Diana Isabel Pacheco de Sousa

Bachelor Degree in Biochemistry

Improving the anti-tumor immune responses against cancer cells

Dissertation to obtain the Master Degree in

Biochemistry for Health

Supervisor: Professor Paula Videira, PhD, FCT/UNL

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Faculty of Sciences and Technology, NOVA University of Lisbon

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Improving the anti-tumor immune responses against cancer cells

Diana Isabel Pacheco de Sousa
FCT-UNL, UNL

The work developed until the present has originated:

- **Two oral presentations:**

- Diana Sousa, Paula Videira, Zélia Silva (2016). *Improving the anti-tumor immune responses against cancer cells.* Jornadas Intercalares das Dissertações anuais dos Mestrados do DQ e do DCV, January 28th-29th, Caparica, Portugal.

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For anyone who thought they couldn't make it.
And then did.

“The thing I really want to emphasize is, I didn't have a choice. I didn't have a choice (…) the dream is something you never knew was going to come into your life. Dreams always come from behind you, not right between your eyes. It sneaks up on you. But when you have a dream, it doesn't often come at you screaming in your face, "This is who you are, this is what you must be for the rest of your life". Sometimes a dream almost whispers. And I've always said to my kids, the hardest thing to listen to - your instincts, your human personal intuition - always whispers; it never shouts. Very hard to hear. So you have to every day of your lives be ready to hear what whispers in your ear; it very rarely shouts. And if you can listen to the whisper, and if it tickles your heart, and it's something you think you want to do for the rest of your life, then that is going to be what you do for the rest of your life, and we will benefit from everything you do.”

Steven Spielberg

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Author's notes:

1. For confidentiality reasons, the names of the hybridoma clones produced by the group and the names of the humanized anti-STn antibodies provided by collaborators were altered for the presentation of the results in this thesis;

2. The terms "cancer/tumor", "T lymphocyte/T cell", "B lymphocyte/B cell" and "antibody/immunoglobulin" will be used interchangeably throughout this thesis;

3. When mentioning DCs on chapters 2, 3 and 4, these are always to be referred as moDCs.

ABSTRACT

Cancer is one of the leading causes of death worldwide. Dendritic cells (DCs) can capture cancer antigens and present them to T lymphocytes, after a maturation process, inducing specific immune responses. Sialyl-Tn (STn) is a tumor-associated carbohydrate antigen that is expressed by several carcinomas. STn downregulates the immune responses towards STn-expressing tumor cells by inducing a tolerogenic phenotype on DCs. For this reason, a successful therapeutic approach against this antigen has to take in consideration the activation of the immune response.

The main goal of this thesis was to assess if the use of anti-STn antibodies to block the STn antigen could improve anti-tumor immune responses by reestablishing the mature phenotype on DCs. To do this, STn-expressing cancer cell lines were cultured and DCs were obtained from differentiation of monocytes. Initially, the work comprised the development and characterization of anti-STn antibodies produced by the group through hybridoma technology, using mainly ELISA and flow cytometry. Three hybridoma clones producing IgM anti-STn antibodies were obtained and will be further characterized. A second part of the work included a functional *in vitro* characterization of humanized anti-STn antibodies. This comprised the establishment of co-cultures between cancer cells and DCs and assessment of the immune response of DCs against STn-expressing cancer cells, with and without STn blockade by those antibodies. The expression of MHC-II and co-stimulatory molecules was assessed by flow cytometry, and the expression of cytokines was assessed by ELISA and RT-PCR. However, the reestablishment of the mature phenotype was not observed.

The work developed contributed to understanding that further improvements and assays are necessary; and also that the production of antibodies comprises many variants that can be modified throughout the development and characterization procedures, thus being a long process and needing many optimizations before success can be achieved.

Keywords:

cancer, sialyl-Tn, anti-STn antibodies, dendritic cells, anti-tumor immune responses, hybridoma technology

RESUMO

O cancro é uma das maiores causas de morte em todo o mundo. As células dendríticas (DCs) capturam antigénios e, após um processo de maturação, apresentam-nos aos linfócitos T, induzindo respostas imunes específicas. O sialil-Tn (STn) é um carbohidrato associado a tumor, sendo expresso por vários tipos de cancro. Este antigénio reduz as respostas imunes contra as células tumorais que o expressam, através da indução de um fenótipo tolerante nas DCs. Por este motivo, terapias de sucesso contra este antigénio têm de ter em conta a activação da resposta imune.

O objectivo principal desta tese consistiu no uso de anticorpos anti-STn para bloqueio do STn, de forma a avaliar se esse bloqueio restabelecia o fenótipo maduro nas DCs, melhorando as respostas imunes anti-tumorais. Para isso foram cultivadas linhas celulares tumorais que expressavam STn, assim como DCs obtidas da diferenciação de monócitos. Inicialmente, o trabalho envolveu o desenvolvimento e caracterização de anticorpos anti-STn produzidos pelo grupo através de tecnologia de hibridoma, principalmente através de técnicas de ELISA e citometria de fluxo. Foram obtidos três hibridomas com capacidade de produzir anticorpos anti-STn, de isotipo IgM, que serão caracterizados no futuro. A segunda parte do trabalho envolveu a caracterização funcional *in vitro* de anticorpos anti-STn humanizados. Para isso foram estabelecidas co-culturas entre células tumorais, bloqueadas ou não com estes anticorpos, e DCs. A resposta imune das DCs foi avaliada através da expressão de moléculas co-estimulatórias e de MHC-II, e também de citocinas, tendo-se para isso utilizado citometria de fluxo, ELISA e RT-PCR. No entanto, o restabelecimento do fenótipo maduro nas DCs não foi observado.

Este trabalho permitiu compreender que são necessários melhoramentos aos protocolos usados e novos ensaios, e também que a produção de anticorpos engloba muitas variantes que podem ser alteradas em várias fases do desenvolvimento, sendo assim um processo demorado e que necessita de bastantes optimizações antes de se ter sucesso.

Palavras-chave:

cancro, sialil-Tn, anticorpos anti-STn, células dendríticas, respostas imunes anti-tumor, tecnologia de hibridoma

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LIST OF ABBREVIATIONS

Abbreviation	Meaning
Ab(s)	Antibody (Antibodies)
ADCC	Antibody-dependent cell-mediated cytotoxicity
Ag(s)	Antigen(s)
APC	Allophycocyanin
APCs	Antigen presenting cells
Asn	Asparagine
BSA	Bovine serum albumin
BSM	Bovine submaxillary mucin
C	Constant region
CD	Cluster of differentiation
CDC	Complement-dependent cytotoxicity
cDNA	Complementary DNA
CDRs	Complementary-determining regions
CFG	Consortium for Functional Glycomics
CMP-sialic acid	Cytidine monophosphate-sialic acid
C_T	Threshold cycle
DC(s)	Dendritic cell(s)
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-Linked Immunosorbent Assay
ER	Endoplasmic reticulum
EV	Empty vector
Fab	Fragment antigen binding
FBS	Fetal bovine serum
Fc	Fragment cristallizable
FHC	Final hybridoma clone
FITC	Fluorescein isothiocyanate
fsc	Forward-scatter
Gal	Galactose
GalNAc	*N*-acetylgalactosamine
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GlcNAc	*N*-acetylglucosamine
GM-CSF	Granulocyte macrophage colony-stimulating factor
H	Heavy chain
HAT	Hypoxanthine-aminopterin-thymidine
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HGPRT	Hypoxanthine-guanine-phosphoribosyltransferase
HIV	Human Immunodeficiency Virus
HLA	Human leukocyte antigen
HRP	Horseradish peroxidase

HTLV	Human T-lymphotropic virus
IC	Isotype control
Ig(s)	Immunoglobulin(s)
IL	Interleukin
IPST	*Instituto Português do Sangue e da Transplantação*
IUPAC	International Union of Pure and Applied Chemistry
L	Light chain
LPS	Lipopolysaccharide
mAb(s)	Monoclonal antibody (antibodies)
MFI	Median intensity fluorescence
MHC	Major histocompatibility complex
MHC-I	Major histocompatibility complex, class I
MHC-II	Major histocompatibility complex, class II
moDCs	Monocyte-derived dendritic cells
mRNA	Messenger ribonucleic acid
Neu	Neuraminic acid
Neu5Ac	*N*-acetylneuraminic acid
NK	Natural killer
OD	Optical density
OSM	Ovine submaxillary mucin
PAMPs	Pathogen associated molecular patterns
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PE	Phycoerythrin
PEG	Polyethylene glycol
PRRs	Pattern recognition receptors
RNA	Ribonucleic acid
RT	Room temperature
RT-PCR	Real-time polymerase chain reaction
Ser	Serine
ssc	Side-scatter
STn	Sialyl-Tn
T or TF	Thomsen–Friedenreich
TACA(s)	Tumor-associated carbohydrate antigen(s)
Tc	Cytotoxic T cells ($CD8^+$)
TE	Trypsin-EDTA
Th	Helper T cells ($CD4^+$)
Thr	Threonine
TMB	3,3',5,5'-tetramethylbenzidine
TNF-α	Tumor necrosis factor-alpha
UDP-GalNAc	Uridine diphosphate *N*-acetylgalactosamine
V	Variable region
V_H	Variable region (heavy chain)
V_L	Variable region (light chain)
WT	Wild type

1. INTRODUCTION

1.1. Cancer

Cancer is one of the leading causes of death worldwide (Figure 1.1), along with cardiovascular and neurodegenerative diseases, diabetes and chronic pulmonary diseases. The number of new cases is expected to increase by about 70% over the next twenty years. Thus, it is important that this disease can be correctly and early diagnosed and that effective treatments that allow its cure or that can considerably prolong the patient's life are made available [1,2].

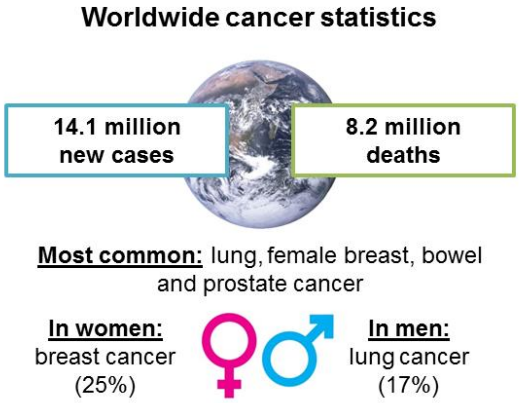

Figure 1.1 – Worldwide cancer statistics: number of new cases and deaths in 2012. Most common cancers (general), and top cancers affecting woman and men. Adapted from [90,91].

This disease results from the accumulation of somatic mutations in the progeny of normal cells, which generates abnormal cells with a selective growth advantage and with an upregulated and uncontrolled capacity of proliferation. Cancer cells can also undergo a process known as metastasizing, in which they gain the ability of invading adjacent normal tissues and organs and spread to distant sites throughout the body, which is the main cause of death related with this disease. These mutations are mostly related with deoxyribonucleic acid (DNA) repair genes, tumor suppressor genes and proto-oncogenes, arising from exposure to physical and chemical agents, from infection with viruses, from genetic predisposition and from behaviors related with known risk factors, like smoking [3,4]. The type of cell from which a tumor arises classifies it: tumors that arise from epithelial cells are named carcinomas, tumors with origin on connective tissues are named sarcomas, tumors that arise from cells of the immune system are called lymphomas and tumors generated from blood-forming cells are named leukemias. They can also be further classified according to their primary site [3].

Several aspects of the cell behavior distinguish cancer cells from their normal equivalents. These aspects, known as the hallmarks of cancer (Figure 1.2), are acquired functional abilities that allow cancer cells to survive, proliferate, and disseminate. Normal tissues ensure the homeostasis of cell growth and proliferation by controlling the production of growth signals. Cancer cells have the

capacity of sustaining chronic proliferation by deregulating those signals, an action that also has an influence on cell survival and energy metabolism. In addition, they have the ability to limit or evade apoptosis and express less surface adhesion molecules, which results in morphological and cytoskeletal modifications. Moreover, cancer cells secrete proteases, such as collagenase, that digest components of the extracellular matrix, allowing them to invade the adjacent normal tissues; they can secrete growth factors as well, thus stimulating angiogenesis (formation of new blood vessels), a process needed so that the proliferating tumor cells can obtain nutrients and oxygen. These properties of cancer cells are associated with their ability of migrating to adjacent tissues and to invade nearby blood and lymphatic vessels, which allows them to colonize distant sites and form metastasis [3–6].

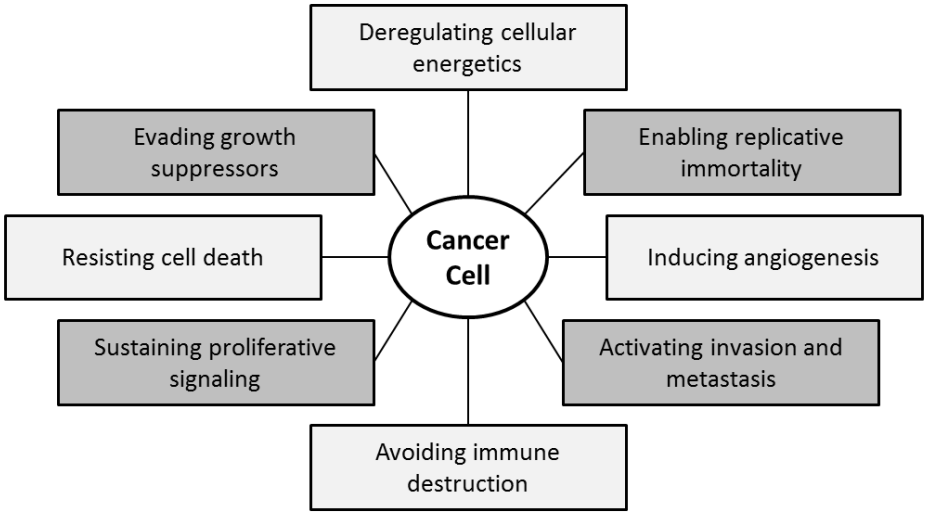

Figure 1.2 – Schematic representation of the hallmarks of cancer. Adapted from [6].

