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Licenciada em Bioquímica

Unravelling the role of alpha 2,6 sialic acid on mouse dendritic cells' functions

Dissertação para obtenção do Grau de Mestre em
Bioquímica para a Saúde

Orientador: Paula Alexandra Quintela Videira, Professora Auxiliar, Nova Medical School – Faculdade de Ciências Médicas e Faculdade de Ciências e Tecnologia da Universidade Nova de Lisboa
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Júri:
A definir

Nova Medical School – Faculdade de Ciências Médicas da Universidade Nova de Lisboa

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Abstract

Dendritic cells (DCs) are vital for immunomodulation and the initiation of adaptive immune responses, whereas sialic acids (Sias) are potential immunomodulators. These cells express high levels of sialyltransferase ST6Gal-1, responsible for transferring Sias to the terminal position of oligosaccharide chains. Indeed, DCs' maturation is associated with decreased cell surface sialylation.

Although its biological significance is unknown, the soluble, extracellular form of ST6Gal-1 increases in cancers and inflammation. However, extracellular ST6Gal-1 was recently identified as modulator of hematopoiesis. Considering that DCs play a crucial role in the initiation of a productive anti-cancer immune response, a link between extrinsic sialylation by the extracellular ST6Gal-1 on DC function needs to be investigated.

We hypothesize that extrinsic α2,6 sialylation of DCs diminishes their maturation features upon lipopolysaccharide (LPS) stimulation. The main goal was to extrinsically α2,6 sialylate mice bone marrow derived DCs (BMDCs) and to evaluate their maturation and cytokine profiles upon LPS stimulation (by Flow Cytometry and ELISA, respectively). Unlike the hypothesis, we observed that BMDCs' profile is not modulated, even using several approaches. In contrast, the consequence of lacking cell surface α2,6 Sias in DC maturation was assessed by analysing: 1) sialidase treated BMDCs, 2) BMDCs from mice lacking ST6Gal-1 and 3) DCs from mice airways, comparing wild type with ST6Gal-1 knockout mice. These results suggest that overall lack in α2,6 Sias is related with increased expression of major histocompatibility class II (MHC-II).

Although appearing to be controversial findings, other intracellular mechanisms might be occurring upon LPS-induced BMDC activation, probably reducing extracellular ST6Gal-1 effect. In opposite, the modification observed in DC profile of ST6Gal-1 knockout mice might be related to its predisposition to a more severe inflammatory status.

With this, the developed work opened future lines of investigation, namely exploring other factors involved in α2,6 (de)sialylation of DC, which might have influence in immunotherapy using DCs.

Key words: dendritic cells, sialidase, ST6Gal-1, extrinsic α2,6 sialylation, major histocompatibility class II

Resumo

As células dendríticas (CDs) são fundamentais na imunomodulação e iniciação de respostas imunes adaptativas, enquanto os ácidos siálicos (Sias) são potenciais imunomoduladores. Estas células expressam níveis elevados da sialiltransferase ST6Gal-1, que transfere Sias para a posição terminal de oligossacáridos. De facto, a maturação de CDs está associada a uma diminuição da sialilação na sua superfície celular.

Apesar de ter função biológica desconhecida, a forma solúvel, extracelular de ST6Gal-1 aumenta em cancros e inflamação. Ainda assim, esta foi recentemente identificada como moduladora da hematopoiese. Considerando o importante papel das CDs na iniciação de respostas anticancerígenas, uma ligação entre a sialilação extrínseca induzida por ST6Gal-1 extracelular e o seu papel na modulação de CDs deve ser identificada.

Neste trabalho hipotetizou-se que a sialilação α2,6 extrínseca de CDs diminui o seu perfil de maturação mediante ativação por lipopolissacarídeo (LPS). O objetivo principal foi sialilar extrinsecamente em α2,6 CDs da medula óssea de murganhos, avaliando os seus perfis de maturação e de libertação de citocinas, após estimulação com LPS (por Citometria de Fluxo e ELISA, respetivamente). Ao contrário da hipótese, o perfil celular não foi modulado, usando várias abordagens. Por outro lado, a consequência da falta de α2,6 Sias na maturação de CDs foi avaliada analisando: 1) CDs da medula óssea de murganhos tratadas com sialidase, 2) CDs da medula óssea e 3) CDs das vias aéreas, ambas de murganhos deficientes em ST6Gal-1, comparando com a estirpe selvagem. Estes resultados sugerem que a perta total de α2,6 Sias se relaciona com o aumento da expressão do complexo de histocompatibilidade principal de classe II.

Apesar de controverso, é provável existirem mecanismos inerentes à ativação por LPS, reduzindo a eficácia de ST6Gal-1 extracelular. Por outro lado, a modificação no perfil de CDs de murganhos deficientes em ST6Gal-1 poderá relacionar-se com uma predisposição para um estado inflamatório severo. Com isto, o trabalho desenvolvido abriu futuras linhas de investigação, nomeadamente explorar outros fatores envolvidos na (de)sialilação α2,6 de CDs, podendo ter impacto em imunoterapia com uso de CDs.

Palavras-chave: células dendríticas (CDs), sialidase, ST6Gal-1, sialilação extrínseca α2,6, complexo de histocompatibilidade principal II

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Abbreviations

AF	Alexa Fluor
AP-1	Activation protein 1
APC	Antigen presenting cells
APC	Allophycocyanin
APR	Acute phase response
BACE	Beta-site-amyloid precursor protein-cleaving enzyme 1
BAL	Bronchoalveolar lavage
BALF	Bronchoalveolar lavage fluid
BCR	B cell receptor
BMDC(s)	Bone marrow derived dendritic cell(s)
BV	Brilliant violet
CCR7	C-C chemokine receptor type 7
cDC(s)	Conventional dendritic cell(s)
CMP-Neu5Ac	Cytidine-5'- monophosphate-N-acyl-neuraminic acid
CMP-Sia	Cytidine monophosphate sialic acid
Cy	Cyanine
DC(s)	Dendritic cell(s)
E. coli	*Escherichia coli*
ELISA	Enzyme linked immunosorbent assay
FBS	Fethal bovine serum
FITC	Fluorescein isothiocyanate
FMO	Fluorescence minus one
FSC	Forward scatter
Gal	Galactose
GalNAc	N-acetyl-galactosamine
Galβ1,4GlcNAc	Galactose β-1,4 N-acetyl-glucosamine
GM-CSF	Granulocyte-macrophage colony stimulating factor
HRP	Horseradish peroxidase
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IP	Intraperitoneal
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibitory motif
LPS	Lipopolysaccharide
MAA	*Maackia amurensis agglutinin*
MAH	*Maackia amurensis hemagglutinin*
MAL	*Maackia amurensis leukoaglutinnin*
MFI	Median Fluorescence Intensity
MHC	Major histocompatibility complex
mo-DC(s)	Monocyte derived dendritic cell(s)

mRNA	Messenger ribonucleic acid
Neu	Neuraminidase
Neu5Ac	N-acetyl neuraminic acid
NF	Nuclear factor
OVA	Ovalbumin
P1	Promotor 1
PAMP(s)	Pathogen associated molecular pattern(s)
PBS	Phosphate buffer saline
pDC(s)	Plasmacytoid DC(s)
PE	Phycoerythrin
PerCP	Peridinin chlorophyll
PRR(s)	Pattern recognition receptor(s)
rST6Gal-1 or rST6	Recombinant murine ST6Gal-1
SAMP	Self-associated molecular patterns
SEM	Standard error of the mean
Sia(s)	Sialic acid(s)
Sial	Sialidase
Siat1 KO	Knockout mice for promotor 1 of ST6Gal-1 gene
Siglec	Sialic-acid-recognizing immunoglobulin-like superfamily
SNA	*Sacumbus Nigra Agglutinin*
SSC	Side Scatter
ST(s)	Sialyltransferase(s)
ST3Gal	β-galactoside α2,3 sialyltransferase
ST6Gal	β-galactoside α2,6 sialyltransferase
ST6Gal-1 KO	β-galactoside α2,6 sialyltransferase I knockout
ST6GalNAc	N-acetyl-galactosamine α2,6 sialyltransferase
ST8Sia	α2,8 sialyltransferase
Th	T helper
TCR	T cell receptor
TLR	Toll like receptor(s)
TMB	3,3',5,5'-tetramethylbenzidine
TNF-α	Tumour necrosis factor alpha
Ut	Untreated
WT	wild type

Chapter I

Introduction

I.1. Immune system

I.1.1 Innate and adaptive immune system

The immune system includes all the cells and molecules that confer immunity, or defense from pathogens or infectious diseases. It includes the innate and the adaptive immune response. The former refers to the first line of defense, present in every multicellular organism, conferring a quick response to a potential pathogen. The innate response includes physical barriers such as epithelia, the action of phagocytic cells, serum proteins (as the complement) and cytokines, involved in inflammation. The adaptive immune response refers to a type of immunity with specific functions and memory. Indeed, the adaptive immune system is only present in vertebrates and is able to distinguish between similar pathogens, inducing a stronger response upon a second contact with the same antigen. In the adaptive immune system, the major players are the lymphocytes (T and B cells), which poses specific receptors, such as T cell receptors and antibodies, allowing the recognition of almost any antigen (Abbas, Lichtman and Pillai, 2012).

I.2. Dendritic cells

I.2.1.1. General functions in the immune system

Dendritic cells (DCs) were first described by Steinmann and colleagues in 1973, found in peripheral lymphoid organs of mice (Steinman and Cohn, 1973). DCs are the most important antigen presenting cells (APC) of the immune system, since they are the only cells that present antigens to naïve T cells (antigen inexperienced cells). Then, DCs are considered the bridge between the innate and the adaptive immune responses (Abbas, Lichtman and Pillai, 2012).

DCs own a set of features enabling them to play this role. First, they differentiate from immature to mature cells, upon a danger signal, adjusting their functions (Steinman and Cohn, 1973). They reside in different organs, such as the skin, the intestine, the lungs and secondary lymphoid organs (like the spleen and thymus), where they recognize and capture antigens, to display in their surface (Abbas, Lichtman and Pillai, 2012). Besides, DCs constantly endocyte self-antigens, contributing to tolerance, which is lost in autoimmune disorders, for example (Merad *et al.*, 2013).

Otherwise, if an antigen is a potential dangerous, DCs phagocyte it and acquire a mature phenotype, migrating to the lymph nodes. There, they present antigens displayed through major histocompatibility complex (MHC) molecules to T cells. MHC can be class I or II. MHC-I is expressed in all nucleated cells, presents endogenous antigens (e.g. self or viral antigens) and may induce the activation of cytotoxic T cells, whereas MHC-II is expressed in every APC, presents phagocytosed antigens, mediating the activation of helper T cells . Other signals, such as the expression of co-stimulatory molecules, like CD80 and CD86, are required to activate T cells (Abbas, Lichtman and Pillai, 2012). Both belong to B7 family and bind to CD28 receptor in T cells, inducing their activation. CD86 is constitutively expressed in DCs at low levels, increasing quickly its expression after DCs' activation. In the opposite, CD80 is only later expressed after DCs' activation (Greenwald, Freeman and Sharpe, 2005). In addition, the release of pro-inflammatory cytokines, like interleukins (IL) 6, 12 and the tumour necrosis factor alpha (TNF-α), is needed to lead T cells towards a certain phenotype and immune response (Abbas, Lichtman and Pillai, 2012), (Merad *et al.*, 2013). Furthermore, DCs also play a role in humoral immunity, by activation of B cells (Palucka and Banchereau, 2012).

Mature or activated DCs are characterized by reduction of the antigen uptake machinery (Granucci *et al.*, 1999), modifications in their morphology (they acquire several dendrites) and the increased expression of receptors, such as C-C chemokine receptor type 7 (CCR7), allowing their migration to the lymph nodes. Mature DCs also have increased expression of MHC, co-stimulatory molecules and enhanced release of pro-inflammatory cytokines. (Banchereau and Steinman, 1998).

In order for DCs to capture antigens, they have a panoply of receptors that recognize pathogen associated molecular patterns (PAMPs). An example of pattern recognition receptors (PRR) is the family Toll like receptor (TLR). They can be found in the extracellular membrane of cells, or within the intracellular nucleus membrane (Takeda, Kaisho and Akira, 2003). Lipopolysaccharide (LPS) is a well-known PAMP, found in the outer membrane of Gram negative bacteria, being a useful tool to study the inflammatory response. Indeed, the engagement of LPS with TLR-4 induces a strong immune response from DCs (Dearman *et al.*, 2009). The intracellular signalling activates transcriptor factors like nuclear factor (NF)-kB, activation protein 1 (AP-1) and interferon (IFN) regulating factors. Consequently, it occurs the expression and release of cytokines,

inducing the adaptive immune response (Takeda, Kaisho and Akira, 2003). (Zanoni and Granucci, 2010).

I.2.1.2. Subsets of DCs

DCs constitute a very heterogeneous population, where a single surface marker is not enough to distinguish them. There are also functional differences found within DCs' subsets, besides the expression of surface markers, which are affected by the inflammatory status (Shortman and Liu, 2002), (Merad *et al.*, 2013).. Nowadays, DCs obtained from mice are better characterized than human DCs, where several studies have the aim to establish homology between them. Because of this, is important to choose appropriate means to obtain DCs, depending on the goals of the investigation (Shortman and Liu, 2002).

Currently is known that myeloid or lymphoid precursors can originate the same subsets of DCs (Satpathy *et al.*, 2012), (Sathe *et al.*, 2013). Conventional DCs (cDCs) are the major subset within DC population and can be classified as migratory or resident (classification only applied in steady state) (Merad *et al.*, 2013). The former includes, for example, dermal DCs and epidermal Langerhans cells, able to migrate to lymph nodes under inflammatory conditions (Shklovskaya, Roediger and Fazekas de St. Groth, 2008). Resident DCs can be found in secondary lymphoid organs, such as the thymus and the spleen (Vremec *et al.*, 2000), constantly screening the blood and lymph (reviewd by Crespo, Lau and Videira, 2013). Conventional DCs express high levels of CD11c and MHC-II, but differentially express other markers, also depending on the tissue distribution and inflammatory status (Abbas, Lichtman and Pillai, 2012), (Merad *et al.*, 2013). Taking this into account, cDCs are further divided in subclasses (Palucka and Banchereau, 2012).

Plasmacytoid DCs (pDCs) constitute a small subset of DCs, mainly found in the bloodstream or in lymphoid organs, whose DCs' features arise upon inflammatory conditions (Abbas, Lichtman and Pillai, 2012). These cells look like plasma cells and express lower levels of CD11c and MHC-II, but express the B cell marker, B220. Their main function is the release of type 1 IFN, during viral infections (Merad *et al.*, 2013).

Under inflammatory conditions, there are also DCs arising from monocytes in circulation (mo-DCs), emphasising the fact that the enormous diversity inherent to DCs' subtypes is influenced by the surrounding microenvironment (Shortman and Liu, 2002).

I.2.1.3. <u>Mice as powerful sources to study DCs</u>

Since DCs are rarest cells, several methodologies were developed, in order to generate DCs *in vitro.* Indeed, the mouse has been a model of excellency to analyse DCs. (Inaba *et al.*, 1992) demonstrated that mouse bone marrow CD34 positive precursors stimulated with granulocyte-macrophage colony stimulating factor (GM-CSF) generate large amounts of DCs. GM-CSF induces the maturation of granulocytes and monocytes (Syme and Glück, 2001). Later, GM-CSF and IL-4 were used to differentiate human monocytes into DCs, where IL-4 avoids the differentiation of monocytes into macrophages (Sallusto and Lanzavecchia, 1994), but also promotes the differentiation of monocytes into DCs lineage (Roy *et al.*, 2004). The protocol developed to generate human DCs is nowadays used to generate mouse bone marrow derived DCs (Inaba *et al.*, 2009). Immature DCs obtained through this protocol express high levels of CD11c, CD11b and medium levels of MHC-II, showing all the major functions related with DCs (Inaba *et al.*, 1992).

Other techniques are useful to study DCs from specific tissues of mice, such as the lungs. DCs in the lungs have been studied in order to unravel the mechanisms underlying in pulmonary or allergic diseases, like allergic asthma (Kim and Lee, 2014). Albeit constituting only a minor population in the lungs, DCs have crucial roles screening inhaled air and migrating to mediastinal lymph nodes upon antigen contact, where they initiate the immune response (Jahnsen *et al.*, 2006), (Hufford *et al.*, 2012). Indeed, in a mouse model of asthma, CD11c positive DCs found in the airways induced the features of allergic asthma, which are abrogated upon their depletion (Julia *et al.*, 2002). Different subsets of DCs are found in different compartments of mouse lungs, with specialized functions (Condon *et al.*, 2011). In order to assess cells from the conducting airways and the alveolar space, bronchoalveolar lavage (BAL) of mouse lungs is performed (Heer, De *et al.*, 2005).This methodology is applied to patients with pulmonary chronic diseases for diagnostic, but also in healthy patients for research purposes, despite only few studies were performed (Reynolds, 2000). In the alveolar compartment, in steady state, cDCs are

the major subset found within DC population, being CD11c high, CD11b positive or CD11c high, CD11b negative, CD103 (langerin) positive cells (Hufford *et al.*, 2012). On the other hand, pDCs are rarest found in alveolar space, but increase under inflammatory conditions. Besides, DCs derived from circulating monocytes migrate to the alveolar compartment, when an inflammation occurs. They are characterized by the high expression of CD11c, expression of CD11b positive and monocyte lineage markers, like Ly6C (GeurtsvanKessel and Lambrecht, 2008).

I.2.1.4. Therapeutic potential of DCs

DCs are potential adjuvants to current therapies, improving the immune response against a certain antigen. Taking advantage of DCs' plasticity, is possible to induce them towards a certain phenotype and different immune response (tolerogenic DCs or DCs activating cytotoxic T cells, for example), (Palucka and Banchereau, 2012).

Indeed, a major field in cancer immunotherapy relies in the administration of DCs obtained from the patient, which are loaded afterwards with cancer antigens (designated *ex vivo* therapy). This protocol elicits tumour specific responses through the expansion of T cells and has proven to be efficient in mice models of cancer, being implemented nowadays in clinical trials (Paczesny *et al.*, 2004), (Steinman, 2008). Despite the vaccines have proven to be safe, several factors need to be improved to reach the success, namely avoiding the inactivation of DCs in the tumour microenvironment and finding the most appropriate antigens to elicit a strong and effective immune response (Palucka and Banchereau, 2012). An example of a promising vaccine relying on this principle is the PROSTVAC, used against prostate cancer, where a survival benefit was observed at Phase II Clinical trials (Kantoff *et al.*, 2010).

I.3. Sialic acids

The concept of Glycobiology was first defined in 1980, to describe the study of the structure, biosynthesis, biology, and evolution of saccharides (sugars or glycans) distributed in nature, as well as proteins that recognize them (Varki *et al.*, 2009). Over the years, the study of glycans in biological systems has gained its importance.

Glycosylation is an important posttranslational modification occurring mainly in the lumen of endoplasmatic reticulum and Golgi apparatus, where glycans are added to proteins and lipids. These modifications are performed by glycosyltransferases, distributed orderly, using a single nucleotide-sugar as a donor substrate. Then, these enzymes are responsible for glycan biosynthesis and structures found in glycoconjugates (Rabinovich and Croci, 2012).

There are many families of glycans, where the family of Sialic acids is one of them. Sialic acids (Sias) are expressed in the cells' surface of all animals from deuterostome lineage (vertebrates and some higher invertebrates) and also in some pathogenic or symbiotic bacteria, which associate with vertebrates (Varki *et al.*, 2009).

The number of members included in Sias family has increased over the years, where more than 50 structures are identified today in nature (Varki, 2010).

One of the most important structures of Sias, found in mammalian cells, is the N-acetyl neuraminic acid (Neu5Ac), which is a nine carbon backbone structure (Varki, 2007), represented in Figure I.1
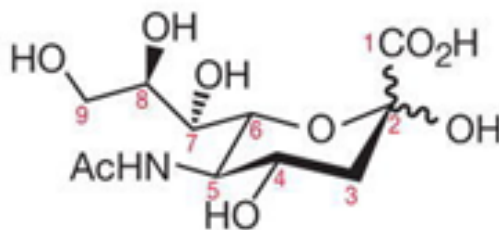
Figure I.1 - **Representation for the chemical structure of Neu5Ac, a structure common to most of Sias.** The carbon numbers are represented, as well as the bonds of the anomeric centre, carbon 2 (C2). Adapted from (Varki *et al.*, 2009).

N-glycans bind to asparagine residues in the sequence motif Asparagine-X-Serine-Threonine (Asn-X-Ser/Thr), whereas O-glycans bind to serine and threonine residues (Varki *et al.*, 2009), as illustrated in Figure I.2. Sias are usually placed at terminal branches of N-glycans, O-glycans, and glycosphingolipids (gangliosides). Due to the type of linkages that Sias form with other molecules and the possible modifications in their primary structure, they increase the variety of structures found in glycoconjugates.
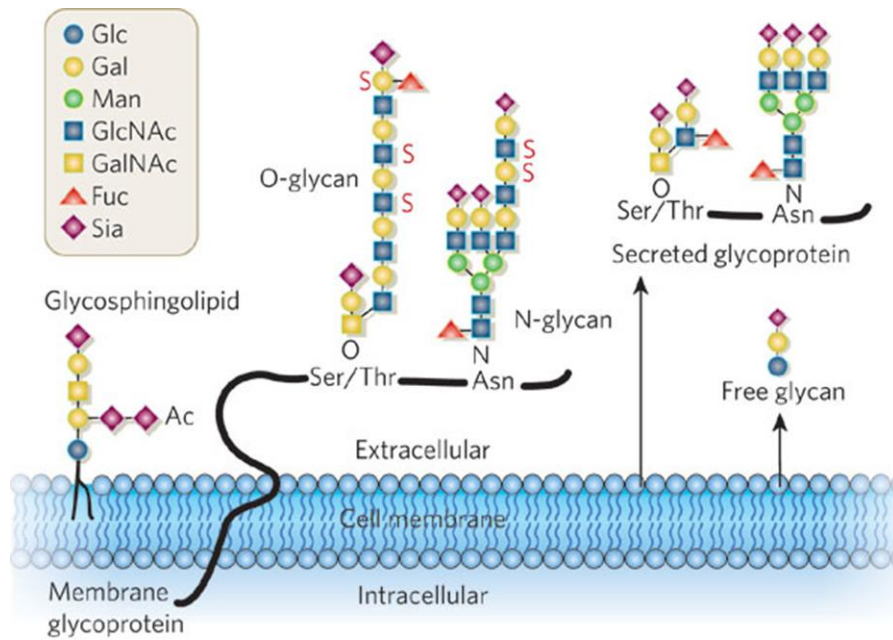
Figure I.2- **Representation of Sias linked to glycosphingolipids or to O or N –glycans in glycoproteins, either in the cellular membrane as in secreted glycoproteins.** The legend for the representation is shown in the upper left side of the chart. Adapted from (Varki, 2007).

Sias can remain in glycans attached to the membrane of cells or decorate secreted glycoproteins, as illustrated in Figure I.2. One of their particularly important features is the negative charge they confer to glycoconjugates, influencing the interactions of the cells with the microenvironment (Varki and Gagneux, 2012).

It is to notice that Sias are linked to other molecules through their anomeric carbon number 2 (C2) and can establish linkages to the carbons 3 or 6 of other glycans or to C8 of another Sia, designated respectively as α2,3, α2,6 and α2,8 Sias (Li and Chen, 2012).

I.4. Sialyltransferases

The attachment of Sias to glycoconjugates implies the existence of an enzyme able to transfer the substrate (donor) to an acceptor. The donor is the sugar cytidine-5'-monophosphate-N-acetyl-neuraminic acid (CMP-Neu5Ac) or CMP-Sia. The substrates can be galactose residues (Gal), N-acetyl-galactosamine (GalNAc) or Sias (Rao *et al.*, 2009).

Sialyltransferases (STs) are enzymes responsible to transfer CMP-Neu5Ac to other glycans. Similarly with other glycosyltransferases, all animal STs are type II membrane proteins with signals for their Golgi localization (Varki *et al.*, 2009). Most motifs of STs are highly conserved in vertebrates, where both mice and humans have 20 known STs. (Li and Chen, 2012). There are 4 classes of STs in mice and human, divided according with their substrate and acceptor specificity. ST3Gal (β-galactoside α2,3 sialyltransferase) family catalyses the transfer of CMP-Sia to terminal galactose through α2,3 linkages, where six subfamilies are identified; ST6Gal (β-galactoside α2,6 sialyltransferase) family has 2 subfamilies, catalysing the transfer of α2,6 Sias to galactose β-1,4 N-acetyl-glucosamine (Galβ1,4GlcNAc); the family ST6GalNAc (N-acetyl-galactosamine α2,6 sialyltransferase) owns six subclasses that transfer α2,6 Sias to N-acetyl-galactosamine (GalNAc); lastly, α2,8 sialyltransferase (ST8Sia) family induces the transfer of Sias in α2,8 linkage to terminal Sias, where 6 subfamilies are identified as well (Takashima, 2008), (Rao *et al.*, 2009), (Harduin-Lepers, 2010).

