer cells as well as to optimise moDCs maturation and efficacy *in vivo*[71,73].

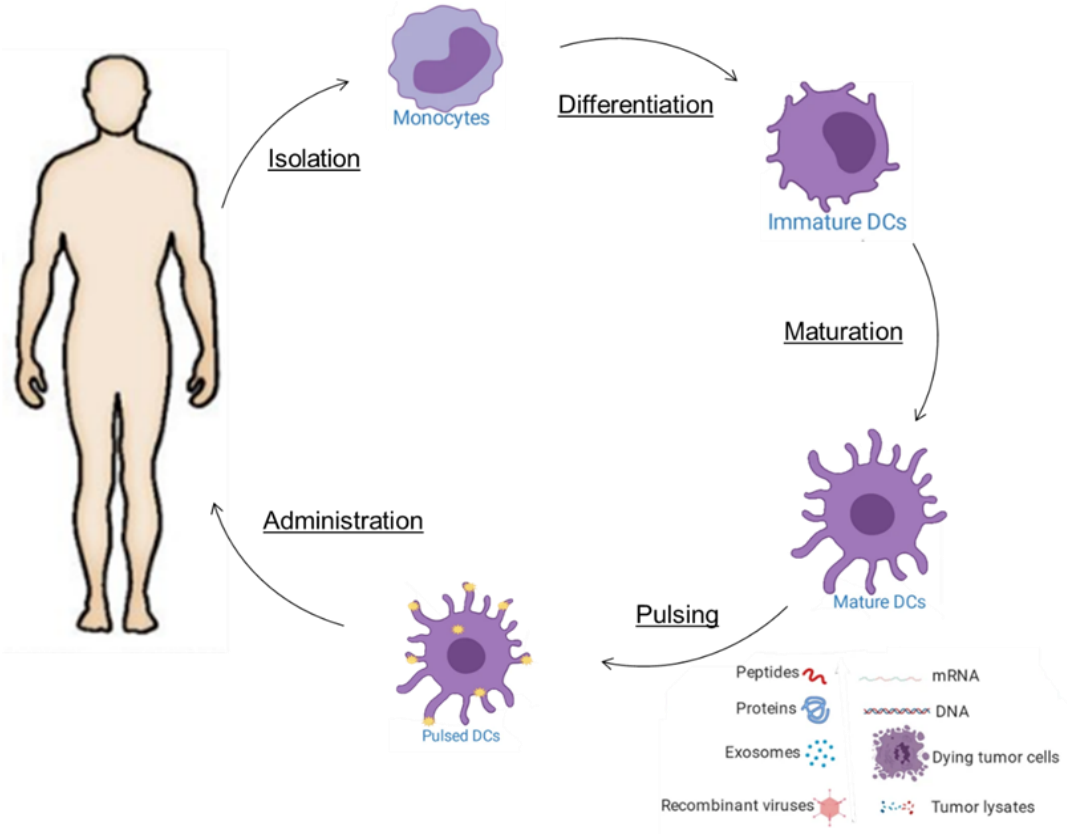

Figure 1.4. Schema of dendritic cell-based vaccine steps. Adapted from Gu *et al.*[72] and Santos and Butterfield.[74]

1.5. Introduction to the aim of the thesis

Glycosylation has been shown to have a preponderant role in human health and disease. Therefore, understating the consequences of its' alteration as well as the pathways in which it is involved is of utmost importance.

Glycosylation is a post-translational modification resulting from the enzymatic attachment of saccharides to either proteins, lipids, or other saccharides, originating glycoconjugates.

Given the privileged location of glycoconjugates at the cell surface and in all secreted proteins, it comes as no surprise that they are important modulators of cellular interactions. Thus, mutations in the genes that orchestrate glycosylation pathways have a major impact on the formed glycoconjugates and their physiological function, leading to diseases like CDG.

PMM2-CDG is a rare disorder and the most prevalent CDG known, with over 1000 patients diagnosed worldwide. It results from mutations in the *PMM2* gene and is characterised by defects in the *N*-glycan biosynthesis, leading to multi-systemic manifestations.

No cure has been found for PMM2-CDG, having only symptomatic treatment available. For that reason, understanding the underlying mechanisms of glycosylation as well as characterising the phenotypic changes that come from altered glycosylation may be a step towards curative treatment and quality of life improvement for this and other CDG types.

In recent years many studies have pointed out the glycosylation role in physiological and pathophysiological states and its association to several immune system functions.

Results from our group already indicated that sialylated glycans have a critical role in the maturation of DCs, negatively modulating the expression of antigen-presenting molecules, co-stimulatory molecules, and cytokine secretion, being these the three signals required to promote T cell activation[33,75,76,77,78]. These findings highlighted the important role of sialic acids in DCs' function and encouraged the proposal of sialidases to manipulate cell surface sialic acid content as a therapeutic strategy. For instance, it could be used to optimise DCs maturation and boost their crucial role in current immunotherapies applied in cancer treatment, like dendritic cell-based vaccines, that still lack effectiveness given the tumour immunosuppressive environment.

In fact, growing evidence that sialic acids may act as immune modulators, and have a major role in cancer progression, gave rise to novel therapeutics combining the use of sialidase and different technologies. The most relevant of these sialic acid-based therapeutics can be seen in some emerging patents, that include, for instance, the fusion of sialidase with therapeutic antibodies, such as anti-HER2 and anti-PD-L1 (Appendix 1).

Here we hypothesised that increased sialylation and *N*-glycosylation defects suppress the proper immune response. Thus, the general aim of this thesis was to better understand the impact and consequences of these glycosylation changes on the immune response.

To pursue this main objective, we have studied monocyte-derived dendritic cells (moDCs) from healthy individuals and compared the impact of different protocols aimed to alter cell surface content in DC immune potency. These protocols included treatment with sialidase, sialidase inhibitors and the standard maturation cytokine cocktail.

Additionally, we also studied the impact of *N*-glycans in immune responses. Here, we used a model already available at our group, namely PMM2-CDG fibroblasts, and compared their response to stimulation with control fibroblasts, by assessing cytokine secretion.

This study may provide new insights on the correlation of glycans and immune functions that can be relevant for understanding immune regulation mechanisms and/or contribute to the identification of new glycan-based targets and to therapy development. This may be relevant to improve dendritic cell-based vaccines in the context of cancer treatment and for treatment of immune-related clinical manifestation in CDG patients.

Chapter 2. Materials and Methods

2.1. Cell Culture

2.1.1. Culture of monocytes and differentiation into dendritic cells

Monocytes, after immunomagnetic separation (section 2.3), were cultured in 24-well plates (Greiner Bio-One) on an incubator (Panasonic) at 37ºC, with 5% CO_2 and a humidified atmosphere. To stimulate the differentiation into monocyte-derived dendritic cells (moDCs), in each well, $1.3x10^6$ cells were cultured in complete Roswell Park Memorial Institute (cRPMI) (Gibco) (composition on Appendix 2), in the presence of 750U/ml of human recombinant interleukin-4 (IL-4) (R&D Systems) and 1000U/ml of human recombinant granulocyte macrophage colony-stimulating factor (GM-CSF) (Miltenyi Biotec), for 5 to 8 days. At the third day of differentiation, the culture medium was supplemented with 750U/ml of IL-4 and 1000U/ml of GM-CSF.

2.1.2. Culture of C1R cell lines

Three different C1R cell lines (human B-cell lymphoblastoid line) transduced to overexpress CD1a, CD1b or CD1c, kindly provided by Mariolina Salio and colleagues, were used. The cells were cultured in cRPMI in T75 culture flasks (Sarstedt) on an incubator at 37ºC, with 5% CO_2 and a humidified atmosphere. For cell passage, as non-adherent cell lines, cells were centrifuged at 200xg for 5 minutes, counted and the medium replaced.

2.1.3. Culture of fibroblasts

Six different skin fibroblasts cell lines, three from PMM2-CDG patients (GM20945, GM27226, GM27386) and three from apparently healthy donors (control) (GM00498, GM00969, GM03349), provided by the Coriell Institute for Medical Research, were used.

Fibroblasts were cultured in complete Dulbecco's Modified Eagle Medium with low glucose (1 g/l) (composition on Appendix 2) in T75 culture flasks on an incubator at 37ºC, with 5% CO_2 and a humidified atmosphere. The medium was replaced every 3 to 4 days, and cells were passaged every 7 to 8 days. To detach the cells from the culture flasks, after removing the medium and washing with phosphate buffered saline (PBS) (composition on Appendix 2), cells were incubated with trypsin-ethylenediamine tetraacetic acid (EDTA) (Gibco) for 5 to 10 minutes at 37ºC. After incubation, medium was added, and cells were centrifuged at 200xg for 5 minutes.

Prior to the lectin staining (section 2.5.2) and ELISA (section 2.6), part of the fibroblasts were stimulated (S) with 1 ng/ml of TNF-α for 24 hours whilst the others remained non-stimulated (NS).

2.2. Isolation of human peripheral blood mononuclear cells (PBMCs) by density gradient centrifugation

Peripheral blood mononuclear cells (PBMCs), for isolation of monocytes and later differentiation into DCs, were obtained from buffy-coats, supplied and ethically approved by the Portuguese Blood Institute (*Instituto Português do Sangue e da Transplantação*).

The buffy-coats were obtained from healthy male volunteers, with age ranging from 18 to 60 years old, and from blood collected the day before usage, to decrease variability between samples.

8 ml of buffy-coat were distributed in two 15 ml falcon tubes (BD Biosciences) and PBS was added up to 15 ml. Both samples were centrifuged at 1100xg for 10 minutes, with brake off, at room temperature (RT). After centrifugation, each leukocyte ring, located between plasma and red blood cells, was collected, and transferred into a new tube. PBS was added up to 12 ml to both tubes containing the leukocyte ring, and the suspension was well mixed. 6 ml from each tube were slowly added to a new tube containing 3 ml of Ficoll (Sigma) at RT, and centrifuged at 1100xg for 30 minutes, with break off. After centrifugation, the interface ring was collected and transferred into a new tube, and PBS was added up to 25 ml. This suspension was centrifuged at 600xg for 10 minutes and the supernatant was discarded. The pellet was resuspended in 10 ml of PBS, and the cells were counted using a Neubauer chamber. The cell suspension was washed to remove platelets by centrifuging at 400xg for 5 minutes, at RT, isolating PBMCs.

2.3. Immunomagnetic isolation of monocytes and differentiation into immature monocyte-derived dendritic cells

PBMCs were resuspended in 80 µl of beads buffer (composition on Appendix 2) and 20 µl of magnetic CD14 MicroBeads (Miltenyi Biotec) for each 10^7 cells and incubated at 4ºC for 15 minutes. 2 ml of beads buffer per 10^7 cells were added, after incubation, and centrifuged at 600xg for 10 minutes, to remove unbound beads. The supernatant was discarded, and the pellet was resuspended in 500 µl per 10^8 cells. After washing a LS column (Miltenyi Biotec),

placed in a MidiMACS Separator on a MACS Multistand (Miltenyi Biotec), with 3 ml of beads buffer, the cell suspension was added to the column. After washing the column three times with 3 ml of beads buffer, it was removed from the action of the magnetic field. 5 ml of beads buffer were added, and the positive fraction ($CD14^+$) was eluted with the help of a plunger. The cells were counted using a Neubauer chamber and centrifuged at 600xg for 10 minutes, at RT. To promote the differentiation into moDCs, monocytes were cultured, as mentioned on section 2.1.1, for 5-8 days.

2.4. Sialidase Treatment

In order to analyse the impact of glycosylation, moDCs and C1R cell lines were treated with sialidase. Cells were centrifuged at 300xg for 5 minutes, the supernatant discarded, and the pellet was washed with simple RPMI medium at 300xg for 4 minutes.

The pellet was resuspended in 200 µl of simple RPMI medium and 20 µl of *Clostridium perfringens* sialidase (Stock concentration: 5 U/ml, Roche), for each 10^6 cells, and incubated at 37ºC for 1h.

After incubation, 1 ml of cRPMI was added to stop the enzymatic reaction, and the cells were centrifuged at 300xg for 4 minutes, discarding the supernatant.

For the cells used as the control group (not treated), the same protocol was used, except for the addition of sialidase.

2.5. Other moDCs Treatments

2.5.1. Cytokine Cocktail Treatment

One of the most common ways to induce the maturation of dendritic cells is the usage of cytokine cocktails[79]. After the differentiation step, the medium was replaced and a cytokine cocktail comprising IL-1β (10 ng/ml), IL-6 (1000 U/ml), Prostaglandin E2 (PGE2) (1 µg/ml) and TNF-α (10 ng/ml) was added to the respective well, for 48 hours.

For the cells used as the control group, the same protocol was used, except for the addition of the cytokine cocktail.