1.2. Glycosylation

Glycosylation is an enzymatic process by which glycosidic linkages are created, allowing saccharides to be bound to other saccharides, lipids or proteins, and generating glycoconjugates. The diversity of glycans (term often applied to name the carbohydrate part of a glycoconjugate) derives from the ways in which monosaccharides can be linked together. Differences in monosaccharide composition, substitutions in the existing glycans, the presence or not of branching structures and the different linkages - to other carbohydrates, to proteins or to lipids – all contribute to an increasing diversity of glycans [7,8]. For this reason, a nomenclature strategy was adopted by the Nomenclature Committee of the Consortium for Functional Glycomics (CFG) [9]. This strategy provides a consistent system to represent glycan structures by both symbols and text (see Appendix I).

In eukaryotic cells, most of the secreted and cell-surface molecules originate in the endoplasmic reticulum (ER). They are then distributed from the *trans*-Golgi network to several destinations and, in the process, proteins and lipids are modified by a series of glycosylation reactions. However, not all glycans are assembled within the ER-Golgi pathway, as some modifications occur in

the cytoplasm and others at the plasma membrane. Nucleotide sugars (activated forms of monosaccharides) are often transferred in glycosylation reactions, which are mediated by glycosyltransferases, to the glycan being synthetized [10]. The biological roles of glycans are broadly classified in four categories, all involving glycan-binding proteins: structural and modulatory roles, intrinsic recognition, extrinsic recognition and molecular mimicry of host glycans [11,12]. The two general classes of protein-bound glycans occur via linkages to oxygen (*O*-glycosylation) or nitrogen (*N*-glycosylation). The process of *N*-glycosylation generates *N*-glycans through the binding of *N*-acetylglucosamine (GlcNAc) residues to the amide groups of asparagine (Asn) side chains. The process of *O*-glycosylation generates *O*-glycans, in which *N*-acetylgalactosamine (GalNAc) residues are linked to the oxygen atom of threonine (Thr) or serine (Ser) side chains (Figure 1.3) [7,8]. Because the work developed and presented in this thesis is more focused on *O*-glycans, more emphasis will be given to these glycans throughout this chapter.

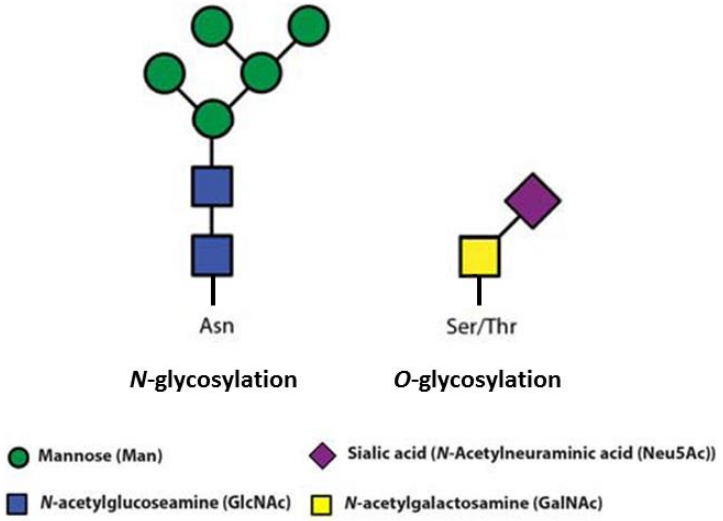

Figure 1.3 – Schematic representation of a *N*-glycosylated glycoprotein (left) and of a *O*-glycosylated glycoprotein (right). Adapted from [92].

1.2.1. *O*-glycosylation

Glycoproteins are often heavily *O*-glycosylated, particularly serine- and threonine-rich mucins that constitute mucous secretions and can also be found on the cell surface as transmembrane glycoproteins. The biosynthesis of *O*-glycan chains occurs in the Golgi apparatus and involves the sequential action of different glycosyltransferases. Thus, the pattern of *O*-glycans that is expressed on a certain cell depends on the level of expression, localization and substrate specificity of the glycosyltransferases. The structures generated by these enzymes can be further modified through processes such as sialylation, acetylation and fucosylation [8,13,14]. The initial step of *O*-glycosylation involves the transfer of a GalNAc residue from uridine diphosphate *N*-acetylgalactosamine (UDP-GalNAc) to a threonine or to a serine in the amino acid chain, generating the Tn antigen (GalNAc-Ser/Thr), which is the simplest mucin *O*-glycan and will be mentioned later in this chapter. The chain is then further elongated [15–17].

1.2.2. Sialic Acids

One of the modifications than can occur on glycan structures is sialylation, through the addition of monosaccharides, named sialic acids, which comprise a group of approximately 50 different chemical derivatives of neuraminic acid (Neu). In mammalian cells, the most common variant is the *N*-acetylneuraminic acid (Neu5Ac), with a *N*-acetyl group on carbon 5 (Figure 1.4) [18,19].

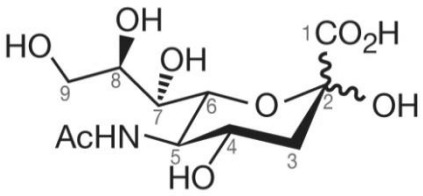

Figure 1.4 – Structure of *N*-acetylneuraminic acid (Neu5Ac) [19].

Sialic acids exhibit a significant diversity due to the different alpha linkages that can be formed. They are commonly found in the terminal positions of *N*- and *O*-glycans of glycoproteins and are added by action of specific sialyltransferases that use cytidine monophosphate-sialic acid (CMP-sialic acid) as donors [18,19]. These monosaccharides have several biological roles, such as the stabilization of membranes and molecules and the modulation of interactions of the molecules and cells with the environment; they can also serve as a biological target, allowing its recognition by a receptor protein, or shield antigenic sites and receptors, protecting molecules from the action of proteases or glycosidases and weakening the immune-reactivity [19,20].

1.2.3. Sialyltransferases and Sialidases

Sialylation of glycoproteins is a modification that occurs in glycans and is associated to cancer. The level of cell surface sialylation is regulated by a diverse group of enzymes. This group includes the enzymes that control the synthesis and availability of the activated donor substrate (CMP-sialic acid); sialyltransferases, the enzymes that add the sialic acids; and sialidases (or neuraminidases), the enzymes that remove the sialic acids. Most of the sialyltransferases are localized in the Golgi apparatus, since sialic acids are added during the biosynthesis of the glycoproteins, while sialidases are usually found in lysosomes or endosomes, for the removal of sialic acids during the degradation of glycoproteins [18].

Sialyltransferases are a family of enzymes that vary in tissue distribution and on the type of sialic acid linkage that they perform. Sialic acids can be added by ST3Gal-I sialyltransferase in an α2-3 linkage to galactose (Gal); by ST6Gal-I and ST6Gal-II sialyltransferases in an α2-6 linkage to galactose or by ST6GalNAc sialyltransferases to *N*-acetylgalactosamine; or by the family of polysialyltransferases in an α2-8 linkage to other sialic acid. Aberrant expression of these enzymes, as well as of sialidases, is observed in cancer [18].

1.3. Glycosylation and Cancer: Tumor-Associated Carbohydrate Antigens

Glycosylation can be essential for the regulation of several physiopathological mechanisms. For this reason, variations in the resulting glycoconjugates can interfere with cancer cell processes, such as inflammation and immune surveillance, and with the tumor microenvironment, as they are associated with oncogenic transformation, cancer growth, progression and metastasis. Cancer cells exhibit a diverse series of differences on the levels and types of carbohydrate structures present on their surfaces. These carbohydrate structures allow them to be distinguished from normal cells and are known as tumor-associated carbohydrate antigens (TACAs). Alterations in the glycans present at the surface of the cell can result from abnormal expression and activity of glycosyltransferases or to their incorrect location in the Golgi apparatus [7,21].

The two main processes of tumor-associated carbohydrate alteration are the neo-synthesis and the incomplete synthesis. The first one is commonly observed in more advanced stages of cancer and results of a cancer-associated induction of genes that are related with the expression of carbohydrate determinants, which leads to the *de novo* expression of antigens in cancer tissue. The second process is more commonly found in early stages of cancer and results of a deficiency on the normal synthesis of complex glycans that are usually expressed in normal epithelial cells, which leads to the generation of truncated structures in cancer tissues [7,22,23].

The most common alterations in glycosylation are fucosylation, sialylation, truncation of *O*-glycans and branching of *O*- and *N*-linked glycans. Although all these alterations have been shown to be related with cancer [24], sialylation and truncated *O*-glycans will be more explored in this thesis. Truncation of *O*-glycans is commonly found in secreted and transmembrane glycoproteins. Sialylated structures of Thomsen-Friedenreich antigens are examples of truncated glycans [25,26]. The increased expression of sialylated glycans promotes cell detachment from the original tumor site, by disrupting cell-cell adhesion processes, leading to metastasis, which is related with poor prognosis for the patients [7].

1.3.1. Thomsen–Friedenreich antigens

Thomsen–Friedenreich antigens are *O*-glycans found mainly on serine- and threonine-rich mucins, thus being present in membrane glycoproteins. These antigens are a result of a defective elongation of the *O*-glycan on the first steps of mucin glycosylation [27]. The first Thomsen–Friedenreich antigen described was called T (or TF) antigen, forms the core 1 structure of mucin *O*-glycans and consists of the disaccharide composed by a galactose β1-3 linked to a *N*-acetylgalactosamine, α-linked to a serine or threonine residue (Galβ1-3GalNAcα1-Ser/Thr). The T antigen is the precursor of core 2 *O*-glycans, but if the cancer cells lose the ability to synthesize the core 2, this antigen can be exposed [28]. The T antigen can, in normal tissues, be substituted by other carbohydrate chains or sialic acids and form more complex glycans. In cancer tissues, however, this antigen is usually unsubstituted, occurring in approximately 90% of human cancers, such as breast, bladder and prostate, thus being considered a pan-carcinoma antigen, and is also correlated with cancer progression and metastasis [27,29]. If, on the contrary, the ability to synthetize the core 1 is

lost, its precursor, the Tn antigen, is exposed. This antigen consists of a single *N*-acetyl-galactosamine α-linked to serine or threonine residue (GalNAcα1-Ser/Thr) [15,28]. The Tn antigen is not usually found on secreted or cell-surface proteins in normal tissues, but is found in most carcinomas, being also a pan-carcinoma antigen [30,31]. As with the T antigen, an increased expression of the Tn antigen is related with an enhancement of cancer invasiveness and cellular proliferation and because they are detected on early-stages of cancer development, they have potential for being studied and used as biomarkers [32]. Both T and Tn antigens can be further modified by, for instance, sialylation, which is how the sialyl-Tn (STn) antigen is generated (Figure 1.5).

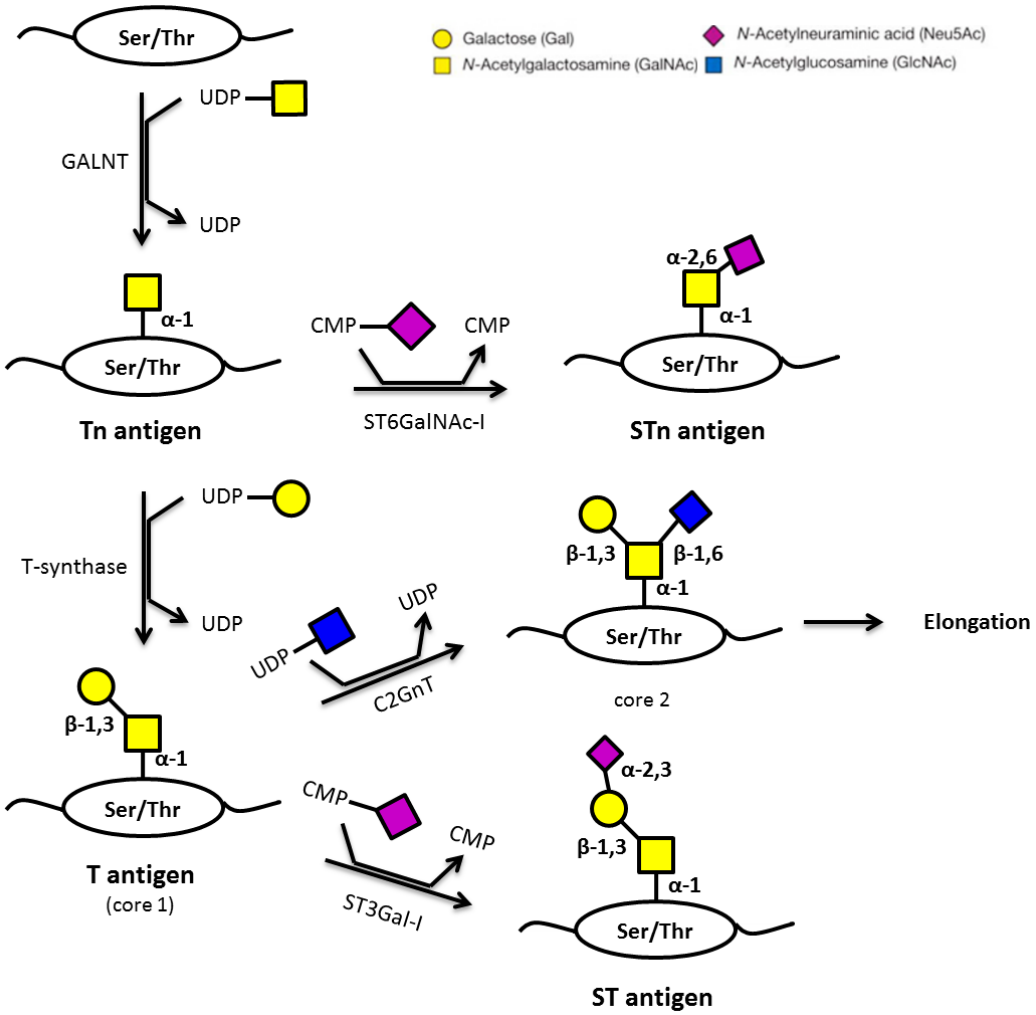

Figure 1.5 – Schematic representation of the biosynthesis of Thomsen-Friedenreich antigens. Adapted from [15,17,61].