ST genetic expression is modified during mammalian development and immune regulation, being also different within different cell types. Besides, ST families are differentially expressed (Rao *et al.*, 2009). Indeed, in mammalian cells, α2,3 is the most common linkage of Sias formed by ST3Gal family, being α2,8 linkage, catalysed by ST8Sia family members, the rarest (Hennet *et al.*, 1998).

In this dissertation we will focus in ST6Gal-1 subfamily (β-galactoside α2,6-sialyltransferase 1), responsible for most of α2,6 Sia linkages in Galβ1,4GlcNAc residues of N-glycans, with widespread tissue distribution, unlike ST6Gal-2 subfamily (Takashima, 2008).

I.4.1 ST6Gal-1: the membrane-anchored and the soluble forms

In humans, ST6Gal-1 gene is placed in chromosome 3, whereas in mice it is located in chromosome 16 (Dalziel *et al.*, 1999). Either in humans as in mice, various and distal promotors control the genetic expression of ST6Gal-1 and have been a target of study (Hennet *et al.*, 1998). Furthermore, ST6Gal-1 is highly expressed in hepatocytes and lymphocytes, where it catalyses α2,6 sialylation of serum glycoproteins and glycoproteins of antigen membrane receptors, respectively (Varki *et al.*, 2009). ST6Gal-1 is usually at Golgi membrane, but can also be present in soluble form due to BACE (beta-site-amyloid precursor protein-cleaving enzyme 1) proteolytic action (Dalziel *et al.*, 1999), (Jones *et al.*, 2010). Liver is the major source of soluble ST6Gal-1, as shown by the creation of a Siat1ΔP1 mice (lacking the liver promotor P1 of ST6Gal-1 gene). These mice presented deficiency in serum levels of ST6Gal-1 under inflammatory status, suggesting that the liver promotor P1 is the most important regulating the levels of soluble ST6Gal-1 (Dalziel *et al.*, 1999), (Appenheimer *et al.*, 2003).

To compare the activity of both forms, the soluble form was deleted for the membrane anchor in different extends, where it retained the enzymatic fold and activity (Legaigneur *et al.*, 2001). Nevertheless, the specificity for glycan acceptor has diminished in the soluble form, compared to the membrane anchored ST6Gal-1 (Legaigneur *et al.*, 2001), (Kuhn *et al.*, 2013).

I.5. Sialidases

In opposite to sialyltransferases, sialidases or neuraminidases are responsible for removing Sias from glycans. In mammals, there are four known neuraminidases, presenting homology between mouse and human: Neu1, 2, 3 and 4. Neu1 is expressed in the majority of cell types and is mainly located in the lysosome, but can be translocated to cell membrane. It regulates molecular adhesion and intracellular signalling, by desialylation of some receptors like TLR in immune responses (Stamat*os et al*, 2010), (Varki and Gagneux, 2012). Neu3 is a membrane associated protein also found in late endosomes, whose major function is to remove Sias from gangliosides. Neu2 is located in cytosol, playing a role in differentiation and malignancy. Lastly, Neu4 is found at lysosomes and mitochondria, but its functions are not well known (Stamatos *et al.*, 2010).

Sialidases from vertebrates are unstable in extracellular fluids, unlike sialidases from bacteria, which are used to perform *in vitro* assays (Varki and Gagneux, 2012).

I.6. Lectins

Lectin is the general term referring to proteins which bind glycans. They are found in animals, plants and also in pathogens or toxins. In addition, these lectins can be intrinsic to an organism, or recognize glycans in other organism (Varki *et al.*,2009). Regarding the lectins that recognize Sias, there are three major families of lectins: Sialic-acid-recognizing Immunoglobulin-like superfamily (Siglec), complement factor H and selectins, which belong to C-type lectins family (Varki *et al.*, 2009).

Siglecs are mostly found in cells of immune system and usually have an intracellular ITIM (immunoreceptor tyrosine-based inhibitory motif) or, not so frequently, ITAM (immunoreceptor tyrosine-based activation motif), regulating immune responses (Crocker, 2002), (Rabinovich and Croci, 2012). They are further divided in subclasses, wherein sialoadhesin, CD22 and CD33 are examples of Siglecs common to all mammals. Regarding CD33 related Siglecs, human and mice have different types expressed (Crocker, Paulson and Varki, 2007). Factor H is a down regulator of complement cascade, binding to Sias in cells' surface and avoiding the constant complement activation. Selectins are present in the endothelia, leukocytes and platelets and induce leukocyte traffic (Varki and Gagneux, 2012).

Regarding lectins found in organisms which do not express Sias, such as plants, they are very useful to evaluate linkages to Sias, because they are specific, soluble and easy to isolate and obtain in higher amounts (Varki *et al.*, 2009). For example, *Sacumbus Nigra Agglutinin* (SNA) and *Maackia amurensis Agglutinin* (MAA) are widely used in Glycobiology field. Both of them have hemagglutination abilities, i.e., they agglutinate red blood cells. SNA recognizes α2,6 Sias in terminal galactose residues (Shibuya *et al.*, 1987), whereas MAA is divided in different classes. *Maackia amurensis leukoaglutinnin I* (MAL-I) preferentially binds Siaα2,3Galβ1-4GlcNAc residues and in a weaker extend Galβ1-4GlcNAc. In opposite, *Maackia amurensis hemagglutinin* (MAL-II) preferentially binds α2,3 Sias, despite some unspecific binding has been observed (Geisler and Jarvis, 2011). However, some controversial regarding MAL-I was reported, since some manufactures reported that MAL-I preferentially binds Galβ1-4GlcNAc, tolerating

instead the substitution of *N*-acetyl-lactosamine with α2,3 Sia binding to Gal (Vector Labs).

I.7. Roles of Sias in the immune system

Regarding the terminal position of Sias in cell membranes, they play important roles in interactions of cells with their microenvironment and in modulation of immune response (Varki *et al.*, 2009), (Varki and Gagneux, 2012).

Sias can have a dual function, either masking important molecules, or being ligands of important receptors, as previously mentioned (Varki and Gagneux, 2012). By masking, Sias protect the host from pathogen interactions, such as proteases action. In addition, Sias prevent galectins binding, a lectin group that regulates several cellular events (Rabinovich and Croci, 2012).

As ligands of important receptors, Sias bind to factor H, recognizing self Sias (*cis* interaction) and avoiding autoimmune responses. As selectin ligands, Sias are involved in leukocyte traffic and chemokine derived migration of immune cells to lymph nodes (Varki and Gagneux, 2012). On the other hand, Sias are a recognition place to pathogens and can even be used as an energy resource, whereas in some cases as a tactic of molecular mimicry (Varki and Gagneux, 2012).

During stages of cellular differentiation and maturation, modifications occur in the genetic expression of STs, correlating with changes in Sias patterns (Crespo, Lau and Videira, 2013). The genetic expression of STs is also changed during embryogenesis, inflammation, some cancers and in autoimmune diseases, emphasizing the roles for Sias in several biological processes and disorders (Varki and Varki, 2007), (Rabinovich and Croci, 2012). In the following two sections, the importance of ST6Gal-1 in the modulation of immune functions will be detailed, namely its influence on DCs' features and functions.

I.7.1 ST6Gal-1 in the modulation of immune functions

The functions of ST6Gal-1 in the immune system are often associated with its biological receptor, CD22 also known as Siglec-2, highly conserved between mice and human forms (Crocker, 2002).

To study the importance of α2,6 Sia in immune system, ST6Gal-1 knockout mice were generated (ST6Gal-1 KO) (Hennet *et al.*, 1998), as this ST family is the major source of α2,6 Sia linkages, either in mice (Jones *et al.*, 2010) as in humans (Varki, 2010). ST6Gal-1 KO mice showed deficient T cell-dependent antibody production, reduced B cell proliferation and antibody production (Varki *et al.*, 2009), modified thymopoiesis and granulopoiesis and disorders on eosinophil and DCs' profiles (Zhuo and Bellis, 2011).

Indeed, B cell functions in ST6Gal-1 KO mice are impaired due to the lack of α2,6 Sias in CD22 surface. In ST6Gal-1 KO mice, the threshold for activation of mature B cell receptor (BCR) increases due to absence of CD22-CD22 interactions, where α2,6 Sias are needed. In this case, CD22 co-localizes with BCR in cell membrane, recruiting phosphatases that will decrease BCR activation. Consequently, the threshold for BCR activation increases and the functions of B cells are supressed (Hennet *et al.*, 1998), (Rabinovich and Croci, 2012).

Another interesting feature about α2,6 Sias is the different tissue distribution of Sias found within different species (Varki, 2010). Actually, the first role reported for Sias was being the receptors for Influenza virus. Interestingly, some Influenza virus preferentially bind to α2,3 Sias, abundant in birds' airways, whereas humans' airways have mostly α2,6 Sias in epithelial cells. This observation explained the initial resistance of humans to the virus, highlighting the protective role of α2,6 Sias, in this case (Varki and Varki, 2007).

Secreted glycoproteins usually have α2,6 Sias in their surface, modifying several of their features: they can have a different conformation, be retained in some cell membranes and clusters of receptors are changed as well. Therefore, events such as cellular migration and adhesion are influenced by α2,6 sialylation (Zhuo and Bellis, 2011). Actually, for immunoglobulin G (IgG) intravenous therapy, used in several autoimmune diseases, the α2,6 sialylation of N-glycans in Fc regions of IgG is required for anti-inflammatory effects (Kaneko, Nimmerjahn and Ravetch, 2006) (Jones *et al.*, 2012).

Confirming the contribution of ST6Gal-1 in several disorders, its expression is up-regulated in some carcinomas as colon, breast, ovarian, gastric, cervix, choriocarcinomas, acute myeloid leukemia, and some brain tumours (Hedlund *et al*, 2008),˙(Park and Lee, 2013). Besides, it promotes tumour cell growth and metastasis. In fact, in early studies, a bigger metastatic phenotype was observed in tumour mutant cell lines, correlating with increased sialylation and pronounced increase in the expression of ST6Gal-1 mRNA (Takano, Muchmore and Dennis, 1994). The presence of Sias in tumour cells' surface also enables complement factor H binding, avoiding complement activation. In addition, α2,6 Sias bind inhibitory Siglecs of immune system cells, which probably dampens their functions helping to eliminate tumour cells (Varki *et al.*, 2009).

I.7.2 Extrinsic α2,6 sialylation: the new concept of distal immune regulation

For several years, ST6Gal-1 soluble was considered a product of metabolically inefficiency, with no biological significance, despite catalytic activity was noticed (Jamieson, McCaffrey and Harder, 1993).

Elevated ST6Gal-1 in the bloodstream of cancer patients is correlated with a bad prognosis. While these observations suggest an important role for extracellular ST6Gal-1 in cancer progression, its role in cancer remains to be elucidated, which will also determine if it can be used as a cancer biomarker (Park and Lee, 2013).

ST6Gal-1 is also released from liver into circulation, under inflammatory conditions, being part of the acute phase response (APR) (Kaplan *et al.*, 1983). In addition, the release of ST6Gal-1 from liver was proved to be IL-6 dependent by using mice models (Dalziel *et al.*, 1999). It is tempting to think that soluble ST6Gal-1 could be a homeostatic modulator, similar to other proteins released into bloodstream upon APR. Nevertheless, the role for ST6Gal-1 role in circulation, under APR, is unknown.

Extrinsic sialylation refers to the sialylation of glycans in distal cells or glycoproteins, occurring through the action of STs extrinsic to it, which could be present in bloodstream. This concept was previously dismissed, due to the absence of enough sugar substrates in circulation for STs to act distally (Zhu *et al.*, 1998). Nevertheless, other roles were proposed for soluble STs, such as the possibility of acting like lectins, through linkages with carbohydrates, modulating then immune functions (Zhu *et al.*, 1998). However, the concept of extrinsic sialylation is nowadays being revised, since appropriate sugar donors

are found in circulation, under certain conditions, like platelets (Wandall *et al.*, 2012). In this case, circulatory STs could extrinsically add Sias in other cells' surface, eventually contributing to the modulation of immune functions (Nasirikenari, Collins and Lau, 2011).

In asthma models of ST6Gal-1 KO, the number of neutrophils is increased comparing with WT mice, upon lung allergen stimulation. Supporting this, downregulation of circulatory ST6Gal-1 activity was noticed in WT mice, during lung allergen challenge (Nasirikenari *et al.*, 2006). In addition, higher eosinophilia was noticed in ST6Gal-1 KO mice subjected to allergen stimulation, suggesting that lack in α2,6 Sia sensitizes these mice (Nasirikenari *et al.*, 2010). These observations have been attributed to increased myelopoiesis (Nasirikenari *et al.*, 2006). Corroborating a potential role for soluble ST6Gal-1, bone marrow myelopoiesis has decreased with *in vitro* treatment of marrow cells in myeloid colony forming assays, by adding physiological concentrations of ST6Gal-1 with appropriate sugar donors (Jones *et al.*, 2010). Moreover, hematopoietic and stem cells numbers have decreased *in vivo*, upon increased levels of circulatory ST6Gal-1, released from the liver (Nasirikenari *et al.*, 2014). The mechanisms underlying are still unknown and may be associated with CD22, since this receptor is related with decreased cellular proliferation, at least regarding B cell function (Crocker, Paulson and Varki, 2007).

I.7.1 ST6Gal-1 in the modulation of DCs functions

In the case of DCs, several changes in the glycosylation patterns are noticed during differentiation and maturation stages (Videira *et al.*, 2008), (Bax *et al.*, 2007).

Indeed, the genetic expression of ST6Gal-1 has increased during differentiation of human mo-DCs, correlating with an increased enzymatic activity and increased SNA binding to cells' surface (Videira *et al.*, 2008). In opposite to this observation, Neu1 and Neu3 genetic expression and activity increase upon differentiation of human monocytes into DCs, which appears to be controversial (Stamatos *et al.*, 2010). Both observations suggest that the mechanisms regulating sialylation patterns of DCs during differentiation are complex, needing further elucidation.

The genetic expression of ST6Gal-1 is dramatically decreased upon DCs' maturation either induced with LPS (activates TLR-4) or with modulators of other TLR or cytokines

(Bax *et al.*, 2007), (Videira *et al.*, 2008). However, upon DCs' maturation, the binding of CD22 to α2,6Sia-Galβ14GlcNAc has increased comparing with immature DCs as well as the binding of SNA, unlike the genetic expression has suggested (Bax *et al.*, 2007). Corroborating the former results, other study has proven that tolerogenic immature human mo-DCs have a higher content in α2,6 Sias. In this case, the genetic expression has also dramatically decreased upon DCs' maturation with pro-inflammatory cytokines, but correlated well with a drastically decrease in SNA binding to mature mo-DCs. (Jenner *et al.*, 2006). Regarding the apparent controversial findings about SNA binding to human mo-DCs upon maturation, is important to understand if these differences at protein level are related with the activation of a specific receptor or if they are dependent on another factor.

Taking into account the modifications in ST6Gal-1 expression upon DCs' maturation, it is likely that removing Sias from DCs' surface could influence related processes. Indeed, neuraminidase treatment of human mo-DCs decreased the number of particles endocytosed by DCs (Videira *et al.*, 2008), where reduced expression of antigen uptake machinery is a common feature inherent to DCs' maturation.

Nevertheless, later was proven that phagocytosis of *Escherichia coli* (*E. coli*) increases upon sialidase treatment. This suggests that this improvement is restricted to pathogens expressing sialylated structures, despite further elucidation is needed (Cabral *et al.*, 2013). Moreover, endogenous Neu1 activity improved the phagocytosis of DCs from mice (Seyrantepe *et al.*, 2010). In fact, DCs from Siat1-null KO mice (unable to produce α2,6 Sias in Gal residues) presented improved phagocytosis of *E. coli*, suggesting the implication of ST6Gal-1 in modulation of this function from DCs (Cabral *et al.*, 2013).

In addition, sialidase treatment of human mo-DCs led to increased expression of MHC-II, MHC-I, CD80 and CD86, to improvement of T cells priming which increased their proliferation, and to increased mRNA expression of pro-inflammatory cytokines, such as IL-6, IL-12, IL-1β and TNF-α (Crespo *et al.*, 2009). Corroborating these observations, endogenous expression of Neu1 and Neu3 increases in DCs stimulated with LPS, suggesting that it may have a role inducing maturation (Stamatos *et al.*, 2010). In fact, in macrophages and DC cell lines, Neu1 activity for desialylation of TLR-4 upon LPS stimulation is required for further cellular activation (Amith *et al.*, 2010). Besides,

DCs grown in the presence of endogenous Neu1 and Neu3 inhibitors decrease the amount of cytokines released from mo-DCs stimulated with LPS. This study also suggests that the hyper sialylation of specific moieties can be related with the specific decrease in maturation of DCs (Stamatos *et al.*, 2010). This later observation highlights the importance of attributing roles for specific types of sialylation, and not only modifications in the general content of Sias by sialidase effect.

Regarding the role of ST6Gal-1 in DCs' maturation, BMDCs from ST6Gal-1 KO mice presented a higher expression of MHC-II compared to WT, upon endocytosis. In addition, ST6Gal-1 KO mice showed a slight, but not impaired, decrease in endocytosis capacity. On the other hand, assessing DCs from blood, spleen and lymph nodes from ST6Gal-1 KO, their numbers and markers expression were similar to WT, whereas only pDCs expressed more MHC-II compared to WT pDCs (Crespo *et al.*, 2009).

Furthermore, the quick changes found in DCs' sialylation profile upon maturation were attributed to the activity of a ST in surface of human mo-DCs, through mechanisms still unknown (Cabral *et al.*, 2010).

I.8. Context and aims of the work

DCs are vital for the initiation of adaptive immune responses and immunomodulation. The elucidation of mechanisms modifying DCs' functions may have potential impact in immunotherapy, where improvements to DCs' based vaccines efficacy need to be achieved (Palucka and Banchereau, 2012).

Previous work from our group demonstrated that sialidase treatment of human mo-DCs increase the expression of MHC-II, CD80, CD86, the genetic expression of released pro-inflammatory cytokines and improves the priming of T cells with their further activation. Moreover, ST6Gal-1 knockout mice presented more mature DCs, regarding MHC-II expression (Crespo *et al.*, 2009). These findings suggest that ST6Gal-1-mediated sialylation might have an immunomodulatory role in DCs. Nonetheless, the mechanism underlying sialidase effect is unknown.

In our group, it was observed that soluble ST6Gal-1 decreases myelopoiesis *in vitro* (Jones *et al.*, 2010) and the numbers of hematopoietic and stem cells in WT mice treated with soluble ST6Gal-1 (Nasirikenari *et al.*, 2014). Moreover, *in vitro* treatment with ST6Gal-1 diminishes the release of pro-inflammatory cytokines in bone marrow derived macrophages (unpublished observations from Mehrab Nasirikenari, Joseph Lau). These findings suggest that extrinsic ST6Gal-1 has a key role in the modulation of immune functions. In addition, the implication of soluble ST6Gal-1 in several cancers (Swindall *et al.*, 2013), (Park and Lee, 2013) emphasises the importance of unravelling its biological role. Considering the results from our groups all together, we hypothesize that DCs extrinsically treated with ST6Gal-1 diminish the expression of maturation markers and the release of pro-inflammatory cytokines.

In order to test this hypothesis, the main goal of this work was to assess the influence of *in vitro* extrinsic α2,6 sialylation in bone marrow derived DCs' features, upon LPS stimulation. Bone marrow cells were chosen as the source of DCs, due to the high amounts of obtained cells, enabling the study of several combined conditions. Moreover, these cells present homology with human conventional DCs, suggesting that findings can be addressed to human DCs (Satpathy *et al.*, 2012).

On the other hand, we intended to study sialidase effect in this type of DCs, establishing a homology with human mo-DCs. Lastly, we intended to address the specific role for ST6Gal-1 in DCs maturation, *in vivo,* namely in lung DCs, due to its importance

in several allergic and pulmonary diseases (Kim and Lee, 2014). To achieve this goal, we used ST6Gal-1 knockout mice stimulated with LPS and assessed, afterwards, the features of cells from their airways. Therefore, with this work we have addressed the effect of ST6Gal-1 in two important and representative DC subtypes.

The present work was first developed in Department of Molecular and Cellular Biology of Roswell Park Cancer Institute, Buffalo, NY, USA and finished in Glycoimmunology group of Chronic Diseases Research Center (CEDOC) from Nova Medical School – Faculdade de Ciências Médicas of Universidade Nova de Lisboa, Portugal.

Chapter II

Materials and Methods

II.1. Extraction of bone marrow cells from mice

All animal experiments of this work were approved by the Institute of Animal Care and Use Committee of Roswell Park Cancer Institute, Buffalo, NY, USA, and by animal facility from Nova Medical School − Faculdade de Ciências Médicas, Universidade Nova de Lisboa.

Wild type (WT) C57BL6 and ST6Gal-1 knockout (ST6Gal-1 KO) mice, 6 to 10 weeks of age, female or male were used. WT mice were purchased from Jackson Laboratories at Bar Harbor, Maine, USA and ST6Gal-1 KO were initially obtained from Dr. Jamey Marth laboratory from Molecular, Cellular, and Developmental Biology University of California, Santa Barbara, USA (Hennet *et al.*, 1998) and successive backcrossed more than 10 generations into C57BL6 background (Roswell Park Cancer Institute, Buffalo, NY, USA). WT mice from animal facility of Nova Medical School − Faculdade de Ciências Médicas, Universidade Nova de Lisboa were kindly given by Dr. José Ramalho, from Molecular Biology group of CEDOC.

The mice were killed by CO_2 asphyxiation. The femurs, tibiae and peroneum were removed, and then, in sterile conditions, the bones were put in a petri dish with PBS 1X (from Corning). The bones were sniped in the ends with scissors. A 3 ml syringe was assembled with a 27 gauge needle and loaded with RPMI complete medium (Appendix I) in 1:10 dilution with PBS 1X. The bones were flushed till become white and then the cells were filtered with a 100 μm strainer. It was added Mouse red blood cell lysis buffer (Appendix I) to the cells in 1:2 proportion, in order to lyse erythrocytes (not needed to further differentiation of bone marrow precursor cells into DCs). Cells were centrifuged at 1400 rpm for 5 minutes. After that, is was discarded the supernatant and the *pellet* was ressuspended in RPMI complete medium. The cells were diluted in PBS 1X and counted using an automatic counter machine (from BioRad), or under the optic microscope with a Neubauer chamber. In the first case, the obtained number was multiplied by the dilution factor and considered the volume where the cells where ressuspended. In the former case, the total cells were counted, applying the following equation:

$$\text{Number of cells} = DF \times 10^4 \times \text{number of cells counted}$$

Where DF is the dilution factor and 10^4 refers to the volume of Neubauer chamber. The number of cells per ml was obtained dividing the result of the equation by total volume where the cells were ressuspended. During this procedure, usually 25 to 30 million bone marrow cells were obtained, per mouse.

II.1.1 Generation of bone marrow derived DCs

The bone marrow cells from both WT and ST6Gal-1 KO mice, obtained as previously described, were cultured with 20 ng/ml (400 U) of recombinant murine GM-CSF (from Peprotech or Immunotools) and 10 ng/ml (50 U) of recombinant murine IL-4 (Biovision), in RPMI complete medium. Together, these cytokines induce the differentiation of bone marrow cells into DCs. Bone marrow cells were plated at 5 x 10^5 cells per 3 ml of medium, in a 6 well plate, and incubated at 37 ºC in humid atmosphere with 5% CO_2. In some assays, the bone marrow cells were cultured with 20 ng/ml of GM-CSF (400 U), without IL-4.

Every two days, the culture medium was changed. Per well, it was discarded 1 ml of the old medium and replaced with 1 ml of fresh RPMI complete medium, supplemented with cytokines, in the same concentrations as mentioned before. By removing the old medium, other types of cells derived from bone marrow are eliminated, as well as metabolites produced by the cells (Inaba *et al.*, 1992).

After 7 to 8 days of culture, the non-adherent cells were harvested, by gently pipetting, dislodging cellular aggregates, since DCs are non-adherent cells. In addition, using this method any eventual contamination of DC population with macrophages is prevented, since these are adherent cells (Inaba *et al.*, 1992). The cells were centrifuged at 900 rpm for 10 minutes. This gentle way to harvest the cells was performed to avoid DCs' activation.

After discarding the supernatant, the pellet was ressuspended in RPMI complete medium. The cells were counted in the same way as previously described and plated in 24 well plates, at 5×10^5 cells per well, each one with 500μL of RPMI complete medium without cytokines. The number of cells obtained at this stage was usually between 5 to 10 million.

II.2. Assays to test the influence of extrinsic α2,6 sialylation in mice BMDCs' activation profile

With the aim of evaluating the influence of α2,6 extrinsic sialylation on bone marrow derived DCs (BMDCs)' activation profile, different conditions upon lipopolysaccharide (LPS) stimulation were tested. These tests were run in the next day after BMDCs were obtained, as previously described in sections II.1 and II.1.1., and this protocol was applied either to WT as to ST6Gal-1 KO BMDCs.