2.5.2. Sialidase Inhibitors Treatment

Another way to address the effects of glycosylation in moDCs was to add sialidase inhibitors in the beginning of the differentiation process. In these conditions, besides IL-4 and GM-CSF,

as previously mentioned, 1 mM of DANA (Sigma) or 1 mM of Zanamivir (Sigma) were also added to the respective wells on the first day of differentiation.

2.6. Flow Cytometry

Flow cytometry is a technique that evaluates several characteristics of particles or single cells in a suspension, namely its relative size and granularity or internal complexity[80]. When the laser light hits the single cells in suspension, the light is scattered and can be measured in different directions. Light scattering is detected and measured in the forward direction (Forward Scatter, FSC), indicating the relative size of the cell, or in higher angles (Side Scatter, SSC), pointing out the cell complexity and granularity[80].

Furthermore, this technique allows the detection of fluorescence, being either from cell autofluorescence or fluorophore conjugated antibodies.

Sometimes, using more than one fluorophore in the same sample can result in an overlap of their emission spectra, resulting in lower data quality. Therefore, compensation must be used, removing mathematically the fluorescence spillover of a certain fluorophore from every other detection channel except its own[81].

For every sample, information from at least $1x10^4$ events was collected using the Attune Acoustic Focusing Cytometer (Applied Biosystems) and the data was later analysed with the Attune Cytometric Software v2.1, Microsoft Office Excel and GraphPad Prism (version 8).

2.6.1. Antibody and Lectin Staining – moDCS

After the treatments previously described (section 2.4 and 2.5), moDCs and C1R cell lines were resuspended in cRPMI, distributed in eppendorf tubes ($1x10^5$ cells/tube) and stained with the antibodies or lectins described in Appendix 3.

Each tube was incubated at 4ºC for 30 minutes in the dark and washed with PBS at 300xg for 4 minutes. In the tubes in which the antibody or lectin was conjugated with biotin, streptavidin-phycoerythrin (PE) was added and incubated at 4ºC for 30 minutes in the dark, then washed with PBS at 300xg for 4 minutes.

After centrifugation, the supernatant was discarded, and the cells were fixed with 300 µl of 2% paraformaldehyde (Polysciences).

2.6.2. Lectin Staining - Fibroblasts

Fibroblasts from PMM2-CDG patients and control cell lines, both stimulated and non-stimulated, were collected and counted. The cells were distributed in each respective well in a 96-well plate ($1x10^5$ cells/well) and 100 µl of FACS buffer (composition on Appendix 2) were added to each well and centrifuged at 300xg for 5 minutes at 4ºC. The supernatant was removed, and the pellet was resuspended in 200 µl of FACS buffer, followed by centrifugation at 300xg for 5 minutes at 4ºC.

The supernatant was discarded, and the cells were stained, in separate wells, with three different lectins: biotin conjugated Concanavalin A (ConA), biotin conjugated *Galanthus nivalis* lectin (GNL) and fluorescein isothiocyanate (FITC) conjugated Peanut Agglutinin (PNA). The cells were incubated for 20 minutes at 4ºC in the dark, then 100 µl of FACS buffer were added to each well and centrifuged as previously described. 100 µl of streptavidin-PE were added to the wells in which biotin conjugated lectins were used and 100 µl of FACS buffer were added to the remaining wells. The cells were incubated for 10 minutes at 4ºC in the dark, 100 µl of FACS buffer were added and centrifuged. The supernatant was removed, the pellet was resuspended in 200 µl of FACS buffer and centrifuged. After discarding the supernatant, the cells were fixed with 300 µl of 2% PFA.

2.7. Enzyme-Linked Immunosorbent Assay (ELISA)

Enzyme-linked immunosorbent assay (ELISA) is a method commonly used to measure analytes, such as proteins, in a biological sample.

For this work, supernatants from both moDCs and PMM2-CDG and control fibroblasts were collected to quantify cytokines of interest using ELISA kits (Immunotools).

According to the cytokine being assessed, 50 µl of capture antibody, diluted 1:100 in PBS, were added to the wells of a 96-well plate (Costar) and incubated at 4ºC overnight. After incubation, the capture antibody was completely removed and 300 µl of blocking buffer (composition on Appendix 2) were added to each well, then incubated for 60 minutes at RT.

For the standards of every cytokine, serial dilutions were prepared with blocking buffer, according to each cytokine kit detection range. Samples, when necessary, were also diluted with blocking buffer to be within the kit detection range.

After the incubation, blocking buffer was completely removed and 100 µl of each sample and standard were added in duplicate to the plate wells, incubating for 2 hours at RT. Then, 200 µl

of washing buffer (composition on Appendix 2) were added to each well and removed, repeating this washing step 5 times.

According to the cytokine, 50 µl of biotinylated detector antibody, diluted 1:100 in blocking buffer, were added to each well and incubated for 2 hours at RT, followed by 5 washing steps. 50 µl of Poly-Horseradish Peroxidase (HRP)-Streptavidin, diluted 1:1000 in blocking buffer, were added to each well, incubated for 30 minutes at RT, and washed 5 times.

After the washing steps, 50 µl of 3,3',5,5'-Tetramethylbenzidine (TMB) substrate at RT were added to the wells and incubated up to 60 minutes in the dark, until the intended development of the colour reaction was achieved.

To stop the reaction, 50 µl of 2M H_2SO_4 were added to each well and the absorbance was read at 450 nm on a SpectraMax 190 Microplate Reader (Molecular Devices). SoftMax Pro software (version 6.4.) was used to collect the data and the results were later analysed with Microsoft Office Excel and GraphPad Prism (version 8).

2.8. Protein Quantification

After the TNF-α stimuli, as previously described (section 2.1.3.), fibroblast lysates were obtained using the Pierce IP Lysis Buffer (Thermo Scientific) in conjunction with Protease Inhibitor Cocktail (Roche). Cells were incubated with these for 30 minutes at 4ºC while vortexing periodically, followed by centrifugation at 17000xg for 2 minutes, collecting the supernatant.

To quantify the protein present in the lysates the Pierce BCA Protein Assay Kit (Thermo Scientific) was used. The bicinchoninic acid (BCA) working reagent can be obtained by adding the reagent A and reagent B (provided by the kit) in a 50:1 proportion, respectively. 10 µl of both the standards (prepared according to the kit) and the lysates were added to a 96-well plate, in duplicate, and 200 µl of the BCA working reagent was added on top.

The plate was incubated at 37ºC for 30 minutes and the absorbance was read at 562 nm on a SpectraMax 190 Microplate Reader and SoftMax Pro software (version 6.4.) was used to collect the data. The results were analysed using Microsoft Office Excel and the protein concentration was estimated based on the calibration curve obtained from the standards.

2.9. Cytokine Gene Expression in moDCS

2.9.1. RNA Extraction and cDNA conversion

In order to address cytokine gene expression in moDCs, RNA extraction and conversion to cDNA was performed, followed by Real Time Quantitative Polymerase Chain Reaction.

For RNA extraction the GenElute Mammalian Total RNA Miniprep Kit (Sigma) was used.

The cells were centrifuged at 300xg for 5 minutes, the supernatant was discarded, and 250 µl of lysis solution, prepared by mixing β-mercaptoethanol (Sigma) and the lysis solution provided by the kit in a 1:100 proportion, were added. Each sample was pipetted thoroughly, and the lysates were centrifuged at 12 000xg for 2 minutes at 4ºC in a filtration column. Then, 250 µl of ethanol (70%) were added to the lysates and pipetted thoroughly. The mixture was added to a binding column and centrifuged at 12 000xg for 15 seconds at 4ºC, discarding the flow-through liquid. The binding column was washed one time with 500 µl of washing solution I and two times with 500 µl of washing solution II (both solutions provided by the kit) by centrifugation at 12 000xg for 15 seconds at 4ºC. The column was later centrifuged at 12 000xg for 1 minute, without any volume, to remove completely any residual ethanol. 50 µl of elution solution (provided by the kit) were added to the binding column and RNA was collected by centrifugation at 12 000xg for 1 minute. RNA was pipetted again to the binding column, 10 µl of elution solution were added and the centrifugation step was repeated in the same conditions.

All RNA extracted was converted to cDNA using the High-Capacity cDNA Transcription Kit (Applied Biosystems) using random primers.

The mix for the conversion to cDNA for each sample comprised 9.8 µl of RNase free water, 8 µl of 10x buffer, 8 µl of 10x random primers, 3.2 µl of 100 mM deoxynucleoside triphosphates (dNTPs) and 1 µl of MultiScribe Reverse Transcriptase enzyme. 50 µl of RNA and the previously prepared mix were added to a PCR tube (VWR) and later placed in a Programmable Thermal Controller PTC-100 (MJ Research), following the PCR program described below (Table 2.1.). The cDNA obtained was stored at -20ºC.

Table 2.1. cDNA synthesis reaction program.

	1st Step	2nd Step	3rd Step	4th Step
Temperature (ºC)	25	37	85	4
Time	10 min	120 min	5 sec	∞

2.9.2. Real Time Quantitative Polymerase Chain Reaction

Real Time Quantitative Polymerase Chain Reaction (RT-qPCR) is a technique that allows the follow up of the amplification product whilst the reaction is still occurring.

RT-qPCR was performed in MicroAmp Fast Optical 96-Well Reaction Plate (Applied Biosystems). In each reaction, prepared in duplicate, 3 µl cDNA, 2 µl of probe, 10 µl of TaqMan® Universal Master Mix II (Applied Biosystems) and 5 µl of molecular biology water (NZYTech) were added. The probes selected for this work included IL-1β, IL-6, IL-10, IL-12, TNF-α as well as glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and β-actin as endogenous controls.

For the RT-qPCR, 7900HT Fast Real-Time PCR System (Applied Biosystems) was used with the conditions detailed below (Table 2.2.).

Table 2.2. RT-qPCR reaction conditions.

	1 cycle	40 – 50 cycles	
Temperature (ºC)	95	95	60
Time (seconds)	20	3	30

The relative gene expression was estimated by the CT method previously described by Livak and Schmittgen[82]. The CT values were obtained with the SDS software (version 2.4) and the relative mRNA levels, normalised using the mean of the endogenous genes expression, were calculated with the equation $2^{-\Delta CT}$ x 1000, and analysed with Microsoft Office Excel and GraphPad (version 8).

Chapter 3. Results and Discussion

3.1. Gating Strategy

To ensure the evaluation of markers in the population of interest as well as guarantee the quality of data, a gating strategy must be defined. In this work, all flow cytometry data was analysed using the gating strategy exemplified on Figure 3.1. Firstly, when reading each sample, a gate was created to select the cells of interest (red gate on Figure 3.1.). Secondly, to guarantee the assessment of single cells as well as the quality of data, multiplets were excluded. This was possible by verifying the proportion between cellular height and area or width. When comparing single cells with multiplets, both will have roughly the same height, but the latter will have an increased width and area in comparison with single cells, allowing its identification. After multiplet exclusion, histograms for all samples were analysed and the median fluorescence intensity (MFI) was calculated. MFI was obtained by subtracting the median intensity of the unstained cells or control samples (cells stained with secondary antibody/streptavidin) from the median intensity of each sample stained with different fluorophores used: fluorescein isothiocyanate (FITC), phycoerythrin (PE) or allophycocyanin (APC).

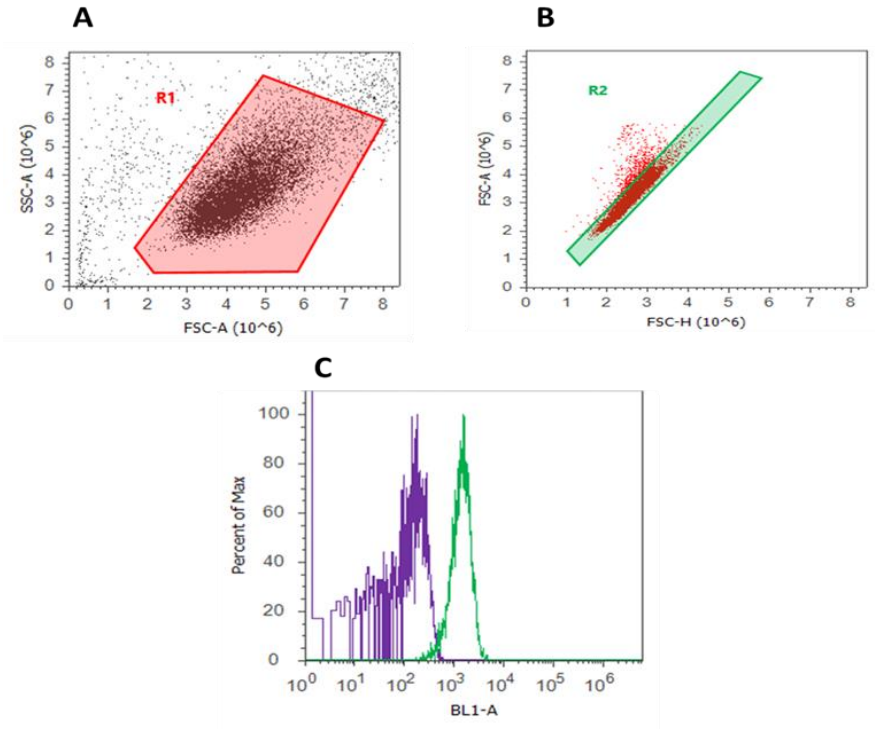

Figure 3.1. Flow cytometry gating strategy. A – Gate selection (R1 - in red) covering the cells of interest (exemplified in C1R CD1a cell line) on a dot plot (SSC vs FSC). **B** - Multiplet exclusion and gate selection (R2 - in green) of single cells in R1, represented on a dot plot (FCS-Area vs FCS-Height). **C** – Histogram exemplification of cells in R2 gate, with *yy* axis representing the normalized cell counts and

xx axis representing the fluorescence intensity of the fluorophore used (in this case FITC). MFI was calculated by subtracting the fluorescence intensity of the unstained/control sample (in purple) from the fluorescence intensity of the samples stained with a fluorophore. Both dot plots and histograms were obtained using Attune Acoustic Focusing Cytometer (Applied Biosystems) and Attune Cytometric Software v2.1.