1.3.2. Sialyl-Tn Antigen

The addition of a sialic acid residue to the carbon 6 of GalNAc (α2-6 linkage) on the Tn antigen originates the disaccharide known as sialyl-Tn (Neu5Acα2-6GalNAcα1-Ser/Thr), also referred to as CD175s [15,17,33]. Similarly, STn is also a pan-carcinoma antigen: is generally not expressed in normal healthy tissues but is frequently detected in most carcinomas, such as pancreatic, ovarian and lung cancers [7,26,33]. Thus, the expression of this antigen is considered pathologic since it is not a normal biosynthetic precursor, as are the T and Tn antigens. In normal cells, the process of *O*-glycosylation generates elongated and branched *O*-glycans that are often modified by sialylation. However, the sialylation of the Tn antigen blocks the normal extension of the *O*-glycan chain, as the STn structure cannot be further elongated, consequently blocking the synthesis of other glycosidic structures [15,18,26] (see Figure 1.5 in the previous section). Its overexpression results of mechanisms related to ST6GalNAc-I upregulation or the re-localization of this enzyme from the Golgi apparatus to the ER [7,15,25,34–37]**.**

As previously mentioned, the expression of the STn antigen is correlated with increased tumor growth and with the disruption of cell-cell adhesion mechanisms, increasing the processes of migration ad invasion and, consequently, modulating a malignant phenotype and thus being associated with a poor prognosis in cancer patients [7,15,22,33,38,39].

Since STn is expressed early in the process of cancer development and is usually not found in healthy cells, this antigen is considered to be a helpful tumor marker in diagnosis [7,26,33]. Tumor markers can be detected in serum, since it is a simple, sensitive and non-invasive method of diagnosis. However, detection of STn in serum is linked to poor prognosis, as it is related with shedding of cells from tumors into the bloodstream. This is consequently correlated with increased tumor masses found in advanced cancers, thus making the STn antigen more useful as a prognosis than as a diagnosis marker [33].

1.4. Immunology and Immune system

Immunology studies the reactions to foreign substances, ranging from microbes to macromolecules, like proteins and carbohydrates, and the cellular and molecular events that occur after an organism encounters them, a process designated as immune response [40]. The immune system comprises different molecules, cell types, tissues and organs that protect the host by identifying potential threats and defending it against them. The process of host defense relies on the action and cooperation of two types of immunity: the innate immunity, which is not antigen-specific, and the adaptive immunity, which is antigen specific [40–42].

1.4.1. Innate immunity

The innate immunity is the first line of defense of the immune system by providing an immediate (hours) response to a stimulus. It consists of biochemical and cellular defense mechanisms that comprise physical and chemical barriers, phagocytic cells, Natural Killer (NK) cells, the

complement system, cytokines and other inflammation mediators. The mechanisms on this type of immunity show a broad specificity and use a variety of pattern recognition receptors (PRRs) to recognize structures that are common in groups of related microbes, known as pathogen associated molecular patterns (PAMPs). One of these structures is, for instance, the lipopolysaccharide (LPS), a component of the external membrane of the cell wall of Gram-negative bacteria. The process of phagocytosis is one of the main mechanisms of innate immunity, enabling the destruction or neutralization of microbes. This process is also important in the activation of the adaptive immunity, as it will be mentioned further in this chapter [40,43].

1.4.2. Adaptive immunity

The adaptive immunity is the second line of defense of the immune system and is present only in vertebrate organisms. It provides a late (days) but specific response to an antigen, after innate signaling. This response is mediated by the action of T and B lymphocytes and can then generate immunological memory and increase in magnitude with repeated exposures to a particular antigen. The antigen can be recognized, in its native conformation, by the receptors of B lymphocytes, whereas the recognition by T lymphocytes occurs via the presentation of antigen-derived peptide fragments in the major histocompatibility complex (MHC) context. The recognition process will then induce the activation and differentiation of B cells, which will produce specific antibodies against the antigen, a mechanism known as humoral immunity; and of T cells, which will generate effector and regulator cells, a mechanism known as cell-mediated immunity. For the development of adaptive immunity it is required that the antigens are captured and presented to lymphocytes, a process performed by antigen presenting cells (APCs). Dendritic cells (DCs) are professional APCs and have a central role in antigen capture and in the induction of T lymphocyte responses against protein antigens, thus being considered an important association between the innate and adaptive immunities [40,43,44]. Due to the significance of these cells in the context of this thesis, they will be further explored in the next section.

1.4.3. Dendritic cells

Dendritic cells comprise a heterogeneous population of cells located on areas of the body that are in contact with the outside environment, such as the skin and respiratory and digestive tracts. On non-lymphoid peripheral tissues, DCs are usually on an immature state, being highly reactive to signals that result of infectious or inflammatory processes. The presence of, for instance, LPS, or of inflammatory cytokines like tumor necrosis factor-alpha (TNF-α), induces on these cells a phenotypical and functional process of maturation. This process results in the production of cytokines, chemokines and on the expression of surface molecules such as co-stimulatory molecules (CD80 and CD86) and molecules of the major histocompatibility complex, both necessary for an efficient presentation of the antigens to T cells. During the maturation process, DCs enter on afferent lymphatic vessels and migrate to the lymphatic nodes, where they will interact with naïve T lymphocytes (that never

contacted with antigens), instruct them by presenting the processed antigen-derived peptides and thus initiate the generation of a specific immunological response [40,45].

Immature DCs are highly endocytic and process endogenous and exogenous antigens, but have a low stimulatory capacity. The mechanisms involved in the uptake of antigens include phagocytosis, through membrane receptors, endocytosis, through Fc and C-type lectin receptors and pinocytosis [45]. Particularly, immature DCs can be obtained *in vitro* by culturing monocytes with granulocyte macrophage colony-stimulating factor (GM-CSF) and interleukin-4 (IL-4) cytokines, thus promoting their differentiation into dendritic cells, being then usually known as monocyte-derived dendritic cells (moDCs) [46,47].

1.4.3.1. Antigen processing and maturation

The mechanism of antigen processing and presentation of antigen-derived peptides is mediated by MHC molecules. In humans, they are referred to as human leukocyte antigen (HLA). These molecules can be of two classes, I and II, designated then MHC-I and MHC-II, respectively. MHC-I is expressed in all nucleated cells and processes and presents endogenous antigens, whereas MHC-II processes and presents exogenous antigens, being expressed by APCs. The activation of T cells occurs through the binding of MHC molecules to the CD28 receptor that is present in those cells [48]. As previously mentioned, DCs possess the ability to capture and process antigens, producing antigenic peptides which will then bind to MHC-I and MHC-II molecules and that will be transported to the cell surface and presented for cellular recognition by T cells. The antigen processing by dendritic cells occurs mainly through two pathways: one cytosolic (endogenous) and one endocytic (exogenous), through which the antigenic peptides are bound to MHC-I and MHC-II molecules, respectively [45,48]**.**

1.4.3.2. Antigen presentation through the cytosolic pathway: MHC-I

In this pathway, the intracellular antigens are bound to molecules of the MHC-I complex and later presented at the cell surface and recognized by $CD8^+$ T cytotoxic (T_c) cells. Therefore, proteins of endogenous origin, from the self or of pathogenic origin are ubiquitinated and degraded in peptides by the proteasome. The resulting peptides are then transported to the ER, where they are bound to the newly-synthetized MHC-I molecules. Then the MHC-I/peptide complexes are transported to the cytoplasmic membrane through the *trans*-Golgi network [45,48].

1.4.3.3. Antigen presentation through the endocytic pathway: MHC-II

In this pathway, the antigens that were captured from the extracellular environment by endocytosis, phagocytosis or pinocytosis are bound to molecules of the MHC-II complex and later presented at the cell surface and recognized by $CD4^+$ T helper (T_h) cells. After being captured, the antigens are processed in endosomes that, in turn, maturate and fuse with lysosomes, where enzymatic cleavage occurs. The antigenic peptides formed are bound to MHC-II molecules and the MHC-II/peptide complexes are transported in exocytic vesicles to the cell surface, where they will be presented to $CD4^+$ lymphocytes. Later, the MHC-II molecules can be recycled [45,48].

1.4.3.4. Maturation of dendritic cells and antigen presentation – therapeutic potential

In immature DCs, the MHC-II molecules and degraded peptides accumulate in intracellular vesicles. The maturation process occurs upon the uptake of antigens, throughout the migration of DCs from peripheral tissues to lymphoid organs and is promoted by inflammatory mediators. During this process, dendritic cells undergo functional and phenotypical alterations. They are characterized by a reduction in the uptake machinery, losing the capacity to capture antigens, and acquire the ability to present the processed antigens to naïve T cells and stimulate them. After receiving an inducing stimulus, the pH in the endosomes of DCs decreases, easing the processing of exogenous antigens, which are then bound to MHC-II molecules and the MHC-II/peptide complexes migrate to the cell surface. Besides these functional alterations, mature dendritic cells suffer a reorganization of their cytoskeleton, increase the expression of adhesion molecules, chemokine receptors, MHC-I and MHC-II molecules, co-stimulatory molecules and pro-inflammatory cytokines. Additionally to the activation of T cells, DCs can also activate B cells, thus having a role in humoral immunity [45,46,48–51]. Since DCs have the potential to initiate both cellular and humoral adaptive responses, their use is currently considered in many anti-cancer therapies in order to improve the immune response against a certain antigen [49]. Immunotherapies regarding DCs often rely on the use of DCs obtained from the patient, which are loaded with cancer antigens *ex vivo* and injected back to the patient with the goal of boosting his immune system to fight the cancer cells via expansion of T cells, eliciting a specific immune response towards the tumor [52].

1.4.4. Antibodies

Antibodies (Abs), or immunoglobulins (Igs), are glycosylated proteins, synthetized by B lymphocytes, that have the ability to recognize and bind to antigens (Ags) with high specificity and affinity. Structurally, antibody molecules share the same characteristics, though displaying significant variability in the antigen-binding regions. Antibodies have a symmetrical core structure, approximately "Y" shaped, that is composed of two identical light (L) chains and of two identical heavy (H) chains. Each light chain is covalently attached to a heavy chain by a disulfide bond. The same type of bond connects the two heavy chains to each other. Functionally, there are two main regions, the N-terminal or variable region (V) which is responsible for antigen recognition, and the C-terminal or constant region (C) which mediates the effector properties. The antigen binding site is formed by the variable region of one light chain (V_L) adjacent to the variable region of one heavy chain (V_H). The heavy chains of constant regions interact with cells of the immune system and other effector molecules, mediating the biological functions of antibodies. The V_L and V_H domains have three hypervariable regions each, also called complementary-determining regions (CDRs). Hence, antibodies consist of three functional units: two Fab (Fragment antigen binding) fragments linked through a flexible region to one Fc (Fragment cristallizable) fragment, which can be obtained after enzymatic cleavage (Figure 1.6) [53,54].

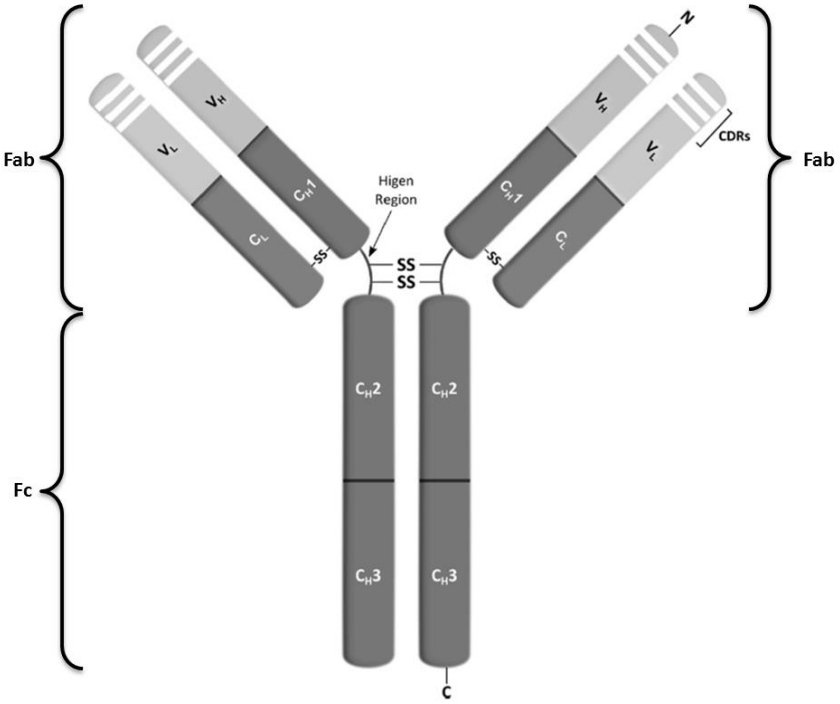

Figure 1.6 – Schematic representation of an immunoglobulin structure (IgG). Adapted from [61].
Abbreviations: C (constant region), V (variable region), L (light chain), H (heavy chain), V_L (variable region of the light chain), V_H (variable region of one heavy chain), CDRs (complementary-determining regions), Fab (fragment antigen binding), Fc (fragment cristallizable).

The differences in the heavy chains of the constant regions of an antibody molecule determine their functional activity. Therefore, antibodies can be divided into five distinct classes or isotypes, named IgA, IgD, IgE, IgG and IgM (Table 1.1). The heavy chains are labelled by the letter of the Greek alphabet that corresponds to the isotype of the antibody: α, δ, ε, γ, and μ, respectively. Also, two types of light chains exist, called κ and λ, being classified by their carboxyl terminal constant regions. The antibody molecule has two κ light chains or two λ light chains [53,54].

Several antibody-mediated effector functions exist, depending on the isotype, besides their ability to specifically bind to target antigens with high affinity. These functions include the neutralization of microbes or toxic microbial products, the opsonization of antigens for enhanced phagocytosis and immediate hypersensitivity [53,56]. Antibodies can also mediate cell death processes by redirecting immune effector cells, a mechanism known as antibody-dependent cell-mediated cytotoxicity (ADCC), or by activating the complement system (known as complement-dependent cytotoxicity, CDC) and even through blocking actions on specific molecules and soluble mediators. The mechanisms of ADCC and CDC depend on the engagement of Fc receptors that are expressed on effector immune cells or of the Fc region of antibodies, respectively, in order to endorse targeted cell death. All of these characteristics are important mechanisms of action and contributed for the application of antibodies in many therapeutic fields, one of them being anti-cancer therapies [53,54,57,58].

Table 1.1 – Human antibody isotype characteristics. Adapted from [53–55].