To run the assays, five solutions were prepared in RPMI complete medium without fethal bovine serum (FBS) (Appendix I) supplemented with:

(1) 400 mU of sialidase/ml per 0.5 million cells, designated here as **Sial** (*Clostridium perfringens* Neuraminidase from Roche);

(2) refers only to RPMI complete medium without FBS, designated as Untreated **(Ut);**

(3) 100 ng/ml of lipopolysaccharide **(LPS)** (from Sigma);

(4) 10 µl/ml of recombinant murine ST6Gal-1 (further detailed in Appendix I and designed here as **rST6Gal-1)** + 100 µM of cytidine-5'- monophosphate-N-acyl-neuraminic acid (from Sigma, described here as **CMP-Sia**);

(5) 10 µl/ml of **rST6Gal-1** + 100 µM of **CMP-Sia** + 100 ng/ml of **LPS**.

In sterile conditions, 400 µl of BMDCs' culture medium was removed, per well, and added straightway 400 µl of the respective solutions. First, solution (1) was added, in the respective wells, incubating for 45 minutes, at 37 ℃, 5% CO_2 .Then, the medium was removed and the other solutions were added to the corresponding wells, as illustrated in Table II.1.

Table II.1- **Representation of the conditions to test in BMDCs' activation assays and respective solutions prepared.** It is shown on the left column the conditions performed and the respective added solutions on the right column. Ut refers to Untreated, rST6Gal-1 to recombinant murine ST6Gal-1, CMP-Sia to cytidine-5'- monophosphate-N-acyl-neuraminic acid and Sial refers to sialidase.

	Conditions	Solutions
Negative controls	Ut	(2)
	rST6Gal-1 + CMP-Sia	(4)
Positive control	LPS	(3)
Tests	Sial	(1), (2)
	rST6Gal-1 + CMP-Sia + LPS	(5)
	Sial + LPS	(1), (3)
	Sial + rST6Gal-1 + CMP-Sia + LPS	(1), (5)

As mentioned in Table II.1, LPS condition was the positive control for BMDCs' activation, whereas Ut and rST6Gal-1 + CMP-Sia conditions were the negative controls. The main goals to achieve with the performed tests will be further detailed in Chapter III, in section III.2. The cells were incubated in the respective conditions for 6 hours at 37 ℃, 5% CO_2, whereas their supernatants were collected for ELISA after 6 or 24 hours. The cells were also collected for Flow Cytometry analysis after 6 or 24 hours.

In some experiments, rST6Gal-1 + CMP-Sia and LPS were added to WT BMDCs in sequential steps, in order to test the influence of each component. In this case, incubation with rST6Gal-1 + CMP-Sia was performed during 4 hours, at 37 ℃, 5% CO_2 followed by addition of RPMI complete medium without FBS with or without LPS. In this case, the supernatants were collected after 6 and 24 hours of BMDCs' incubation at 37 ℃, 5% CO_2 (for ELISA), whereas the cells were collected for Flow Cytometry analysis after 24 hours.

In some experiments, BMDCs treated with Sialidase or ST6GalI were collected after 1 hour of the respective treatments, for Flow Cytometry assays with lectins.

Modifications to the protocol were applied to test different hypothesis, further detailed in the following sections.

II.2.1 Assays to test the influence of extrinsic α2,6 sialylation during bone-marrow cells' differentiation into DCs

The influence of extrinsic α2,6 sialylation during WT bone-marrow cells' differentiation into DCs was assessed. In this case, half of BMDCs were generated as described in II.1.1., whereas the other BMDCs were generated with RPMI complete medium supplemented with cytokines, but with 10 µl/ml of rST6Gal-1 + 100 µM of CMP-Sia, added every two days. After 8 days of differentiation, BMDCs were treated with RPMI complete medium without FBS with or without LPS (solutions 2 and 3, previously mentioned) and incubated for 6 hours, at 37 ℃, 5% CO_2. Their supernatants were collected after this time, similar to the cells, for ELISA and Flow Cytometry assays, respectively.

II.2.2 Assays to test the influence of extrinsic α2,6 sialylation performed in a concentrated cellular volume

In this case, BMDCs were also obtained as described in II.1.1 and after 7 days of differentiation the cells were harvested, where each 2 million cells were treated with solutions (2) and (4), in a final volume of 1 ml. In this case, solution (1) was replaced by RPMI complete medium without FBS + 100 mU/ml of sialidase, which was also added to 2 million cells.

The incubation in the respective conditions was performed at 37 ℃, 5% CO_2, during 1 hour, while the cells were shaken every 20 minutes. After this incubation, solution (2) was added to half of the cells and solution (3) to the others. Then 5×10^5 BMDCs were plated in 500µL per well (previously described in section II.1.1.), incubating for 48 hours. The supernatants were collected at 6, 24 and 48 hours for ELISA assays. The cells were collected for Flow Cytometry analysis after 24 and 48 hours.

II.3. Evaluation of BMDCs' surface markers by Flow Cytometry

Flow Cytometry was used in this project to identify DCs and characterize them. Flow Cytometry is a very powerful technique in the field of Immunology, allowing the analysis of single cells in suspension. It works in the basis of cells or particles passing through a flow chamber, where they scatter the light from lasers in different directions. The light scattered in the same direction than the laser refers to Forward Scatter (FSC) and at 90º related to the laser path refers to Side Scatter (SSC). The former provides information about the relative size of the cell, whereas the later refers to its granularity. In addition, when the cells are stained with a fluorophore, the lasers excite it and the light emitted in a certain wavelength is filtered, collect, amplified and converted to digital data, providing useful information (*Flow Cytometry Analysis from Applied Cytometry makers of VenturiOne & StarStation*, 2012). In this work, the use of specific antibodies with fluorophores allowed the measurement of CD11c and maturation markers in BMDCs' surface.

During the course of the work, LSR II (BD Bioscience) and the Attune® Acoustic Focusing Cytometer were used. The former owns 5 different lasers, enabling an 18 colour panel of fluorescent antibodies to be used, with the appropriate compensations, whereas the later has two lasers enabling the use of a panel constituted by 6 different fluorophores.

After BMDCs' activation assays, the cells were incubated at 37 ºC, 5% CO_2, with Accutase (Sigma), during 7 to 10 minutes, with the aim of harvesting the cells (upon LPS stimulation BMDCs tend to become adherent). Then, DCs were removed from the wells by scrapping and centrifuged at 1200 rpm for 5 minutes. The supernatants were removed and the cells found in each tube were ressuspended at a concentration of 0.5 million cells / 200µl of Flow Cytometry buffer (see Appendix I to further details) or PBS 1X (Corning).

Half million BMDCs were kept at 4 ºC, to be used as the unstained sample (Us), becoming the negative control of the experiment. The cells were incubated for 5 minutes with 5 µl of purified anti-mouse CD16/CD32 antibody (Biolegend), to block Fc-receptors and avoid unspecific binding of other antibodies added later. In the case of assays performed with lectins and antibodies in the same tube, the staining with lectins was performed in the first place, whereas different lectins were not mixed with each other. BMDCs were incubated with: 1 µl of fluorescein isothiocyanate (FITC)-SNA or 5 µl of FITC-SNA diluted 1:10 in PBS 1X; 1 µl of FITC-MAL-I (Vector labs) and with 5 µl of biotinylated MAL-II, diluted 1:10 in PBS 1X (all the lectins are from Vector). The incubation was performed during 10 minutes, at 4 ºC, in the dark. Then, the cells were washed with 2 ml of Flow Cytometry buffer and centrifuged at 1400 rpm for 5 minutes. The supernatant was removed, once again, and the cells ressuspended in 200 µl of Flow Cytometry buffer or PBS 1X.

BMDCs were incubated during 20 minutes, at 4 ºC in the dark, with 5 µl of the respective antibodies, first diluted in PBS 1X, according with the instructions of the manufacturer: Phycoerythrin (PE)-CD11c (Biolegend) or FITC-CD11c (Vector labs), Peridinin chlorophyll (PerCP) cyanine (Cy) 5.5-I-A^{b} or Alexa Fluor 647-I-A^{b} (both from Biolegend), Allophycocyanin (APC)-CD11b, Brilliant violet (BV) 605-CD80 or PerCPCy5.5-CD80, APCCy7-CD86 or PE-CD86 (all from BioLegend) and with FITC-streptavidin (BioLegend), in the case of assays where biotinylated MAL-II was first used.

Taking into account that usually 4 or 5 different fluorophores were added to the same sample, compensations were performed later in order to avoid cross-talk between channels of multicolours. Then, single colour controls were prepared, incubating 30 µl of anti-mouse beads particles (Spherotech) with 1 µl of the respective antibody. The negative control was made with 30 µl of blank beads particles (Spherotech). Afterwards, 200 µl of Flow Cytometry buffer was added to each control and beads were incubated in

the dark at 4 ºC, over the course of the experiment. Under certain circumstances, the single cell controls were prepared using 0.5 million BMDCs, incubated with the respective antibody, in the same conditions as previously mentioned.

After incubation, the cells were washed with 2 ml of Flow Cytometry buffer in each tube and centrifuged at 1400 rpm for 5 minutes. The supernatants were removed and BMDCs ressuspended once again in 200 µl of Flow Cytometry buffer, when using LSR II and in 1 mL of PBS 1X, in the case of Attune® Acoustic Focusing Cytometer.

Following this protocol, the percentage of BMDCs expressing CD11c and I-A^b was evaluated. I-A^b corresponds to MHC-II in the case C57BL6 strain of mice, the one used over the project (Biolegend, 2015). In addition, the expression of CD80 and CD86 maturation markers was determined, as well as the linkages to α2,6 Sias, α2,3 Sias and Galβ1-4GlcNAc, using the lectins SNA, MAL-II and MAL-I, respectively.

During Flow Cytometry analysis, usually 5.000 to 10.000 events were acquired. The compensations were performed after the acquisition, using FlowJo software.

II.4. Evaluation of cytokines by ELISA

Enzyme linked Immunosorbent assay, usually referred as ELISA, was performed in this work to evaluate the concentrations of TNF-α, IL-6 and IL-10 released from BMDCs in the cellular medium, upon the activation assays described in section II.2 of this Chapter. The assays were performed through ELISA sandwich method, where both capture and detection antibodies are used, in order to improve the sensitivity and specificity (PeproTech, 2015). Over the project, two different kits were used: ELISA Ready-Set-Go!® kit, from eBioscience Affymetrix, for TNF-α, IL-6 and IL-10 evaluation and Peprotech mouse kit, for TNF-α and IL-6 evaluation.

First, a polystyrene plate with 96 wells, provided by the respective manufacturers, was covered with 100 µl/well of capture antibody, diluted 1:250 in Coating buffer, in the case of Affymetrix kit, or diluted 1:100 in PBS 1X, in the case of Peprotech kit (see Appendix I for further details). The plate was incubated at 4 ºC, overnight.

The content of the wells was removed and washed four to five times with 200 µl/well of Wash buffer (Appendix I). Then, 200 µl/ well of Diluent were added (Affymetrix kit). In the case of Peprotech kit, 200 µl/well of Block buffer (Appendix I) were added. The plate was incubated for 1 hour at room temperature, in the dark. This

step enables the blocking of unspecific protein surface binding, in the next steps. The Diluent was removed and washed, once more, with Wash buffer.

Two fold serial dilutions of standards in Diluent were prepared for each cytokine, with a total of 8 points (Affymetrix kit) or a total of 12 points (Peprotech kit), following the recommendations of the manufacturers. The aim of using standards for each cytokine was to create a calibration curve, later used to determine the concentrations of the respective cytokines, for each sample. After that, 100 µl of diluted samples were added in each well, incubating either for two hours at room temperature or overnight at 4ºC (for maximum sensitivity). The content of wells was removed and washed four to five times with Wash buffer, as described before. It was added 100 µl/well of detection antibody with Diluent in the proportions 1:250 (Affymetrix kit), or 1:200 (IL-6) or 1:400 (TNF-α), in the case of Peprotech kit. The plate was incubated for 1 hour at room temperature, in the dark. Once more, the content of wells was removed and washed four to five times with Wash buffer.

It was added 100 µl/well of Avidin-HRP (horseradish peroxidase) diluted 1:250 in Diluent (Affymetrix kit) or Streptavidin-HRP (Immunotools) diluted 1:1000 in Diluent (in the case of Peprotech kit). The plate was incubated for 30 minutes at room temperature, in the dark. The content of wells was removed and washed with Wash buffer, 4 to 7 times, to avoid unspecific binding of the enzyme to the substrate in the next step. It was added 100 µl/well of the substrate (3,3',5,5'-tetramethylbenzidine or TMB) and incubated for 15 minutes, in the dark, at room temperature. When the cytokine that we are analysing is present in the supernatants, TMB is converted by HRP enzymatic reaction to a blue product. To stop the reaction, 100 µl of H_3PO_4 1 M or HCl 1 M were added and the absorbencies of the resulting yellow product were read at 450 nm. In the case of Affymetrix kit, the absorbencies at 570 nm were also determined, in order be subtracted to 450 absorbencies, eliminating the background. In the case of Peprotech kit, the mean absorbencies of wells with Block buffer were subtracted to the absorbencies of the samples, to discount the background.

II.5. Analysis of cells from bronchoalveolar lavage fluid of WT and ST6Gal-1 KO mice

II.5.1 Intra-tracheal injections

WT and ST6Gal-1 KO mice were anesthetized with isoflurane inhalation. They were placed in a vertical platform, hanging by his front teeth with a string. The tong was gently pulled off the side, and 50 µl of sterile PBS 1X (Corning) or 50 µl of 2g/ml sterile LPS (from Sigma) were administrated in the open cavity. The mouse was kept upright for a while, to guarantee that the lungs were properly filled and when waking up from the anaesthesia, they were kept at room temperature.

II.5.2 Bronchoalveolar lavage

After 24 hours of PBS or LPS administration through intra-tracheal injection, the mice were sacrificed by intraperitoneal (IP) injection with 1 to 1.5 mL of avertin work solution (Appendix I), using a 6 ml syringe (27 or 30 gauge needle). Avertin was used to replace CO_2 asphyxiation to sacrifice the animals, as the later would block and compromise the mice airways.

The skin was dissected away and a string was placed below the trachea of the mouse, where the first white ring of the trachea was found. It was perforated with a BD Insyte Autoguard Winged catheter (22 gauge needle, 1.00 inches). The needle was slowly pushed towards the lungs direction (perforating about 1 cm) and then carefully removed, remaining there the plastic cover of it. The string was now tied around the plastic catheter. Using a plastic syringe, 1 ml of Flow Cytometry buffer was assembled in the catheter and the syringe was pushed, filling the lungs, and then pulled, recovering the fluid. This procedure was repeated twice, where usually 2 ml of bronchoalveolar lavage fluid (BALF) were obtained, in the end of the procedure.

BALF was kept on ice, while the protocol was performed with other mice. In the end, Mouse red blood cell lysis buffer (1:2 dilution) was added, in order to lyse the erythrocytes. Then, the cells were centrifuged at 1400 rpm for 5 minutes and afterwards the supernatants were removed, whereas the cells were ressuspended in 200 µl of Flow Cytometry buffer.

II.5.3 Assess bronchoalveolar lavage fluid by Flow Cytometry

After centrifuging, 5 µl of purified anti-mouse CD16/CD32 antibody (Biolegend), was added, incubating for 5 minutes at 4 ºC, as previously mentioned. Then, 5 µl of the following antibodies (diluted 1:10 in PBS 1X) were added to the samples: APC-Ly6G, BV711-CD11b, FITC-CD11c, PECy7-B220, PerCPCy5.5-I-A^b, BV605-CD80 and APCCy7-CD86 (all from Biolegend). The cells were incubated during 20 minutes, at 4 ºC, in the dark. This antibody panel was established in order to distinguish different cell types and DCs' maturation markers.

Once more, 0.5 million cells were saved from each mice (ST6Gal-1 KO and WT mice) to be used later as unstained samples. In this case, single colour controls were performed with beads particles. In addition, fluorescence minus one (FMO) controls were performed to PECy7-B220, PerCPCy5.5-I-A^b, BV605-CD80 antibodies, using beads particles as well. This type of control includes all used antibodies at the same time, except one of them. It is performed to exclude the interference of the other fluorophores while setting the negative population of a specific fluorescence upon Flow Cytometry analysis. Therefore, the antibodies corresponding to surface markers where it was expected a small expression were chosen to perform these controls. Both type of controls were made incubating 30 µl of anti-mouse beads particles (Spherotech) with 1 µl of the respective antibody. The negative control was made with 30 µl of blank beads particles (Spherotech) and then 200 µl of Flow Cytometry buffer were added to every tube. Afterwards, the cells were washed with 2 ml of Flow cytometry buffer and centrifuged at 1400 rpm for 5 minutes. The supernatant was removed and 200 µl of Flow Cytometry buffer were added in each tube, proceeding to Flow Cytometry analysis.

II.6. Analysis of data

The results obtained from ELISA assays were evaluated by Excel software, whereas Flow Cytometry results were assessed by FlowJo software, including the previously mentioned compensations. The statistical analysis was performed using GraphPad software. Regarding statistic tests, paired student t-tests or unpaired student t-tests were performed to each condition, according with the most appropriate statistic test to execute in every experiment. Tests were considered statistically significant when p-value was inferior to 0.05 (*), 0.01 (**) and 0.001 (***).

Chapter III

Results

III.1. General Introduction

This project was developed in two parts, presented in the following sections: 1) Activation profile of mice bone marrow derived dendritic cells (BMDCs) upon α2,6 extrinsic sialylation and 2) Characterization of ST6Gal-1 knockout (KO) and WT DCs from the airways of mice.

In this work, we hypothesize that modulation of DCs with α2,6 Sias decrease their immune response, upon LPS stimulation.

Several observations led to formulate and to test this hypothesis, like the fact that tolerogenic DCs express high levels of α2,6 Sias in their surface (Jenner *et al.*, 2006), which could justify their low immunogenic phenotype, by the inhibition of inflammatory pathways. In addition, it is well known that the genetic expression of ST6Gal-1 decreases upon cellular activation, suggesting a modulator role for ST6Gal-1 (Jenner *et al.*, 2006), (Videira *et al.*, 2008). Sialidase corresponds to an opposite action of adding Sias in DCs' surface known to increase DC maturation. Indeed, our group observed that the *in vitro* treatment of human monocyte derived DCs (mo-DCs) with sialidase increases the expression of maturation markers, their genetic expression of pro-inflammatory cytokines and the priming and activation of T cells (Crespo *et al.*, 2009), In agreement with this, it was also reported that sialidase inhibiters decrease the release of cytokines from DCs upon LPS stimulation (Stamatos *et al.*, 2010).

The first aim was to understand if BMDCs' maturation markers and released cytokines are influenced by α2,6 Sias extrinsically added by ST6Gal-1 upon lipopolysaccharide (LPS) stimulation. LPS engagement with TLR-4 leads to DCs' activation, where an increased release of pro-inflammatory cytokines (Granucci *et al.*, 1999) and increased expression of maturation markers is known to occur (Dearman *et al.*, 2009).

Within the formulated hypothesis, the second aim was to identify and compare DCs from the airways of ST6Gal-1 KO and WT mice upon LPS stimulation, regarding their maturation markers. It was predicted that DCs lacking α2,6 Sias (from ST6Gal-1 KO mice) present a more mature phenotype comparative to WT DCs in mice airways.

With these goals we intended to obtain a better insight in the influence of α2,6 Sias modulating BMDCs, but also to understand if lung DCs' features are modified in mice lacking α2,6 Sias, upon LPS stimulation.

III.2. Activation profile of mice BMDCs upon α2,6 extrinsic sialylation

As previously mentioned, the first goal of this work was to understand if extrinsic α2,6 sialylation modulates BMDCs' maturation profile, by treating the cells with recombinant murine ST6Gal-1 (which will be designated over this Chapter as **rST6**) and the substrate cytidine-5'-N-acetyl-neuraminic-acid (**CMP-Sia**), *in vitro*.

These analyses of BMDCs' profile were performed after submitting the cells to the conditions mentioned in Chapter II, topic II.2, namely cells treated with ST6Gal-1 and sialidase, adding or not LPS.

Cells only treated with RPMI complete medium (Untreated, designated here as **Ut**) were set as a negative control. Treatment with ST6Gal-1 was designated over this Chapter as **rST6 + CMP-Sia.** This condition was used to evaluate the degree of extrinsic α2,6 sialylation of BMDCs, induced by the treatment. **LPS** was used to induce BMDCs' activation, being the positive control.

To test the major goal of the experiment, the condition **rST6 + CMP-Sia + LPS** was performed. Moreover, different conditions were run to test the effect of sialidase. In previous studies of our group, sialidase treatment has improved the maturation of human monocyte derived DCs (mo-DCs) (Cabral *et al.*, 2013), whereas in ST6Gal-1 KO BMDCs a higher expression of MHC-II was reported, comparing with WT BMDCs (Crespo *et al.*, 2009). In these studies, sialidase has induced the opposite effect expected to observe upon α2,6 extrinsic sialylation. Then, with the aim of evaluating sialidase effect on BMDCs, the condition Sialidase (designated here as **Sial**) was performed.

Furthermore, the condition **Sial + rST6 + CMP-Sia + LPS** was performed in some experiments. In this treatment, sialidase was added in the first place to remove sialylated structures from BMDCs' surface. The sialidase used in the experiments cleaves mostly α2,3 sialylated structures, followed by α2,6 and α2,8 linkages at the same rate (*Neuraminidase* - Roche). Afterwards, ST6Gal-1 plus LPS were added at the same time to improve the extrinsic α2,6 sialylation and activate the cells, respectively. The idea of treating cells previously with sialidase arises from the fact that DCs express high levels of sialylated structures on its cell surface, which are increased during their differentiation (Videira *et al.*, 2008). Because of this, extrinsic sialylation could have a smaller effect by itself, due to absence or reduced available ligands to be sialylated. Therefore, the pre-

treatment with sialidase could remove these structures (mostly α2,3 Sias) and improve α2,6 extrinsic sialylation of DCs' surface, boosting the effect of ST6Gal-1.

III.2.1 Optimization of BMDCs' generation

To achieve the first goal of this work, the conditions to grow and harvest BMDCs were optimized and criteria to define BMDCs' populations by Flow Cytometry were set.

Several protocols can be adopted to grow BMDCs, where the number of days spent in the cellular differentiation can influence the degree of differentiation reached. In this work, BMDCs were harvested with 7 to 8 days of differentiation. A pure population of BMDCs was not expected to be reached after differentiation, but the non-adherent cell fraction usually corresponded to 70-90% BMDCs, evaluated by Flow Cytometry. The mean obtained with 10 independent experiments (10 mice) was 76.00%, standard error of the mean (SEM) of ± 3.43, regarding CD11c positive cells, which justifies the absence of an expensive purification step before the assays.

Regarding the criteria to define BMDCs, they are characterized as large cells and express CD11c in their surface, which is the most important marker to distinguish them from other cells (Banchereau *et al.*, 2000). Although macrophages share similar features with DCs, such as the expression of CD11c and CD11b, BMDCs usually express higher levels of CD11c comparing with macrophages. MHC-II is expressed at basal levels in DCs, but can be considered a maturation marker since it significantly increases upon DCs' activation (Dearman *et al.*, 2009).