3.2. Monocyte-derived Dendritic cells

Dendritic cells have been shown to be crucial players in the crosstalk between the innate and adaptive immunity, by capturing and processing antigens and later presenting them to T lymphocytes, eliciting a more specific immune response.

Given their important role in the immune system, DCs have been used in immunotherapies against cancer, such as dendritic cell-based vaccines. Although this therapeutic has shown promising results, in numerous cases it still exhibits limited clinical benefit[70]. One of the reasons for the reduced efficacy is probably the tumour immunosuppressive environment. Thus, the development of novel strategies to boost its efficacy is essential, being one of them a more efficient DC maturation.

Previous results from the group showed that the removal of sialic acids by sialidase treatment can induce DCs maturation and increase the expression of antigen-presenting and co-stimulatory molecules and cytokine secretion, the three necessary signals for T cell activation. Here, the treatment of DCs with sialidase was compared to the standard cytokine cocktail used in DCs maturation, regarding the expression of antigen-presenting and co-stimulatory molecules and cytokine secretion.

Also, since it is known that monocyte differentiation into DCs is characterized by an upregulated expression of Neu1 and Neu3, which have an important role during differentiation and later maturation[45], we addressed the impact of altering sialylated molecules, by using inhibitors for these sialidases, DANA (Neu1 and Neu3 inhibitor) and Zanamivir (Neu3 inhibitor). All these results will be further explored in the next topics.

3.2.1. Lectin Staining

In this part, we aimed to have a better characterization of the glycosylation profile resultant from different treatments. To accomplish that, cells were stained with PNA and *Sambucus nigra* lectin (SNA) (see Appendix 4, Figure 6.1.). The former binds to galactose residues linked to GalNAc in a β-1,3 bound, also referred as T-antigen, whilst the latter binds preferentially to sialic acids linked to galactose or GalNAc through an α2,6 linkage, and through an α2,3 linkage

to a lesser degree. Therefore, the results obtained with these lectins elucidate the extent of cell surface sialylation, as PNA cannot bind to T-antigen when the latter is sialylated, whilst SNA requires sialic acids to bind.

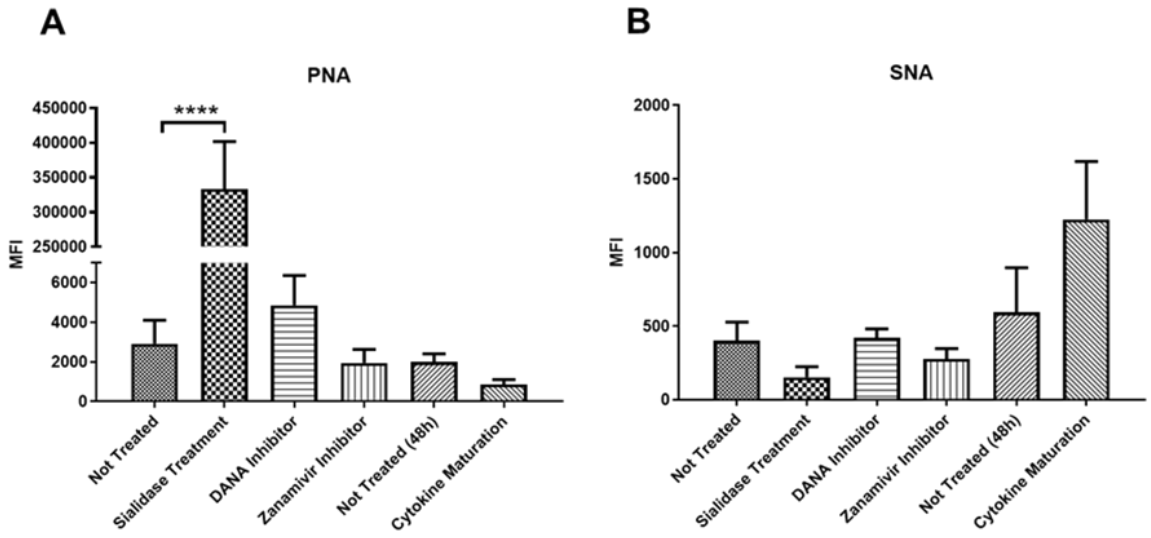

Figure 3.2. Changes in lectin binding on moDCs' surface. Values represent median fluorescence intensity (MFI) (mean ± SEM) for two different lectins (**A** – PNA, **B** – SNA) after distinct treatments (N ≥ 5). Sialidase Treatment stands for moDCs treated with *Clostridium perfringens* sialidase for 1h. DANA and Zanamivir Inhibitor represent moDCs differentiated in the presence of DANA or Zanamivir, respectively. Cytokine Maturation stands for moDCs incubated with IL-1β, IL-6, PGE2 and TNF-α for 48 hours. Not treated represents the control for sialidase treatment, DANA and Zanamivir Inhibitor, whilst Not Treated (48h) stands for Cytokine Maturation control. Statistical significance was obtained using Ordinary one-way ANOVA and is indicated by asterisks (**** - $p \leq 0.0001$)

Overall, there were opposing tendencies when comparing the results obtained with PNA and SNA staining. And, considering the nature of the structures recognized by these two lectins, it was expected, since PNA binds to non-sialylated molecules and SNA to α2,6 and α2,3 linked sialic acids.

Firstly, it is important to highlight the statistically significant difference for PNA staining, around 100-fold MFI increase, between sialidase treated DCs and those not treated (Figure 3.2. A). Furthermore, it was observed for SNA staining (Figure 3.2. B), a decrease in MFI with the sialidase treatment. These two results are essential to corroborate the effectiveness of the sialidase treatment protocol used.

Considering the conditions in which sialidase inhibitors (DANA and Zanamivir) were used, a lesser PNA staining and an increased SNA staining were expected, resulting from the increased presence of sialylated glycoconjugates. However, little changes in MFI were noted in these two different conditions. This result might point out the complexity of the sialylation

process, orchestrated by a great variety of sialyltransferases and sialidases[83], maintaining a tight regulation in sialic acid content.

It is interesting to notice that the use of the maturation cytokine cocktail leads to opposing results from sialidase treatment when it comes to surface sialic acid content. This can be noted by less MFI with PNA staining and the increased binding with SNA, when compared to the respective control (Not treated 48h). These opposing results might suggest different maturation mechanisms induced by these two treatments.

3.2.2. Antigen-Presenting Molecules

As mentioned above, antigen-presenting molecules are essential to present captured antigens to T cells and activate them. Besides MHC-I and MHC-II, peptide-presenting molecules, we also used antibodies for CD1a and CD1c, both lipid-presenting molecules.

All these molecules were assessed in moDCs after the treatments referred before, and the results can be seen in Figure 3.3.

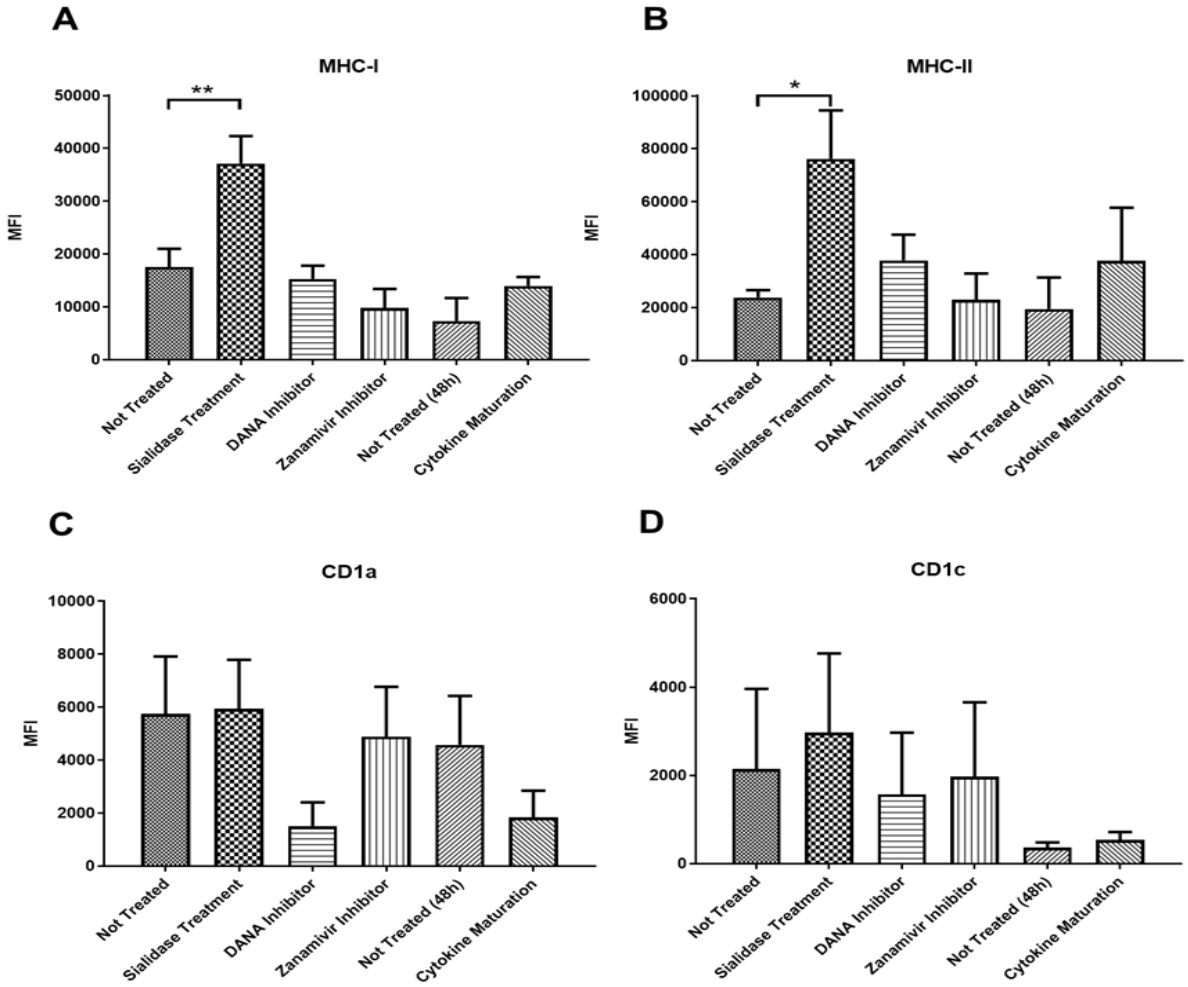

Figure 3.3. Changes in the expression of antigen-presenting molecules on moDCs' surface. Values represent median fluorescence intensity (MFI) (mean ± SEM) of different antigen-presenting molecules (**A** – MHC-I, **B** – MHC-II, **C** – CD1a, **D** – CD1c) after distinct treatments (N ≥ 5). Sialidase Treatment stands for moDCs treated with *Clostridium perfringens* sialidase for 1h. DANA and Zanamivir Inhibitor represent moDCs differentiated in the presence of DANA or Zanamivir, respectively. Cytokine Maturation stands for moDCs incubated with IL-1β, IL-6, PGE2 and TNF-α for 48 hours. Not treated represents the control for sialidase treatment, DANA and Zanamivir Inhibitor, whilst Not Treated (48h) stands for Cytokine Maturation control. Statistical significance was obtained using Ordinary one-way ANOVA and is indicated by asterisks (* - $p \leq 0.05$, ** - $p \leq 0.01$).

Regarding MHC-I and MHC-II (Figure 3.3. A and B) a significant increase was noted with sialidase treatment in comparison to its control. This specific result indicates that the removal of sialic acids from the cell surface leads to an increased presence of these markers in the cellular membrane.