Isotype	Subtypes	Heavy chain	Light chain	Molecular weight (kDa)	Serum half-life (days)	General structure	Functions
IgA	IgA_1 IgA_2	α1 α2	λ or κ	150-600	5-7	Monomer, dimer, trimer	Mucosal immunity
IgD	None	δ	λ or κ	150	2-8	Monomer	Naïve B cell antigen receptor
IgE	None	ε	λ or κ	190	1-5	Monomer	Immediate hypersensitivity, protection against parasite worms
IgG	IgG_1 IgG_2 IgG_3 IgG_4	γ1 γ2 γ3 γ4	λ or κ	150	21-24	Monomer	Opsonization, complement activation, antibody-dependent cell-mediated cytotoxicity, neonatal immunity
IgM	None	μ	λ or κ	750-900	5-10	Pentamer, Hexamer	Naïve B cell antigen receptor, complement activation

1.4.4.1. Antibody production *in vitro*: Hybridoma Technology

Köhler and Milstein originally established, in 1975, a technique that allowed the generation of monoclonal antibodies (mAbs) with high specificity and affinity [59]. Nowadays, hybridoma technology, as it is known, is still a widely used tool for the production of monoclonal antibodies against antigens of interest. This technology is based on the immortalization of the production of antibodies by fusing B lymphocytes from the spleen of immunized mice with myeloma cells. Initially, the immune system of the mouse (or other suitable host) is stimulated with the antigen of interest, which can be mixed with adjuvants in order to boost the immune response and therefore enhance the antibody production. Then, antibody-secreting B lymphocytes are isolated from the mouse's spleen and fused with myeloma cells, by using polyethylene glycol (PEG). The myeloma cell line used is immortalized, does not secrete immunoglobulins and has the gene that codes hypoxanthine-guanine-phosphoribosyltransferase (HGPRT) absent. The cells are cultured in hypoxanthine-aminopterin-thymidine (HAT) selection medium. In this medium, only correctly fused cells (hybridoma cells) are able to survive and proliferate, as they inherited the immortality from the myeloma cells and the selective resistance of the B lymphocytes to survive in a medium with aminopterin. Afterwards, the screening for antibody-producing cells with the desired reactivity is performed by analyzing the culture supernatant. The hybridoma cells that are considered as positive in the screening process (this is, the

hybridoma cells that have antibodies in their culture supernatants that show reactivity towards the antigen of interest) are then cloned and grown so that monoclonal antibodies can in that way be produced, continuously, *in vitro* (Figure 1.7) [60,61].

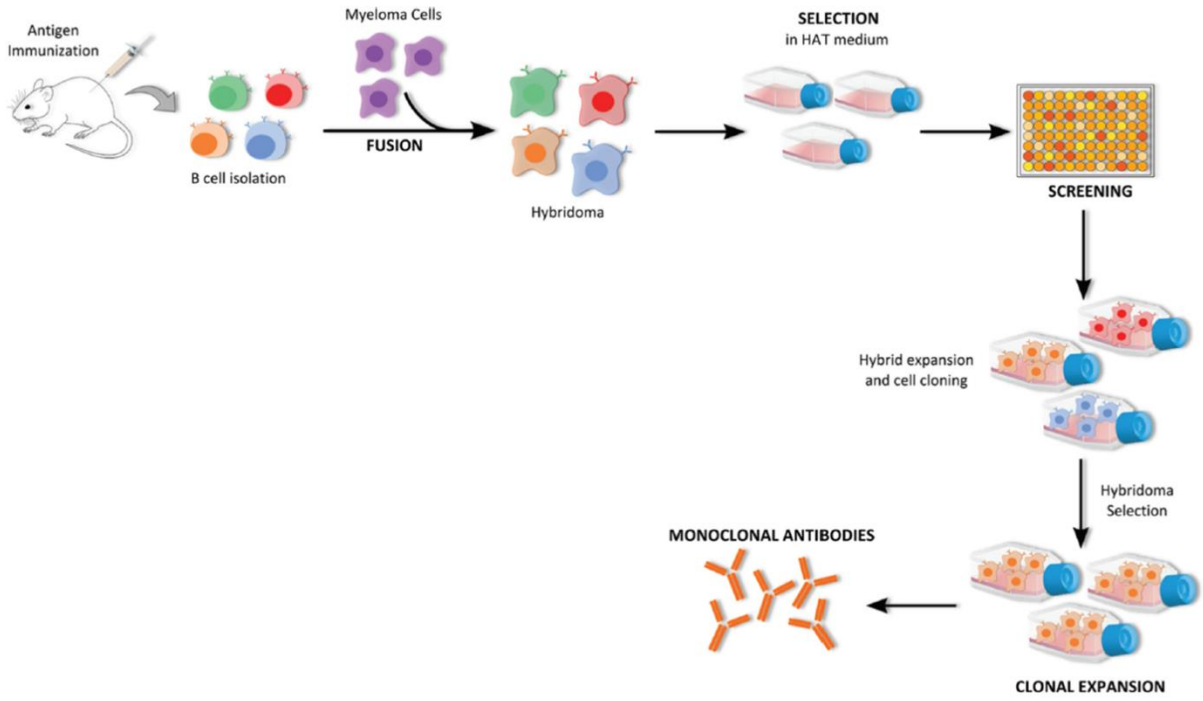

Figure 1.7 – Schematic representation of hybridoma technology [61].

1.4.5. Anti-tumor immunity

An appropriate anti-tumor immune response involves the action of both cellular and humoral immune responses. Using the patient's own immune system for the combat of cancer is becoming more common. This is mainly because the immune system has the potential to destroy specifically cancer cells without offering toxicity to normal tissues, while inducing long-term memory that will allow the prevention of cancer recurrence. Besides having this potential for cancer destruction, is it also known that cancer can suppress the anti-tumor immune responses, leading to cancer cells not being eliminated and continuing to proliferate. This immune suppression is associated with the aberrant expression of TACAs by the cancer cells [33,61,62].

Cytotoxic T cells and NK cells are activated in cellular immune responses in order to eliminate cancer cells by apoptosis. However, as previously mentioned, activation of cytotoxic T cells requires antigen-derived peptides to be presented via MHC-I, which can be a disadvantage on the development of a strong immune response against carbohydrate antigens. Humoral immune responses consist of tumor-specific antibodies, which can then assist on effector functions like antigen neutralization, phagocytosis, ADCC mediated by NK cells and complement-dependent cytotoxicity. Also, as opposite to T cells, B cells can directly recognize carbohydrate antigens through their

receptors, which enables their activation and antibody secretion, a process named T cell-independent B cell activation. T cell-dependent B cell activation, however, requires the involvement of antigen-specific T helper cells. These are activated by APCs via the presentation of antigen-derived peptides through MHC-II. The isotype switching from the initially produced IgM antibodies to high-affinity IgG antibodies is mediated through the secretion of cytokines by the helper T cells. This enables the generation of long-lasting anti-tumor humoral responses, as carbohydrate-specific plasma B cells differentiate into memory B cells and the IgG antibodies produced can bind to the target antigen on cancer cells, signaling them for destruction [33,38].

1.5. Anti-cancer immunotherapy

The main goal of anti-cancer immunotherapy is to neutralize or eliminate cancer cells and/or factors that enable the survival and proliferation of those cells, by using components or mechanisms of the patient's immune system. The interest in this kind of therapy has increased in the last years, mainly due to its therapeutic potential: it relies on adaptive immunity for specific and effective anti-tumor responses and it can establish immunological memory, thus eventually being able to prevent the recurrence of the tumor. It is also an attractive strategy against cancer as it presents less side-effects and toxicity when compared to conventional therapies [33,63]. Examples of immunotherapies currently being applied against cancer are dendritic-cell based vaccines and monoclonal antibodies against TACAs.

Dendritic-cell based vaccines rely on the use of DCs as APCs and as the source of the tumor antigens to be presented to the immune system. Monocytes are collected from the patient and differentiated into DCs *ex vivo*, which are loaded with one or more tumor antigens, being then injected back into the patient. Ideally, this vaccine initiates a local inflammatory response, which then results in the specific activation of T cells [63].

The use of monoclonal antibodies is usually combined with drugs in a mechanism of drug-delivery by which antibodies with specificity towards a certain antigen are conjugated to a drug and transport it to the tumor site, thus making the action of that drug more specific and localized. However, antibodies can also be used as therapy on their own, due to their effector characteristics (mentioned previously in section 1.4.4). Another goal towards the use of monoclonal antibodies is the reversion of the immunologic suppression or tolerance often caused by cancer cells, which prevents the activation of specific responses from T cells [63].

1.6. Introduction to the aim of the thesis

Cancer is currently one of the leading causes of death worldwide with the number of new cases being expected to increase by about 70% in the next two decades [1]. Though several therapies exist and are currently used, such as surgery and radio- and chemotherapy, they tend to fail on the removal of residual cancer cells, and thus on the prevention of recurrence and of metastasis. For this reason, it is important to develop new therapeutic strategies. Immunotherapies boost the patient's own

immune system in order to fight cancer cells and can not only eliminate cancer cells, but also provide immunological memory to prevent recurrence and metastasis. These therapies decrease the adverse effects often observed on conventional cancer therapies, offering in that way an advantage over those.

Over the last decades the role of the immune system and its influence on cancer, as well as several cancer mechanisms, became better understood. In addition, new therapeutic and diagnostic targets were discovered, leading to an increased importance of the relevance of the field of glycobiology in cancer research [7]. In fact, cancer cells exhibit glycosylation-related alterations which are now considered interesting candidates for not only diagnostic but also as therapeutic targets. One example is the sialyl-Tn antigen, which became even more interesting due to its low or absent expression in normal cells, while being overexpressed on cancer tissues. Nonetheless, aberrantly expressed glycans may contribute for cancer cells to escape from immunological surveillance. For instance, it was shown that STn-expressing cancer cells have the ability to induce a tolerogenic phenotype on dendritic cells, impairing their maturation process [64]. These results have highlighted the importance of the STn antigen as a diagnostic marker and potential target for anti-cancer therapies. It also highlights the importance of developing new ways to block the tolerogenic immunomodulatory role of these cancer antigens.

Antibodies have been being broadly used as therapeutic strategies due to their high specificity and possibility of altering the immune system through recognition by immune cells. Additionally, there have been great improvements in the development and optimization of the methods involved in antibody production and engineering, which promoted the introduction of antibodies into clinics [65]. The group has shown that antibodies against STn may override the tolerogenic profile of dendritic cells [64]. Therefore, it was hypothesized that the development of targeted therapies based on antibodies against STn may offer effective means to improve the immune responses against cancer cells, thus being an attractive approach for immunotherapy.

The main aim of this thesis consisted in assessing whether antibodies against STn could improve the anti-tumor immune responses. This general aim comprised two specific aims: 1) development of anti-STn antibodies, in which this work has specifically contributed for the screening of the specificity of antibodies produced by the group using hybridoma technology. In this work the reactivity of hybridoma supernatants towards the STn antigen was assessed, and their characterization was performed (Part A); 2) functional characterization of humanized anti-STn antibodies provided by collaborators. For this purpose, humanized anti-STn monoclonal antibodies provided by collaborators were used in co-cultures of cancer cells with moDCs to block the STn antigen. The STn blockade was then tested if capable of preventing the tolerogenic phenotype imposed on moDCs by STn-expressing cancer cells (Part B). The work plan proposed for this thesis is represented on Figure 1.8.

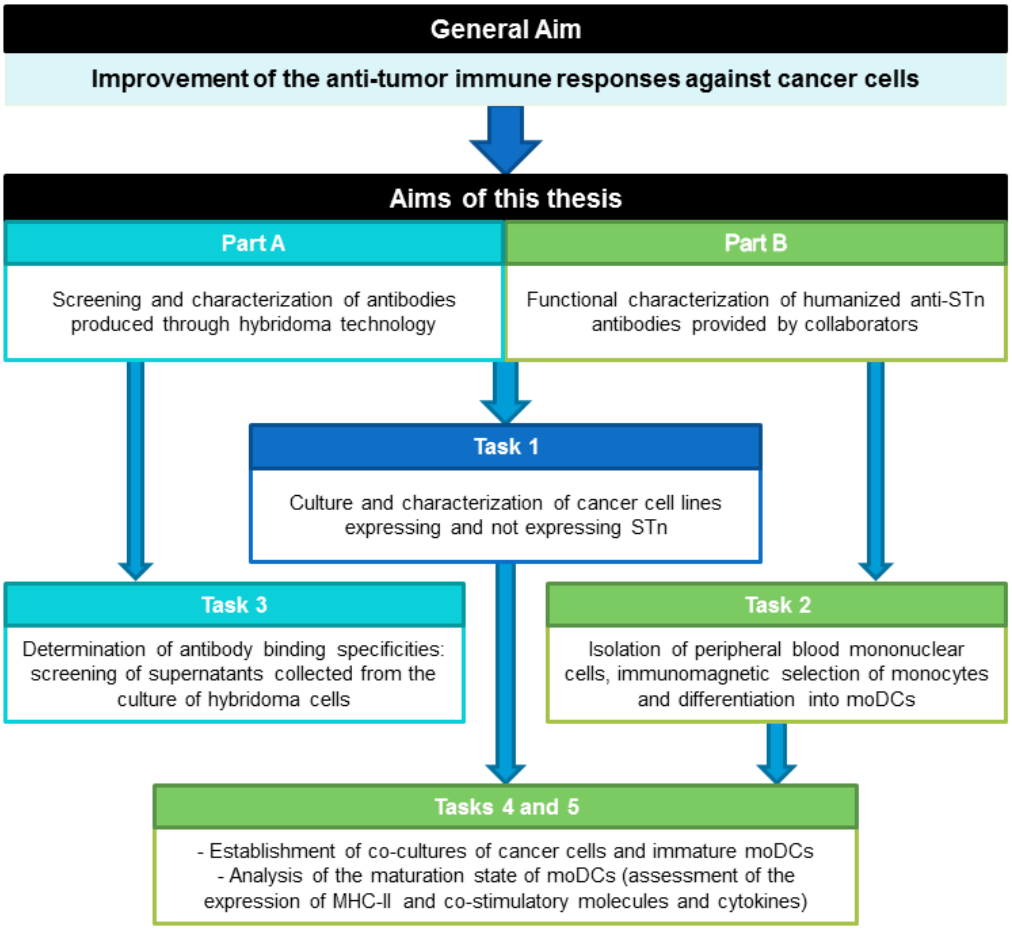

Figure 1.8 – Representation of the work-plan proposed for this thesis.