In this project, the major criteria to define BMDCs by Flow Cytometry was the high Forward Scatter (FSC) and Side Scatter (SSC) levels and the expression of CD11c, whereas MHC-II could have different levels of expression, depending if the cells were stimulated with LPS or not. Then, MHC-II was set as a maturation marker as well as CD80 and CD86 co-stimulatory molecules since their expression is known to dramatically increase upon LPS stimulation (Dearman *et al.*, 2009). In Figure III.1 is shown a significant example of BMDCs obtained from a WT mouse with 8 days of differentiation, evaluated by Flow Cytometry.
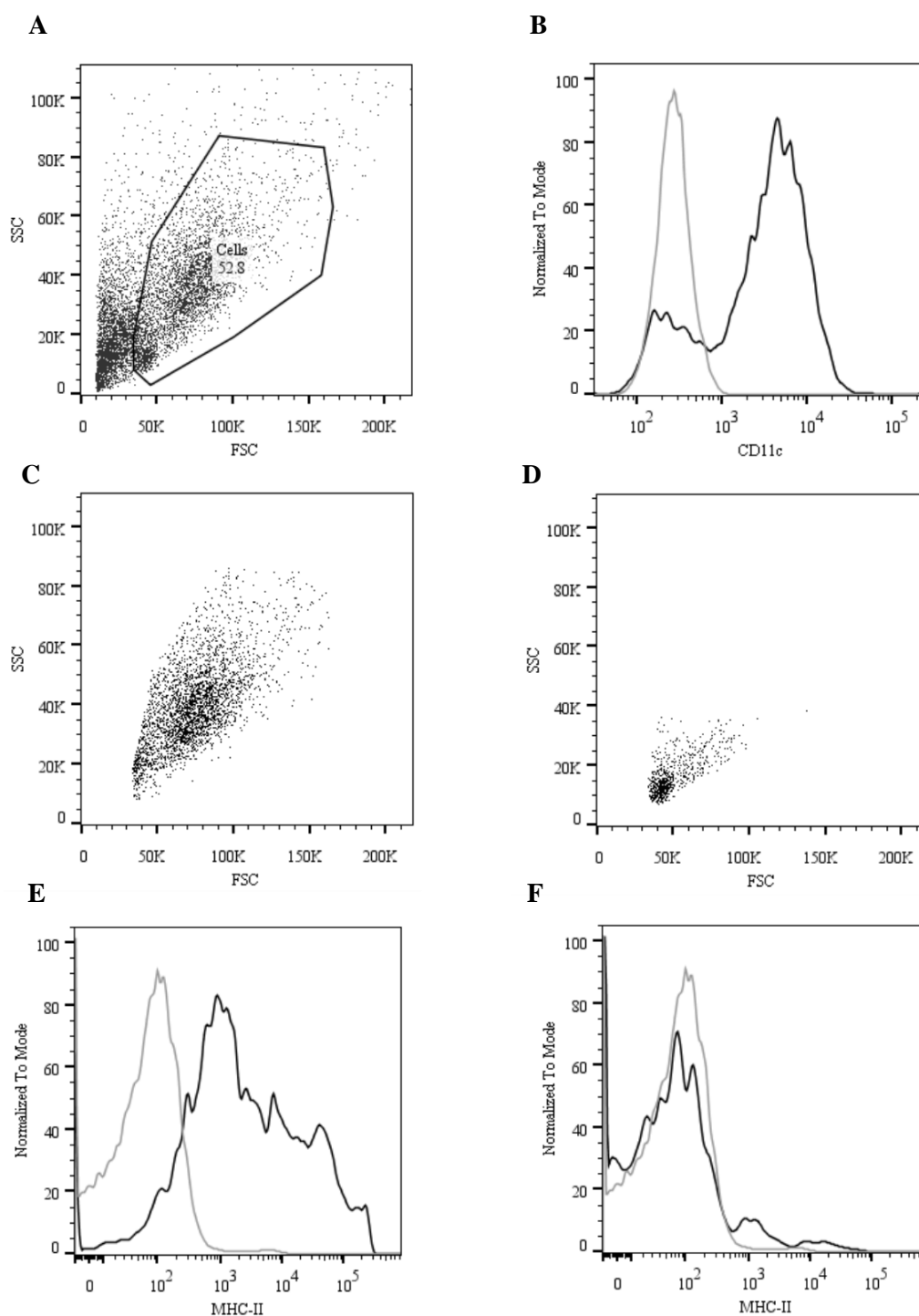
Figure III.1- **Identification of BMDCs obtained within 8 days of differentiation, through Flow Cytometry analysis.** A) FSC vs SSC dot plot of BMDCs, where is shown the gate acquired cells, excluding dead cells and debris. B) Histogram of gated cells with anti-CD11c staining, with 82.00 % CD11c positive cells (black solid line), determined by setting the negative population with basis on the unstained sample (grey solid line). C) FSC vs SSC dot plot of CD11c positive cells. D) FSC vs SSC dot plot of CD11c negative cells. E) Histogram of CD11c positive cells about MHC-II fluorescence (black solid line), where 87.90% express different levels of MHC-II. F) Histogram of CD11c negative cells about MHC-II fluorescence (black solid line), where only 14.00% express MHC-II.

After gating the cells (Figure III.1 A), the CD11c positive population was determined, as showed in Figure III.1 B, corresponding to 82.00% of cells. FSC evaluates the size of cells, whereas SCC corresponds to cells' granularity. By gating this population, we observe that cells are presented in higher levels of FSC vs SCC (Figure III.1 C), as expected about DCs' size. In addition, these cells also stain for MHC-II (black solid line in Figure III.1 D), in different levels. On the other hand, CD11c negative cells arise in lower levels of FSC vs SSC (Figure III.1 E) and do not stain for MHC-II, since the sample overlaps with unstained cells (Figure III.1 F).

CD11c positive cells, arising in higher levels of FSC and SSC and expressing MHC-II, are most likely to be BMDCs that we intended to analyse. Taking this into account, this gating strategy was applied to all the following experiments.

III.2.2 Influence of α2,6 extrinsic sialylation in BMDCs' profile upon LPS stimulation

Upon BMDCs' activation, they release pro-inflammatory cytokines, such as TNF-α and IL-6. In Figure III.2 is present the amount of TNF-α and IL-6 cytokines released by BMDCs after 6 hours in the conditions described in section III.2. These results were obtained by ELISA.
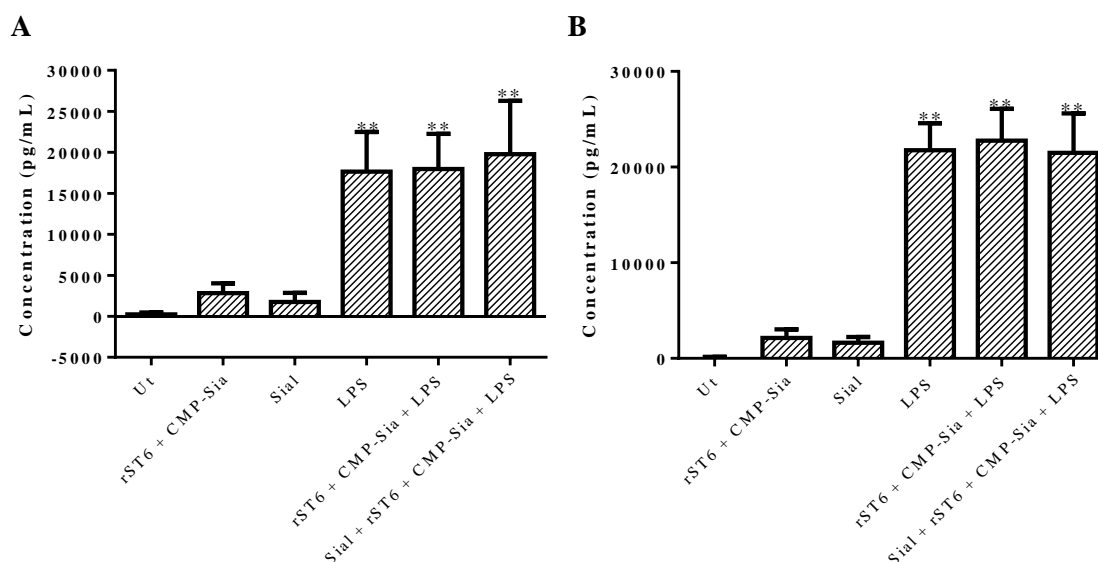
Figure III.2- **Concentrations of TNF-α (A) and IL-6 (B) released by BMDCs, determined by ELISA**. The respective conditions are mentioned at x axis. To evaluate Ut, LPS and rST6 + CMP-Sia + LPS conditions, 8 mice were used, whereas for rST6 + CMP-Sia control 4 mice were used. To perform conditions Sial and Sial + rST6 + CMP-Sia + LPS 6 animals were used. For each condition it is presented the mean + SEM (standard error of the mean). Unpaired t-tests were performed comparing every condition with each other, where statistical significant differences comparing Ut with LPS, rST6 + CMP-Sia + LPS and Sial rST6 + CMP-Sia + LPS, are presented as **. The respective p-values were: 0.0028, 0.0010 and 0.0043, for both TNF-α and IL-6. No significant differences were found between the negative controls and Sial, neither comparing the conditions where LPS was added with each other.

As one can see in Figure III.2, all the conditions where LPS was added present increased amounts of TNF-α and IL-6 released, in comparison with Ut BMDCs. The unpaired t-tests confirm this significant difference (**) comparing Ut with LPS, rST6 + CMP-Sia + LPS and Sial + rST6 + CMP-Sia + LPS conditions (p-values <0.01 for both cytokines). Moreover, the additional negative control rST6 + CMP-Sia is also significantly different from the conditions where LPS was added (not shown), demonstrating that despite the slight increase in the amount of cytokines released in this control, it does not activate the cells *per se.*

These observations indicate an effective activation of BMDCs upon LPS stimulation, as expected. However, sialidase did not lead to any significant increase in the amount of cytokines released comparing with untreated cells.

In opposite to the hypothesis we have proposed, ST6GalI plus LPS has not diminished BMDCs' release of pro-inflammatory cytokines since this is very similar to LPS, for both cytokines. Furthermore, the condition Sial + rST6 + CMP-Sia + LPS also did not occurred the way hypothesize, since the amount of cytokines released by BMDCs is alike in LPS treated BMDCs.

On the other hand, when an anti-inflammatory response occurs it is usually related to the cytokine IL-10. In this work this cytokine was also assessed by ELISA, as presented in Figure III.3.
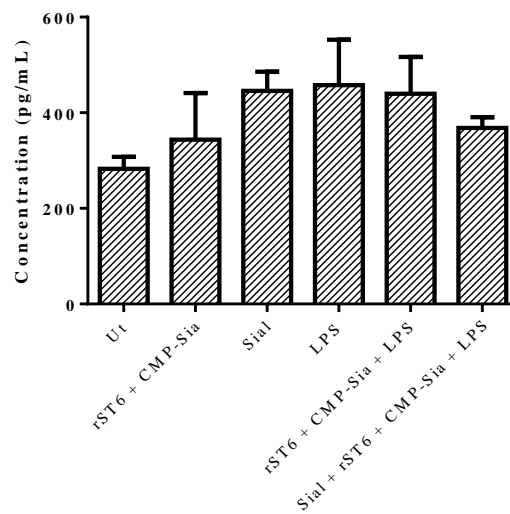
Figure III.3-**Concentrations of IL-10 released by BMDCs, determined by ELISA**. The respective conditions are mentioned at x axis. For each condition it is presented the mean + SEM of three independent experiments, where no significant differences were found between them, by performing unpaired t-tests.

IL-10 is usually released after a pro-inflammatory response has occurred, to downregulate the induced immune response. In this case, the effect of LPS engagement on TLR-4 was assessed after 6 hours. Therefore, it is not expected to have a relevant release of IL-10 due to LPS, since the anti-inflammatory response usually occurs later (Abbas, Lichtman and Pillai, 2012). Nevertheless, we intended to understand if extrinsic α2,6 sialylation could lead DCs towards an anti-inflammatory profile upon LPS stimulation.

As noticeable in Figure III.3 a small amount of IL-10 was released by BMDCs in every condition, where no significant differences between them were noticed. This suggests that an anti-inflammatory response from BMDCs was not induced in any case.

BMDCs' maturation markers were assessed after 6 hours of the same treatments, by Flow Cytometry. The unstained samples were used to set the negative population to each marker. Then, the populations with different levels of expression of each maturation marker were defined based on their fluorescence levels.

In the case of CD86 and MHC-II, two or three populations were usually found, corresponding to negative, medium and high levels of expression, whereas in the case of CD80, a negative and a positive population were determined. In Figure II.4 is summarized the percentage of BMDCs' populations with high levels of expression for CD86 and MHC-II and positive for CD80.
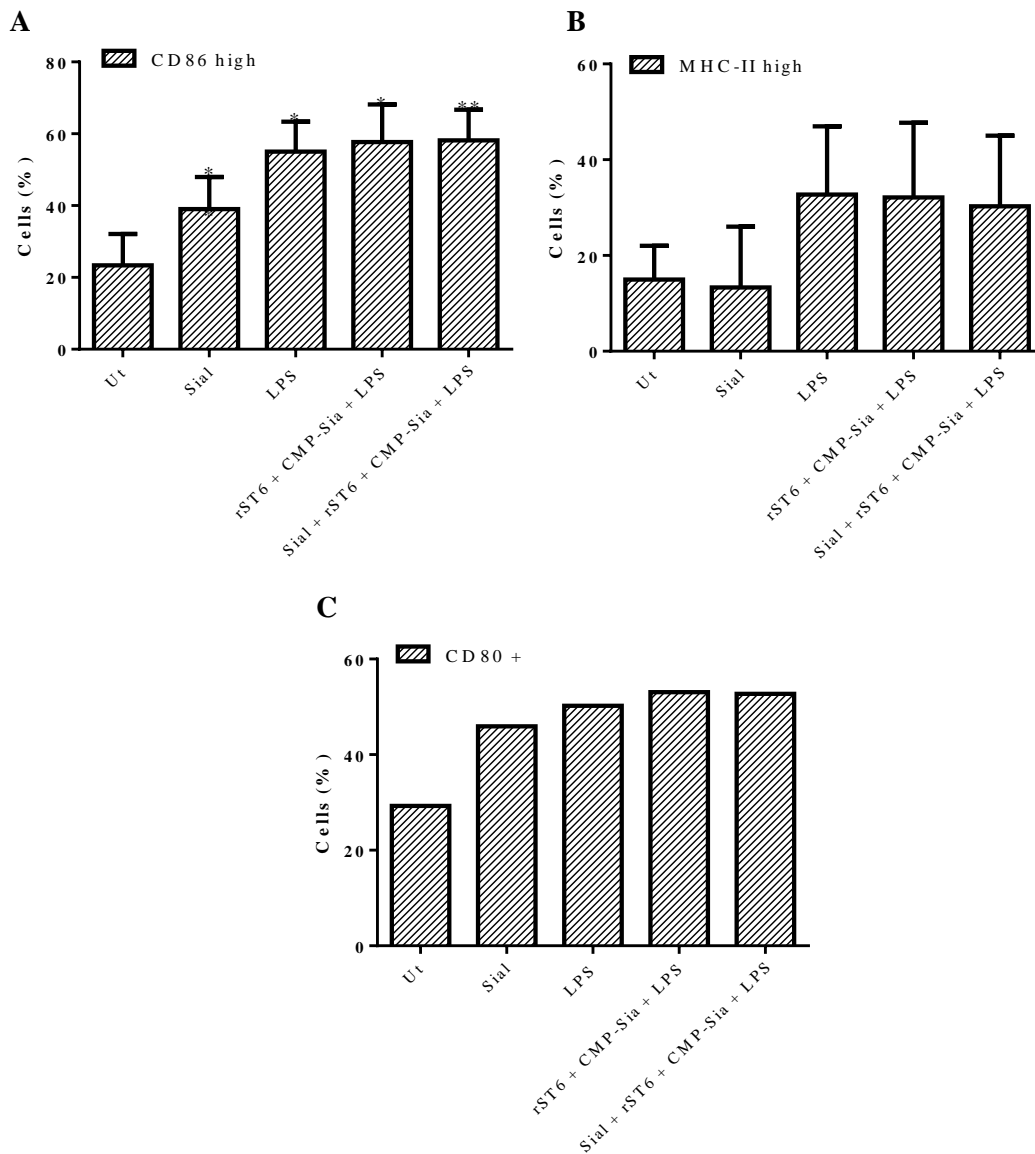
Figure III.4- **Percentage of cells within CD11c positive cells, which express high levels of CD86 (A), MHC-II (B) and are positive for CD80 (C), obtained by Flow Cytometry.** 3 mice were used to perform every condition about CD86 (A) and MHC-II (B), whereas one mouse was used to evaluate CD80 (C). Bars show the mean + SEM, where it was evaluated the statistical significance for CD86 and MHC-II, by paired t-tests. A) Ut was significant different from Sial (*), LPS (*), rST6 + CMP-Sia + LPS (**) and Sial + rST6 + CMP-Sia + LPS (**), whose p-values were respectively: 0.0482, 0.0312, 0.0201 and 0.0063. No other significant differences were noticeable in B and C.

Moreover, the Median Fluorescence Intensity (MFI) was determined to obtain a better insight in the cellular maturation, in the same experiment. The MFIs were normalized to unstained samples, by subtracting its MFI value for each acquired fluorescence. The fold increase for each MFI, comparatively to untreated cells was also determined, as shown in Figure III.5.
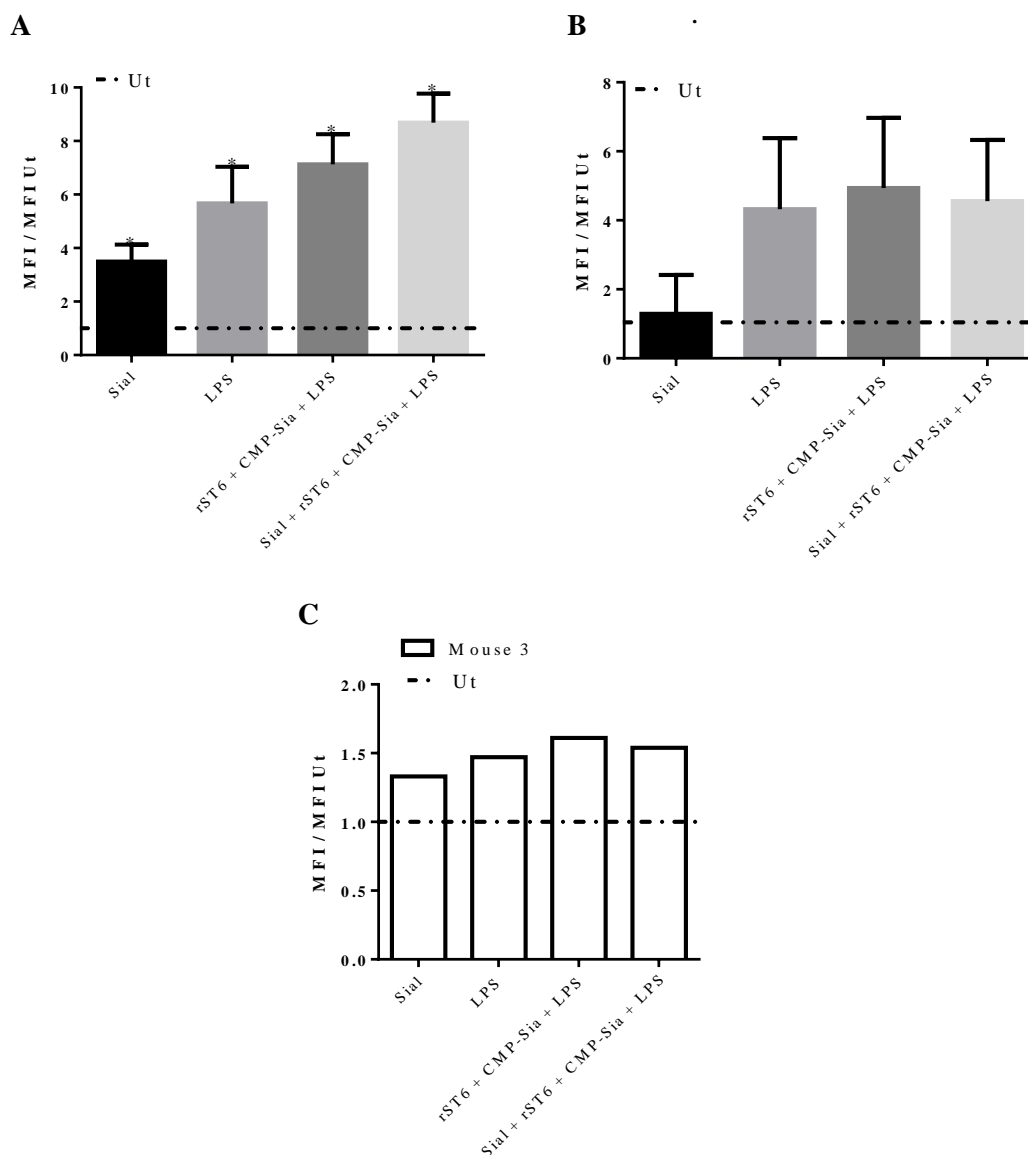
Figure III.5- **Fold increase of the MFIs for each condition comparatively to Ut, regarding the maturation markers CD86 (A), MHC-II (B) and CD80 (C), obtained by Flow Cytometry.** The maturation markers for each mouse are distinguished in the charts and the same 3 animals were used for CD86 and MHC-II and 1 mouse for CD80 evaluation. A) Statistical significance was found by paired t-tests, comparing Ut with rST6 + CMP-Sia + LPS (*) and Sial + rST6 + CMP-Sia + LPS (*), whose p-values were respectively: 0.0316 and 0.0194. No significant differences were detected neither in B or C.

Regarding CD86 high populations, untreated BMDCs were significantly different from Sial (*), LPS (*), rST6 + CMP-Sia + LPS (**) and Sial + rST6 + CMP-Sia + LPS (**), whose p-values were respectively: 0.0482, 0.0312, 0.0201 and 0.0063, calculated by paired t-tests. This observation suggests that these conditions shifted the cells from lower levels of CD86 expression to high levels of CD86 expression (Figure III.4 A).

In addition, the fold increase in the MFI of CD86 related to untreated cells was bigger than 1 to every condition (Figure III.5 A), despite that in different extents. By performing paired t-tests, only rST6 + CMP-Sia + LPS and Sial + rST6 + CMP-Sia + LPS were significant different from Ut, whose p-values were respectively: 0.0316 and 0.0194. This observation suggests that the biggest differences regarding CD86 expression were found in the conditions where both LPS and ST6Gal-1 were added, in opposite to what we expected.

Sialidase significantly increased the percentage of cells expressing CD86 (Figure III.4 A), confirmed by the fold increase in its MFI (mean of 3.5 ± SEM 0.6, N=3, Figure III.5 A), despite no differences were detected before about the amount of cytokines released in the same condition.

About MHC-II, there is a tendency for more cells to achieve a MHC-II high population whenever LPS was added (Figure III.4 B), despite there were no significant differences by performing paired t-tests. In this case, sialidase effect did not increase MHC-II expression, as seen in Figure III.5 B. Regarding all the conditions where LPS was added, mean fold increases around 4 were noticed, although with discrepancies between mice (Figure III.5 B). Nevertheless, the conditions where LPS was added were similar with each other about MHC-II expression, suggesting once again that extrinsic α2,6 sialylation was not able to modulate LPS-induced activation of BMDCs.

CD80 was evaluated only for mice 3, to discard the hypothesis that α2,6 extrinsic sialylation could influence later expressed maturation markers upon LPS stimulation. However, as shown in Figure III.4 C, there is a tendency to increase the expression of CD80 on sialidase and all the conditions with LPS, just like occurred to CD86. There is also a slight fold increase in the MFI of CD80 (between 1.2 and 1.5) for every condition comparing with untreated cells, as presented in Figure III.5 C.

Since no differences were observed comparing LPS with ST6Gal-1 plus LPS treated cells, we have questioned if the treatment with rST6 + CMP-Sia was efficient in

the improvement of the extrinsic α2,6 sialylation in BMDCs. Then, *Sacumbus Nigra Agglutinin* (SNA) binding assays were performed to assess the content in α2,6 Sias binding to terminal galactose residues of BMDCs (Shibuya *et al.*, 1987). Besides, human DCs are known to have a high content of sialylated structures in their surface, owning about 10^{18} Sias per cell (Stamatos *et al.*, 2010), which are further increased during monocytes differentiation into DCs (Videira *et al.*, 2008), (Bax *et al.*, 2007). This effect can lead to the absence of available ligands in cells' surface to be further extrinsically sialylated. However, as far as we know, there is no available information regarding the content in Sias of mice BMDCs. Therefore, we also assessed α2,6 Sias content in unstimulated BMDCs. In Figure III.6 A is presented the fold increase calculated for each MFI of SNA in comparison with untreated cells, for the previous shown 3 independent experiments, also obtained after 6 hours of the respective treatments, by Flow Cytometry. In Figure III.6 B is presented a significant example of SNA binding to untreated BMDCs.
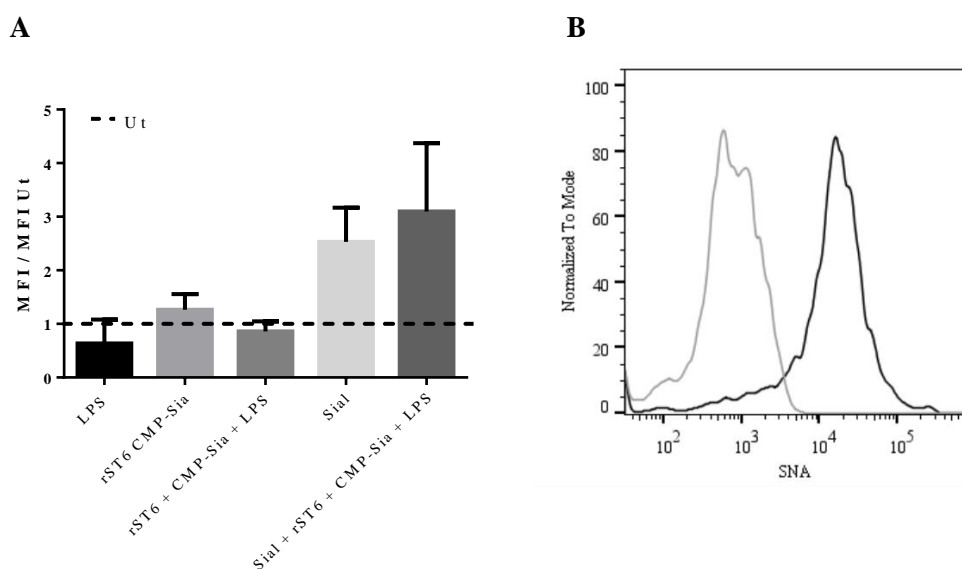
Figure III.6- A) **Fold increase for the MFI of SNA to each condition comparatively to Ut. B) Histograms of unstained sample (grey solid line) and Ut sample (black solid line), representing a significant example of three independent experiments.** Both results were obtained within gated CD11c positive cells, by Flow Cytometry. In A, the same 3 mice from the Flow Cytometry experiments previously shown were used, where value is presented as the mean + SEM. No significant differences comparing any condition with each other were found by performing paired t-tests.

As noticed in Figure III.6 A, ST6Gal-1 treatment led to a slight increase in SNA binding to BMDCs, compared to untreated (fold increase of $1.3 \pm$ SEM 0.3, N=3). Since BMDCs revealed a big shift in SNA staining compared to unstained cells (black solid line

in Figure III.6 B), we conclude that they own a high content in α2,6 sialylated structures *per se*, like human DCs.