Although MHC-II regulation is still unclear, the underlying mechanism for the increment of MHC-I at cell surface has been previously tested by our group and it was observed that desialylation increases the stability of MHC-I, reducing its turnover rate and retaining it more time at the cell surface[78]. In fact, since the MHC molecules are sialylated[84], it is possible that the stabilization mechanism is a direct consequence of the sialic acid removal from MHC-I itself, from other neighbour molecules or both.

Another possible explanation for the higher expression of MHC molecules is that sialic acid removal from the cell surface might lead to an indirect upregulation through increased synthesis of these molecules. One other possible mechanism would be through sialic acid-Siglec binding. Since sialic acids act as ligands for several molecules, such as Siglecs, which are mostly inhibitory and can bind in *cis*, the loss of sialylated structures could lead to less inhibitory signals and consequently to a more activated profile.

In terms of sialidase inhibitors, DANA and Zanamivir, no great changes were observed in MHC-I and MHC-II, although there is a slight decrease in MFI for MHC-I, especially with Zanamivir, and an increase in MFI for MHC-II, especially with DANA.

Given that the cells are treated with these sialidase inhibitors during the differentiation stage (for 5 to 8 days), perhaps at this time point we are not seeing the direct influence of the inhibitors, since no noticeable changes were also seen in glycosylation with the lectin staining (previous topic).

When it comes to the cytokine cocktail, an increase in MHC-I and MHC-II was expected, since dendritic cell maturation is accompanied with an upregulation of these markers. However little changes were noticed when comparing cytokine maturation to its control (Not treated

48h). It might be possible that moDCs are not yet fully maturated with 48 hours exposure to the cytokine cocktail, or that this treatment has less effect on these molecules when compared to sialidase treatment.

Regarding CD1 molecules, CD1a and CD1c, it was observed overall a big variation in MFI values, as can be seen by the error bars in Figure 3.3. This variation appeared to be related to the variability between the donors from which monocytes were isolated.

Nonetheless, no big differences in MFI were noted with the different treatments, except for DANA and cytokine cocktail in CD1a staining, that although not significant, in both cases, a decrease in MFI was seen when compared to their respective controls.

It might be important to highlight that a decrease in CD1a was only noted when the cells were treated with DANA, and not with Zanamivir, possibly indicating a potential regulatory role of Neu1 in this molecule.

Overall, and given the little data available for these molecules, further studies will be necessary to fully understand their mechanisms and regulation.

3.2.3. Co-stimulatory Molecules

Co-stimulatory molecules, referred as the second signal, are indispensable to activate T cells, as their absence leads to T lymphocyte anergy. Therefore, we aimed to understand the impact glycosylation has on CD80, CD86 and CD40, molecules that are crucial in DC-T cell crosstalk, and that have been shown to be upregulated in mature DCs[85,86]. The results obtained are represented in Figure 3.4.

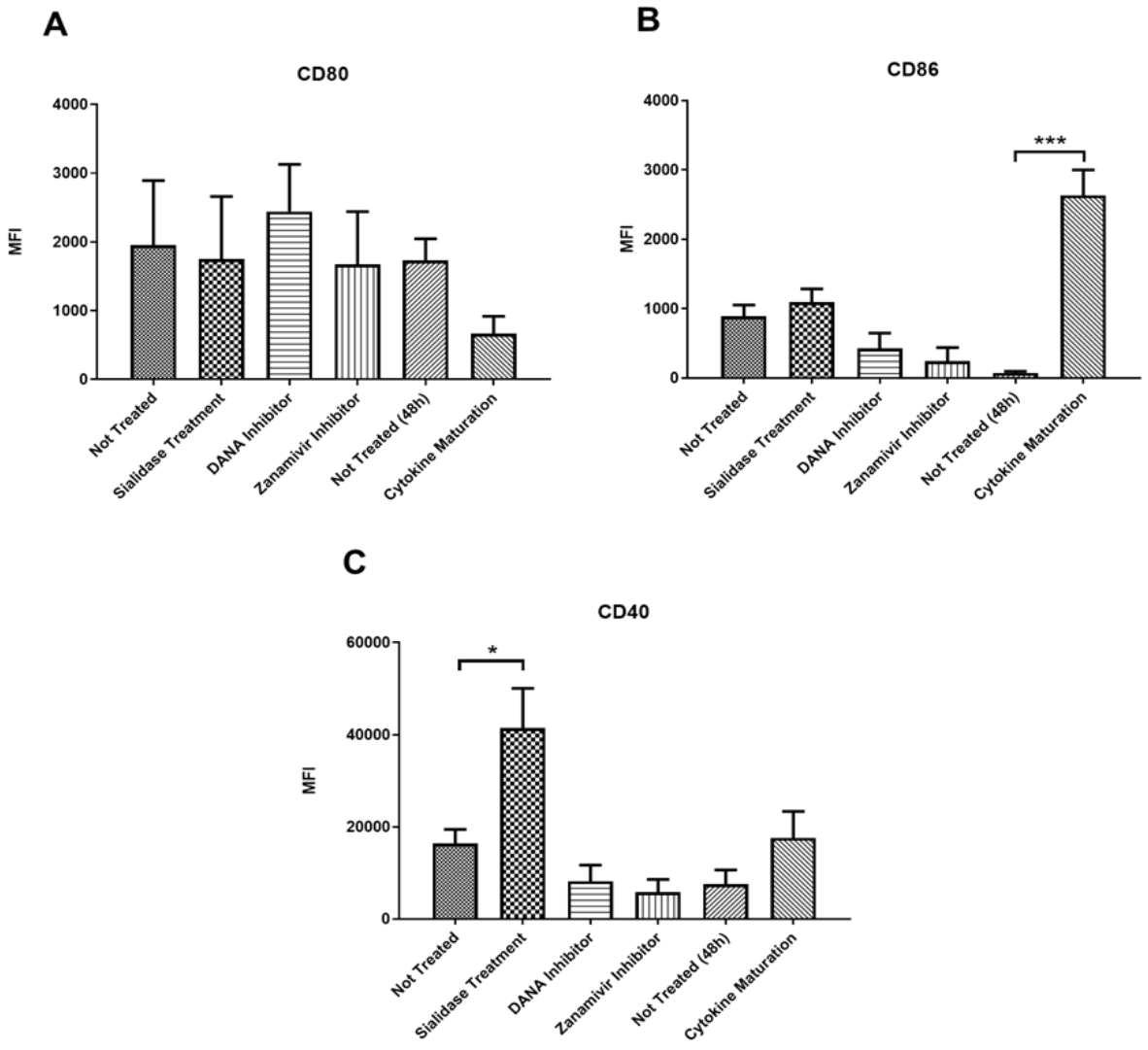

Figure 3.4. Changes in the expression of co-stimulatory molecules on moDCs' surface. Values represent median fluorescence intensity (MFI) (mean ± SEM) of different co-stimulatory molecules (**A** – CD80, **B** – CD86, **C** – CD40) after distinct treatments (N ≥ 5). Sialidase Treatment stands for moDCs treated with *Clostridium perfringens* sialidase for 1h. DANA and Zanamivir Inhibitor represent moDCs differentiated in the presence of DANA or Zanamivir, respectively. Cytokine Maturation stands for moDCs incubated with IL-1β, IL-6, PGE2 and TNF-α for 48 hours. Not treated represents the control for sialidase treatment, DANA and Zanamivir Inhibitor, whilst Not Treated (48h) stands for Cytokine Maturation control. Statistical significance was obtained using Ordinary one-way ANOVA and is indicated by asterisks (* - $p \leq 0.05$, *** - $p \leq 0.001$).

Regarding CD80, Figure 3.4. A, relevant changes in MFI were not noted with the different treatments, apart from the cytokine maturation cocktail, which surprisingly showed a decreased MFI. Despite previous group results showing an increased expression of CD80 after sialidase treatment, here, little changes in MFI were observed, possibly pointing out a big variation of this molecule expression and regulation between different donors.

With DANA and Zanamivir inhibitors almost no changes were obtained, which might indicate, once again, a late time point to see changes in this molecule.

In terms of CD86 staining (Figure 3.4. B), only the maturation cocktail revealed a statistically significant increase in MFI, as we would expect given the known upregulation of CD86 in DCs maturation. Regarding sialidase treatment we can see a slight increase in the expression of this molecule, and with DANA and Zanamivir we noticed an opposing tendency, as the MFI decreased to some extent in these conditions.

In CD40 staining, Figure 3.4. C, a significant increase in MFI with sialidase treatment was observed, whilst DANA and Zanamivir showed an opposing trend. Although not statistically significant, the use of the cytokine cocktail resulted in a higher expression of CD40.

Overall, it seems that the modulation of these co-stimulatory molecules differs greatly between one another, possibly having different regulation mechanisms. This could help explain, for instance, a decrease in CD80 expression with cytokine cocktail, treatment that also led to an increase in the expression of CD86 and CD40. Furthermore, it might be possible that a different time point was required to see some changes in the expression of these molecules.

3.2.4. Cytokine Production

As previously mentioned, cytokines are important immune modulators, essential for T cell activation. To assess cytokine production by moDCs after the exposure to the previously referred treatments two different methods were used. Firstly, ELISA was used to measure IL-12 secretion in the cell culture supernatant. Since IL-1β, IL-6 and TNF-α were used in the maturation cytokine cocktail we suspected that their presence in the medium would affect the results obtained by ELISA. Therefore, genetic expression obtained by RT-qPCR was the method selected to analyse the production of these cytokines, as well as IL-12 and IL-10. All the referred cytokines play a major role in the immune response, being all pro-inflammatory cytokines, except for IL-10, an anti-inflammatory cytokine.

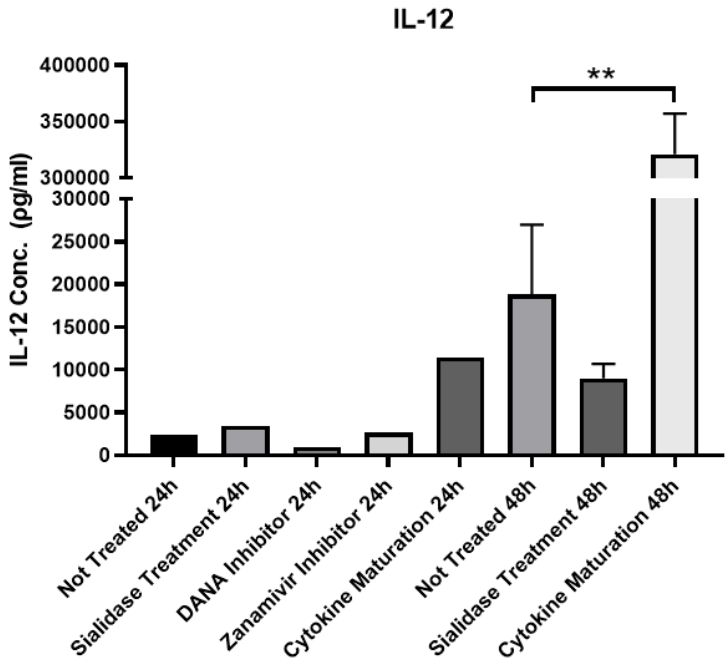

Figure 3.5. IL-12 secretion in moDCs treated with different treatments. Values represent pg of IL-12 secreted per ml of medium (mean ± SEM) by moDCs after different treatments. After moDCs differentiation the medium was replaced and each treatment was performed, collecting the medium after 24 (N = 1) and 48 hours (N = 2). Sialidase Treatment stands for moDCs treated with *Clostridium perfringens* sialidase for 1h. DANA and Zanamivir Inhibitor represent moDCs differentiated in the presence of DANA or Zanamivir, respectively. Cytokine Maturation stands for moDCs incubated with IL-1β, IL-6, PGE2 and TNF-α for 48 hours. Not Treated represents cells that were not exposed to any treatment. Statistical significance was obtained using Ordinary one-way ANOVA and is indicated by asterisks (** - $p \leq 0.01$).

IL-12 secretion (Figure 3.5.) was measured by ELISA at two different time points (24 and 48 hours) to have a better characterization over time. At 24 hours, no great changes were noticed in the levels of this cytokine, apart from the condition in which the cytokine cocktail was used, where a noticeable increase was observed. Sialidase treatment induced slightly the production of IL-12, whilst DANA and Zanamivir appeared to slightly hinder the secretion of this cytokine.

Regarding the 48 hours timepoint, as expected, higher levels of cytokine were measured when compared to 24 hours, since cytokines were accumulated in the medium for another day. Surprisingly, we noted a decrease in IL-12 secretion with sialidase treatment, which differed from previous literature results[76,77]. Nonetheless, a significant increase in IL-12 levels were obtained when the cells were treated with the cytokine cocktail, which corroborated literature

results[87]. However, it is important to reinforce that more replicates need to be done to confirm the data obtained.