The results obtained in these tasks and the accomplishment of the proposed aims would then aid in providing insights on the use of antibodies in order to improve anti-tumor responses against cancer cells. This would be accomplished by trying to overcome the immunosuppressive effect of the STn antigen, so that dendritic cells and T cells can be used in the future as a cellular therapy against cancer.

2. MATERIALS AND METHODS

2.1 Cell culture

2.1.1 Culture of breast and bladder cancer cell lines

Several cancer cell lines were used during this thesis, specifically from breast and bladder cancer. The cell lines used were of three variants - the wild type (WT), the empty vector (EV) and the STn positive (STn). The breast cancer cell lines used were MDA-MB-231 WT, MDA-MB-231 STn, MCF-7 WT and MCF-7 STn and the bladder cancer cell lines used were MCR WT, MCR EV and MCR STn.

The cell line MDA-MB-231 was originally established from a pleural effusion of a 51 year old woman with breast adenocarcinoma [66]. The cell line MCF-7 was originally established from a pleural effusion of a 69 year old woman with breast adenocarcinoma [67]. Both of these cell lines were, previously, transfected with the plasmid pRc-CMV on which the complementary DNA (cDNA) of the gene that codes the enzyme ST6GalNAc-I had been inserted, generating the cell lines MDA-MB-231 STn and MCF-7 STn [36]. The cell line MCR was originally established from a subcutaneous metastatic lesion of a 51 year old man diagnosed with grade III transitional cell bladder carcinoma [68]. This cell line was previously transduced with a lentiviral vector, the pLenti6/V5 Directional TOPO in which the cDNA of the human gene that codes the enzyme ST6GalNAc-I had been inserted, generating the cell line MCR STn. Also, the same cell line was transduced with the same lentiviral vector without the cDNA insertion, generating the cell line MCR EV, which is used as a negative control of STn expression [35].

All the cancer cell lines used are adherent and were cultured on T25 and/or T75 culture flasks (Sarstedt) on an incubator (Panasonic) at 37^{o}C, with a humidified atmosphere and 5% CO_2. Both bladder and breast cancer cells were cultivated in complete Dulbecco's Modified Eagle Medium (DMEM) (Gibco) – see composition on Appendix II. All the variants of the MCR and MCF-7 cells were supplemented with 1 µl/ml of recombinant human insulin (Sigma) every two passages. The cells were detached from the culture flasks with Trypsin-EDTA (TE) (Gibco) when a confluence of 80-90% was observed, washed with phosphate buffered saline (PBS) (see composition on Appendix II) by centrifugation (Eppendorf) at 200x*g* for 5 minutes and subcultured according to the desired dilutions for subsequent uses. Besides being subcultured, the cell lines were also stored at -80^{o}C by resuspending the pellet in complete DMEM with 10% (v/v) of dimethyl sulfoxide (DMSO).

2.1.2 Culture of hybridoma cells

As it will be mentioned further in this chapter, and also in chapter 3, part of the work developed involved the screening of supernatants collected from hybridoma cell cultures, as one of the aims of this thesis was the development of anti-STn antibodies. The hybridoma cells that were screened for anti-STn antibody production were obtained through hybridoma technology, a technique commonly used to produce antibodies *in vitro*, as it was mentioned on section 1.4.4.1 of chapter 1. Some steps

involved in this technique had already been performed, namely the steps of mice immunization up to the initial screening of hybridoma cell clones. The mice had been immunized with seven antigens, all containing STn: ovine submaxillary mucin (OSM), a STn-rich natural mucin; membrane extracts of the STn^+ variant of MDA-MB-231, MCF-7 and MCR cell lines; cell lysates of the STn^+ variant of MDA-MB-231 and MCR cell lines; and a mixture of cell lysates of both MDA-MB-231 STn and MCR STn cell lines. Following immunization, the reactivity of the serums was assessed by ELISA and, according to the serum analysis, B cells were isolated from the mice's spleen and fused with Sp2/0 myeloma cells to generate hybridoma cells. These were selected in HAT medium and cultured in microplates, for further screening of the reactivity of the supernatants, selection of the clones of interest, expansion and limiting cloning steps in order to obtain hybridoma cells producing monoclonal antibodies. An overview of the procedure performed is illustrated on Figure 6.3 (Appendix III).

The hybridoma cells were mainly adherent and were maintained on T25 and/or T75 culture flasks (Sarstedt) on an incubator at 37^{o}C, with a humidified atmosphere and 5% CO_2, and cultivated in complete RPMI-1640 medium (Gibco) - see composition on Appendix II. The hybridoma cells were detached from the culture flasks with a scraper whenever their confluence was approximately of 80%. After centrifugation at 200x*g* for 5 minutes, the supernatant was collected and stored for further analysis. The corresponding pellet was resuspended in complete medium for subculture or resuspended in fetal bovine serum (FBS) (Gibco) with 10% (v/v) of DMSO (Sigma) for storage in liquid nitrogen.

2.1.3 Culture of monocytes and monocyte-derived dendritic cells (moDCs)

Monocytes were cultured in 6-well plates (Orange Scientific) on an incubator at 37^{o}C, with a humidified atmosphere and 5% CO_2. In each well, $5x10^6$ cells were cultured in complete RPMI-1640 medium supplemented with 1 µl/ml of recombinant human GM-CSF (Miltenyi Biotec) and 0.75 µl/ml of recombinant human IL-4 (R&D Systems), for 5-7 days, to promote the differentiation of monocytes into dendritic cells. The culture medium was changed on the third day of differentiation, by removing half of the culture medium on each well and adding the equivalent volume of fresh culture medium supplemented with GM-CSF and IL-4.

2.2 Isolation of human peripheral blood mononuclear cells by density gradient centrifugation

Part of the work that was developed, namely the one presented on section 3.3 of chapter 3, required the use of human peripheral blood mononuclear cells (PBMCs) in order to obtain monocytes which would later be differentiated into DCs. These cells were isolated from leuco-platelet concentrates, also known as buffy-coats, and each concentrate was obtained from a single blood donation (approximately 450-500 ml of blood) of healthy volunteers. The buffy-coats were provided and ethically approved by the Portuguese Blood Institute (*Instituto Português do Sangue e da Transplantação*, IPST). The donated blood is submitted to a serological test for the absence/presence of *Treponema pallidum*, Human Immunodeficiency Virus (HIV), hepatitis C and hepatitis B viruses (HCV and HBV, respectively) and human T-lymphotropic virus (HTLV). The blood collection, separation of blood components, serological testing and other necessary processing steps were

performed by IPST. Each buffy-coat comprises approximately 60 ml of leuco-platelet concentrate. In order to reduce inter-sample variability, the buffy-coats were requested to be from male donors aged between 18 years old and 60 years old and from donations of blood collected on the day before it was used for the work of this thesis.

The PBMCs were separated from the remaining erythrocytes, granulocytes and plasma by density gradient centrifugation, using a Biocoll separating solution (Biochrom AG). This solution has a higher density than the lymphocytes and monocytes but is less dense than the erythrocytes and granulocytes. Approximately 30 ml of leuco-platelet concentrate were centrifuged at room temperature (RT), for 10 minutes at 1100x*g*, with no break. After centrifugation, the plasma and leukocytes were collected and transferred into a new tube. The remaining solution was discarded. PBS was added up to 40 ml, in order to dilute the previous mixture of leukocytes and plasma. The Biocoll separating solution, previously stored at room temperature, was added to two new 50 ml falcon tubes (12 ml/tube) and 20 ml of the previously prepared cell suspension was slowly added to each tube, in order not to disturb the Biocoll gradient. The samples were centrifuged at RT for 30 minutes, at 1100x*g*, with no break. The plasma was discarded, the mononuclear cell rings were added to a new tube and PBS was added up to 40 ml. Then, the sample was centrifuged at RT for 10 minutes, at 800x*g*. The supernatant was removed, the pellet was resuspended in PBS and after resuspension, PBS was added up to 40 ml. A sample of the PBMCs suspension was collected and the cells were counted using a Neubauer chamber. The sample was centrifuged at RT for 10 minutes, at 400x*g*. After this step the PBMCs were obtained, which could either be resuspended in culture medium and cultured or further separated and used for several different assays. In this thesis, the isolation of monocytes and their differentiation into dendritic cells was performed, as will be described on the next section.

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

In order to isolate monocytes from PBMCs, these were resuspended in 80 µl of cold beads buffer (see Appendix II for the composition) for each $3x10^7$ cells and 20 µl of human CD14 magnetic microbeads (Miltenyi.Biotec) were added to the suspension in the same proportion. The cell suspension was incubated at 4^oC for 15 to 30 minutes. After the incubation, 2 ml of cold beads buffer were added for each $1x10^7$ cells and the sample was centrifuged at RT for 10 minutes, at 300x*g*. After centrifugation, the pellet was resuspended in 500 µl of cold beads buffer for each $1x10^7$ cells and the suspension was filtered using a 30 µm nylon strainer (BD Biosciences) to remove cell aggregates. The suspension was then added to a LS column (Miltenyi Biotec) (previously engaged on a MidiMACS Separator (Miltenyi Biotec) by using a MACS Multistand (Miltenyi Biotec) and washed with 3 ml of cold beads buffer). Due to the magnetic field being applied in the column, the labelled cells, ($CD14^+$ fraction), were immobilized on the column and the unlabeled cells ($CD14^-$ fraction) were eluted from the column to a 15 ml falcon tube. The column was then washed three times with 3 ml of cold beads buffer. After the washing steps, the column was removed from the action of the magnetic field and 5 ml of cold beads buffer were added to the column. The $CD14^+$ fraction was eluted by insertion of the plunger into the column. A sample of each of the fractions was collected and the cells were counted

using a Neubauer chamber. The samples were centrifuged at RT for 10 minutes, at 400x*g*. Monocytes were cultured as previously described on section 2.1.3, promoting their differentiation into immature moDCs for 5-7 days.

2.4 Magnetic selection of STn positive cells using anti-mouse IgG_1 magnetic beads

Whenever it was observed that the overall percentage of cells expressing STn was reduced, a selection of the cells expressing STn was performed, in order to enrich the cell culture on STn^+ cells. This procedure was performed on MCF-7 STn, MDA-MB-231 STn and MCR STn cell lines.

The cells were detached from the flasks when a confluence of approximately 80-90% was observed and were washed with PBS by centrifugation at 290x*g* for 5 minutes. After centrifugation, the cells were counted, resuspended in 300 µl of PBS (for $5x10^6$ cells) and stained for 30 minutes at RT with 15 µl of anti-STn antibody B72.3 (kindly provided by collaborators). The cells were washed with PBS by centrifugation at 290x*g* for 5 minutes and the cell pellet was resuspended in 120 µl of cold beads buffer (for $5x10^6$ cells); 60 µl of anti-mouse IgG_1 magnetic microbeads (Miltenyi Biotec) (for $5x10^6$ cells) were added and the cells were incubated at 4^oC for 30 minutes. The cells were washed with cold beads buffer by centrifugation at 290x*g* for 5 minutes and the pellet resuspended in 2 ml of cold beads buffer. The suspension was filtered using a 30 µm nylon strainer (BD Biosciences) to remove cell aggregates. A LS column (Miltenyi Biotec) was engaged on a MidiMACS Separator (Miltenyi Biotec) by using a MACS Multistand (Miltenyi Biotec) and washed with 3 ml of cold beads buffer and the suspension was then added to the column. Due to the magnetic field being applied in the column, the labelled cells (STn^+ fraction), were immobilized on the column and the unlabeled cells, (STn^- fraction) were eluted from the column to a 15 ml falcon tube. The column was washed three times with 3 ml of cold beads buffer. After the washing steps, the column was removed from the separator, thus interrupting the magnetic field, and 5 ml of cold beads buffer were added to the column. The STn^+ fraction was eluted by insertion of the plunger into the column. The STn^- fraction was discarded and the STn^+ fraction was centrifuged at 290x*g* for 5 minutes. The pellet was resuspended in 1 ml of cold beads buffer and the previous steps – magnetic separation – were repeated with a new column. Again, the STn^- fraction was discarded and the STn^+ fraction was centrifuged at 290x*g* for 5 minutes. The pellet was resuspended in complete culture medium with two times more antibiotic, to prevent contaminations, and cultured on a T25 flask in the same conditions mentioned on section 2.1.1. The expression of STn was assessed by flow cytometry after 4-5 days in culture.

2.5 Techniques

2.5.1 Flow cytometry

Flow cytometry is a widely used technique that allows the measurement of characteristics of a suspension of particles, such as cells, in a flow system, in a short period of time. The suspension of cells flows rapidly through a beam of light, usually a laser, and ideally each cell will be analyzed individually. Several characteristics of the cell can be analyzed, such as the light scattered at different angles and the fluorescence intensity of one or more fluorophores, which can be used to identify

different subsets of cells on a single suspension. The amount of scattered light that is measured is affected by the size and shape of the cells, this is, by their structural complexity. This light can be scattered at larger angles, thus known as side-scatter (ssc), or at lower angles in a forward direction, thus known as forward-scatter (fsc). Therefore, the side-scatter is mostly influenced by the structural complexity, irregularities and texture of the surface or interior of the cells, while the forward-scatter is mostly influenced by their size and area, and as a result the scattered light will provide information about the physical characteristics of the cells being analyzed. Besides, cytometers can also detect fluorescent light. Fluorescent light is a light of a long wavelength that is emitted when a molecule absorbs high energy light and then emits that energy as photons of a lower energy. This fluorescence can be either derived from natural molecules that the cells possess or via the use of fluorescent chemicals that are used to stain non-fluorescent molecules that are, in this way, undetectable in the cytometer. These chemicals absorb light of specific wavelengths and emit light of different wavelengths and for that reason the cytometer must have the appropriate filters and detectors so that they can be detected correctly, allowing the determination of the median intensity fluorescence (MFI). Sometimes these chemicals can be used to label different cell components directly, or they can be conjugated to other probes, such as antibodies, thus allowing the detection of a certain specific molecule. The MFI obtained is proportional to the amount of antibody that is binding to the cells, which is in turn proportional to the amount of molecules expressed. In this way, a phenotypical analysis of the cells and functional assays can be easily performed [69,70].