In contrast, LPS decreased the content in α2,6 Sias, where the mean fold increase calculated for three independent experiments was lower than 1 (Figure III.6 A). This has been observed before and is in agreement with the fact that during mo-DCs' maturation, the genetic expression of ST6Gal-1 decreases (Videira *et al.*, 2008). The LPS effect is remarkable in ST6Gal-1 plus LPS treated cells, where not even a slight increase of α2,6 Sias content occurred. Considering these three conditions, ST6Gal-1 treatment had a small impact increasing α2,6 Sias' content to BMDCs' surface, which was diminished by the addition of LPS.

About sialidase, we expected a decrease in the content of α2,6 Sias, even that in a smaller extend comparing with α2,3 sialylated structures. Interestingly, the opposite effect has occurred, with a fold increase around 3.0 in the MFI of SNA for this condition. Nevertheless, this effect can be explained by the sialidase mediated cleavage in a higher rate of α2,3 Sias than α2,6 Sias, exposing more α2,6 Sias hidden before. This allows SNA to bind to more of these structures, leading to the false conclusion that α2,6 Sias content has increased.

According with this, Sial + rST6 + CMP-Sia + LPS condition presents the biggest mean fold increase in the MFI of SNA (3.1 ± SEM 1.3, N=3, Figure III.6 A). This effect is probably a combination between sialidase effect, exposing more α2,6 Sias, but also ST6Gal-1 action. Since there are probably more available ligands to add α2,6 Sias in BMDCs' surface, due to Sial cleavage of α2,3 Sias, it is likely that rST6 enzymatic action induced a more pronounced effect in this last condition, as expected.

Even though, we wanted to assure that sialidase treatment was efficient, at least removing α2,3 Sias from BMDCs' surface. Then, *Maackia Amurensis leukoagglutinin* (MAL-II), which binds α2,3 sialylated structures (Siaα2,3Galβ1,4GlcNAc) (Geisler and Jarvis, 2011) was used to assess the content in α2,3 Sias. SNA binding assays were also performed in parallel, in order to understand if the previously observed effect would be the same. These assays were performed right after the treatment with sialidase, instead of 6 hours after it, in order to avoid the influence of any intracellular sialylation mechanism. The MFIs for sialidase comparatively to untreated are presented in Figure III.7, regarding SNA and MAL-II binding assays.
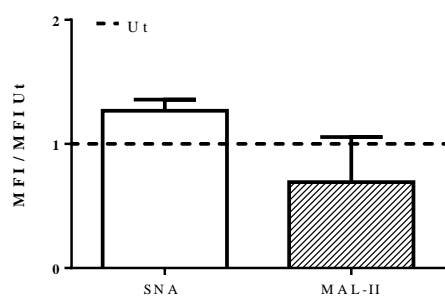
Figure III.7- **Fold increase of Sial comparatively to Ut in the MFI of SNA and MAL-II, obtained within CD11c positive cells, by Flow Cytometry.** 3 independent experiments were performed right after sialidase treatment. For both lectins no significant differences were noticed, performing paired t-tests.

As shown in Figure III.7, none of the lectins presented a significant different fold increase about sialidase comparatively to untreated. However, it was still noticed an increase in the SNA binding right after sialidase treatment (Figure III.7 A), despite it was smaller than the fold increase noticed before to the same condition after 6 hours (Figure III.6 A). Once over, this suggests that α2,6 sialylated structures were exposed upon sialidase treatment, increasing the binding to SNA.

Even though, sialidase action was confirmed by analysis of the MFI of MAL-II, which has decreased upon this treatment, as seen in Figure III.7. This observation suggests that sialidase treatment has efficiently removed α2,3 sialylated structures, despite no statistical significance was reached by performing paired t-tests with 3 independent experiments.

III.2.3 Influence of extrinsic α2,6 sialylation during bone-marrow cells' differentiation into DCs in the modulation of their profile upon LPS stimulation

Considering that BMDCs presented a high content in α2,6 Sias *per se* and no significant differences were found comparing ST6Gal-1 plus LPS with LPS treated DCs, we tried a different approach of α2,6 extrinsic sialylation. In order to increase the effectiveness of α2,6 extrinsic sialylation, rST6 + CMP-Sia were added to cell culture every 2 days during bone marrow cells' differentiation into DCs (described in Chapter II, section II.2.1). Moreover, by using this protocol, α2,3 Sia linkages created during differentiation by ST3Gal could be diminished, since α2,6 Sia linkage would be favoured. After differentiation, untreated cells were compared with ST6Gal-1 treated cells, either adding or not LPS.

BMDCs' maturation markers CD86 and MHC-II were evaluated by Flow Cytometry, to verify if this treatment influenced their expression on BMDCs. The percentage of populations expressing both maturation markers and the MFIs for each condition are shown in Figure III.8.
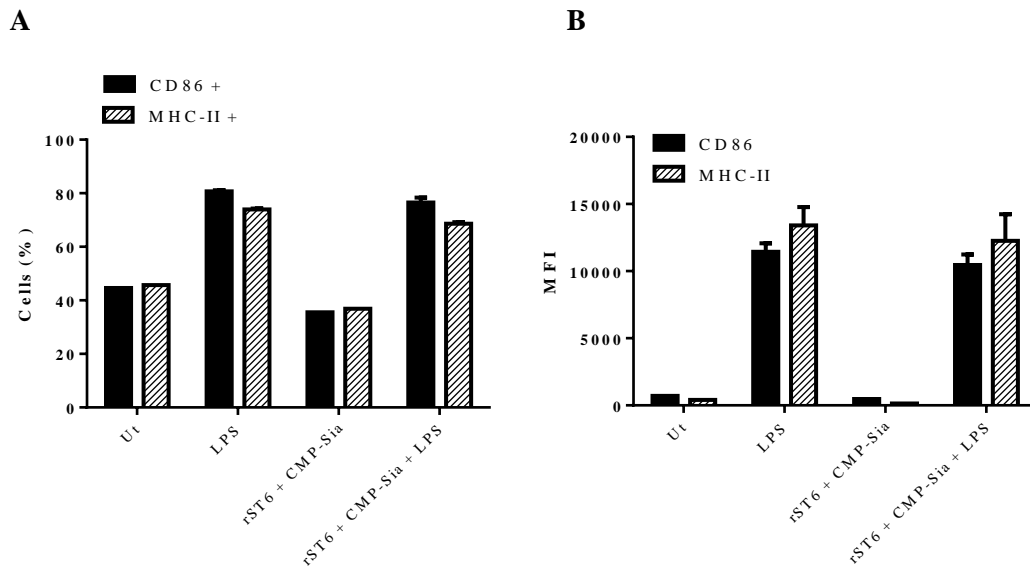
Figure III.8- **A) Percentage of cells expressing CD86 (black solid bars) and MHC-II (black dashed bars), within CD11c positive cells, obtained by Flow Cytometry. B) MFI of CD86 (black solid bars) and MHC-II (black dashed bars), obtained within CD11c positive cells, by Flow Cytometry.** The respective conditions are mentioned in x axis. In the case of LPS and rST6 + CMP-Sia + LPS conditions, the experiment was performed in duplicates and is presented the mean + SEM. It were not found differences comparing LPS and rST6 + CMP-Sia + LPS conditions, about maturation markers' percentage (A) and the correspondent MFIs (B).

It is evident an increased percentage of cells expressing both CD86 and MHC-II to every condition where LPS was added, comparing with untrated and ST6Gal-1 treated cells, as presented in Figure III.8 A. However, the positive cellular populations for CD86 and MHC-II are similar comparing LPS with ST6Gal-1 plus LPS treatments, suggesting that this treatment also did not influence DCs' maturation profile upon LPS stimulation. Furthermore, the correspondent MFIs are in agreement with this observation, as shown in Figure III.8 B.

Although CD86 and MHC-II expression was not affected by ST6Gal-1 added during cellular differentiation, the amount of TNF-α and IL-6 released by BMDCs in each of the previous conditions was measured and compared by ELISA, as shown in Figure III.9.

.
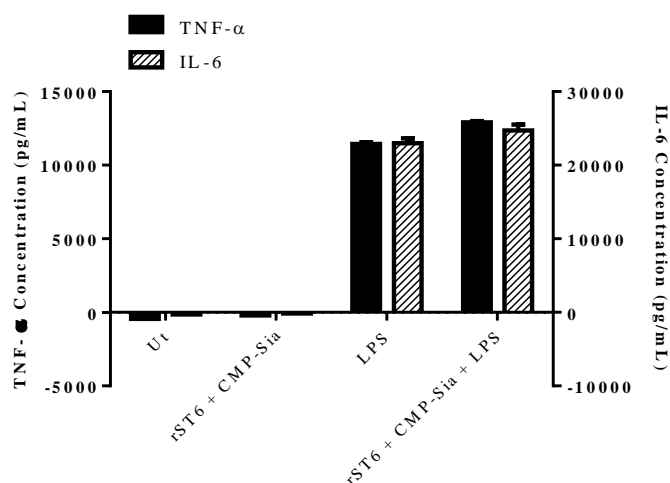
Figure III.9- **Concentrations of TNF-α (black solid bars) and IL-6 (black dashed bars) released by BMDCs, determined by ELISA.** The respective conditions are mentioned at x axis**,** where LPS and rST6 + CMP-Sia + LPS conditions were performed in duplicates. In this case, it is presented the mean of the duplicates + SEM. No significant differences were found comparing the conditions where LPS was added with each other.

As one can see in Figure III.9, both conditions where LPS was added led to increased amounts of TNF-α and IL-6 released, comparing with untreated or ST6Gal-1 treated BMDCs. However, LPS and ST6Gal-1 plus LPS present similar profiles, suggesting that ST6Gal-1 treatment also did not modify the release of cytokines from BMDCs upon LPS stimulation.

In order to verify if this apparent lack of effect was due to a defeat in the effectiveness of ST6Gal-1 treatment, SNA binding to BMDCs was evaluated 6 hours after adding LPS, by Flow Cytometry, as shown in Figure III.10.
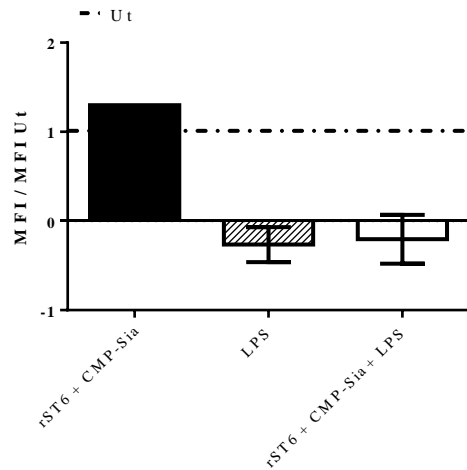
Figure III.10 – **Fold increase of the MFI of SNA comparatively to Ut cells, obtained within CD11c positive cells, by Flow Cytometry.** The respective conditions are mentioned in the x axis and for LPS and rST6 + CMP-Sia + LPS conditions the means of the duplicates ± SEM are shown.

Untreated cells appear to have a high content on α2,6 Sia *per se*, as mentioned before. ST6Gal-1 treatment during bone marrow cells' differentiation into DCs did not improve α2,6 extrinsic sialylation, as expected, since a slight increase in the MFI of SNA was found once more, for this condition (fold increase of 1.3), as shown in Figure III.10.

Nevertheless, LPS led to a remarkable decrease in the MFI of SNA, being lower than untreated cells, which was more pronounced in this experiment than in the previously shown. Despite that, it was expected that ST6Gal-1 added during differentiation could reinforce the content in α2,6 Sias in ST6Gal-1 plus LPS condition, which did not occur, since this condition was alike LPS, as presented in Figure III.10.

According with these data, ST6Gal-1 treatment during bone marrow cells' differentiation into DCs was not efficient improving α2,6 sialylation of BMDCs and the expression of important DCs' maturation markers and released cytokines has not decreased, as we hypothesize. These results are in agreement with the ones previously shown, since we still do not observe an evident increase in α2,6 extrinsic sialylation or any difference between LPS and ST6Gal-1 plus LPS treatments.

III.2.4 Influence of sequential addition of ST6Gal-1 followed by LPS in the role of extrinsic α2,6 sialylation modulating DCs' profile upon LPS stimulation

Regarding that the previous results did not improve α2,6 Sias content of BMDCs, we propose that a pre increment in α2,6 Sias content needs to be reached in BMDCs' surface before addition of LPS, in order to DC modulation occur. Indeed, LPS engagement in TLR-4 of DCs induces a strong immune response (Dearman *et al.*, 2009), which is probably not easily dampened. Considering this, over the course of the previous experiments, it is likely that adding simultaneously ST6Gal-1 plus LPS induced DCs' activation by LPS in the first place, rather than extrinsic α2,6 sialylation. In this case, rST6 action might be happening too late to restore the features of unstimulated BMDCs.

Physiologically, DCs would probably contact an antigen and the soluble form of ST6Gal-1 at the same time, during inflammation. This preview led to perform ST6Gal-1 plus LPS condition in the previous assays with all the components added at the same time, in order to approach the conditions *in vitro* to a more physiologic environment.

Despite we attempt to unravel the role of soluble ST6Gal-1 form as close as possible to a physiological condition, we designed here an experiment where all the components were added in separate, to evaluate each effect by itself. Nevertheless, there is a possible scenario in physiological conditions where this is likely to happen, namely in patients with cancer. Soluble ST6Gal-1 is increased in the serum of patients with several cancer types (Swindall *et al.*, 2013), which might enable a constant level of ST6Gal-1 in their bloodstream. This increased level of ST6Gal-1, together with the appropriate sugar donors in the bloodstream, might extrinsically sialylate DCs before any stimulus occurs. Therefore, this possible physiologic condition can be replicated *in vitro* by adding ST6Gal-1 to BMDCs followed by LPS.

. In addition, we decided to assess the inflammatory response after 24 hours, to understand if any effect of ST6Gal-1 treatment would occur later.

Therefore, the protocol mentioned at Chapter II, section II.2 was followed, where ST6Gal-1 treatment was performed during 4 hours, before addition of LPS.

The amount of TNF-α and IL-6 evaluated 6 and 24 hours after adding LPS is presented in Figure III.11.
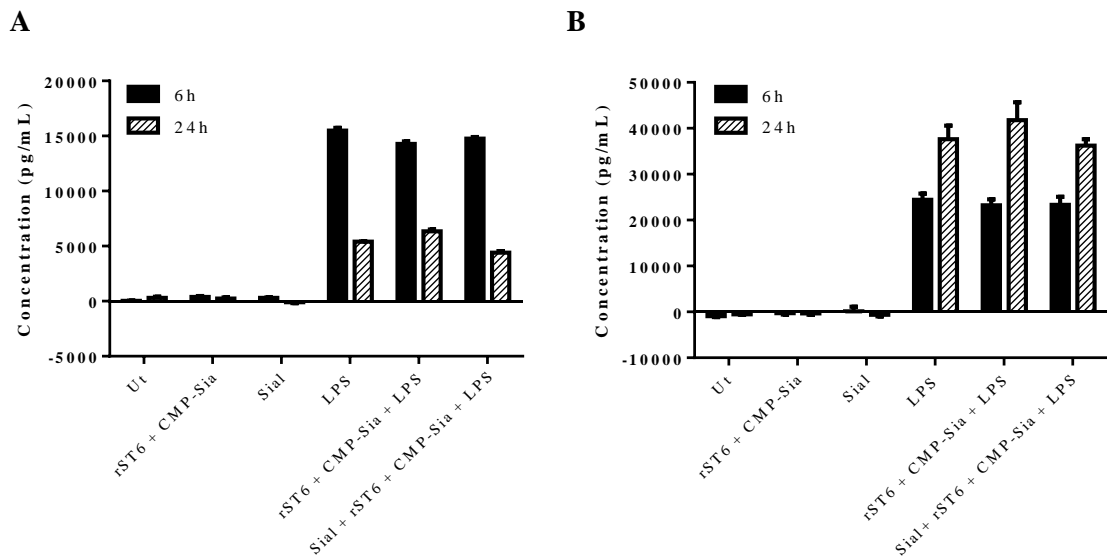
Figure III.11- **Concentrations of TNF-α (A) and IL-6 (B) released by BMDCs after 6 (black solids bars) and 24 hours (black dashed bars) of LPS stimulation, determined by ELISA**. The respective conditions are mentioned at x axis. 1 mouse was used to perform the experiment, despite the samples were run in duplicate in ELISA. It is presented the mean + SEM of the duplicates for each condition.

As shown in Figure III.11, the amount of both cytokines has increased whenever LPS was added, comparing with untreated, but still no differences were seen comparing the conditions where LPS was added with each other.

Interestingly, BMDCs released less amounts of TNF-α within 24 hours of LPS stimulation comparatively to 6 hours, where a fold decrease bigger than 2 has occurred. In opposite, the amount of IL-6 tends to increase after 24 hours of stimulation. This observed decrease for TNF-α at 24 hours corresponds to the biologic roles for this cytokine, since it stimulates the production of IL-6 (Abbas, Lichtman and Pillai, 2012). This might possibly have increased IL-6 levels at 24 hours, as observed in Figure III.11 B, in opposite to TNF-α concentration (Figure III.11 A).

Regarding sialidase, it was not observed any increase in the amount of both cytokines neither at 6 nor at 24 hours. Lastly, Sial + rST6 + CMP-Sia + LPS presents a very similar profile comparing with LPS about the released cytokines, suggesting that has not occurred modulation of BMDCs' profile.

Once more, the effectiveness of ST6Gal-1 and sialidase treatments under these conditions was evaluated, in this case after 24 hours, as presented in Figure III.12.
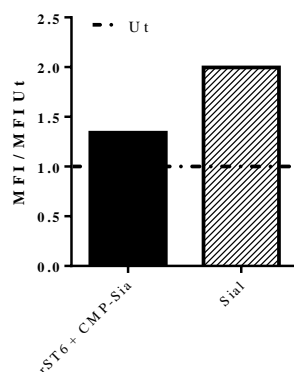
Figure III.12- **Fold increase calculated for the MFIs of SNA about rST6 + CMP-Sia treatment performed during 4 hours and Sial treatment performed during 1 hour, comparatively to Ut and obtained by Flow Cytometry after 24 hours.** 1 mouse was used to perform the experiment.

Alike the previous experiments, the fold increase in the MFI of SNA upon ST6Gal-1 treatment was 1.3 comparing with untreated, as shown in Figure III.12, suggesting a small increase in the content of $\alpha 2,6$ Sias. In addition, sialidase increased the binding of BMDCs to SNA (fold increase of 2.0), where the most likely explanation is a bigger exposition of $\alpha 2,6$ sialylated structures, due to the cleavage of $\alpha 2,3$ Sias in a higher rate than $\alpha 2,6$ Sias, as mentioned before. Moreover, it is possible that other sialylation mechanisms were induced in the cells within 24 hours of treatment.

Lastly, the maturation markers CD86, MHC-II and CD80 were assessed after 24 hours, by Flow Cytometry, where the fold increases comparatively to untreated are presented in Figure III.13.

A

B

C
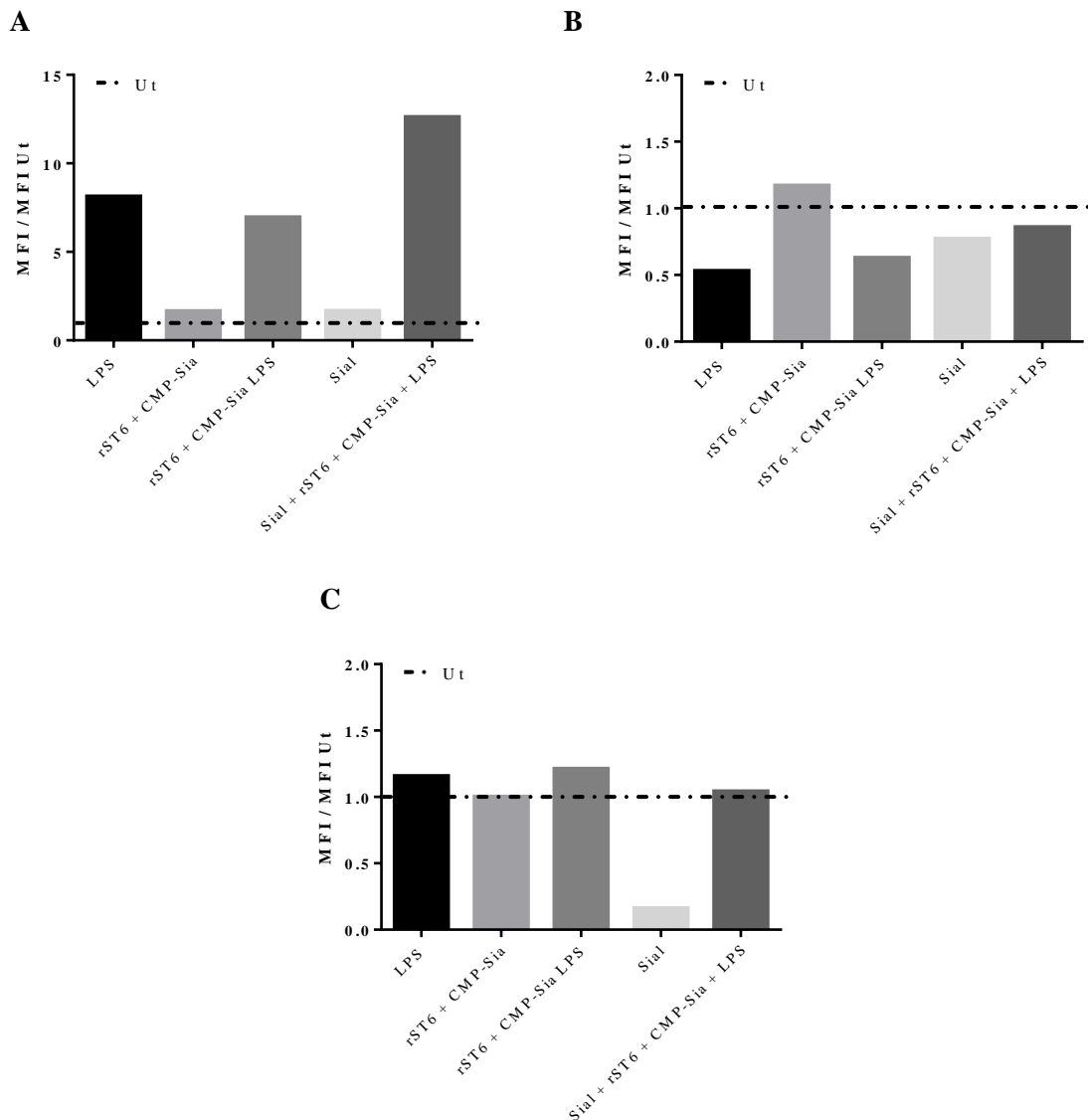
Figure III.13- **Fold increase of the MFIs for each condition comparatively to Ut, regarding the maturation markers CD86 (A), MHC-II (B) and CD80 (C), obtained within CD11c positive cells, by Flow Cytometry.** 1 mouse was used to perform the experiment.

As observed in Figure III.13 A, LPS, rST6 + CMP-Sia + LPS and Sial + rST6 + CMP-Sia + LPS led to fold increases comparatively to untreated cells superior to 1 (8.1, 6.7 and 12.6, respectively).

Unlike the previous experiments performed after 6 hours, MHC-II expression after 24 hours has decreased in the conditions where the cells have been activated. This decrease was less pronounced in Sial + rST6 + CMP-Sia + LPS, possibly because this condition appears to have induced the most mature phenotype in BMDCs, at least in terms

of CD86 expression. In sialidase treated cells, a decrease in MHC-II expression comparing with untreated is also noticeable but in a small level.

Lastly, CD80 expression was alike untreated in every condition, except Sial, where it presents a very pronounced decrease (fold increase 0.2). However, the percentages of CD80 positive cells in both untreated and sialidase conditions were similar (8.68% and 7.85%, respectively), suggesting that this decrease in the MFI is probably related to the degree of CD80 expression within CD80 positive cells.

All together, these data suggest that the mechanism proposed in this section has not occurred. We were also able to verify an opposite behaviour about TNF-α and IL-6 comparing 6 and 24 hours after stimulation with LPS, in agreement with the biological functions described for these cytokines. Regarding sialidase treatment, it appears to do not activate DCs after 24 hours of LPS stimulation.

III.2.5 Influence of a concentrated cell volume at ST6Gal-1 treatment in the role of extrinsic α2,6 sialylation modulating DCs' profile upon LPS stimulation

Considering all the previous results, it was not obtained a substantial increase in the content of α2,6 Sias in BMDCs' surface derived from ST6Gal-1 treatment. Moreover, sialidase added in the first place appears to expose more α2,6 Sias, which makes it difficult to say if Sial + rST6 + CMP-Sia + LPS induced a real increase in α2,6 Sias content or not. Regarding this, it is important to perform different approaches about ST6Gal-1 treatment in BMDCs, to understand if it influences their content in α2,6 Sias.