As previously said, to further characterize changes in cytokine production, when cells were exposed to different treatments, RT-qPCR was used. Like described on topic 2.8.2., an adaptation of Livak and Schmittgen method[82] was used to quantify the genetic expression of different cytokines. Here, the relative expression was normalised by endogenous genes, constitutively expressed, obtaining the number of messenger RNA molecules of interest per 1000 molecules of the endogenous controls. For the mentioned cytokines, relative gene expression was assessed after 3 hours of each treatment, apart from IL-12 where results after 3 and 6 hours were obtained (Figure 3.6.).

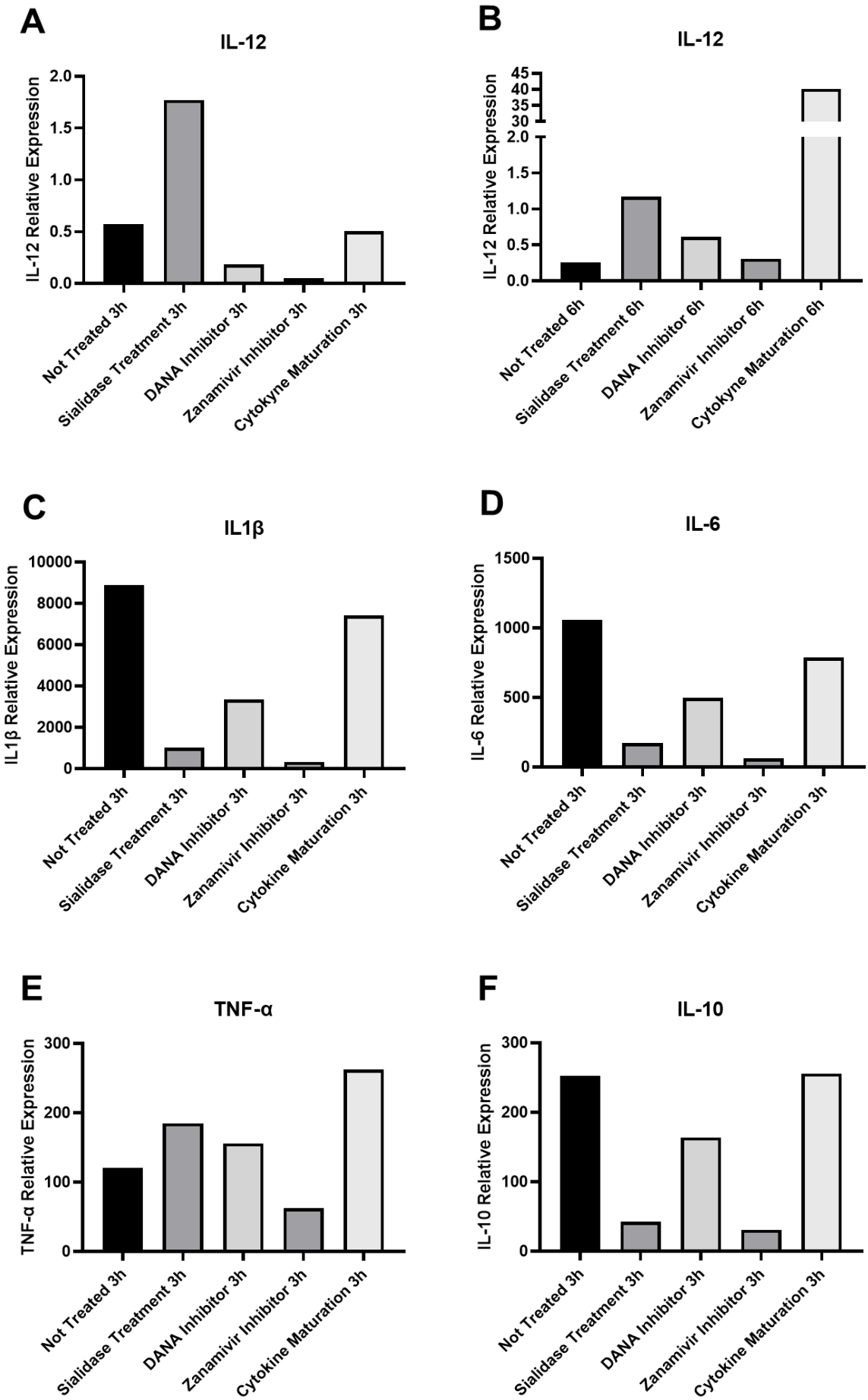
A
IL-12
IL-12 Relative Expression
2.0
1.5
1.0
0.5
0.0
Not Treated 3h
Sialidase Treatment 3h
DANA Inhibitor 3h
Zanamivir Inhibitor 3h
Cytokyne Maturation 3h
B
IL-12
IL-12 Relative Expression
45
40
35
30
2.0
1.5
1.0
0.5
0.0
Not Treated 6h
Sialidase Treatment 6h
DANA Inhibitor 6h
Zanamivir Inhibitor 6h
Cytokyne Maturation 6h
C
IL1β
IL1β Relative Expression
10000
8000
6000
4000
2000
0
Not Treated 3h
Sialidase Treatment 3h
DANA Inhibitor 3h
Zanamivir Inhibitor 3h
Cytokine Maturation 3h
D
IL-6
IL-6 Relative Expression
1500
1000
500
0
Not Treated 3h
Sialidase Treatment 3h
DANA Inhibitor 3h
Zanamivir Inhibitor 3h
Cytokine Maturation 3h
E
TNF-α
TNF-α Relative Expression
300
200
100
0
Not Treated 3h
Sialidase Treatment 3h
DANA Inhibitor 3h
Zanamivir Inhibitor 3h
Cytokine Maturation 3h
F
IL-10
IL-10 Relative Expression
300
200
100
0
Not Treated 3h
Sialidase Treatment 3h
DANA Inhibitor 3h
Zanamivir Inhibitor 3h
Cytokine Maturation 3h

Figure 3.6. Cytokines genetic expression analysis in moDCs treated with different treatments. Values represent relative expression of **A** – IL-12 (3h), **B** – IL-12 (6h), **C** – IL-1β, **D** – IL-6, **E** – TNF-α, and **F** – IL-10 in moDCs after different treatments. After moDCs differentiation, cells were treated, and genetic expression was assessed after 3 hours and 6 hours (only IL-12) (N =1). Sialidase Treatment stands for moDCs treated with *Clostridium perfringens* sialidase for 1h. DANA and Zanamivir Inhibitor represent moDCs differentiated in the presence of DANA or Zanamivir, respectively. Cytokine Maturation stands for moDCs incubated with IL-1β, IL-6, PGE2 and TNF-α. Not Treated represents cells that were not exposed to any treatment.

Regarding IL-12 gene expression (Figure 3.6. A and B) it was noted an increase over time, except for the not treated and sialidase treated cells. Nonetheless, it is important to note that at 3h, sialidase treatment seems to be the condition with a higher gene expression, suggesting that sialic acids modulation causes a faster response in the cells.

The fact that sialidase treatment shows a higher gene expression than not treated cells, at both timepoints, appears to be contradictory to the data obtained by ELISA, where at 48 hours lower levels with sialidase treatment were detected. This reinforces the need for further experiments.

Whilst DANA and Zanamivir treated cells appear to have an increasing expression with time, a noticeable increase, around 40-fold, with the cytokine cocktail was observed. This relates with the results obtained by ELISA, in which the cytokine maturation cocktail revealed a high production of IL-12.

For the IL-1β, IL-6 and IL-10 gene expression (Figure 3.6. C, D and F, respectively), and although the latter has an opposing role in the immune system, similar results were obtained. This can suggest that all these cytokines are regulated in similar ways. It may be possible that more time would be required to notice higher gene expression changes in the referred cytokines, since they showed lower values of expression in all conditions when compared to the control (Not treated).

Considering TNF-α gene expression (Figure 3.6. E), all conditions showed higher gene expression when compared to control, except for cells treated with Zanamivir.

In fact, for all cytokines, the presence of Zanamivir appears to exert a negative regulation.

Although all these results can point out some tendencies, more replicates and further studies need to be done for a better understanding of the mechanisms involved in cytokine regulation.

3.2.5. Possible synergy between treatments

Given the results for cytokine production, in which the cytokine cocktail appeared to have a leading role, we aimed to investigate the possible synergy between cytokine cocktail and

sialidase treatment and DANA and Zanamivir inhibitors. For that, moDCs were treated with sialidase or differentiated in the presence of DANA or Zanamivir and posteriorly incubated with the referred cytokine cocktail. Cytokine production on these different conditions was assessed like in the previous topic (3.2.4).

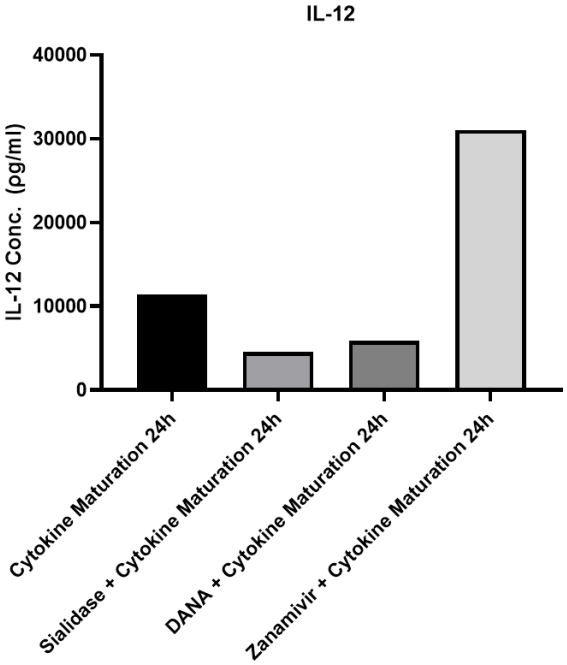

Figure 3.7. IL-12 secretion in moDCs treated with cytokine maturation cocktail in conjunction with other treatments. Values represent pg of IL-12 secreted per ml of medium (mean ± SEM) by moDCs after different treatments. After moDCs differentiation the medium was replaced, and sialidase treatment was performed. Afterwards, all cells were incubated IL-1β, IL-6, PGE2 and TNF-α and medium was collected after 24 hours (N = 1). Cytokine Maturation stands for moDCs incubated with IL-1β, IL-6, PGE2 and TNF-α for 24 hours. Sialidase stands for moDCs treated with *Clostridium perfringens* sialidase for 1h. DANA and Zanamivir represent moDCs differentiated in the presence of DANA or Zanamivir, respectively.

Results obtained by ELISA for IL-12 secretion (Figure 3.7.) revealed that the combined use of cytokine cocktail and sialidase treatment or DANA, appear to hinder the secretion of this cytokine. This could suggest that both these treatments prevent the maximum cellular response to cytokine maturation cocktail. However, it appears that Zanamivir might have a potentiating effect when combined with cytokine cocktail, as higher IL-12 levels were detected when comparing to cytokine maturation on its own. Nonetheless, given the low sample size, more replicates and further experiments need to be planned to confirm and understand the possible synergy between these treatments.