In all the procedures of this thesis that required analysis in a flow cytometer the data was collected using an Attune Acoustic Focusing Cytometer (Applied Biosystems). This cytometer is constituted by a blue (488 nm) and a red (638 nm) lasers, which allow the use of up to six different fluorescences during the analysis. Four of them are detected by the blue laser (BL1, green; BL2, orange; BL3, red; BL4, red) and the remaining two are detected by the red laser (RL1, light red; RL2, dark red) [71]. On all experiments, information of at least $1x10^4$ events was acquired with the use of the Attune Cytometric Software (version 2.1). The data were then analyzed with the software FlowJo (version 10) and GraphPad Prism (version 7).

2.5.1.1 General flow cytometry protocol

Most of the data acquisition performed during this thesis was done with the use of flow cytometry. For this reason, a general protocol was used, which will be described next. However, in some experiments, some alterations had to be made and will be mentioned on the respective sections, to prevent an exhaustive repetition of the protocol throughout the chapter.

The desired cancer cells were detached from the culture flasks and washed with PBS by centrifugation at 200x*g* for 5 minutes. The pellet was resuspended in 1 ml of culture medium and the cells were counted. The desired number of cells to be stained and analyzed by flow cytometry ($2x10^5$ to $3x10^5$ cells/condition) was collected into an eppendorf tube. From this point on, the cells could either be directly used for antibody staining or, depending on the assay, treated with sialidase (as referred on the next section). After collection of the desired cellular concentration, 500 µl of PBS were added and the samples were centrifuged at 1500x*g* for 2 minutes. The supernatant was discarded and the

previous step was repeated. The pellets were resuspended in the desired volume of PBS, in order to then have 100 µl of cellular suspension per condition being tested. The primary antibodies were added and the samples were incubated at 4°C for 30 minutes. After the incubation, the cells were washed with 500 µl of PBS by centrifugation at 1500x*g* for 2 minutes. The supernatant was discarded and the pellets were resuspended in 100 µl of PBS. The secondary antibody (anti-mouse Ig conjugated with fluorescein isothiocyanate (FITC)) (Dako) was diluted 1:10 in PBS and 5 µl of this diluted antibody were added. The samples were incubated at RT, in the dark, for 15 minutes and the cells were washed with 500 µl of PBS by centrifugation at 1500x*g* for 2 minutes. The pellets were then resuspended in 1 ml of PBS and the cells were analyzed in the flow cytometer.

2.5.1.2 Sialidase treatment

When treatment with sialidase was performed, the cells were washed with PBS by centrifugation at 200x*g* for 5 minutes. The supernatant was discarded and the pellet was resuspended in 400 µl of sialidase buffer (see Appendix II for the composition), diluted in PBS. These 400 µl of cell suspension were divided into two eppendorf tubes, one labelled as non-treated (NT) and the other labelled as treated (T). For the treated sample, sialidase (from *Clostridium perfringens*, Roche) was added to a final concentration of 100 mU/ml. This enzyme is responsible for the hydrolysis of the α2-6- and α2-3-linked sialic acids of sialylated structures. Both samples were then incubated at 37°C, 5% CO_2 for 90 minutes. After the incubation, the protocol was performed as described above.

2.5.1.3 Characterization of MDA-MB-231, MCF-7 and MCR cell lines – phenotypical analysis of the expression of STn and assessment of the binding of antibodies produced by hybridoma technology

In order to perform most of the work of this thesis it was necessary to evaluate the expression of STn on the cell lines being used. This antigen was expected to be expressed in the STn variants and not on the EV or WT variants. To do this, the flow cytometry protocol was performed as described on section 2.5.1.1. Since the goal was to detect the STn antigen and to determine the percentage of cells expressing it, anti-STn antibodies were used for this detection. Therefore, one of the following mouse anti-STn antibodies was used as primary antibody: TKH2 (15 µl), 3F1 (2.5 µl) or B72.3 (1.5 µl) (all of them kindly provided by collaborators). The remaining steps of the protocol were performed as described.

When assessing the binding of antibodies present in the hybridoma supernatants to cancer cell lines, the same protocol was followed, with the exception of two steps. After the initial distribution of the cell suspension on the tubes for different conditions, these cells were pelleted by centrifugation at 290x*g* for 2 minutes and then were resuspended in 200 µl of each supernatant. In parallel, a positive control condition for STn binding was performed as described above, using one of the three mouse anti-STn antibodies. The remaining steps of the protocol were performed as described. Sialidase treatment of the cells was also performed in parallel to evaluate the specificity of the binding of the antibodies.

2.5.1.4 Characterization of MDA-MB-231 cells – phenotypical analysis of the expression of MHC-II and co-stimulatory molecules; assessment of the binding of humanized anti-STn antibodies and of human $IgG_1κ$ isotype control

The binding of the humanized anti-STn antibodies (provided by collaborators) and of their respective isotype control (IC) was also assessed, as well as the expression of other molecules, such as MHC-II, co-stimulatory molecules and the common leukocyte antigen (usually identified by the CD45 molecule). The flow cytometry protocol was performed as described on section 2.5.1.1. To assess the binding of humanized anti-STn antibodies and isotype control, the cells were treated with sialidase, distributed into eppendorf tubes and pelleted by centrifugation at 1500x*g* for 2 minutes. Then the cells were resuspended in 100 µl of humanized anti-STn antibody and in 100 µl of human $IgG_1κ$ isotype control (SouthernBiotech). In parallel, a positive control condition for STn binding was performed, using one of the three mouse anti-STn antibodies previously mentioned. Two different secondary antibodies were used according to the primary antibody to be targeted: anti-human, to target humanized primary antibodies, or anti-mouse, to target mouse primary antibodies. On the cells stained with humanized anti-STn antibody and human isotype control the secondary antibody used was an anti-human IgG (Fc specific) (Sigma) conjugated with FITC. On the cells stained with mouse anti-STn antibody, the secondary antibody used was an anti-mouse Ig (Fab specific) (Dako) conjugated with FITC. The volumes and dilutions of both secondary antibodies were the same as mentioned in the general protocol. The remaining steps of the protocol were performed as described.

Since all the antibodies used on the evaluation of the expression of MHC-II, CD45 and co-stimulatory molecules were already conjugated with a fluorophore, the protocol was followed as described up to the wash after the incubation with primary antibody, as the step of staining with secondary antibody was not necessary and the cells were immediately resuspended in 1 ml of PBS and analyzed by flow cytometry. To stain these molecules the volumes and antibodies used were: 3 µl of anti-human HLA-DR conjugated with allophycocyanin (APC) (Immunostep, clone GRB-1) for MHC-II; 3 µl of anti-human CD45 conjugated with phycoerythrin (PE) (Immunotools, clone MEM-28) for common leukocyte antigen; 5 µl of anti-human CD80 conjugated with PE (Immunotools, clone MEM-233) and 5 µl of anti-human CD86 conjugated with FITC (Immunotools, clone BU63) for CD80 and CD86 co-stimulatory molecules, respectively.

2.5.1.5 Antibodies titration

Three humanized monoclonal anti-STn antibodies, with known concentrations, were provided by collaborators. In this thesis they were named as mAb1, mAb2 and mAb3. All of them have the $IgG_1κ$ isotype, reason why the isotype control used was also human $IgG_1κ$.

With the aim of determining which antibody concentration would be used for co-culture experiments with STn blockade (as it will be mentioned in the next section), a set of titration experiments had to be performed to test several concentrations. The cell line MDA-MB-231 STn was used and the flow cytometry protocol was performed as earlier described, without sialidase treatment, though resuspending the cell pellets in 100 µl of each antibody concentration. The incubations followed as described, in which the secondary antibody used was an anti-human IgG (Fc specific)

conjugated with FITC. Three titration experiments were performed, each one differing in the concentrations for reasons that will be mentioned in chapter 3. On the first experiment, the three antibodies were tested on a set of eight concentrations: 0.0144, 0.072, 0.360, 1.8, 9, 19.5, 45 and 60 µg/ml. On the second experiment, the three antibodies were tested on a set of five concentrations: 0.8, 1.2, 2, 3.5 and 5.5 µg/ml. On the third experiment, only mAb2 was tested on a set of five concentrations: 0.036, 0.072, 0.1, 0.36 and 0.8 µg/ml.

2.5.1.6 Establishment of co-cultures of cancer cell lines and immature monocyte-derived dendritic cells

Following results previously obtained by the group [64,72], co-cultures of the MDA-MB-231 cell line with immature moDCs were initially established in order to evaluate the maturation state of moDCs before and after culture with cancer cells. The variants MDA-MB-231 WT (as a STn negative control) and MDA-MB-231 STn were used, as well as moDCs on their 6^{th} day of differentiation. The cancer cells were detached from the culture flasks, washed with PBS by centrifugation at 200x*g* for 5 minutes and counted. Then, $3x10^5$ cells/ml were cultured in complete DMEM on the wells of a 12-well plate for 24h, so that they would adhere and stabilize prior to the co-culture. After 24h, the culture medium of the cancer cells was removed and the adhered cells were washed with PBS. In order to observe the effect of the blockade of STn by humanized anti-STn antibodies in the maturation of the moDCs, the cancer cells were incubated with the anti-STn antibodies and isotype control (previously diluted in adhesion buffer - see Appendix II for the composition) at 37°C, 5% CO_2, for 30 minutes. During this thesis two out of the three humanized anti-STn mAbs provided (mAb1 and mAb2) were used. A human $IgG_1κ$ isotype control was also used, always in the same concentration and diluted in the same buffer as the mAb used for the assay. The first humanized anti-STn mAb to be used was mAb2, at a concentration of 0.1 µg/ml. This concentration was then increased to 1.8 µg/ml when using mAb1. The moDCs were removed from the wells and washed with PBS by centrifugation at 200x*g* for 10 minutes. The supernatant was discarded, the pellet of the moDCs was resuspended in adhesion buffer and the cells were counted. Then, the moDCs were added to the wells containing cancer cells (or antibody-coated cancer cells, when using anti-STn mAbs for STn blockade) in a proportion of 5:1 (moDCs:cancer cell). As control and to assess the maturation state before contact with cancer cells, the same number of moDCs was added to an empty well. Additionally, as a positive stimulation control and as a mean to verify that the moDCs were responsive to stimuli, the same number of moDCs was stimulated with 5 µg/ml of LPS (Sigma). The co-cultures were then incubated at 37°C, 5% CO_2 for 2 hours. A schematic representation of these co-cultures can be found in Figure 2.1. After the incubation, the adhesion buffer was collected from the wells and stored at -20°C for posterior cytokine analysis (see section 2.6.4) and the wells were washed with PBS, in order to remove the remaining moDCs that did not adhere to the cancer cells. The co-cultures, containing the cancer cells and the moDCs adhered to them, were removed from the wells by trypsinization and the cells were washed with PBS by centrifugation at 200x*g* for 10 minutes. The pellet was resuspended in PBS and the suspension was divided in two parts: one part was used for antibody staining and analysis by flow cytometry (as it will be mentioned further in this section); the remaining suspension was pelleted by

centrifugation at 200x*g* for 10 minutes and stored at -80°C for posterior genetic expression analysis (see section 2.7.1). The maturation state was assessed by flow cytometry, via staining with antibodies against MHC-II and co-stimulatory molecules, and by ELISA and RT-PCR, via cytokine expression, secretion and quantification.

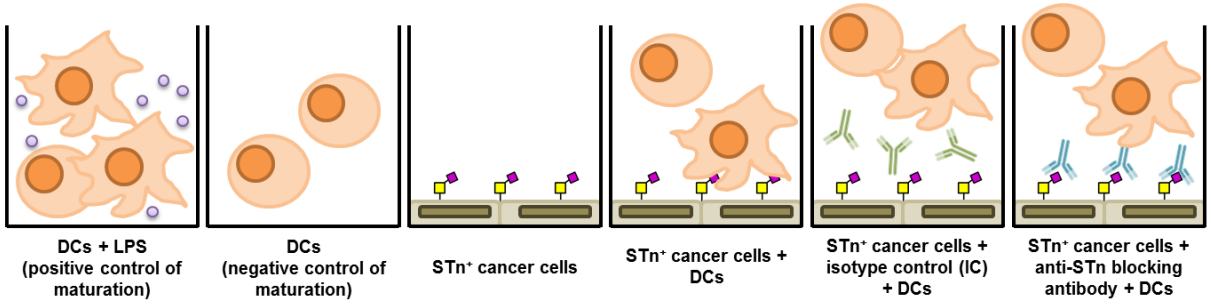

Figure 2.1 – Schematic representation of the conditions of the co-culture experiments performed.

Though the method previously described was initially used, soon adjustments were made in the protocol in order to adapt it to the experiments that needed to be performed according with the results being obtained. One of the first modifications was the co-culture time-points, which were increased from one experiment of 2 hours to two parallel experiments with the cells being co-cultured for 6 and 24 hours. This time alteration however, brought along the need to change the solution in which the cells were co-cultured and in which the antibodies and isotype control were diluted, in order to maintain cell viability during that time. For those reasons, when using co-culture time-points longer than 2 hours, the adhesion buffer was replaced by complete RPMI-1640 medium (with only 5% FBS), supplemented with GM-CSF and IL-4 in the proportion mentioned in section 2.1.3. Despite these alterations, the protocol was followed as previously described. The cells were stained with antibodies and analyzed by flow cytometry, the supernatants were collected and analyzed by ELISA and the genetic expression of cytokines was analyzed by RT-PCR.

For the analysis by flow cytometry, the cells were washed with PBS and pelleted by centrifugation at 200x*g* for 10 minutes. The samples referring to each co-culture condition were stained with 3 µl of anti-human HLA-DR conjugated with APC (Immunostep, clone GRB-1), 3 µl of anti-human CD45 conjugated with PE (Immunotools, clone MEM-28) and 5 µl of anti-human CD86 conjugated with FITC (Immunotools, clone BU63) or 5 µl of anti-human CD80 conjugated with PE (Immunotools, clone MEM-233). For the condition of moDCs that were not in contact with cancer cells, a tube with unstained cells (one for each time-point) was used to adjust the side-scatter and forward-scatter settings prior to data acquisition. The samples were incubated at 4°C, in the dark, for 30 minutes and then washed with PBS by centrifugation at 200x*g* for 10 minutes. When using a time-point of 2 hours, the pellets were resuspended in 1 ml of PBS and the data was acquired shortly after. When using the time-points of 6 and 24 hours, the pellets were resuspended in 300 µl of a 2% paraformaldehyde fixation buffer (Polysciences, Inc.) and incubated at 4°C for at least 20 minutes. Finally, to analyze the samples by flow cytometry, 700 µl of PBS were added to each sample and the

data was acquired shortly after. To prevent fluorescence overlays and interferences due to the use of different fluorochromes in the same tube, a compensation step was also performed in parallel with the desired antibody staining. For this compensation step, the moDCs that were not in contact with cancer cells were distributed in four tubes: one unstained and one for each fluorophore being used (in this case, APC, PE and FITC). The cells were incubated with the antibodies as previously described and the compensation step was performed by following the instructions on the cytometer software.