In this section, the results about BMDCs' activation assays were obtained applying the protocol described in Chapter II, section II.2.2, where ST6Gal-1 and sialidase treatments were performed before addition of LPS, like the previous one, but the cells were concentrated and shaken every 20 minutes. Eventually, rST6 needs to acquire the proper conformation to catalyse the reaction, contacting with the acceptor and the substrate donor, where it needs a correct position of its active centre (Kuhn *et al.*, 2013). Therefore, this protocol aimed to improve the contact of rST6 with the cells, possibly improving the extrinsic α2,6 sialylation, and also of improving sialidase action.

Despite this condition resembles a less physiologic environment, it was important to attribute the absence of DCs' modulation upon ST6Gal-1 treatment was due to a lack of enough extrinsic α2,6 sialylation, or to a different mechanism than the hypothesis we

have formulated. In Figure III.14 is shown the amount of TNF-α and IL-6 released by BMDCs after 6, 24 and 48 hours (only to IL-6) of LPS stimulation.
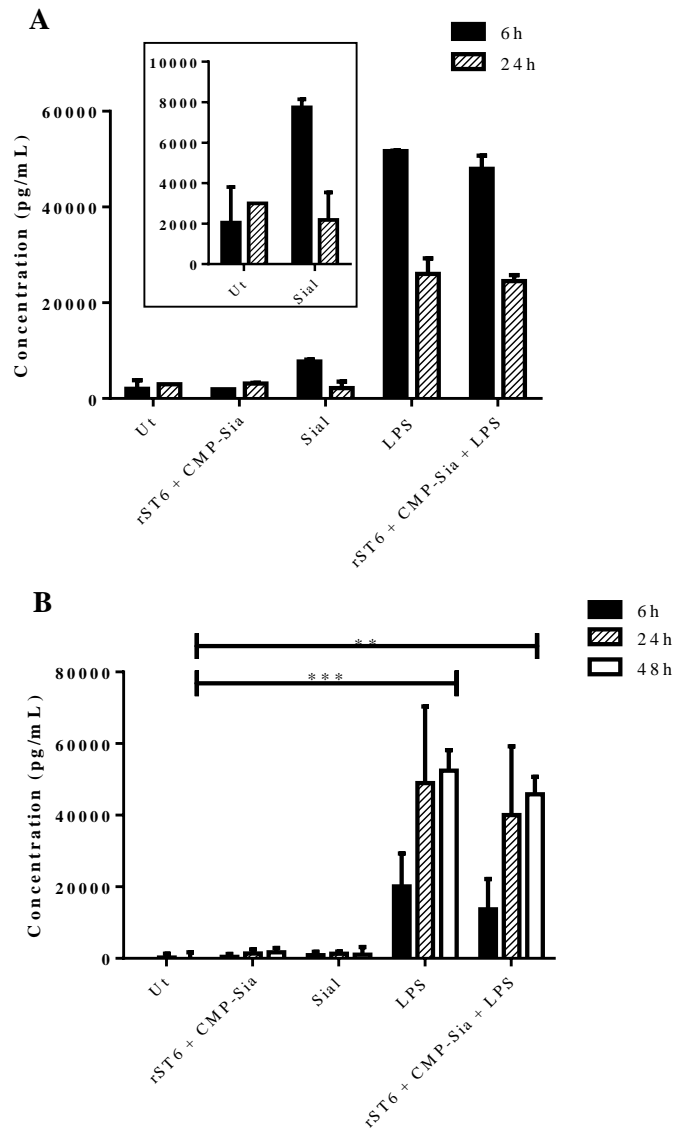
Figure III.14- **Concentrations of TNF-α (A) and IL-6 (B) released by BMDCs within 6 (black solids bars), 24 (black dashed bars) and 48 hours (white solid bars, only performed for IL-6) after LPS stimulation, determined by ELISA**. The respective conditions are mentioned at x axis, where is shown the mean + SEM for each one. 1 mouse was used to perform the experiment about TNF-α and 3 mice were used to evaluate IL-6, where all the samples were run in duplicates in ELISA. Unpaired t-tests were performed for IL-6, where only LPS (***) and rST6+ CMP-Sia + LPS (**) at 48 hours were significant different from Ut. The obtained p-values were respectively: 0.0010 and 0.0018.

As seen in Figure III.14, the release of both cytokines has increased in every condition where LPS was added, comparing with untreated cells, but no differences were noticed comparing them with each other.

Sialidase led to a 2-fold increase in the amount of released TNF-α after 6 hours, which was not significant in the previous experiments with N=3 (section III.2.2, Figure III.2 A). Nevertheless, this protocol is different than the one previously applied, regarding sialidase treatment (detailed in section II.2.2), suggesting that the concentrated cell volume probably improved sialidase action.

Once more, in the conditions where LPS was added, a 2-fold decrease in the release of TNF-α after 24 hours has occurred, in comparison with 6 hours (Figure III.14 A), whereas for IL-6 the opposite situation has occurred, as predicted (Figure III.14 B). Moreover, after 48 hours, the concentrations of released IL-6 were similar to the ones at 24 hours, suggesting a stable production and release of this cytokine.

Regarding IL-6, unpaired t-tests about 3 independent experiments were performed, where only LPS and ST6Gal-1 plus LPS at 48 hours were significantly different from untreated (p-values of 0.0010 and 0.0018, respectively, by unpaired t-tests).

BMDCs' maturation markers were also evaluated after 24 and 48 hours of LPS stimulation, whose fold increases comparing their MFIs with the MFI of untreated are presented in Figure III.15.

A

B

C
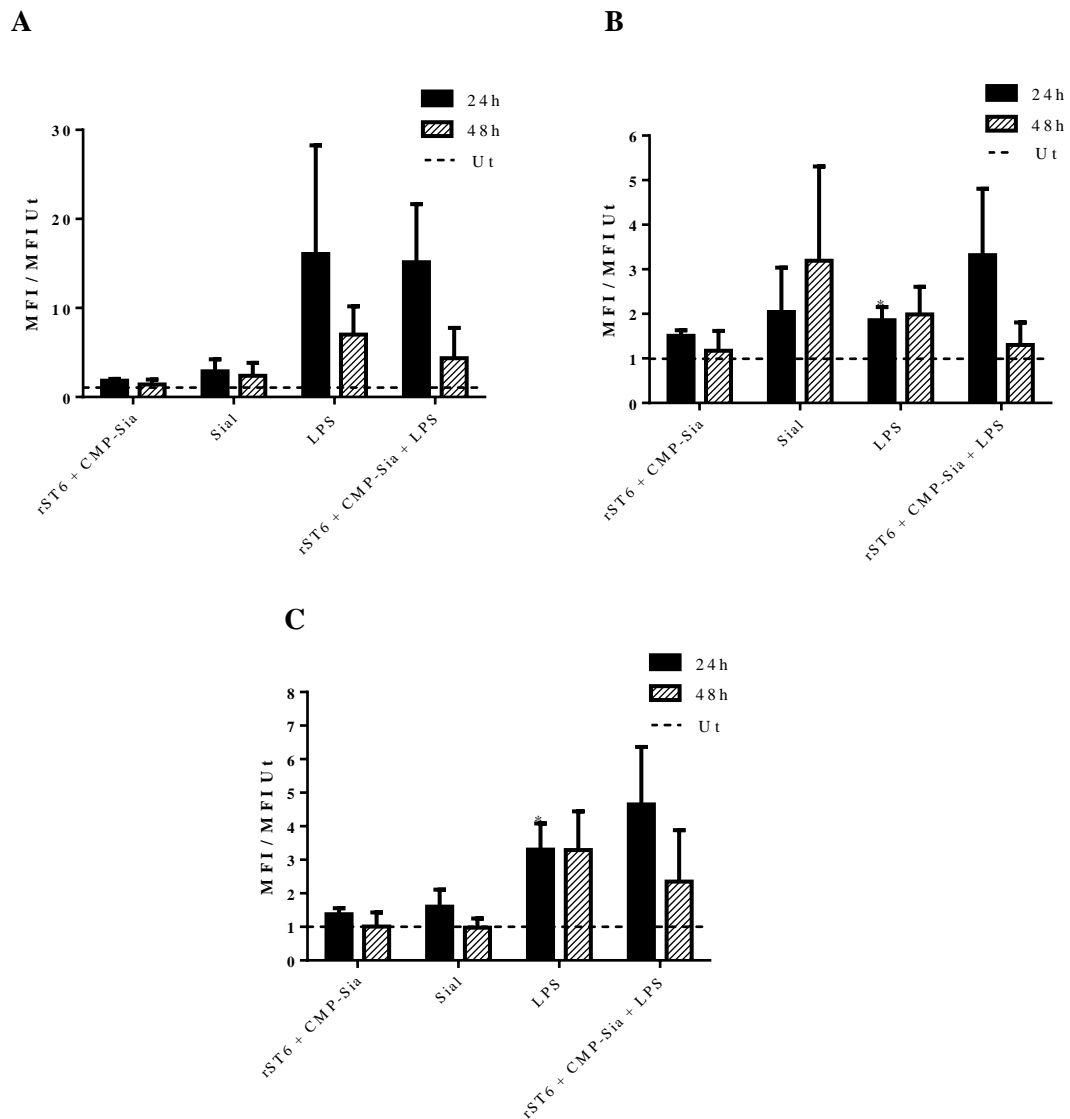
Figure III.15- **Fold increase of the MFIs for each condition comparatively to Ut, regarding the maturation markers CD86 (A), MHC-II (B) and CD80 (C), obtained within CD11c positive cells, by Flow Cytometry.** 2 or 3 independent experiments were performed. A) None of the conditions was significant different from each other. B) LPS condition at 24 hours was significantly different from Ut (*), whose p-value was 0.0437. C) LPS at 24 hours presented a significant difference (*), p-value of 0.0411.

Regarding CD86 (Figure III.15 A), none of the conditions was significantly different from each other, although LPS and ST6Gal-1 plus LPS presented a mean fold increase compared to untreated of $16.0 \pm$ SEM 12.2 and $15.0 \pm$ SEM=6.5, respectively, 24 hours after LPS stimulation. After 48 hours, both conditions led to a decrease in CD86 expression despite the fold increase was still superior to 1.

About MHC-II, only LPS was significantly different from untreated after 24 hours with a mean fold increase of $1.9 \pm$ SEM 0.3 (p-value= 0.0437, obtained through unpaired

t-tests), as shown in Figure III.15 B. Nevertheless, ST6Gal-1 plus LPS induced a mean fold increase of $3.3 \pm$ SEM 1.5 after 24 hours, which appears to have diminished after 48 hours ($1.3 \pm$ SEM 0.5). Sialidase induced an increase in the expression of MHC-II compared with untreated, either after 24 and 48 hours, but it was not observed in every mice.

Lastly, CD80 expression has significantly increased for LPS condition at 24 hours, (p-value= 0.0411, obtained through unpaired t-tests, Figure III.15 C). After 48 hours, the level of CD80 expression was similar to 24 hours. ST6Gal-1 plus LPS led to an increased expression of CD80 related to untreated (mean of $4.7 \pm$ SEM 1.7), which has decreased after 48 hours (mean of $2.4 \pm$ SEM 1.5), despite no significant differences were found comparing this condition at 24 and 48 hours. Sialidase does not appear to significantly influence the expression of CD80 in spite of a 1.6 fold increase $\pm$ SEM 0.5 has occurred at 24 hours, as shown in Figure III.15 C.

SNA binding was assessed to evaluate the treatments with ST6Gal-1 and sialidase. In the case of sialidase, MAL-I binding assays were performed as well. MAL-I preferentially binds glycoproteins with Galβ-1,4GlcNAc residues, according with the manufacturer (VECTOR LABS - Fluorescein labeled *Maackia Amurensis Lectin I (MAL I)*). These residues are exposed upon sialidase treatment, inducing MAL-I binding to these structures. Both results are presented in Figure III.16.
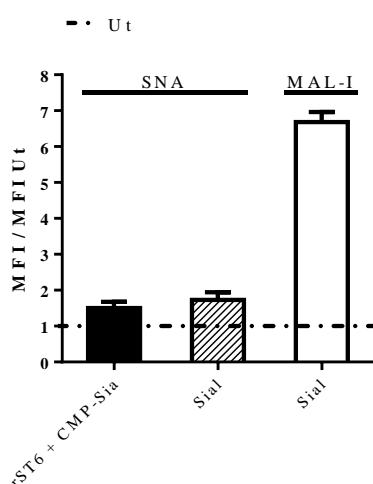
Figure III.16- **Fold increase of the MFIs for rST6 + CMP-Sia and Sial conditions comparatively to Ut, obtained for SNA and MAL-I after 1h of the respective treatments, by Flow Cytometry.** 2 animals were used to evaluate rST6 + CMP-Sia treatment under the mentioned conditions (black solid bar). Sial condition was evaluated by SNA binding assays with 3 independent experiments (black dashed bar). To assess MAL-I binding, 2 animals were used (white solid bar).

As presented in Figure III.16, this type of ST6Gal-1 treatment has induced once more a slight increase in the binding of SNA to BMDCs comparing with Ut (fold increase of 1.5 ± SEM 0.2). Besides, sialidase treatment continued to increase the binding to α2,6 Sias, unlike the expected (1.7 ± SEM 0.2). This is likely to happen due to an increased exposition of α2,6 Sias, upon the cleavage of α2,3 Sias, as mentioned before.

Sialidase led to an increased MFI of MAL-I in comparison with Ut (6.7 ± SEM 0.3), as observed in Figure III.16, suggesting that α2,3 Sias were effectively removed with this treatment. This enforces the idea that an effective cleaving of α2,3 could be exposing more α2,6 Sias in BMDCs' surface, leading to the observed increase in SNA binding upon this treatment.

In summary, this attempt to improve α2,6 extrinsic sialylation was not achieved, which can be the main reason for the similarity found between LPS and ST6Gal-1 plus LPS treated BMDCs, over this work.

III.2.6 ST6Gal-1 KO BMDCs' profile upon extrinsic α2,6 sialylation upon LPS stimulation

Taking into consideration that ST6Gal-1 treatments do not modulate BMDCs' profile, as shown in the previous sections of this Chapter, it was a goal to understand if the total absence of α2,6 Sias in BMDCs' surface influences their activation status. Then, the same experiment using ST6Gal-1 KO BMDCs was performed in order to assess their activation profile.

This approach can confirm if KO BMDCs display a more mature phenotype *per se* comparing with WT BMDCs or if their response to LPS is stronger, since they do not express α2,6 Sia in their surface. It was also a goal to know whether extrinsic α2,6 sialylation can occur in these cells and what are the consequences in the cellular activation upon LPS stimulation.

First of all, the concentrations of TNF-α and IL-6 released by WT and ST6Gal-1 KO BMDCs were determined by ELISA, as shown in Figure III.17.
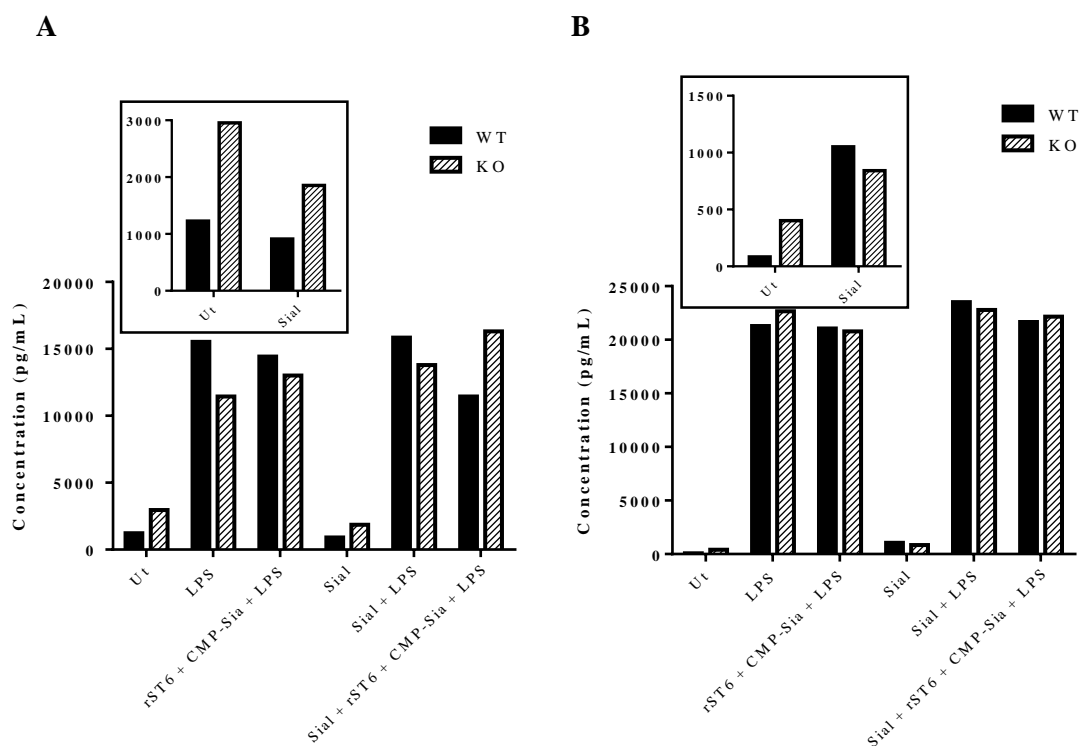
Figure III.17- **Concentrations of TNF-α (A) and IL-6 (B), comparing WT with KO BMDCs, in the respective conditions, determined by ELISA**. 1 WT and 1 KO mouse were used to perform the experiment. The respective conditions are mentioned at x axis. WT (black solid bars) and KO (black dashed bars) were compared, where no significant differences were found. Ut and Sial conditions are emphasized in both charts in a proper scale.

No significant differences were found comparing TNF-α and IL-6 released from WT and KO BMDCs, as presented in Figure III.17. Both cytokines released by KO BMDCs increased in every condition where LPS was added, comparing with Ut, identical to WT BMDCs.

However, untreated KO BMDCs appear to release more cytokines on its basal state than WT BMDCs, which is more evident for TNF-α, where a 2-fold increase has occurred (Figure III.17 A). Sialidase also led to a 2- fold increase in TNF-α release by KO BMDCs, in comparison with WT BMDCs. Even though, all the other conditions where LPS was added presented similar levels of released cytokines comparing WT and KO BMDCs, suggesting a similar response upon LPS stimulation and α2,6 extrinsic sialylation.

With the aim of verifying if the absence of α2,6 Sia on DCs' surface could influence BMDCs' maturation profile, CD86 and MHC-II maturation markers from KO BMDCs were assessed by Flow Cytometry. It was determined the percentage of cells expressing high levels of CD86 and MHC-II to every condition, as presented in Figure III.18 A, but also the MFI of the respective conditions, shown in Figure III.18 B.
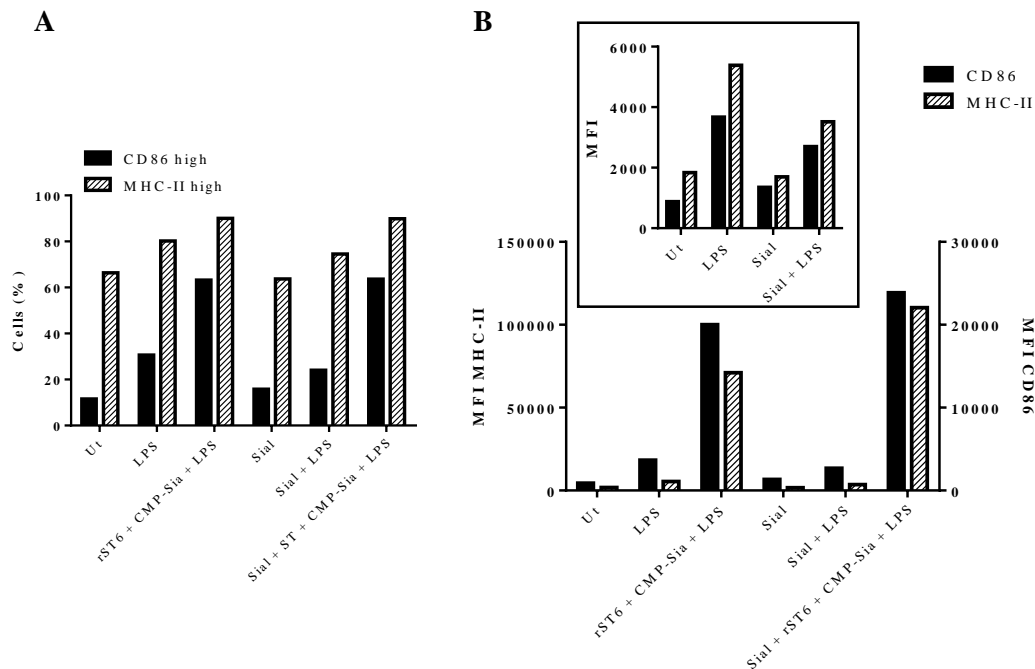
Figure III.18- **A) Percentage of KO BMDCs expressing high levels of CD86 (black solid bars) and MHC-II (black dashed bars). B) MFI of CD86 (black solid bars) and MHC-II (black dashed bars), both within CD11c positive cells gate, obtained by Flow Cytometry.** 1 mouse was used to perform the experiment. In B, the conditions Ut, LPS, Sial and Sial + LPS are emphasized in a proper scale.

As represented in Figure III.18 A, the percentage of KO BMDCs expressing high levels of both CD86 and MHC-II has increased in every condition where LPS was added, comparing with untreated. This observation is similar to WT BMDCs' profile upon LPS stimulation, previously shown over this Chapter. However, KO BMDCs on its basal state appear to express more MHC-II (64.40%), comparing with previous results of MHC-II positive cells for WT untreated BMDCs (32.62% ± SEM 12.58, N=3) (Figure III.4).

This increased expression of both CD86 and MHC-II was more pronounced to the conditions rST6 + CMP-Sia + LPS and Sial + rST6 + CMP-Sia + LPS, where about 80% of BMDCs express high MHC-II and around 65% express high levels of CD86, for both. The same conclusion was achieved through MFI analysis (Figure III.18 B), where these conditions revealed the biggest increase for both CD86 and MHC-II MFIs.

Lastly, it was assessed the MFI of SNA 6 hours after these treatments, in order to evaluate their efficiency, presented in Figure III.19.

A

B
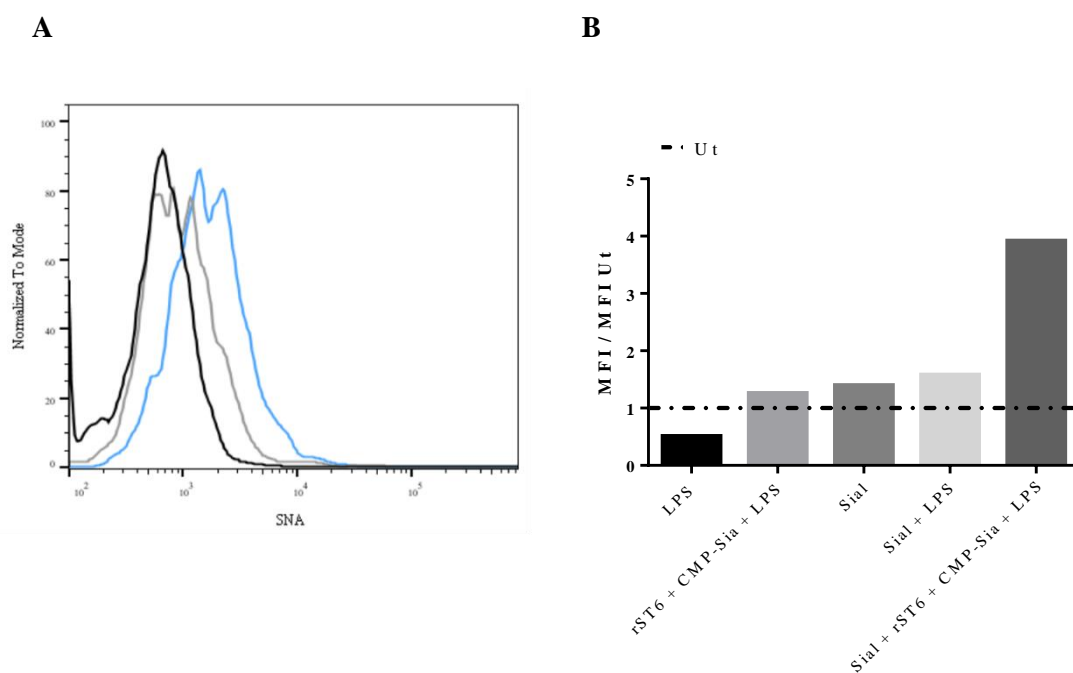
Figure III.19- **A) Histograms of SNA fluorescence in KO BMDCs for unstained (black line), Ut (grey line) and Sial + rST6 + CMP-Sia + LPS (blue line), obtained within CD11c positive cells, by Flow Cytometry. B) Fold increase in the MFI of SNA comparatively to Ut, from KO BMDCs in the respective conditions, also obtained within CD11c positive cells, by Flow Cytometry.** 1 KO mouse was used to perform the experiment.

As expected, KO BMDCs do not bind SNA, since they do not express α2,6 Sias linked to Galβ1,4GlcNAc in their surface, as seen in Figure III.19 A, where untreated (grey line) almost overlaps the unstained sample (black line). Even so, there was not a

complete overlap between the two histograms, which has probably occurred due to unspecific binding of a small amount of α2,6 Sias in O-linked chains (Hennet *et al.*, 1998) or produced by ST6GalNAc or ST6Gal-1I (Rao *et al.*, 2009). As noticeable in Figure III.19 B, ST6Gal-1 plus LPS did not improve extrinsic α2,6 sialylation, since the fold increase in the MFI of SNA comparing with untreated was close to 1.