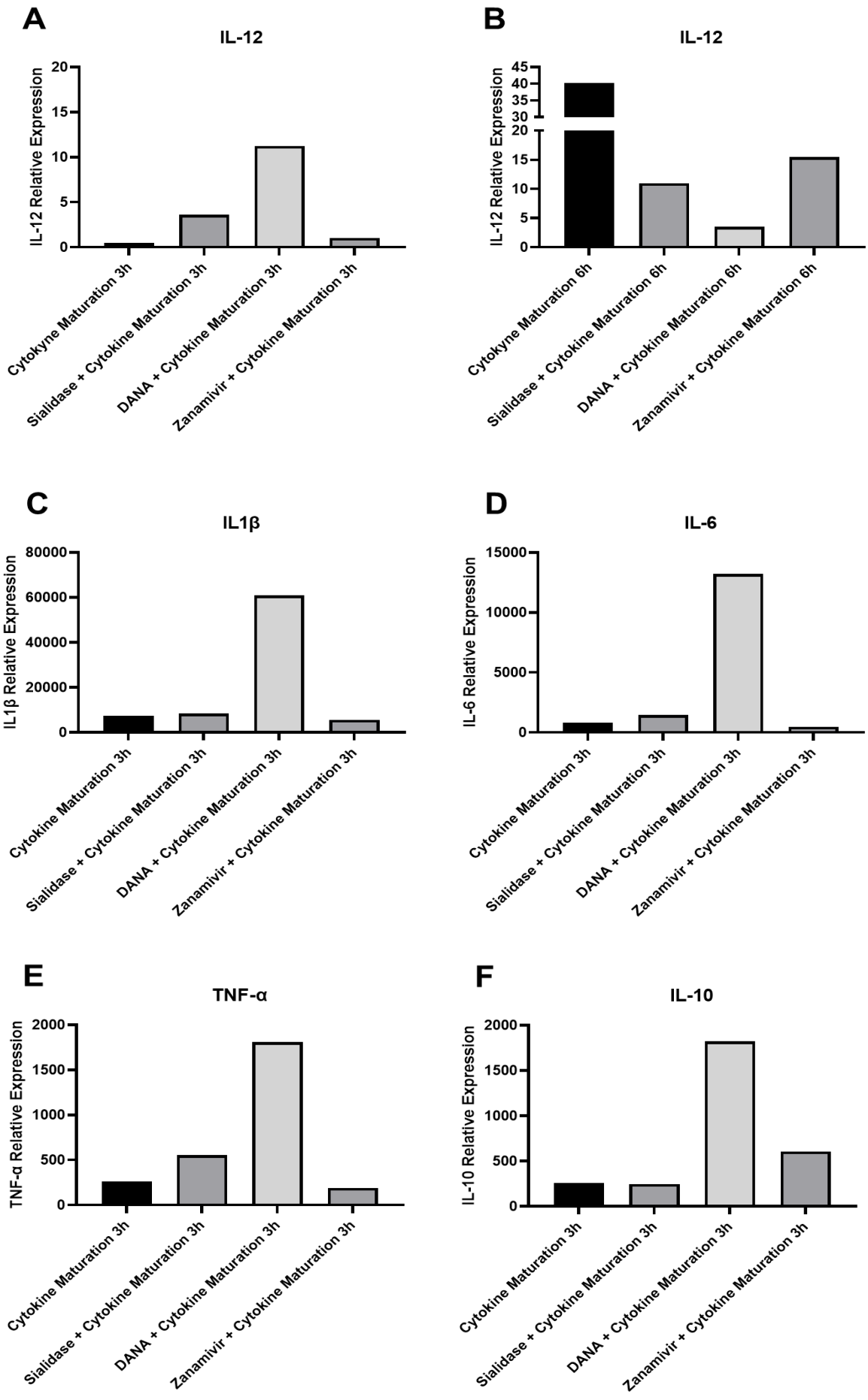
A
IL-12
IL-12 Relative Expression
20
15
10
5
0
Cytokyne Maturation 3h
Sialidase + Cytokine Maturation 3h
DANA + Cytokine Maturation 3h
Zanamivir + Cytokine Maturation 3h
B
IL-12
IL-12 Relative Expression
45
40
35
30
20
15
10
5
0
Cytokyne Maturation 6h
Sialidase + Cytokine Maturation 6h
DANA + Cytokine Maturation 6h
Zanamivir + Cytokine Maturation 6h
C
IL1β
IL1β Relative Expression
80000
60000
40000
20000
0
Cytokine Maturation 3h
Sialidase + Cytokine Maturation 3h
DANA + Cytokine Maturation 3h
Zanamivir + Cytokine Maturation 3h
D
IL-6
IL-6 Relative Expression
15000
10000
5000
0
Cytokine Maturation 3h
Sialidase + Cytokine Maturation 3h
DANA + Cytokine Maturation 3h
Zanamivir + Cytokine Maturation 3h
E
TNF-α
TNF-α Relative Expression
2000
1500
1000
500
0
Cytokine Maturation 3h
Sialidase + Cytokine Maturation 3h
DANA + Cytokine Maturation 3h
Zanamivir + Cytokine Maturation 3h
F
IL-10
IL-10 Relative Expression
2000
1500
1000
500
0
Cytokine Maturation 3h
Sialidase + Cytokine Maturation 3h
DANA + Cytokine Maturation 3h
Zanamivir + Cytokine Maturation 3h

Figure 3.8. Cytokines genetic expression analysis in moDCs treated with cytokine maturation cocktail in conjunction with other treatments. Values represent relative expression of **A –** IL-12 (3h), **B –** IL-12 (6h), **C –** IL-1β, **D –** IL-6, **E –** TNF-α, and **F –** IL-10 in moDCs after different treatments. After moDCs differentiation sialidase treatment was performed. Afterwards, all cells were incubated IL-1β, IL-6, PGE2 and TNF-α, and genetic expression was assessed after 3 hours and 6 hours (only IL-12) (N =1). Cytokine Maturation stands for moDCs incubated with IL-1β, IL-6, PGE2 and TNF-α. Sialidase stands for moDCs treated with *Clostridium perfringens* sialidase for 1h. DANA and Zanamivir represent moDCs differentiated in the presence of DANA or Zanamivir, respectively.

Considering IL-12 gene expression (Figure 3.8. A and B), an increasing tendency over time was noted in all conditions, apart from DANA + cocktail. However, it is interesting to point out that at 3 hours, DANA + cocktail, and sialidase treatment to a lesser extent, showed higher gene expression when compared to the cytokine cocktail alone. Perhaps this could suggest an earlier response with both these treatments. Nonetheless, at 6 hours, no condition revealed gene expression levels similar to cytokine cocktail on its own. Even Zanamivir + cocktail, that had higher levels of IL-12 in medium after 24h (obtained by ELISA) in comparison to only cytokine cocktail, had less gene expression at 6 hours. Although this can suggest contradictory results to ELISA, it might be important to understand how IL-12 gene expression changes after the 6 hours.

All the other cytokines (Figure 3.8. C, D, E and F) showed very similar gene expression profiles, with DANA + cocktail standing out with higher expression levels than the other conditions. This can suggest that Neu1 inhibition might potentiate an earlier effect of cytokine cocktail in cytokine regulation. Nonetheless, we are not sure how gene expression changes overtime, as we only have data regarding the 3 hours timepoint.

Once again, it is important to highlight that further studies need to be performed to a better characterization of the cytokine production regulation.

3.3. C1R cell lines overexpressing CD1a, b or c

Here, we used C1R cell lines overexpressing CD1a, CD1b or CD1c (referred here by C1R CD1a, C1R CD1b and C1R CD1c, respectively) to have a better grasp on the possible modulation of CD1 molecules resulting from changes in sialic acid content. The use of these cell lines would rule out the variability in these markers observed in moDCs from different donors. For that purpose, cells were treated with sialidase and changes in several markers were observed by flow cytometry, namely MHC-I and β2M (Figure 3.9.) and CD1a, CD1b and CD1c (Figure 3.10.).

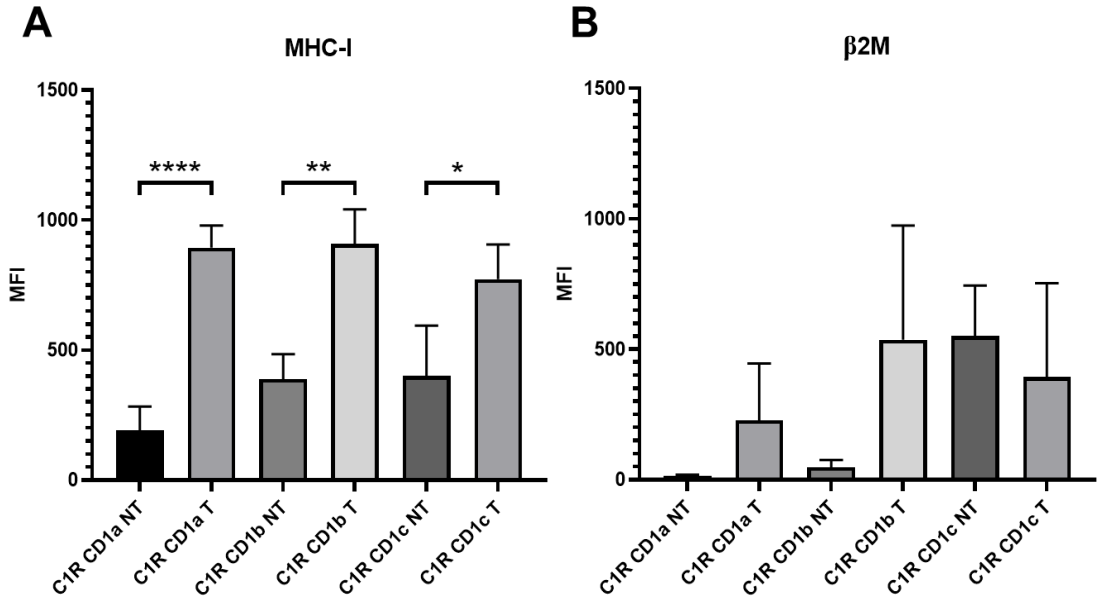

Figure 3.9. Changes in the expression of MHC-I and β2-microglobulin on C1R cell lines overexpressing CD1a, CD1b or CD1c. Values represent median fluorescence intensity (MFI) (mean ± SEM) of **A** - MHC-I and **B** - β2-microglobulin (β2M) after sialidase treatment (N ≥ 3). NT stands for cells that were not treated and T stands for cells treated with *Clostridium perfringens* sialidase for 1h. Statistical significance was obtained using Ordinary one-way ANOVA and is indicated by asterisks (* - $p \leq 0.05$, ** - $p \leq 0.01$, *** - $p \leq 0.001$).

Attending MHC-I staining (Figure 3.9. A) it was noted a significant increase in MFI in the three different cell lines when comparing sialidase treated to control cells. This result reinforces the ones obtained with moDCs, as we can see the same tendency when treating the cells with sialidase. Just as in moDCs, it is likely that sialidase either increases the expression of this antigen-presenting molecule, or increases its stability at cell surface, resulting in higher MFIs. Regarding β2-microglobulin (Figure 3.9. B), although with big variability, an increase in sialidase treated C1R CD1a and C1R CD1b was noticed, whilst C1R CD1c remained unchanged with sialidase treatment. As mentioned before, β2-microglobulin is part of the MHC-I heterodimer and of CD1 molecules as well. The antibody used (BBM.1) can either bind to β2-microglobulin in its free form or when complexed with MHC-I or CD1 heavy chains. Therefore, it appears that the increase in MFI observed might be correlated with the increase also verified in MHC-I.

As mentioned before, CD1a, CD1b and CD1c were also stained in C1R cell lines overexpressing these molecules and on moDCs (Figure 3.10.).

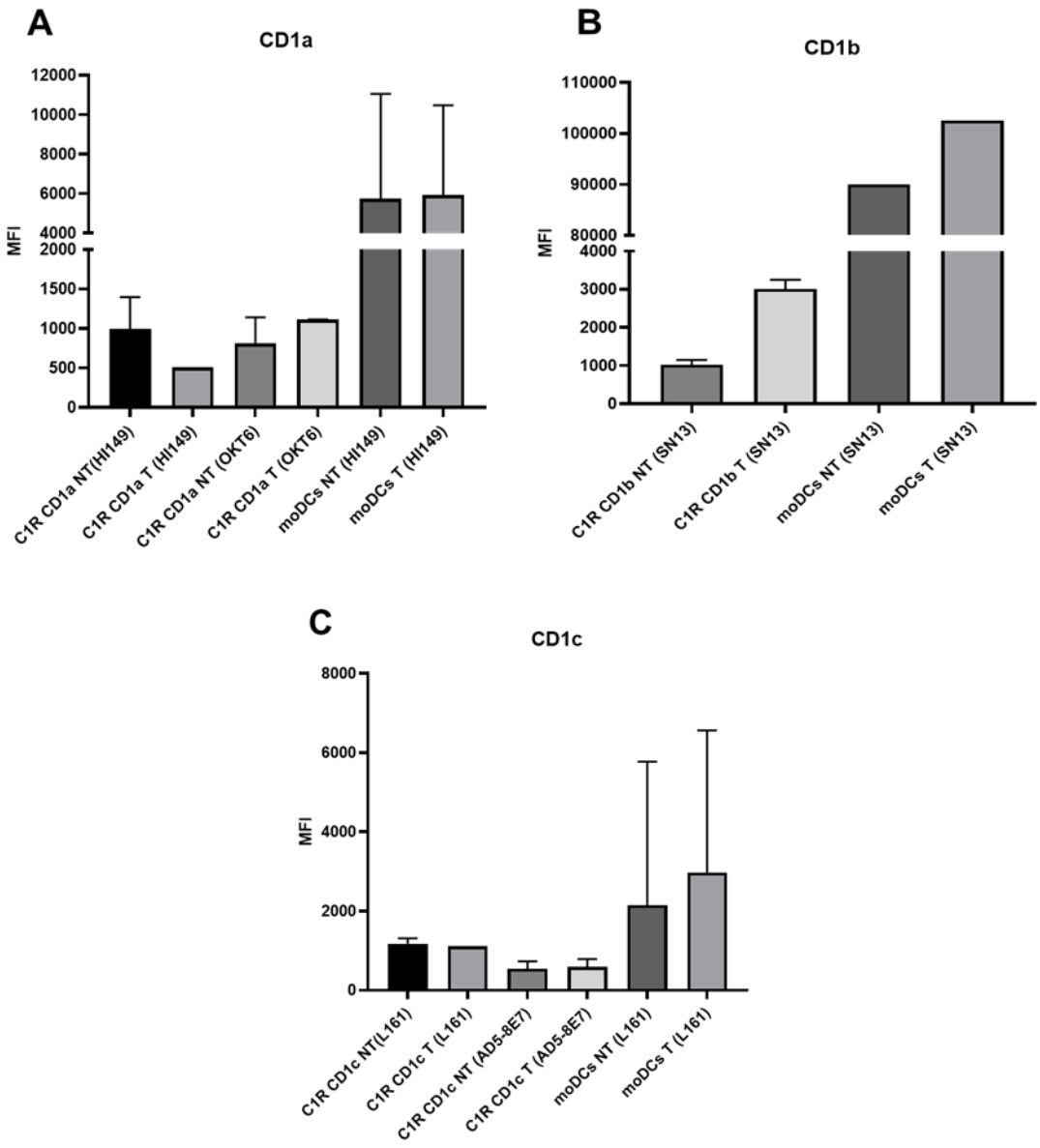

Figure 3.10. Changes in the expression of CD1a, CD1b and CD1c on C1R cell lines overexpressing CD1a, CD1b or CD1c and moDCs. Values represent median fluorescence intensity (MFI) (mean ± SEM) of **A** - CD1a (N ≥ 1), **B** - CD1b (N ≥ 1), and **C** - CD1c (N ≥ 1) on C1R cell lines overexpressing CD1a, CD1b or CD1c and moDCs. For CD1a staining two different antibody clones, HI149 and OKT6, were used. For CD1b staining one antibody clone, SN13, was used. For CD1c staining two different antibody clones, L161 and AD5-8E7, were used. NT stands for cells that were not treated and T stands for cells treated with *Clostridium perfringens* sialidase for 1h.