2.5.1.7 Assessment of the internalization of the humanized anti-STn antibodies

Another experiment performed was the assessment of the internalization of the humanized anti-STn antibodies by cancer cells. In this experiment, the cells were prepared as mentioned in the flow cytometry protocol, without sialidase treatment, and the pellets were resuspended in 100 µl of mAb2 at a concentration of 0.1 µg/ml (diluted in adhesion buffer). The cells were incubated for 15, 30, 60, 120 and 150 minutes, at 37^{o}C, 5% CO_2. In parallel, as a control, another set of cells was incubated in the same conditions, at 4^{o}C. At this temperature, the cellular metabolism is reduced to a minimum and no internalization should occur, so the MFI obtained should be approximately the same on all the time-points. A tube of unstained cells was also prepared to adjust the side and forward scatters, as well as the fluorescence intensity considered negative. All the protocol was performed in adhesion buffer, to simulate the conditions of the co-cultures. After each incubation, the cells were washed with PBS and stained with anti-human IgG-FITC secondary antibody as described. However, this staining was performed at 4^{o}C to guarantee that the cells would not internalize the secondary antibody. After being washed, the cells were then fixed in 300 µl of a 2% paraformaldehyde solution and incubated at 4^{o}C for at least 20 minutes. When the samples were to be analyzed by flow cytometry, 700 µl of PBS were added to the samples and the data was acquired.

2.6 Enzyme-Linked Immunosorbent Assay (ELISA)

The Enzyme-Linked Immunosorbent Assay (ELISA), besides being a biochemical technique widely used nowadays as diagnostic tool in medicine or as quality-control method in several types of industries, is also an analytical tool widely used in biomedical research. This technique can be used for qualitative and quantitative measurements based on enzyme-mediated colorimetric changes, allowing the detection and quantification of specific antibodies or antigens in a sample, such as biological fluids. Some of the most common enzymes are alkaline phosphatase and horseradish peroxidase (HRP). The antigen is immobilized on a 96-well microplate. Then, a specific antibody is used to bind the antigen already immobilized, and this antibody is later detected by a secondary antibody, usually enzyme-conjugated. A color change is achieved by addition of a chromogenic substrate, for example 3,3',5,5'-tetramethylbenzidine (TMB), which indicates the presence of the antigen [73–75]. According to the variations performed in the procedure, different types of ELISA can be used. On this thesis, indirect ELISA and also another variant, named capture ELISA, were used. The capture ELISA follows the same principle of the indirect ELISA, with the exception that this variant is used for the detection of a specific antigen in a sample by initially immobilizing a specific capture antibody on the plate. Ideally, only the antigen of interest will bind to the immobilized antibody, and

then a specific detection antibody will bind to that antigen of interest, thus “capturing” the antigen and allowing its detection [73].

For the work developed in this thesis, namely regarding its first part, an anti-mouse secondary antibody HRP-conjugated was used. TMB was used as chromogenic substrate, which yields a soluble blue colored product when detecting HRP. The color changes to yellow after addition of an acid solution and has a maximum absorbance at 450 nm, detectable on a spectrophotometric plate reader [76]. The plates were read on a SpectraMax 190 Microplate Reader (Molecular Devices) and the data were acquired using the software SoftMax Pro (version 6.4.). The results were analyzed using Microsoft Office Excel and GraphPad Prism (version 7).

2.6.1 Screening of hybridoma clones by indirect ELISA – assessment of antibody production and reactivity against BSM

To assess if the hybridoma clones were producing antibodies with reactivity towards STn, the wells of 96-well plates were coated with 50 µl of bovine submaxillary mucin (BSM) at 10 µg/ml and the plate was incubated overnight at 4^{o}C. Duplicates were always used. The coating solution was removed and 50 µl of sialidase at 25 mU/ml (in sialidase buffer, diluted in PBS) were added to half of the coated wells and 50 µl of PBS were added to the remaining wells. The plate was incubated at 37^{o}C for 90 minutes. The content of the plate was discarded, 100 µl of blocking buffer (see composition on Appendix II) were added to the wells and the plate was incubated for 60 minutes at RT. The blocking buffer was discarded and the wells were washed four times. The washing steps were always performed using 130 µl of washing buffer (0.1% Tween 20; see composition on Appendix II). Then, 50 µl of the samples to analyze – hybridoma supernatants – were added to the corresponding wells. In the initial screenings, these supernatants were analyzed neat, but later a dilution of 1:15 (in washing buffer) was adopted. As positive control, an anti-STn antibody was used out of the three mouse anti-STn antibodies previously referred, and their dilutions (in washing buffer) were optimized to the following: TKH2 (1:500), 3F1 (1:750) and B72.3 (1:3000). Also, 50 µl of washing buffer were added to the wells designed as a control of the secondary antibody binding. The plate was incubated at RT for 90 minutes. The contents on the wells were discarded and the wells were washed three times. The secondary antibody (anti-mouse Ig-HRP, BD Biosciences) was diluted 1:1000 in washing buffer, 50 µl of diluted secondary antibody were added to all the wells and the plate was incubated at RT for 60 minutes. Prior to its use, the substrate, TMB, was incubated in the dark at RT. After the incubation with the secondary antibody, the content of the plate was discarded and the wells were washed four times; 50 µl of TMB were added to all the wells and the plate was incubated at RT, in the dark, for 15 to 30 minutes; 50 µl of HCl at 1M were added to stop the reaction. The optical density was read at 450 nm.

2.6.2 Screening of hybridoma clones by indirect ELISA – assessment the production of antibodies by clones that showed no reactivity against BSM

After the assessment of antibody production with reactivity towards STn, the hybridoma clones whose supernatants did not show reactivity towards STn were evaluated for the production of any other type of antibody. This evaluation was performed by ELISA. The wells were coated with 50 µl of the "negative" hybridoma supernatants. Also, 50 µl, of one "positive" supernatant were used as coating for a positive control of antibody production and 50 µl of complete RPMI-1640 medium were used for the coating of the secondary antibody control wells. Duplicates were always used. The plate was incubated overnight at 4^{o}C. The coating solutions were discarded; the addition of blocking buffer and the remaining steps of the protocol were performed as mentioned in the previous section.

2.6.3 Isotyping assessment by capture ELISA

The wells of a 96-well plate were coated with 100 µl of each of the isotype specific antibodies (goat anti-mouse IgG_1, IgG_{2a}, IgG_{2b}, IgG_3, IgM and IgA, previously diluted 1:1000 in PBS), in duplicate, according to the number of samples to be tested. The plate was incubated for 60 minutes at 37^{o}C. After the incubation, the coating solution was removed and the plate was washed three times with washing buffer (0.05% Tween 20; see composition in Appendix II); 100 µl of the samples to be tested were added to the wells and incubated for 60 minutes at RT, as well as the positive and negative controls. The samples were removed and the plate was washed three times. The peroxidase-labeled goat anti-mouse IgG (Fab specific) (Sigma) antibody was diluted 1:600 in washing buffer, 100 µl of this antibody were added into each well and the plate was incubated at RT for 30 minutes. The non-bound peroxidase-labeled antibody was removed and the plate was washed three times; 100 µl of TMB were added to the wells and the plate was incubated at RT, in the dark, for 20-30 minutes. The development of a blue color indicated a positive result and the reaction was stopped by addition of 50 µl of a 1M solution of HCl. The plate was read at 450 nm.

2.6.4 Assessment of cytokines in co-culture supernatants by capture ELISA

The assessment of cytokine production, secretion and relative quantification was performed by capture ELISA, following the instructions of the ELISA kit for each cytokine tested. The volumes mentioned in the ELISA kits used had been previously optimized by the group and cut to half. Initially, the presence of four cytokines (IL-6, IL-10, IL-12 and TNF-α) was to be assessed. However, due to the existence of issues in the remaining kits or lack of reagents, it was only possible to assess the presence of IL-10 (Human Interleukin-10 ELISA Kit, Immunotools) and IL-12 (Total Human IL-12/IL-23 p40 ELISA Kit, Immunotools). According to the cytokine being assessed, the wells of a 96-well plate were coated with 50 µl of capture antibody, previously diluted 1:100 in PBS. The plate was incubated overnight at 4^{o}C. After the incubation, the capture antibody solution was removed completely and 200 µl of blocking buffer (0.05% Tween 20; see composition in Appendix II) were added to all the wells and the plate was incubated at RT for 60 minutes. The standards were serially diluted in blocking buffer from a range of 4000 pg/ml to 0 pg/ml. The blocking buffer was removed completely and 50 µl of each

standard dilution, as well as 50 µl of each sample were added to the wells. Duplicates were always used. The plate was incubated at RT for two hours. The contents of the wells were discarded and the plate was washed five times. All the washing steps were performed using 200 µl of washing buffer (see composition in Appendix II). The biotinylated detector-antibody was diluted 1:100 in blocking buffer and 50 µl of the diluted detector antibody were added to each well. The plate was incubated at RT for two hours. The contents of the wells were discarded and the plate was washed five times. The poly-HRP-streptavidin conjugate was diluted 1:1000 in blocking buffer; 50 µl of this solution were added to the wells and the plate was incubated for 20 minutes (IL-12) or 30 minutes (IL-10) at RT. The contents of the wells were discarded and the plate was washed five times. Prior to its use, the TMB substrate was incubated at room temperature; 50 µl of TMB were added to each well and the plate was incubated at RT, in the dark, for 40-50 minutes. The reaction was stopped with the addition of 25 µl of a HCl 1M solution. The optical density was read at 450 nm.

2.7 Real-Time Polymerase Chain Reaction (RT-PCR)

Real-Time Polymerase Chain Reaction (RT-PCR) is used to quickly and efficiently analyze and quantify the expression of a certain gene. It allows the combination of the processes of amplification and detection in one single step, as the data is collected during the polymerase chain reaction (PCR) process as it occurs. That is attained with the use of fluorescent chemistries in which the fluorescence intensity is correlated with the concentration of PCR product [77]. For the detection of specific amplification products in the RT-PCR experiments that were performed in this thesis, TaqMan chemistry, also known as fluorogenic 5' nuclease chemistry, was used. It consists on the use of oligonucleotide hydrolysis probes that are dual labelled: a reporter dye is linked to the 5' end of the probe and a non-fluorescent quencher is linked to the 3' end of the probe. While the probe is intact, the fluorescence emitted by the reporter dye is reduced by the quencher. When the target sequence is present, the probe specifically hybridizes to it and, during the extension cycle, is cleaved by the 5' nuclease activity of the taq DNA polymerase, which separates the reporter dye from the quencher and promotes the increase of the signal of the reporter dye, which is detected by the device. At each cycle, new reporter dyes are cleaved from the probes, and this results in an increase of fluorescence intensity that is proportional to the amplicon formed [77,78]. For quantification purposes, a method of relative quantification was used, which describes the change in expression of the target gene relatively to a reference gene, an endogenous control. An ideal control gene should have a stable expression regardless of experimental conditions, such as different tissues or cell types, developmental stage, or sample treatment, thus being a gene that is constitutively expressed. For this endogenous control, two housekeeping genes, *GAPDH* (that codes glyceraldehyde-3-phosphate dehydrogenase) and *ACTB* (that codes β-actin) were used. The relative expression of the genes of interest was analyzed according to the mathematical model that was developed by Livak and Schmittgen, the $2^{-\Delta\Delta CT}$ method, also called the comparative C_T method. The amount of target, when normalized to an endogenous reference control and relative to a calibrator is given by: $2^{-\Delta\Delta CT}$, in which C_T (threshold cycle) corresponds to the cycle number at which the amount of amplified target reaches a fixed threshold, this is, the cycle at which the fluorescence intensity of the amplified target is greater than the

background fluorescence; ΔC_T corresponds to the variation between the C_T value of amplification of the target gene and the C_T value of the amplification of an endogenous control. Thus, $\Delta\Delta C_T$ corresponds to the variation between the ΔC_T value of the sample of interest and the ΔC_T value of the calibrator sample. In this way, the data are presented as the fold change in the gene expression, normalized to an endogenous reference gene, and relatively to a calibrator control [77,79,80].

2.7.1 Analysis, by RT-PCR, of the genetic expression of the cells recovered from the co-culture experiments

In order to analyze the genetic expression of the cells recovered from the co-cultures, a ribonucleic acid (RNA) extraction and its posterior conversion to cDNA were performed.

The GenElute Mammalian Total RNA Miniprep Kit (Sigma Aldrich) was used for the RNA extraction. The lysis mixture was prepared by adding 10 µl of 2-mercaptoethanol (Sigma) per 1 ml of lysis solution. Then, 250 µl of this mixture were added to each tube and the cells were lysed by thorough pipetting. The lysate was then collected and filtered with a filtration column by centrifugation. All the centrifugation steps were performed at 12000x*g* for 2 minutes, at 4^{o}C. The filtration column was discarded and 250 µl of 70% ethanol were added to the filtered lysate and thoroughly mixed by pipetting. The previous mixture was then added onto a binding column and RNA was bound to the column by centrifugation. The flow-through liquid was discarded. The column, now with the bound RNA, was washed with 250 µl of wash solution 1 by centrifugation and the flow-through liquid was discarded. The DNase I (Quiagen) was prepared by mixing 10 µl of DNase I with 70 µl of digest buffer by inversion, for each sample tube. These 80 µl were added directly onto the filter in the binding column and incubated at RT for 15 minutes. After the incubation, 250 µl of wash solution 1 were added to the column and the column was washed by centrifugation. The flow-through liquid was discarded, 500 µl of wash solution 2 were added into the column and the column was washed by centrifugation. The flow-through liquid was discarded, a second volume of 500 µl of wash solution 2 was added into the column and the column was washed by centrifugation. The flow-through liquid was discarded and the remaining ethanol was removed from the column by centrifugation of the dry column. The flow-through liquid and the collection tube were discarded and the column was placed on a new collection tube. The RNA was eluted from the column by addition of 55 µl of elution solution into the binding column and centrifugation. The flow-through liquid was recovered and added a second time into the binding column and the column was centrifuged. The binding column was discarded and the purified RNA was then recovered in the flow-through liquid.