All the other conditions revealed similar profiles with Ut, except Sial + rST6 + CMP-Sia + LPS. In Figure III.19 A, Sial + rST6 + CMP-Sia + LPS (blue line) has two peaks indicating two different populations of SNA binding. In this case, α2,6 extrinsic sialylation has probably occurred, supported by the fact that this condition presented a 4-fold increase in the MFI of SNA in comparison with Ut, as shown in Figure III.19 B. This is the most likely condition to induce α2,6 sialylation of KO BMDCs, since sialidase might have removed α2,3 Sias creating available ligands to α2,6 Sias. Despite this increase in SNA binding appears to be relevant, it is to notice that WT DCs always express higher amounts of α2,6 Sias *per se* than KO BMDCs with this treatment (see section III.2.2 of this Chapter, Figure III.6 B).

Nevertheless, Sial + rST6 + CMP-Sia + LPS from KO BMDCs was analysed for SNA positive population and SNA negative population, in terms of their maturation markers. In Figure III.20 is represented a counter plot for CD86 expression vs SSC, within the gate SNA negative population (A) and the gate SNA positive population (B).

A **B**
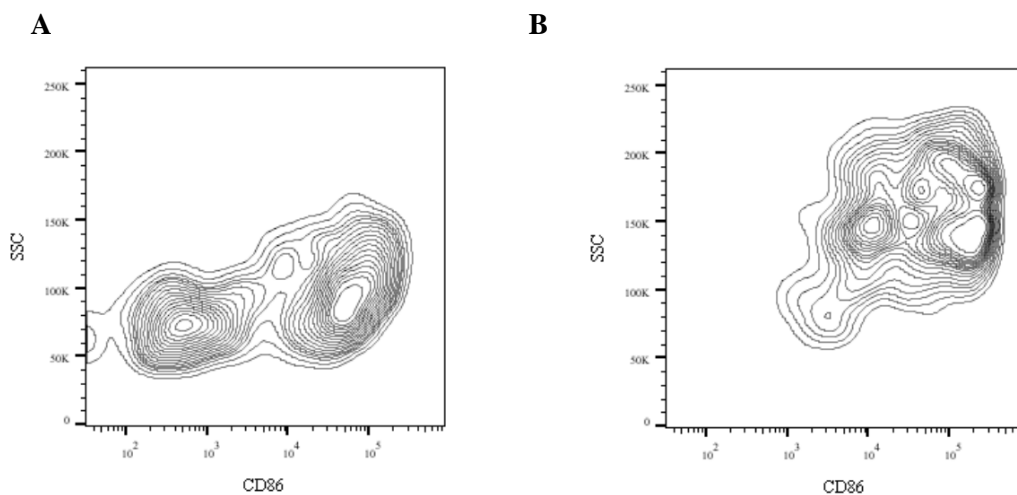
Figure III.20- **Counter plots regarding CD86 vs SSC for Sial + rST6 + CMP-Sia + LPS conditions from KO BMDCs, obtained within CD11c positive cells gate, by Flow Cytometry.** In A is presented the SNA negative population, whereas in B is presented the SNA positive population.

Regarding MHC-II, no significant differences between SNA positive and negative populations were noticed, within Sial + rST6 + CMP-Sia + LPS from KO BMDCs (not shown). As observed in Figure III.20 B, for SNA positive population, has occurred a shift from lower levels of CD86 expression towards high levels of CD86 expression, in opposite to what happened in SNA negative population (Figure III.20 A). In addition, the SNA positive cells appear to have higher levels of SSC, also suggesting a more mature phenotype.

This observation is the opposite effect that we predicted about ST6Gal-1 role in the modulation of DCs' functions. Furthermore, it is supported by the data presented in this section, where every conditions where ST6Gal-1 plus LPS were added together presented the most mature phenotype (see Figure III.18 A and B).

In summary, comparing WT with KO BMDCs upon LPS stimulation, no significant differences were noticed, despite KO BMDCs tend to release more cytokines *per se* than WT (as shown in Figure III.17), suggesting a more activated status for these cells. Alike WT BMDCs, KO BMDCs have increase the expression of their maturation markers upon LPS stimulation.

III.3. Characterization of DCs from the airways of ST6Gal-1 KO and WT mice

The role of DCs in the lungs has been studied by different groups, in order to understand the mechanisms underlying in certain pulmonary and allergic diseases (GeurtsvanKessel and Lambrecht, 2008). To study these disorders, a common technique to harvest cells from the airways is the bronchoalveolar lavage (BAL), as described in Materials and Methods section II.5.2.

In the present study we intended to identify DCs in the airways from mice upon LPS stimulation, induced by intra-tracheal injection. Therefore, we intended to distinguish DCs' maturation markers (MHC-II, CD86 and CD80) comparing their expression between ST6Gal-1 KO and WT mice.

The main goal was to test the hypothesis that ST6Gal-1 KO mice own DCs with a more mature phenotype in their airways upon inflammation than WT mice. As far as we know, the maturation of DCs in the airways from ST6Gal-1 KO mice upon LPS stimulation was not assessed before. Nevertheless, our group has previously shown that upon ovalbumin (OVA)-induced allergic pulmonary inflammation, the number of DCs and macrophages tends to increase in the lungs of Siat1ΔP1 KO mice (mice lacking P1, the liver promotor for ST6Gal-1 gene), compared to WT mice, although not statistical significant differences were noticed (Nasirikenari *et al.*, 2010). Nevertheless, these observations suggest a role for ST6Gal-1 controlling the severity of inflammation, where DCs may also have different features in ST6Gal-1 KO mice.

To all the following results, a gate excluding debris and dead cells was performed. Through Flow Cytometry assays, FSC vs SSC parameters were used to distinguish the major populations obtained in BAL fluid (BALF), under steady state conditions (PBS) and inflammatory conditions (LPS), as shown in Figure III.21.

A   B
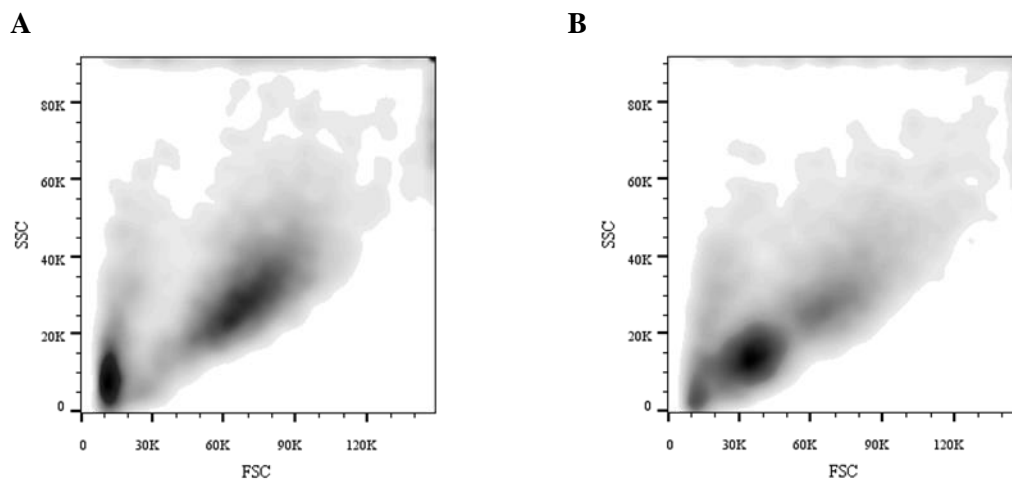
Figure III.21- **FSC vs SSC density plots representing a significant example of BALF from ST6Gal-1 KO mice where PBS (A) and LPS (B) intra-tracheal injection were performed, assessed by Flow Cytometry after 24 hours.** One WT mouse and one KO mouse were used to evaluate PBS, whereas 3 KO mice and 2 WT mice were used to assess FSC vs SSC features upon LPS stimulation. No significant differences were found comparing PBS treated mice with each other and LPS treated mice with each other.

As seen in Figure III.21 A, corresponding to PBS treated mice, there is a population found in the lowest FSC vs SSC levels which probably corresponds to lymphocytes. There is another population placed in upper levels of FSC vs SSC probably including alveolar macrophages and DCs found in the steady state condition, in the alveolar compartment. Placed in between these populations there are only few cells, which are probably neutrophils. In Figure III.21 B, corresponding to LPS stimulation, the major population corresponds to neutrophil settings, whereas smaller densities are found in upper FSC vs SSC levels and lower FSC vs SSC levels. This observation suggests that neutrophils are the major population in BALF upon LPS stimulation. To confirm the presence of neutrophils under inflammatory conditions, Ly6G was used in combination with CD11b, since they are both expressed in these cells (Abbas, Lichtman and Pillai, 2012). In Figure III.22 is shown a significant example regarding the expression of Ly6G and CD11b for 3 KO and 2 WT mice, assessed 24 hours after LPS stimulation.

A

B
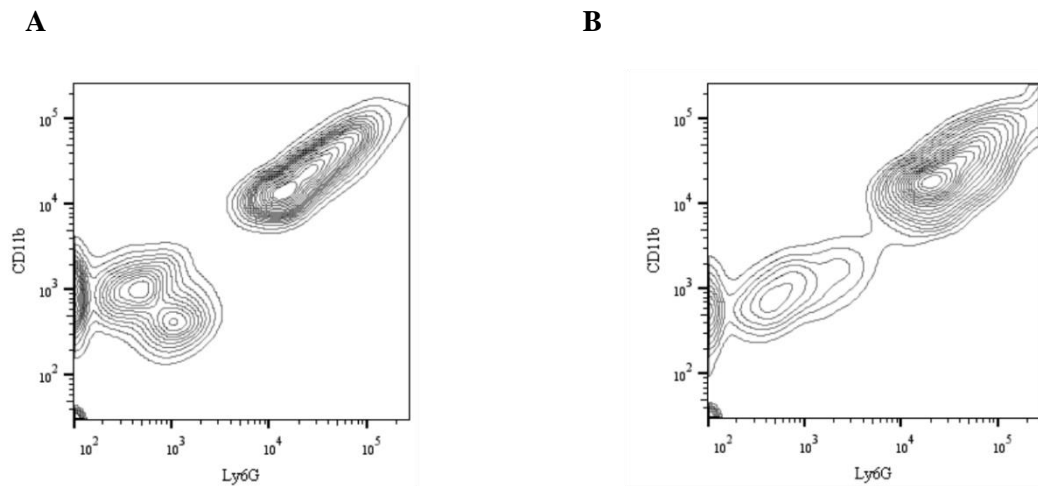
Figure III.22- **Counter plots of a significant example from 2 WT (A) and 3 KO mice (B), regarding Ly6G and CD11b markers, obtained by Flow Cytometry after 24 hours of LPS stimulation.** No significant differences were found comparing WT and KO mice with each other.

As noticeable in Figure III.22, a major population was found upon LPS stimulation for both WT and KO mice. This cellular population is placed in high levels of CD11b and is positive for Ly6G, suggesting that neutrophils are, indeed, the major population in the BALF under inflammatory state. This observation is supported by (Asti *et al.*, 2000) and is in agreement with FSC vs SSC features previously shown in Figure III.21 B.

The mean percentage of neutrophils found for WT was 73.95 % ± SEM 16.55, N=2 and for KO it was 83.83% ± SEM 4.12, N=3, which were not significantly different from each other performing unpaired t-tests. Nevertheless, a slight tendency for KO mice present a higher number of neutrophils was found, suggesting a more severe type of inflammation for KO mice. Indeed, our group has previously shown that ST6Gal-1 KO mice and Siat1ΔP1 mice own a more severe inflammation than WT upon ovalbumin (OVA)-induced allergic pulmonary inflammation due to a significant increased number of cells recovered from BALF, particularly eosinophils (Nasirikenari *et al.*, 2010).

As mentioned in Chapter I, conventional DCs (cDCs) resident in the lungs can be positive or negative for CD11b expression, but express high levels of CD11c. However, monocyte derived DCs (mo-DCs), which migrate to the lungs upon inflammatory conditions, express high levels of both CD11c and CD11b. Then, CD11c positive, CD11b positive cDCs resident in the lungs can be confounded with inflammatory mo-DCs if not used other markers to distinguish them. Another confounding aspect is that B220 expression is induced in conventional CD11b positive cDCs upon inflammation,

hindering the identification of pDCs and inflammatory cDCs, if not considered the fact that pDCs do not express CD11b. It is also to notice that alveolar macrophages have high levels of CD11c expression, similar to cDCs and mo-DCs. Since alveolar macrophages express lower levels of CD11b, they can be confounded with cDCs which are CD11c high, CD11b negative (GeurtsvanKessel and Lambrecht, 2008).

It is to notice that it was not a goal for this experiment to distinguish between cDCs, pDCs and mo-DCs that have migrated to the lungs due to inflammatory status, but to clarify if DCs from KO mice present a more mature phenotype than DCs from WT mice, upon LPS stimulation.

In order to identify DCs, CD11c expression was analysed within Ly6G negative population gated. Three levels of CD11c expression were found, as shown in Figure III.23.
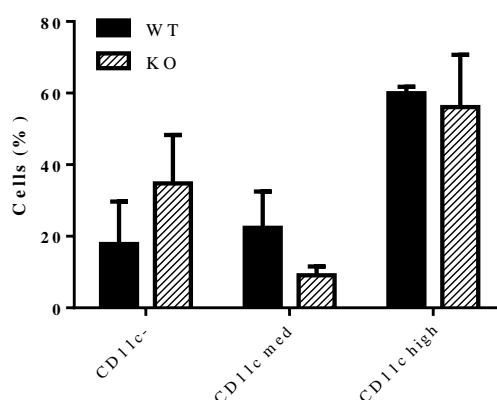
Figure III.23- **Percentage of cells within Ly6G negative population expressing different levels of CD11c, for WT (black solid bars) and KO mice (black dashed bars) after 24 hours of LPS stimulation, obtained by Flow Cytometry**. 3 KO mice and 2 WT mice were used to perform the experiment, where no significant differences between KO and WT mice were found performing unpaired t-tests.

As noticeable in Figure III.23, most of the Ly6G negative cells express high levels of CD11c, suggesting that within CD11c high population we will probably find cDCs, mo-DCs and alveolar macrophages. In addition, KO and WT mice presented similar percentages of cells expressing CD11c high. However, CD11c medium population tends to be lower in KO compared to WT, in opposite to CD11c negative population, despite no significant differences were noticed between them performing unpaired t-tests.

Regarding CD11c medium population, it is likely that pDCs could be found within this gate, together with a gate in B220 positive population and excluding CD11b positive cells. Nevertheless, Siglec-H was not used to distinguish pDCs from IFN-producing

killing DCs (GeurtsvanKessel and Lambrecht, 2008), suggesting that CD11c medium population could include both cell types. Even though, no significant differences about the percentages of CD11c medium, B220 positive, CD11b negative population were found, comparing WT with KO mice. Regarding Ly6G negative population within CD11c negative gate, it could include lymphocytes or eosinophils, which were not assigned in this experiment.

Focusing in Ly6G negative, CD11c high population, the expression of CD11b, CD80, CD86 and MHC-II expression was evaluated, as shown in Figure III.24.
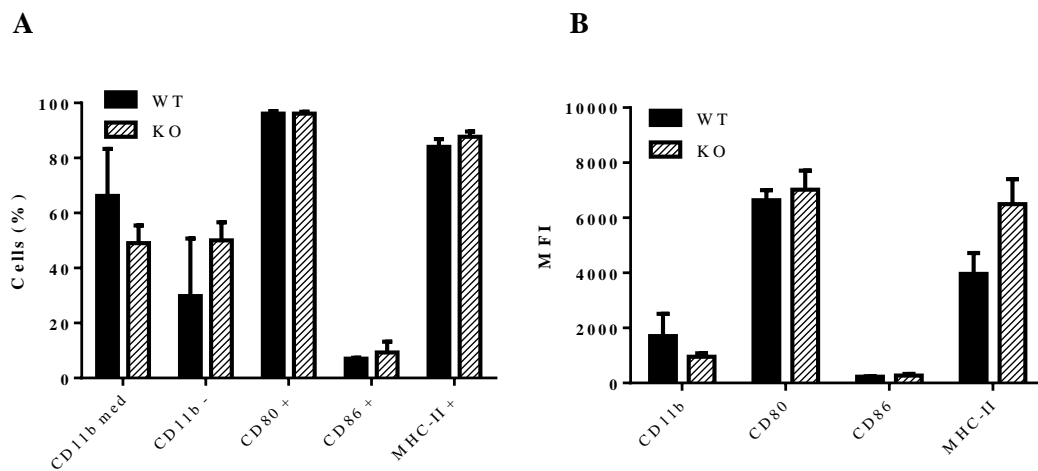
Figure III.24- **Evaluation of Ly6G negative, CD11c high population about the percentage of cells expressing CD11b, CD80, CD86 and MHC-II (A), with the respective MFIs (B), for WT (black solid bars) and KO (black dashed bars), after 24 hours of LPS stimulation, obtained by Flow Cytometry.** No significant differences were found comparing WT and KO mice in the respective conditions, performing unpaired t-tests.

As expected, CD11c high population is classified in two sub-populations, regarding CD11b levels, as shown in Figure III.24 A, corresponding to cDCs CD11b negative or positive or to mo-DCs CD11b positive (Kim and Lee, 2014), (GeurtsvanKessel and Lambrecht, 2008). It is to notice that the classification of CD11b medium is due to the fact that neutrophils presented superior levels of CD11b, as shown before in Figure III.22.

Almost all cells express MHC-II and CD80 for both WT and KO mice, unlike CD86, whose percentages of positive populations are 9.29% ± SEM 3.96, N=3 and 7.06% ± SEM 0.30, N=2, for KO and WT mice, respectively. This observation may be due to the expression of later maturation markers like CD80, instead of the early maturation marker CD86, since the BALF was performed after 24 hours (Abbas, Lichtman and Pillai, 2012). In contrast, DCs CD11c medium, B220 positive, CD11b negative were mostly CD86 positive populations (81.97% ± SEM 7.85, N=3 for KO mice and 87.85% ± SEM

5.85, N=2 for WT mice), whereas CD80 was not expressed for most of the cells (12.73% ± SEM 1.56, N=3 for KO mice and 19.89% ± SEM 11.21, N=2 for WT mice, regarding CD80 positive populations - not shown).This suggests that pDCs or IFN-killing DCs express CD86 rather than CD80, under these conditions.

Nevertheless, CD80 expression and CD86 expression was similar, comparing WT with KO mice. MHC-II appears to be expressed in every Ly6G negative, CD11c high cells (Figure III.24 A), but presents a superior MFI in KO mice compared to WT mice (Figure III.24 B).

In order to assign the increased expression of MHC-II observed for KO mice to mo-DCs / cDCs CD11b positive or to alveolar macrophages / cDCs CD11b negative, the CD11c high populations were distinguished with basis on CD11b levels of expression. In addition, it was also found a sub-population within the MHC-II positive cells, where higher expression of MHC-II was found, named MHC-II high population. Both results are presented in Figure III.25.
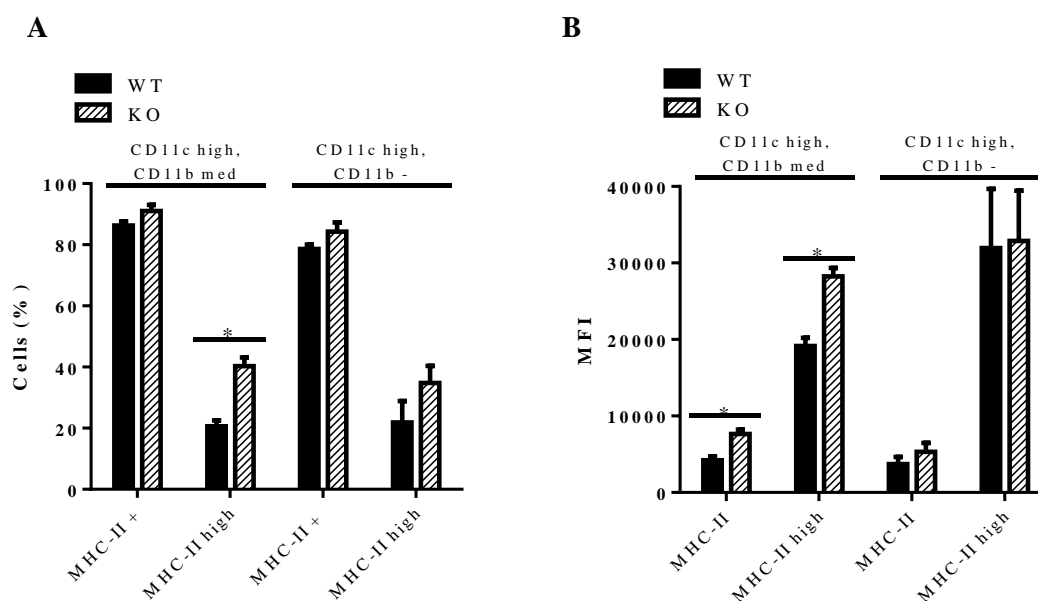
Figure III.25- **Percentage of MHC-II + and MHC-II high populations within CD11c high, CD11b med population and CD11c high, CD11b – population (A), for WT mice (black solid bars) and KO mice (black dashed bars), whose respective MFIs are presented in B and were both obtained by Flow Cytometry after 24 hours of LPS stimulation.** A) Within CD11c high, CD11b medium population, KO and WT MHC-II high populations were significantly different from each other, by unpaired t-tests (*), whose p-value was 0.0148. B) CD11c high, CD11b med population presents significantly increased MFIs for MHC-II and MHC-II high populations of KO, compared with WT (*), whose p-values obtained through unpaired t-tests were respectively 0.0247 and 0.0108.

As observed in Figure III.25, the biggest differences about MHC-II expression between KO and WT mice have occurred to CD11c high, CD11b medium population. In this case, there are more ST6Gal-1 KO cells expressing high levels of MHC-II than WT cells (Figure III.25 A), in agreement with the significant increases found in the MFIs for KO within this population (Figure III.25 B). In this case, we conclude that either mo-DCs or cDCs CD11b positive presented a significant increased expression of MHC-II, namely in MHC-II high population for KO mice in comparison with WT mice. This observation suggests that KO mice own more mature DCs in terms of MHC-II marker.

Whether CD11c high, CD11b negative population corresponds to cDCs CD11b negative or to alveolar macrophages is not distinguished here, since other markers or techniques were not used to distinguish them. Nevertheless, the cells from KO and WT mice within this population look alike in terms of the expression of maturation markers.

Chapter IV

Discussion of Results and Conclusions

IV.1. General discussion of the Results

In this project, we hypothesize that extracellular ST6Gal-1 is able to decrease DCs' maturation profile upon LPS stimulation, by extrinsically adding α2,6 Sias to their surface. Taking this into account, we will now discuss all the obtained results over this hypothesis.

In the first experiment (topic III.2) we saw that extrinsically added ST6Gal-1 upon LPS stimulation has not decreased the release of pro-inflammatory cytokines (Figure III.2 of section III.2.2), neither the expression of maturation markers (Figures III.4 and 5 of section III.2.2) from WT BMDCs, as first hypothesized. Moreover, the release of the anti-inflammatory cytokine IL-10 from WT BMDCs was not induced by ST6Gal-1 treatment, assessed 6 hours after LPS stimulation (Figures III.3, section III.2.2). In addition, the experiment designed to increase the amount of extrinsically added α2,6 Sias, where Sias are first removed with sialidase followed by addition of ST6Gal-1 with LPS, led to similar results, as demonstrated over the topic III.2.2.

In this context, we evaluated the **effectiveness of ST6Gal-1 treatment** by assessing SNA binding to BMDCs. In this, we have observed a slight increase in α2,6 Sias' content in ST6Gal-1 treated cells, comparatively to untreated cells (fold increase of 1.3 ± SEM 0.3, N=3), assessed 6 hours after treatment (Figure III.6 A). In addition, we also evaluated the **efficacy of sialidase** cleaving sialylated structures. In spite of an efficient removal of α2,3 Sias by sialidase suggested by MAL-II binding assays, an increased SNA binding to α2,6 Sias in BMDCs' surface was observed in comparison with untreated cells, unlike the expected (Figure III.7). This effect is likely to occur due to an exposure of these residues upon the removal of α2,3 Sias. Moreover, right after the treatment, sialidase led to a lower SNA binding (Figure III.7), relatively to that observed after 6 hours (Figure III.6 A). This suggests that the remaining sialidase in culture might continue its action, cleaving more α2,3 Sias and exposing α2,6 Sias. Furthermore, there is also the possibility that an up-regulation in α2,6 Sias' content has occurred within this time, replacing the removed sialylated structures. However, this mechanism was not confirmed and, as far as we know, the regulation of endogenous ST6Gal-1 upon extrinsic modulation of Sias' content has not been reported. Also, comparing with the other performed conditions, sialidase pre-treatment followed by ST6Gal-1 plus LPS led to the biggest fold increase in the SNA binding relatively to untreated cells (Figure III.6 A).