In terms of CD1a expression, similar results were obtained in C1R CD1a and moDCs, maintaining the same, or even a little lower expression (HI149 clone), after sialidase treatment.

This could suggest that sialidase does not affect or modulate the expression of this molecule. However, for CD1b staining, an increase was noted after sialidase treatment, both in moDCs and C1R CD1b. If this tendency is maintained with more replicates, it could suggest that sialic acid content modulation has an important role in CD1b expression at cell surface.
Regarding CD1c, both C1R CD1c and moDCs revealed no noticeable differences in MFI with sialidase treatment, pointing out that removal of sialic acids at cell surface do not impact the expression of this marker.
Nonetheless, it is important to note that more experiments need to be done to confirm the tendencies here shown, since little or none replicates were made for some of the stainings.

3.4. Control and PMM2-CDG Fibroblasts

As mentioned before, fibroblasts are considered to have a support role in the immune system, mainly by the production of pro-inflammatory cytokines and chemokines. Fibroblasts can either be activated by recognition of PAMPs or by other immune cells, such as macrophages. One of the most prominent cytokines secreted by macrophages is TNF-α, which has shown to lead to fibroblast activation and induce downstream signaling, culminating in the secretion of pro-inflammatory cytokines like IL-6[88].
For this part, fibroblasts obtained from PMM2-CDG patients and apparently healthy donors were used. Here, we aimed to better characterize the impact of defective glycosylation in fibroblasts and how it can affect the immune response. For that, cell surface glycosylation and cytokine secretion were analysed in non-stimulated and TNF-α-stimulated PMM2-CDG and control fibroblasts, as further elaborated on the next topics.

3.4.1. Lectin Staining

In order to address surface glycosylation in control and PMM2-CDG fibroblasts, lectin staining was performed. Given that PMM2-CDG affects *N*-glycosylation pathway, two different lectins, ConA and GNL, that recognize structures associated with this modification (see Appendix 4, Figure 6.2.), were used. Although this CDG type has not been described to affect *O*-glycosylation, PNA was also used in this assay, to better understand the length and repercussions in glycosylation that arise from the mutations in the *PMM2* gene.

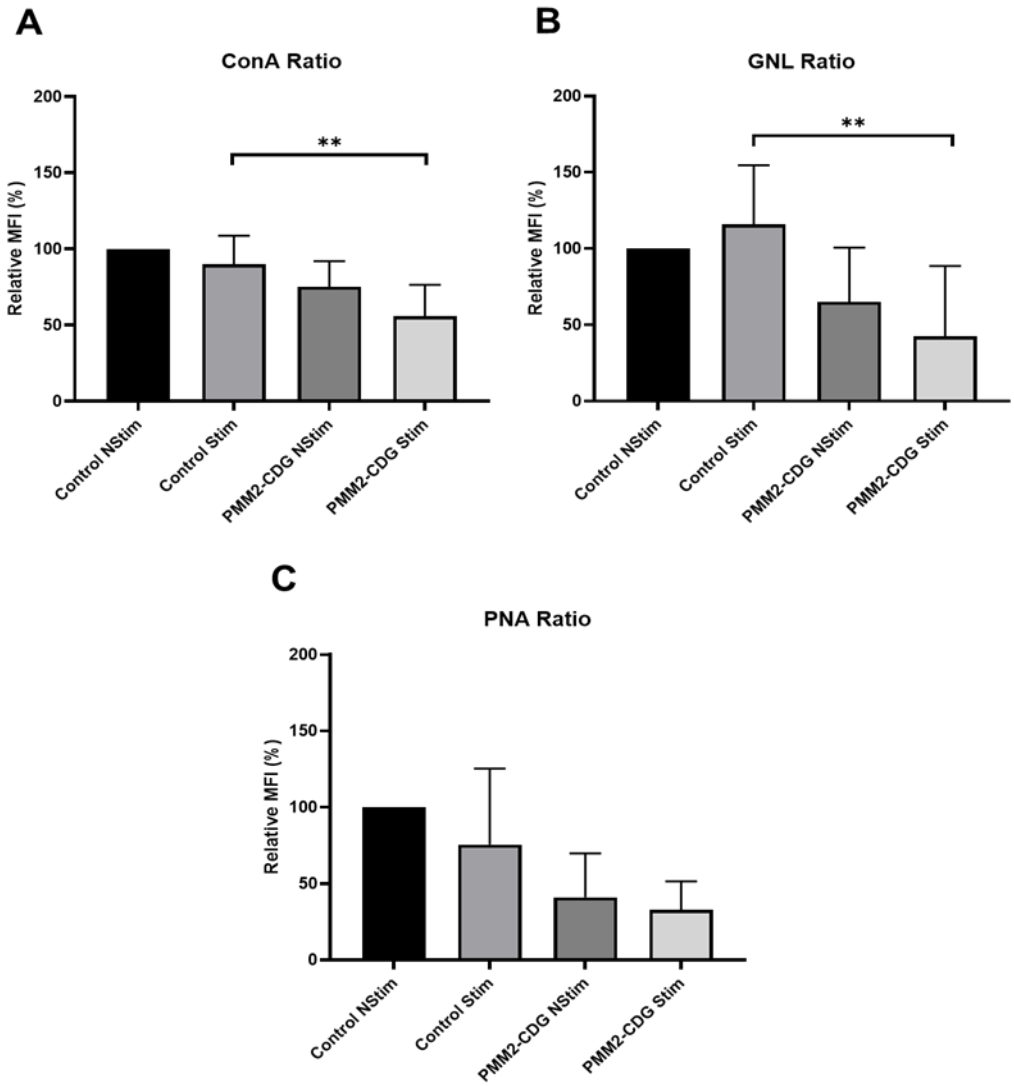

Figure 3.11. Changes in lectin binding in PMM2-CDG and control fibroblasts surface. Values represent the relative median fluorescence intensity (MFI) (mean ± SEM) for three different lectins (**A** – ConA, **B** – GNL, **C** – PNA) in control and PMM2-CDG fibroblasts, non-stimulated (NStim) or stimulated (Stim) with TNF-α (N ≥ 2). The relative MFI was calculated by dividing each condition by the non-stimulated control and shown in percentage. Statistical significance was obtained using Ordinary one-way ANOVA and is indicated by asterisks (** - $p \leq 0.01$)

Overall, it was noted a lesser staining with the three different lectins when comparing the PMM2-CDG to control fibroblasts (Figure 3.11.), not only between the non-stimulated conditions, but also when stimulated with TNF-α for 24 hours.

As PMM2-CDG is characterised by a defective *N*-glycosylation, in these cells it would be expected a lower level of *N*-glycans and consequently less lectin binding, specifically ConA and GNL, when comparing to control fibroblasts. In fact, it is worth highlighting the significant difference in *N*-glycosylation, assessed by ConA and GNL staining, between

stimulated control and PMM2-CDG fibroblasts, as it corroborates the impact of this CDG type in the patients' cells.

When it comes to *O*-glycosylation, there has been no report on PMM2-CDG directly affecting this modification, however, and although not statistically significant, it is visible an around 2-fold decrease in PNA staining in PMM2-CDG fibroblasts compared to control fibroblasts.

Furthermore, the results above also suggest that fibroblast stimulation with TNF-α on its own might lead to glycosylation changes, as can be seen by some variations in MFI. As a matter of fact, literature already suggested that TNF modulates the expression of sialyltransferases[89], so it might be a possible regulator of other glycosylation modifications.

3.4.2. Cytokine Secretion

To further understand the importance of a defective glycosylation, cytokine secretion was assessed by ELISA. For that, several cytokines were tested, namely IL-1β, IL-6, IFN-γ and monocyte chemokine protein-3 (MCP-3).

The first three mentioned are pro-inflammatory cytokines with a major role in the activation of immune cells and pathogen elimination[90,91]. MCP-3 is a relevant chemokine responsible for the recruitment of APC, namely DCs and macrophage[92].

However, for all cytokines, except IL-6, no signal was detected, even in the stimulated conditions, indicating none or low secretion of these cytokines. Thus, only IL-6 values were analysed in this topic (Figure 3.12.).

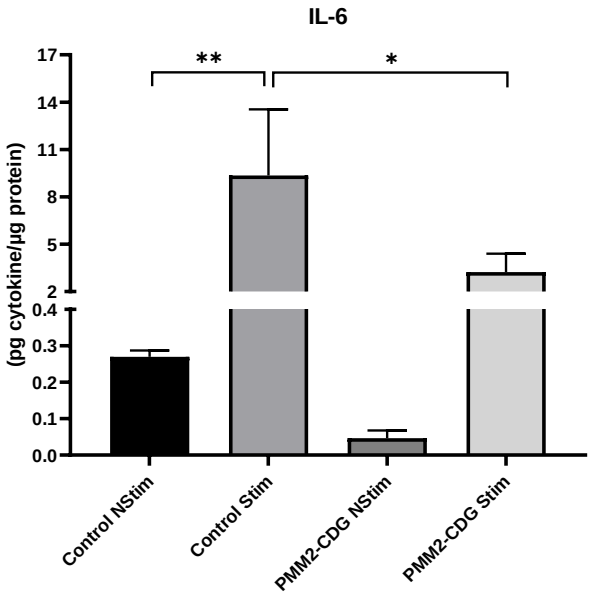

Figure 3.12. IL-6 secretion in PMM2-CDG and control fibroblasts. Values represent pg of IL-6 secreted per μg of total protein (mean ± SEM) by control and PMM2-CDG fibroblasts, non-stimulated (NStim) or stimulated (Stim) with TNF-α (N = 3). Statistical significance was obtained using Ordinary one-way ANOVA and is indicated by asterisks (* - $p \leq 0.05$, ** - $p \leq 0.01$)

Here, we noticed a considerable increase in cytokine secretion after stimulation with TNF-α, especially in control fibroblasts. Although in PMM2-CDG fibroblasts an increase was also detected, it was to a lesser extent. This significant difference in secretion between control and PMM2-CDG cells is likely related to the disparities observed in glycosylation.

As mentioned above, IL-6 has a preponderant role in the immune system, especially in inflammation and infection[93]. In fact, it has been shown to activate a great variety of immune cells and induce the production of more cytokines, upregulating an inflammatory process, relevant in pathogen elimination[93].

Although still not clear why changes in glycosylation alter IL-6 production in these cells, it is apparent that PMM2-CDG fibroblasts respond differently when stimulated, in comparison with control fibroblasts. This could suggest a deregulated immune response in PMM2-CDG patients. In fact, this deregulation may correlate with the higher infection rate, and higher rates of hospitalization resulting from infection, described in PMM2-CDG patients[94].

Chapter 4. Conclusions and Future Perspectives

There has been growing evidence of glycosylation's role in human health and disease. In fact, cancer, and other diseases, like Congenital Disorders of Glycosylation (CDG) are known to have altered glycosylation. Therefore, exploring the role of glycosylation is necessary for developing higher-efficacy therapeutics and improving quality of life.

For that reason, we aimed to better understand the impact and consequences of altered glycosylation on the immune response.

Attending the results obtained, we noted that sialidase treatment led to an increase in the expression of certain antigen-presenting molecules. Both MHC-I and MHC-II showed higher levels in moDCs and in C1R cell lines (only MHC-I was assessed) after sialic acid removal, when compared to control cells or cells exposed to other treatments. This can corroborate the MHC-I stabilization at cell surface, already reported in the literature, and indicate that sialic acid removal may, as well, modulate MHC-II expression at cell surface. However, for CD1 molecules, no change was noted with sialidase treatment, except for CD1b. Taking into consideration the low sample size (N = 1), more experiments are required to verify the tendency in CD1b expression, shown by our results.

Regarding co-stimulatory molecules, in cells treated with sialidase, CD80 and CD86 remained unchanged, but a significant increase was noted in CD40. It might be interesting to assess in future experiments, whether changes in the expression of CD80 and CD86 can be seen if observed in different time points, and not right after sialidase treatment.

Nonetheless, it is worth mentioning that the use of cytokine maturation cocktail led to significantly higher levels of CD86.

Considering cytokine secretion assessed by ELISA, although after 24h a slight increase was noted, after 48h removal of sialic acids did not appear to modulate positively IL-12 production. However, RT-qPCR data revealed increased IL-12 gene expression with sialidase treatment, when compared to control, which corroborates previous results from literature. For the other cytokines, analysed by RT-qPCR, only TNF-α had higher gene expression with this treatment, in comparison to control.

Overall, for cytokine production, the incubation with cytokine maturation cocktail appeared to be the most effective treatment, with a great increase in IL-12 production and higher levels of TNF-α gene expression.

Nevertheless, the data obtained, is not, on its own, sufficient to fully understand cytokine regulation, with more experiments being needed, as well as the assessment of cytokine production at different timepoints.

The noticeable increase in IL-12 using the cytokine maturation cocktail left us wondering if the treatments used in this work could be used together and potentially synergize.

Even though more experiments are required, results obtained from ELISA and RT-qPCR, suggested that the differentiation of moDCs in the presence of Zanamivir or DANA, respectively, might help potentiate the effects of the cytokine cocktail.

It is important to highlight that all the results obtained in this work indicated that after the differentiation period, no relevant changes were noted with the use of sialidase inhibitors alone. Therefore, it might be interesting to test the markers during the differentiation step or use the sialidase inhibitors in combination with different stimuli.

Regarding PMM2-CDG fibroblasts, we confirmed a defective *N*-glycosylation, characteristic of this CDG type, and we noticed differential IL-6 secretion after stimulation, when compared with control fibroblasts. Given the support role that fibroblasts have in our immune system, these results might be relevant in a better characterization of PMM2-CDG clinical manifestations.

Overall, and although in this work some tendencies were observed, it is important to reinforce that further studies need to be planned to take a better grasp at the complex role glycosylation has in the immune system. Nevertheless, these results might be a stepping stone towards new glycan-based therapeutics.

In fact, since the use of sialidase appears to increase the expression of some maturation markers in DCs, its application in the maturation step of dendritic cell-based vaccines might optimize this process and potentiate the efficacy of this immunotherapy in cancer and other diseases. Furthermore, the removal of surface sialic acids, on its own, may prevent Siglecs recognition and consequently avoid the induction of inhibitory signals.

In addition, a further understanding of glycosylation's impact on the immune system may lead to novel therapeutics in CDG patients, improving their quality of life, or potentially unravelling a curative treatment.

Chapter 5. References

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Chapter 6. Appendix

Appendix 1: Sialidase related patents

The search for patents related with sialidase was conducted in the Espacenet database on the 26th of January 2021 using the keyword "sialidase" and 273 results were obtained. The claims of each patent were read and excluded the ones that provided no claims or in which the claims were not written in English. Furthermore, only patents related to cancer treatment were selected, obtaining the table below.

Table 6.1. List of sialidase related patents.

Title	Publication Number/Date	Mechanism of Action
RECOMBINANT HUMAN SIALIDASES, SIALIDASE FUSION PROTEINS, AND METHODS OF USING THE SAME	WO2021003469 (A2) 07.01.21	Method of **treating cancer** by administering a **fusion protein** comprising: recombinant mutant human **sialidase** and an **immunoglobulin Fc domain** and/or an **immunoglobulin antigen-binding domain**
SIALIDASE-HER2-ANTIBODY FUSION PROTEINS AND METHODS OF USE THEREOF	WO2021003465 (A1) 07.01.21	Method of **treating cancer** by administering a **fusion protein** comprising: a **sialidase** enzyme and an **anti-HER2 immunoglobulin antigen-binding domain**
SIALIDASE-CD20-ANTIBODY FUSION PROTEINS AND METHODS OF USE THEREOF	WO2021003463 (A1) 07.01.21	Method of **treating cancer** by administering a **fusion protein** comprising: a **sialidase** enzyme and an **anti-CD20 immunoglobulin antigen-binding domain**
SIALIDASE-PD-L1-ANTIBODY FUSION PROTEINS AND METHODS OF USE THEREOF	WO2021003464 (A1) 07.01.21	Method of **treating cancer** by administering a **fusion protein** comprising: a **sialidase** enzyme and an **anti-PD-L1 immunoglobulin antigen-binding domain**

ENGINEERED EXPRESSION OF CELL SURFACE AND SECRETED SIALIDASE BY CAR T CELLS FOR INCREASED EFFICACY IN SOLID TUMORS	WO2020236964 (A1) 26.11.20	Method of **treating cancer** by administering **immune cell** (**T cell or NK cell**) modified (chimeric cell surface **sialidase** (extracellular portion)) and comprising a **chimeric antigen receptor (CAR) and/or a T cell receptor (TCR)**
METHODS AND COMPOSITIONS FOR TREATING CANCER WITH IMMUNE CELLS	WO2020142727 (A1) 09.07.20	Method of **treating cancer** or **inhibiting tumor growth** by administering **immune cell** (**T-cell, a Tumor Infiltrating Lymphocyte (TIL), and a natural killer (NK) cell**) modified (exogenous nucleotide sequence encoding a **sialidase**), wherein the immune cell further comprises an exogenous nucleotide sequence encoding a **chimeric antigen receptor (CAR)** that binds a **cancer antigen**
DELIVERY OF SIALIDASE TO CANCER CELLS, IMMUNE CELLS AND THE TUMOR MICROENVIRONMENT	WO2020018996 (A2) 23.01.20	Method of **treating solid tumor** by administering recombinant **oncolytic virus** comprising a nucleotide sequence encoding a polypeptide comprising a **sialidase domain**
METHODS AND REAGENTS TO TREAT TUMOR AND CANCER	WO2018231661 (A1) 20.12.18	Method of **treating and/or inhibiting a tumor** by administering a **conjugate** comprising a **sialidase** and an **affinity ligand** (antibody or aptamer) that can **bind to immune cell** (**T cell or NK cell**) surface"
A VIABLE CELL POPULATION, METHOD FOR PRODUCTION AND USES THEREOF	WO2017002045 (A1) 05.01.17	A **vaccine** comprising a viable cell population obtainable by the **maturation of the dendritic cells or T2 cells** with **sialidase** and comprising an **antigen** for use in the treatment or **prevention of cancer**, immune diseases, viral infections or bacterial infections"

Appendix 2: Solutions and reagents used

Beads buffer: 2mM of EDTA and 0.5% of bovine serum albumin (BSA) (w/v), in PBS

Blocking buffer/reagent diluent buffer: 5% of BSA (w/v) and 0.05% (v/v) of Tween-20 in PBS

Complete DMEM culture medium: Simple RPMI-1640 medium with low glucose (1g/l), supplemented with 10% (v/v) FBS, 2 mM L-glutamine, 100 µg/ml of streptomycin and 100 U/ml of penicillin.

Complete RPMI-1640 culture medium: Simple RPMI-1640 medium, supplemented with 10% (v/v) FBS, 2 mM L-glutamine, 100 µg/ml of streptomycin, 100 U/ml of penicillin, 1 mM sodium pyruvate and 1% (v/v) non-essential amino acids

FACS buffer: PBS 1X and 10% (v/v) FBS

Phosphate buffered saline (PBS) 1x: 1.47 mM of KH_2PO_4, 4.29 mM of $Na_2HPO_4.7H_2O$, 2.68 mM of KCl and 137 mM of NaCl in distilled water, with a pH of 7.4

Washing Buffer (ELISA): 0.05% (v/v) of Tween-20 in PBS

Appendix 3: Supplementary Tables

Table 6.2. List of antibodies and lectins used in moDCs and/or C1R cell line staining.

Antibodies & Lectins	Clone	Fluorophore
Anti-MHC-I (HLA-ABC)	W6/32	FITC
Anti-MHC-II	GRB-a	APC
Anti-CD1a	HI149	FITC
	OKT6	-
Anti-CD1b	SN13 (K5-1B8)	APC
Anti-CD1c	L161	PE
	AD5-8E7	PE
Anti-CD86	BU63	FITC
Anti-CD80	MEM-233	PE
Anti-CD40	HI40a	APC
PNA	-	FITC
SNA	-	-

Appendix 4: Supplementary Figures

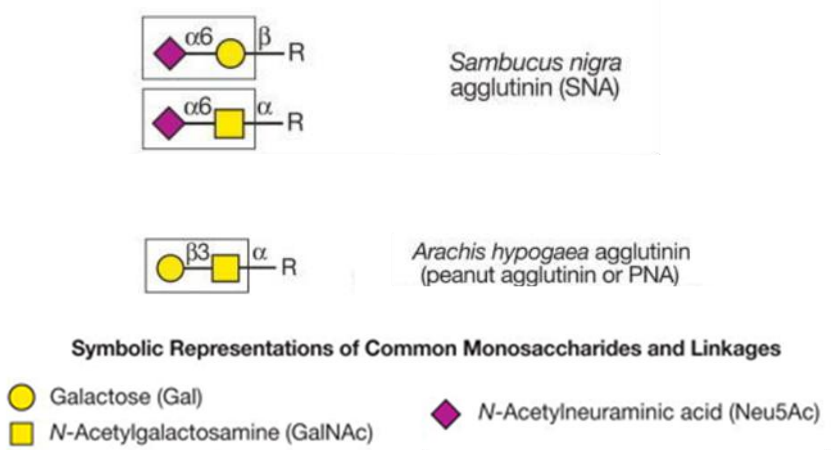

Figure 6.1. Examples of glycans that are recognized by SNA and PNA. *N*-Acetylneuraminic acid is the predominant sialic acid found in human cells. From Cummings and Etzler[95].

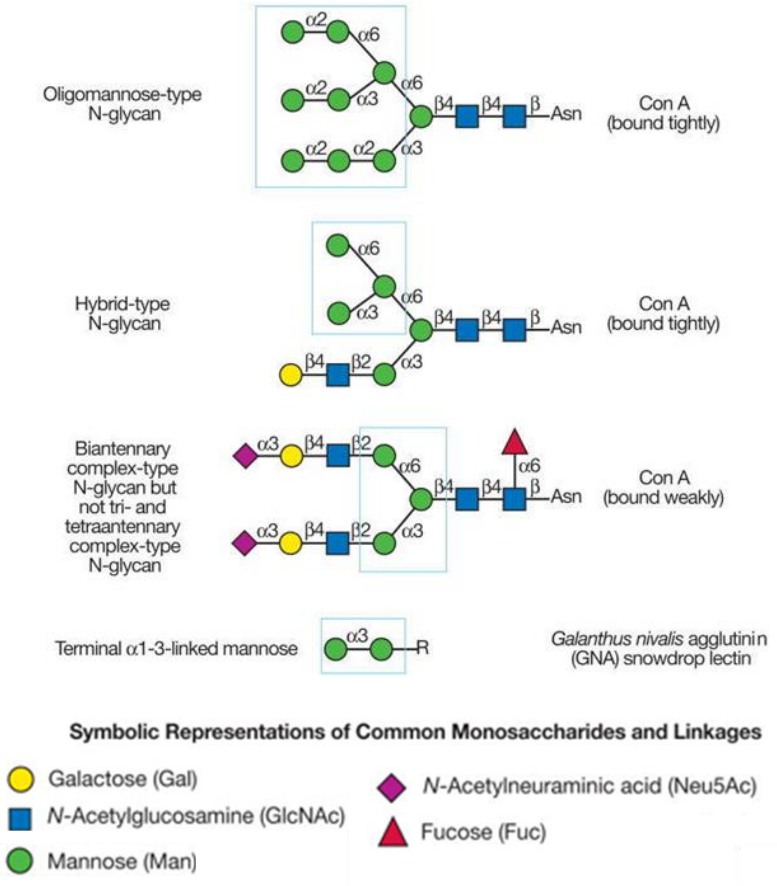

Figure 6.2. Examples of N-glycans that are recognized by ConA and GNL. In the figure GNL is represented by GNA. *N*-Acetylneuraminic acid is the predominant sialic acid found in human cells. From Cummings and Etzler[95].

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EXPLORING THE EFFECTS OF ALTERED GLYCOSYLATION ON THE IMMUNE RESPONSE

NOVA SCHOOL OF SCIENCE & TECHNOLOGY