This effect is probably due to a combination between sialidase and ST6Gal-1 action, since we have noticed that the first cleaves α2,3 Sias, which might expose more α2,6 Sias and, at the same time, creates available sites for ST6Gal-1 to add α2,6 Sias in BMDCs' surface. Therefore, this combined effect can explain the fact that rST6 enzymatic action has induced a more pronounced outcome in this last condition, as we have predicted.

These unexpected results can be related to the fact that extrinsic sialylation in BMDCs has not occurred in a significant extent. Also, we cannot ignore the fact that rST6 could have lost part of its enzymatic activity, since this parameter was not evaluated before the assays. It is also possible that CMP-Sia could have been degraded to other sub-products (such as cytidine) over the experiments, jeopardizing the catalytic activity of rST6. Nevertheless, even if this has happened, it is not likely that the enzymatic activity would be completely lost, therefore these hypothesis were set aside.

Assuming that rST6 can efficiently add α2,6 Sias using CMP-Sia as a substrate, it will need **available ligands** on BMDCs' surface to act. In this context, he have seen that most of untreated mice BMDCs presented positive SNA binding, as depicted in Figure III.6 B, suggesting that unstimulated cells have high content in α2,6 Sias *per se*, similar to human DCs. This effect can lead to the absence of available ligands in cells' surface to be further extrinsically sialylated. Therefore, it is likely that there are not enough available ligands to be extrinsically α2,6 sialylated, which might be related with the absence of BMDCs' profile modulation.

Regarding the only slight increase in SNA binding to BMDCs upon ST6Gal-1 treatment, in addition to the natural high content in α2,6 Sias seen in BMDCs, several approaches to increase even more the α2,6 extrinsic sialylation were performed, namely: addition of ST6Gal-1 during the **differentiation** of bone marrow cells into DCs (section III.2.3), **sequential addition** of ST6Gal-1 followed by LPS (section III.2.4) and using a **concentrated cell volume** at the ST6Gal-1 treatment (section III.2.5). Oppositely to the initial hypothesis, it was clear in all performed assay that α2,6 extrinsic sialylation upon LPS stimulation was not able to diminish the maturation profile of BMDCs, regarding either the released pro-inflammatory cytokines and the expression of maturation markers.

Regarding the addition of ST6Gal-1 during the **differentiation** of bone marrow cells into DCs (section III.2.3), we aimed to increase α2,6 Sias' content instead of favouring other sialylated structures to be added to cells' surface. Even though, this

treatment had no improvement over the α2,6 Sias content in ST6Gal-1 treated DCs' precursors, comparing with untreated cells (fold increase of 1.3, as depicted in Figure III.10). Besides the already known increased sialylation occurring during DC differentiation, increased endogenous neuraminidase (Neu) 1 and Neu3 activity during their differentiation into DCs has also been reported in human monocytes (Stamatos *et al.*, 2010). If this is the case in BMDCs, α2,6 Sias incorporated on their surface by rST6, during differentiation, could have been removed by Neu activity. In opposite, since ST genes are differentially up-regulated during DC differentiation, with the consequent increase in Sias content (Videira *et al.*, 2008), this might have masked the effect of extrinsic ST6Gal-1. However, the net sialylation effects in BMDCs during differentiation are not well understood, which makes it difficult to say if endogenous Neu action would overwhelm the effect of increased sialylation. Unravel the net result of both STs and endogenous Neu activities during differentiation of BMDCs would be an important line of investigation in order to provide a better understanding in the regulation of Sias' content in BMDCs, during this stage.

Another possible explanation for the absence of BMDCs' profile modulation is that a pre-increment in α2,6 Sias status needs to be reached in BMDCs' surface before addition of LPS, as proposed in **sequential addition** of ST6Gal-1 followed by LPS, topic III.2.4. In fact, LPS treated BMDCs always presented significant release of pro-inflammatory cytokines and increased expression of maturation markers, as demonstrated over Chapter III. Taking this into account, extrinsic α2,6 sialylation was induced for 4 hours before addition of LPS. Nevertheless, and once again, the improvement in the content of α2,6 Sias was only slight (fold increase of 1.3 in the MFI of SNA comparatively to untreated cells, assessed 24 hours after this treatment, as presented in section III.2.4, Figure III.12). The released cytokines and the maturation markers were similar comparing with LPS (Figures III.11 and III.13, respectively), again suggesting that α2,6 extrinsic sialylation was unable to modulate DCs' profile in these conditions.

Extracellular ST6Gal-1 activity has been shown to decrease myelopoiesis in *ex vivo* colony formation (Jones *et al.*, 2010) and to occur in marrow hematopoietic stem and progenitor cells as a potential immunomodulator (Nasirikenari *et al.*, 2014). However, the unsuccessful extrinsic ST6Gal-1 action might be due to a lower contact between rST6 and the cells in the previous experiments (section III.2.2, III.2.3 and

III.2.4). Therefore, a **concentrated cell volume** at the ST6Gal-1 treatment was performed (section III.2.5). A fold increase of 1.503 ± SEM 0.1728, N=2 was obtained as the MFI of SNA for ST6Gal-1 treated cells, in comparison with untreated cells, assessed right after the treatment (Figure III.16). An increased SNA binding compared to the previous assays was found, although still not significant. This suggests that there are probably other influencing factors upon LPS stimulation abrogating ST6Gal-1 action,

Considering these results all together, it is possible that extrinsic α2,6 sialylation indeed occurs, since an increase in α2,6 Sias' content was noticed in every approaches, although in a small extent. However, ST6Gal-1 is not able modulate DC functions in this scenario. Some reasons that might lead to these results are now going to be discussed.

It is tempting to think that under activation of BMDCs with LPS engagement in TLR-4, a possible way of rST6 to act would be the sialylation of TLR-4 itself, reducing the intracellular signalling through this pathway. In fact, TLR are highly glycosylated, namely in N-glycans structures (Takeda, Kaisho and Akira, 2003) and endogenous Neu1 activity is essential for LPS activation of several DCs lines and macrophages, removing Sias from TLR-4 (Amith *et al.*, 2010). This observation suggests that the opposite action (sialylation) should be able to revert the scenario of cellular activation, restoring the anti-inflammatory status (Amith *et al.*, 2010). However, if removal of sialylated structures by **continuous endogenous Neu activity** occurs, it is likely that ST6Gal-1 might not be able to add enough α2,6 Sias in TLR-4. Therefore, α2,6 Sias' potential immunomodulator role might be prevented to take place. Whether this mechanism happens in BMDCs, has not been reported, as far as we know. Nevertheless, if endogenous Neu1-induced cleavage of α2,6 Sias upon LPS stimulation has happened in BMDCs, it might explain the observed decrease in the SNA binding to cells' surface in every LPS condition, as demonstrated over Chapter III. Besides, LPS-induced decrease in SNA binding to human DCs has also been reported (Jenner *et al.*, 2006). Understanding the regulation of endogenous Neu in BMDCs, either in differentiation and upon LPS-induced activation, would be an important future line of investigation, in order to understand if underlying desialylation mechanisms could influence the effectiveness of extrinsic α2,6 sialylation by ST6Gal-1 in BMDCs.

Another issue is that the **content in inhibitory Siglecs** which have ITIM motifs in BMDCs can probably influence DCs' immune response to extrinsic addition of α2,6

Sias. Indeed, human mo-DCs and blood circulating DCs are known to express a variety of Siglecs, which can bind in *cis* or *trans* conformation and have potential roles in the regulation of their immune response (Crespo, Lau and Videira, 2013). Actually, due to the increased number of sialylated structures in immune cells' surface, *cis* interactions are usually preferred to *trans* linkages (Razi and Varki, 1999). Nevertheless, cis linkages are not strong and can be easily abrogated by sialidase or endogenous Neu action (Crocker, Paulson and Varki, 2007). In fact, in our initial hypothesis, it was expected that upon LPS-induced BMDCs' activation, Sias in *cis* conformation could be removed in order to stop the inhibitory features associated with ITIM motifs of Siglecs. Then, by extrinsically adding ST6Gal-1, new available sites might be filled with α2,6 Sias, restoring the inhibitory features associated with Siglecs. Moreover, we expected to boost this effect using sialidase first and then ST6Gal-1. However, if the content in the inhibitory Siglec of BMDCs' surface was lower, the extracellular ST6Gal-1 might not have a significant impact controlling DC's immune responses. Furthermore, if *cis* interactions were not abrogated upon LPS stimulation, as predicted, the extracellular ST6Gal-1 might not be able to replace α2,6 Sias in *trans* conformation and restore the non-inflammatory status. Considering this, exploring the content of Siglecs in BMDCs, namely the human homologue CD22, which binds α2,6 Sias in terminal galactose residues(Crocker, 2002), would be an important field of study. In addition, it is important to address whether sialylation available places in inhibitory Siglecs might have influence in changing their conformation, which consequently modify the intracellular signalling (Ravetch and Lanier, 2000), (Avril *et al.*, 2004).

In any case, is possible that **DCs' activation cannot be reverted** anyway. In terms of their biological function, DCs are known to enter in apoptosis after its activation has been induced. Moreover, different types of DCs from mice presented a short turnover, mainly after arrival to lymph nodes (Kamath *et al.*, 2002). This appears to be a remarkable difference between DCs and macrophages, since macrophages are able to restore their functions after activation (Banchereau *et al.*, 2000), (Zanoni and Granucci, 2010). Nonetheless, complex mechanisms which avoid apoptotic pathways and prolong macrophages' life span are not totally understood (Parihar, Eubank and Doseff, 2010). Unlike BMDCs, in our laboratory we have seen that bone marrow derived macrophages treated with ST6Gal-1 decreased the release of pro-inflammatory cytokines upon LPS

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stimulation (unpublished observations from Mehrab Nasirikenari, Joseph Lau). Corroborating with this, a different mechanism for conventional DCs and macrophages was recently found regarding LPS engagement with TLR-4. Although both cells have high content in CD11b, in DCs it promotes TLR-4 activation facilitating LPS engagement, which does not occur in macrophages (Ling *et al.*, 2014), (Banchereau *et al.*, 2000). Therefore, this might improve the LPS engagement on TLR-4 in BMDCs, which could induce a very effective and irreversible response.

Lastly, it would be important to address if **other BMDCs' surface markers** are affected by different contents in α2,6 Sias. For example, MHC-I is highly sialylated in terminal N-glycans (Ryan and Cobb, 2012), where sialylation events can be related with proper folding and traffic to cell membrane (Gunten, von and Bochner, 2008). Indeed, increased sialylation in T cell receptor (TCR) decreases their interaction with MHC-I, under flow conditions, suggesting that sialylation events are able to decrease activation, in a contact-dependent way (Daniels *et al.*, 2001). However, in the case of MHC-II, sialylation roles in cell activation are not so well studied (Ryan and Cobb, 2012). In this work, BMDCs' priming with T cells was not evaluated, but is possible that extrinsic sialylation events could only influence events in a contact-dependent way, like MHC interactions with TCR.

Besides the extrinsic modulation of BMDCs' with ST6Gal-1, the opposite effect was assessed, either by **sialidase** treatment of BMDCs, as by assessing the features of **BMDCs and cells from the airways of ST6Gal-1 KO mice**, which have an overall lack in α2,6 Sias.

Upon LPS stimulation, the release of pro-inflammatory cytokines from ST6Gal-1 KO BMDCs was similar to WT BMDCs, under the same conditions, as observed in section III.2.6, Figure III.17. However, ST6Gal-1 KO BMDCs released twice more TNF-α and IL-6 compared to untreated WT BMDCs (Figure III.17), appearing to be more mature *per se*. This observation is also supported by the increased expression of MHC-II on ST6Gal-1 KO BMDCs' basal state (64.40% cells expressed MHC-II), whereas 32.62% ± SEM 12.58, N=3 of WT BMDCs expressed this marker (topic III.2.2).

Another interesting feature is that 2,6 Sias appears to be a strange component to ST6Gal-1 KO cells, since both ST6Gal-1 plus LPS and sialidase followed by ST6Gal-1 plus LPS led to their most mature phenotype, comparing with LPS treated cells (Figure

III.18). Moreover, the positive population for SNA staining in sialidase followed by ST6Gal-1 plus LPS condition from ST6Gal-1 KO BMDCs presented more cells expressing high levels of CD86, as depicted in Figure III.19. In this case, a likely explanation is that ST6Gal-1 KO BMDCs perceive α2,6 Sias as non-self. Also, receptors could have recognized α2,6 Sias as PAMP instead of SAMP (self-associated molecular patterns), changing conformation and inducing activating intracellular signalling pathways (Crocker, 2002). Furthermore, certain Siglecs that are involved in the phagocytosis requires Sias to promote it (Gunten, von and Bochner, 2008), boosting phagocytosis and LPS-induced BMDCs' activation. Nonetheless, this consideration needs further elucidation.

Sialidase treatment was not consistent increasing the expression of all maturation markers, despite MAL-II and MAL-I binding assays suggest that this treatment was effective removing α2,3 Sias, as shown in sections III.2.2 and III.2.5 (Figures III.7 and III.16, respectively). Only an increased number of cells expressing CD86 upon sialidase treatment showed statistical significant in three independent experiments, after 6 hours upon LPS treatment (section III.2.2, Figure III.5 A). The data regarding sialidase treatments suggest that sialidase does not induce a significant modulation of BMDCs' features, despite some modifications have occurred (presented over Chapter III). These modifications could be related with the type of treatment performed, or with a specific maturation marker or released cytokine affected, which needs further elucidation.

The results from our group with human mo-DCs suggest that neuraminidase treatment has improved the maturation of mo-DCs, in terms of MHC-II, CD80 and CD86 expression upon endocytosis, whether the genetic expression of pro-inflammatory cytokines has also increased (Crespo *et al.*, 2009). It is important to address if the differences in terms of maturation markers found between mice BMDCs and human mo-DCs are related to a specific mechanism within the cell types. This can help to unravel the mechanism, underlying the effect of sialidase in cellular maturation. In this context, it is also important to consider Siglec differences between human and mice, since less CD33-related Siglecs are found in mice (Siglecs E, F, G and H) than in human cells (Siglecs 5, 6, 7, 8, 9, 10, 11 and 14) (Crocker, Paulson and Varki, 2007). Besides, they do not always share homology whose functional implications are unknown, needing further elucidation. Moreover, the protein levels are not always co-related with their genetic

expression, which can also be the reason why the cytokines released from mice BMDCs have not significantly increased upon sialidase treatment. Nevertheless, and supporting the observations from this work, other authors were not able to see increased amounts of released cytokines in human immature mo-DCs pre-treated with sialidase and then exposed to LPS (Stamatos *et al.*, 2010). Considering this, the genetic expression of cytokines from mice BMDCs should be evaluated upon this treatment, in future experiments.

It is also important to consider that α2,6 Sias were not effectively removed by sialidase, as SNA binding assays suggest over the sections III.2.2, III.2.4 and III.2.5. In this case, **α2,6 Sias might be the key modulators for sialidase effect**, where the observed effect in human mo-DCs could be enhanced by specific removal of α2,6 Sias. Actually, BMDCs from ST6Gal-1 KO mice appear to have a more mature phenotype *per se*, comparing with BMDCs from WT, at least in terms of released pro-inflammatory cytokines and MHC-II expression (as shown in section III.2.6). In other studies from our group, BMDCs from both ST6Gal-1 and ST3Gal-1 (the major source of α2,3 Sias) KO mice presented a higher expression of MHC-II, when compared with WT BMDCs. However, DCs obtained from blood, spleen and lymph nodes from ST3Gal-1 KO mice had no significant increases in the expression of MHC-II and CD86, whereas cells obtained from ST6Gal-1 KO mice presented significant increased expression of MHC-II, comparing with WT cells (Crespo *et al.*, 2009). This observation alerts to possible specific roles within different types of Sia linkages, but also to different behaviours within different types of DCs, since these cells constitute a very heterogeneous population.

Corroborating these observations, CD11c high, CD11b medium cells from BALF of ST6Gal-1 KO mice under LPS stimulation, presented a significant increased expression of MHC-II, in comparison with WT, as depicted in section III.3, Figure III.25. These cells are likely to be cDCs CD11b positive or inflammatory mo-DCs, whereas CD11c high, CD11b negative cells can be alveolar macrophages or cDCs CD11b negative. In the later, no significant increases were found regarding MHC-II expression, which can be related to the fact that two different cell types were not distinguished in this analysis. In opposite, it is possible that MHC-II expression in both cellular populations is indeed not affected. In future experiments, it can be useful to distinguish between alveolar macrophages and cDCs CD11b negative, assessing the levels of cellular autofluorescence

by Flow Cytometry, since alveolar macrophages present higher autofluorescence compared to DCs, (Rijt, Van *et al.*, 2004), (Geurtsvan, Kessel and Lambrecht, 2008). Despite no significant differences were found comparing WT with ST6Gal-1 KO mice within CD11c medium, B220 positive, CD11b negative population, regarding their maturation markers, it is important to use anti Siglec-H in future experiments to distinguish between pDCs and IFN-producing killing DCs in the BALF (Geurtsvan, Kessel and Lambrecht, 2008).

Another interesting feature about BALF results is that CD86 was only slight expressed in CD11c high populations (positive CD86 populations of 9.29% ± SEM 3.96, N=3 and 7.06 ± SEM 0.30, N=2, for KO and WT mice, respectively), whereas CD80 was expressed for most of these cells. This observation suggests that later maturation markers were expressed in these cells, after 24 hours. In opposite, most of CD11c medium, B220 positive, CD11b negative DCs expressed CD86 (81.97% ± SEM 7.85, N=3 and 87.85% ± SEM 5.85, N=2 for KO and WT mice, respectively), whereas CD80 was barely expressed (12.73% ± SEM 1.56, N=3 and 19.89% ± SEM 11.22, N=2 for KO and WT mice, respectively).

CD11c high, CD11b medium population may include both cDCs CD11b positive and mo-DCs. However, considering that cDCs CD11b positive are sentinels in the lungs, it is likely that these cells were activated within the first 24 hours, expressing the later maturation marker CD80 before other DCs (Condon *et al.*, 2011). Indeed, pDCs (included in CD11c medium, B220 positive and CD11b negative population) only arise under inflammatory conditions, being activated after cDCs (Kim and Lee, 2014). Therefore, it is likely that the early maturation marker CD86 was still being expressed in these cells 24 hours after LPS was administered to mice. This is also an important line of investigation, regarding the described specialized functions of DCs in the lungs (GeurtsvanKessel and Lambrecht, 2008).

Nevertheless, considering that only MHC-II presented significant differences between WT and ST6Gal-1 KO mice, it is likely that α2,6 desialylation has specifically influenced the expression of this surface marker. In fact, the increased MHC-II expression was also observed in this work in untreated BMDCs from ST6Gal-1 KO mice. Besides, this observation is in agreement with previous observations from our group, regarding increased MHC-II expression in BMDC and blood, spleen and lymph nodes DCs from

ST6Gal-1 KO mice (Crespo *et al.*, 2009). These considerations are important to be evaluate in future experiments, repeating the protocols with an increased number of experiments. Moreover, the fact that sialidase treatment of WT mice BMDCs only significantly increased CD86 mainly due to α2,3 Sias removal, supports the concept of **specialized functions to attribute to specific Sia linkages**.

The functional implications in DC immune response, regarding MHC-II increased expression when α2,6 Sias are absent, also needs further elucidation namely unravelling the consequences for DC-induced T cell priming. These findings can be important in DC therapy to boost an immune response, wherein a more efficient removal of α2,6 Sias might be required to increase MHC-II expression, improving then DC function.

IV.2. Conclusions

With this project we found, by the use of different conditions, that extrinsic action of ST6Gal-1 leads to a slightly increase in the content of α2,6 Sias in BMDCs' surface. In addition, this slight modulation do not modify DCs' profile, regarding the expression of CD80, CD86 and MHC-II and the release of IL-10, IL-6 and TNF-α, unlike the formulated hypothesis.

The most likely explanations for the absence of modulation in BMDCs' features upon ST6Gal-1 plus LPS treatment are: **1)** endogenous Neu action might have continued either during DC differentiation and upon LPS stimulation, which could have reduced the sialylation induced by extracellular ST6Gal-1 and **2)** the lack of enough Siglecs with available sites to be sialylated might have occurred, which would prevent conformational changes to happen, not influencing the intracellular signalling. In future studies, both of these considerations are crucial to be explored, in order to understand which mechanisms underlie in the control of α2,6 Sias content upon LPS stimulation. In addition, understanding these underlying mechanisms might help to unravel the importance of α2,6 Sias in the control of DCs' immune response.

In the second part of the work, sialidase treatment of BMDCs mainly removing α2,3 Sias, has only significantly enhanced CD86 marker. However, the lack in α2,6 Sias of ST6Gal-1 KO mice led to a significant increased MHC-II expression in DCs from mice

airways. These observations suggest the possibility of α2,6 Sias specifically influence the expression of MHC-II in DCs.

In this work, an overall lack in α2,6 Sias influenced MHC-II expression, whereas the opposite action (addition of α2,6 Sias by ST6Gal-1) has not influenced DCs' profile, which appear to be controversial findings. Nonetheless, an overall lack in α2,6 sialylation might have pronounced implications in DC functions, namely in their surface markers' expression. Indeed, sialylation is known to affect proper folding of proteins and even the transport of receptors to cell membrane (Gunten, von e Bochner, 2008), (Ajit Varki *et al.* 2009). In addition, ST6Gal-1 KO mice have a predisposition to a more inflammatory status, although the underlying mechanisms are not completely understood (Hennet *et al.*, 1998). In spite of extrinsic addition of α2,6 Sias intends to simulate the opposite action of loss in α2,6 Sias, this might not be a robust enough stimulus to dampen an immune response. Furthermore, it is likely that other sialylation events might take place in WT BMDCs' surface upon LPS stimulation, jeopardizing the effectiveness of α2,6 Sias addition in BMDCs' surface. These several possible constraints might prevent an immunomodulator role for extrinsically added α2,6 Sias, needing further elucidation.

This work is, as far as we know, the first time that the role of extrinsic α2,6 sialylation was assessed in DCs. The developed work has opened an important line of research in Glycobiology field: the extrinsic α2,6 sialylation as a key modulator in DCs' immune response. This is a vital subject to explore, due to soluble ST6Gal-1 increased expression in several cancers, which can also have an impact in cancer immunotherapy using DCs.

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Appendix

I.   Solutions prepared in the work

Cell culture

- **RPMI complete medium:** RPMI-1640 medium with Phenol Red (Gibco, Life Technologies) supplemented with 10% (v/v) heat inactivated FBS (Premium select- from Atlanta Biologicals), 2 mM L-glutamine (Invitrogen), 1% (v/v) penicillin/streptomycin (Corning), 1% (v/v) sodium pyruvate and 1% (v/v) non-essential aminoacids, both from Corning.

- **RPMI complete medium without FBS:** RPMI-1640 medium with Phenol Red (Gibco, Life Technologies) supplemented with 2 mM L-glutamine (Invitrogen), 1% (v/v) penicillin/streptomycin (Corning) and 1% (v/v) sodium pyruvate and 1% (v/v) (Corning).

- **Mouse Red blood cells lysis buffer:** 0.8% (m/v) NH_4Cl_3 in H_2O, 0.1 mM of Ethylenediamine tetraacetic acid (EDTA), buffered with $KHCO_3$ to pH 7.3

Flow Cytometry

- **Flow Cytometry buffer:** 2 mM EDTA, 0.02% (v/v) azide, 0.05% (m/v) BSA, in Dulbecco's Phosphate Buffered Saline (DPBS) 1X

ELISA solutions

- **ELISA Wash Buffer:** 0.05% (v/v) PBS Tween (Sigma) in PBS 1X

    Solutions prepared for use in Affymetrix eBioscience mouse kit:

- **Coating buffer:** the coating buffer provided by Affymetrix kit was diluted 1:10 with deionized water.

- **Diluent:** the assay diluent provided in the kit was first diluted 1:5 with deionized water.

    Solutions prepared for use in Peprotech mouse kit:

- **PBS 1X:** commercial PBS from NZYTech was diluted 1:10 in mili-Q water

- **Diluent:** 0.01% (m/v) BSA (Sigma), 0.05% PBS Tween in PBS 1X

- **Blocking buffer:** 0.5% BSA (m/v) in PBS 1X

<u>BAL solutions:</u>

- **Avertin work solution:** The stock solution was prepared at a final concentration of 1.6 g / mL, with 25 g avertin (2, 2, 2-Tribromoethanol), (Sigma) and with 15.5 ml tert-amyl alcohol (2-methyl-2-butanol) (Fisher). The solution was mixed at room temperature overnight in a dark bottle. The work solution was prepared in PBS 1X in a final concentration of 20 mg/ml, filtered in a 0.2 micron filter and stored at 4 ℃ in the dark, as recommended.

<u>Other reagents:</u>

- **Recombinant murine ST6Gal-1:** obtained from Dr. Kelley Moremen (University of Georgia, USA), whose lab purified it in Chinese hamster ovarian (CHO) cell line. The His tag used for purification was cleaved and the separation between α2,6 and α2,3 ST linkages was performed with SNA agarose beads. The enzymatic activity was calculated through N-acetyl-D-lactosamine II assay, where an activity of 210,500 fmol/hr*μg was determined. It was used a concentration of 10 μg/ ml of medium.