The conversion of RNA to cDNA performed was based on the strategy that random primers hybridize to several regions of RNA, thus allowing its subsequent transcription via the action of the reverse transcriptase enzyme. The RNA was reverse transcribed to cDNA by using the High-Capacity cDNA Transcription Kit (Applied Biosystems). To do this, 50 µl of the purified RNA were added to a PCR tube. The mix for the conversion was prepared by adding 10 µl of buffer 10x, 10 µl of random primers 10x, 4 µl of 100 mM deoxynucleoside triphosphate (dNTPs) mixture, 7.5 µl of reverse transcriptase enzyme and 18.5 µl RNase free H_2O (Nzytech), to a final volume of 50 µl of mix per sample. This mix was added to the RNA samples on a proportion of 1:1. The cDNA synthesis was

performed on a thermocycler (MJ Research), according to the program described in Table 2.1. After the conversion reaction, the cDNA samples were stored at -20^{o}C until further use.

Table 2.1 – Program used in the cDNA synthesis reaction.

	Step 1	**Step 2**	**Step 3**	**Step 4**
Temperature (^{o}C)	25	37	85	4
Time	10 min	120 min	5 sec	∞

For the RT-PCR experiments, each PCR reaction was performed on a volume of 10 µl. The samples were prepared by adding, in duplicate, 2 µl of probe (β-actin and GAPDH, for endogenous controls; IL-6, IL-10, IL-12 and TNF-α, for the genes of interest) (Applied Biosystems), per tube, diluted 1:4, 3 µl of cDNA and 5 µl of TaqMan Fast Universal PCR Master Mix 2x (Applied Biosystems). The reactions were performed on a Rotor-Gene 6000 Series (Corbett) according to Table 2.2.

Table 2.2 – Reaction conditions used in the RT-PCR experiments.

	Step 1 (1 cycle)	**Step 2 (40-50 cycles)**	
Temperature (^{o}C)	95	95	60
Time	20 seconds	3 seconds	30 seconds

The C_T was determined by analysis of the curves using the Rotor-Gene 6000 series software (version 1.7). Microsoft Office Excel was used to perform the following analysis and calculations needed for applying the comparative C_T method. The relative mRNA levels were normalized against the arithmetic mean of the expression of the *GAPDH* and *ACTB* genes and calculated with the equation $2^{-\Delta CT}x1000$, which allows to infer the number of messenger RNA (mRNA) molecules of each gene of interest per 1000 molecules of the endogenous controls. The relative quantification was performed by applying the comparative C_T method as described above and results were analyzed using GraphPad Prism (version 7).

2.8 Statistical analysis

The statistical analysis of the experimental results obtained was performed using the software GraphPad Prism 7. The data were statistically analyzed with the t-Student test and a value of $p<0.05$ was considered to be statistically significant (“ * “ means that the p-value was inferior than 0.05, “ ** ” means that the p-value was inferior than 0.01 and “ *** “ means that the p-value was inferior to 0.0001).

3. RESULTS AND DISCUSSION

The main goals of this thesis comprised the development/screening and characterization of anti-STn antibodies, either developed by the group or provided by collaborators. For this reason, and since the whole process of antibody production is quite extensive and was already in progress when this thesis started, the results presented in this chapter are divided in two main parts: the development and screening of anti-STn antibodies produced using hybridoma technology (here referred as Part A); and the characterization of humanized anti-STn monoclonal antibodies produced by a biotechnology company (here referred as Part B). Another part of the work, common to both parts A and B, involved the phenotypical characterization of cancer cell lines. Part A consisted mostly on the screening of hybridoma supernatants to assess their antibody production capacity and the reactivity of those antibodies towards the STn antigen, as well as their specificity. An initial characterization of the antibodies was also performed. Part B consisted on a functional characterization of humanized anti-STn antibodies provided by collaborators. The group previously reported that the STn antigen induced a tolerogenic phenotype on dendritic cells. DCs, when in contact with cancer lines expressing the STn antigen, showed a less mature profile and reduced ability to activate T cells [64]. Thus, the second part of this thesis involved testing the efficiency of the humanized anti-STn antibodies on the blockade of the STn antigen on cancer cell lines and evaluation of whether that blockade would reestablish the mature phenotype of DCs.

3.1. Phenotypical characterization of cancer cell lines by flow cytometry

To perform the procedures needed on both part A and part B of this thesis, different cancer cell lines were initially cultured, as it was previously mentioned in chapter 2. A phenotypical characterization was performed by flow cytometry, with and/or without sialidase treatment. In this characterization, the focus was mostly on evaluation of the expression of STn, the antigen of interest, both in STn^+ and WT/EV (STn^-) cell lines. As expected, it was observed that all the WT/EV variants of the cell lines were not expressing this antigen. To guarantee that the cells maintained the phenotype of interest, this is, that the expression of STn was maintained, this procedure was performed often. This allowed to observe that, of the three STn^+ cell lines that were being cultured (MDA-MB-231 STn, MCF-7 STn and MCR STn), only the cell line MDA-MB-231 STn showed to have approximately 70-85% of the cells expressing STn in a stable way throughout several passages. On the contrary, the percentage of cells expressing STn on the MCF-7 STn cell line rapidly decreased. Therefore, it was necessary to perform an enrichment procedure to increase the STn^+ population in this cell line. However, using flow cytometry, it was observed that this cell line had almost no cells expressing STn and, because of this, the cell line was discarded and further procedures were performed using only the MDA-MB-231 and MCR cell lines, as well as their variants. The MCR STn cell line showed a stable expression on 70-80% of the cells for 2-3 passages, yet it dropped to approximately 30-50% after 5-6 passages. The observed loss of STn expression on MCF-7 STn and MCR STn cell lines and the

stability of the expression on the MDA-MB-231 STn cell line has already been reported before by the group [81]. However, in this thesis it was observed that the loss or decrease in STn expression occurred in the early passages of these cell lines. It was also previously reported that constitutive expression of ST6GalNAc-I in MCR cells resulted in a down-regulation of several genes involved in different mechanisms of DNA repair and chromosomal segregation [82]. This is possibly why the cells show a decrease in the expression of STn, since the transfection/transduction process causes genetic instability and for that reason the cells tend to seek stability by losing the inserted plasmid throughout their growth and proliferation process. Since that instability was observed on both MCF-7 STn and MCR STn cell lines, the work of this thesis, which will be presented further in this chapter, was performed using mainly MDA-MB-231 WT and MDA-MB-231 STn cells.

Besides the assessment of STn expression, the expression of MHC-II molecules via HLA-DR staining, of CD45, common leukocyte antigen marker, and of co-stimulatory molecules, CD80 and CD86 was also assessed, by flow cytometry, on the MDA-MB-231 STn cell line. This phenotypical evaluation, though expected to be negative, was performed to assure that, when analyzing co-cultured cells, it would be possible to discriminate cancer cells from DCs, based on these markers. The binding capacity of the isotype control to be used on those co-culture assays was also assessed. These results are shown on Figure 3.1.

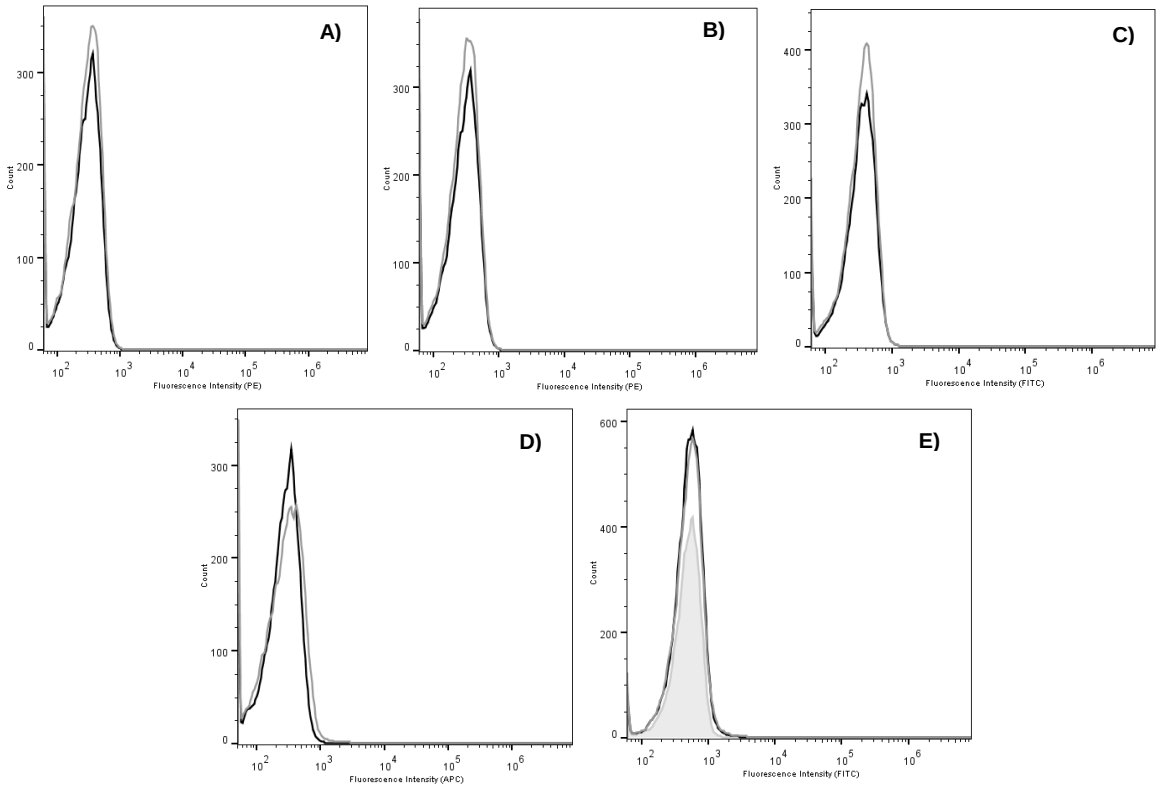

Figure 3.1 - Phenotypical analysis performed, by flow cytometry, on the MDA-MB-231 STn cells. On all histograms, the *yy* axis represents the number of cells and the *xx* axis represents the fluorescence intensity of each fluorophore used. On figures A-D, the black line represents the negative control (unstained cells) and the grey line represents the staining of anti-CD80-PE (A), anti-CD45-PE (B), anti-CD86-FITC (C) and anti-HLA-DR-APC (D). On figure E, the negative control (cells stained only with the secondary antibody) is represented by the grey filled histogram, as well as the staining of human $IgG_1\kappa$ isotype control in cells treated with sialidase (grey line) and in cells non-treated with sialidase (black line).

It was observed, as expected, that the MDA-MB-231 STn cell line does not express CD45, CD80, CD86 or HLA-DR [83], which means that, when these cells are co-cultured with dendritic cells, all the staining obtained for these molecules will be due only to the DCs and that the cancer cells will not have contribution in that staining. Also, it was observed that the isotype control does not bind to the cancer cells, either with or without previous treatment with sialidase, which was also expected [84].

3.2. Part A: Development and screening of anti-STn antibodies using hybridoma technology

As mentioned in the beginning of this chapter, this part of the work consisted mostly on the screening of hybridoma supernatants and analysis of their reactivity towards STn; this was performed by ELISA, using BSM as target-antigen, and by flow cytometry, using cells modified to express STn and their negative controls.

3.2.1. Analysis of the reactivity of the hybridoma supernatants by indirect ELISA and flow cytometry

The hybridoma technology was used in order to produce anti-STn antibodies. Work regarding mice immunization, serum screening and fusion of B cells with myeloma cells to generate hybridoma cells had already been performed, as well as an initial screening of the culture supernatants of the hybridoma cells obtained. BSM was used as target for the screening of the supernatants collected from the hybridoma cell cultures by ELISA, since it has a STn content of approximately 50% [85]. BSM was either in its native form or treated with sialidase. The enzyme used cleaves α2-3- and α2-6-linked sialic acids. Since STn is a truncated *O*-glycan containing a sialic acid α2-6-linked, this treatment allowed the assessment of the presence of antibodies with sialic acid-dependent binding. When antibodies that showed this dependent binding were present in the supernatants being tested, a reduction of optical density would be obtained for the samples tested after the BSM had been previously treated with sialidase. This strategy was used in order to direct the selection of hybridoma clones towards the ones that were producing antibodies that would bind specifically to sialic acids, since the main target was the STn antigen.

From the seven antigens used to immunize the mice (see section 2.1.2 of chapter 2), only OSM-derived hybridoma cells seemed to produce antibodies with reactivity towards BSM and to simultaneously present sialic acid-dependent binding. The fact that OSM was the only antigen that later originated antibody-producing hybridoma cells with the desired reactivity may be due to its high STn content, of approximately 90% [86], and probably much higher than the one found in cell lysates or membrane extracts. Though the production of antibodies with that reactivity was assessed, a decrease in the production of these antibodies was observed in some of the initial selected hybridoma clones, which eventually reached a point of no antibody production. Since hybridoma cells are generated from the fusion of two different types of cell, they are frequently unstable, which could explain this loss of antibody production during cell culture. This is probably related with their genomic instability, as reported before [87]. Taking that into account, the work proceeded with only one of the hybridoma clones, derived from immunization with OSM, here named 2C5. When testing the 2C5

supernatant by flow cytometry on STn^+ cells treated or not with sialidase it was observed that the antibodies present in the supernatant showed an increased staining of the cells treated with sialidase (that had their sialic acids removed), as seen in Figure 3.2, B)). A pattern similar to the positive control, an anti-STn antibody (3F1), was expected, in which a decreased binding on sialidase-treated cells was observed (Figure 3.2 A) and 3.3 A)). For this reason, it was hypothesized that this hybridoma clone was probably producing a mixture of antibodies that bound either to structures that were exposed after treatment with sialidase, such as T and Tn antigens, or to non-sialylated structures. Nevertheless, this hybridoma clone was still at an initial stage of cloning and therefore prone to produce antibodies with different specificities.

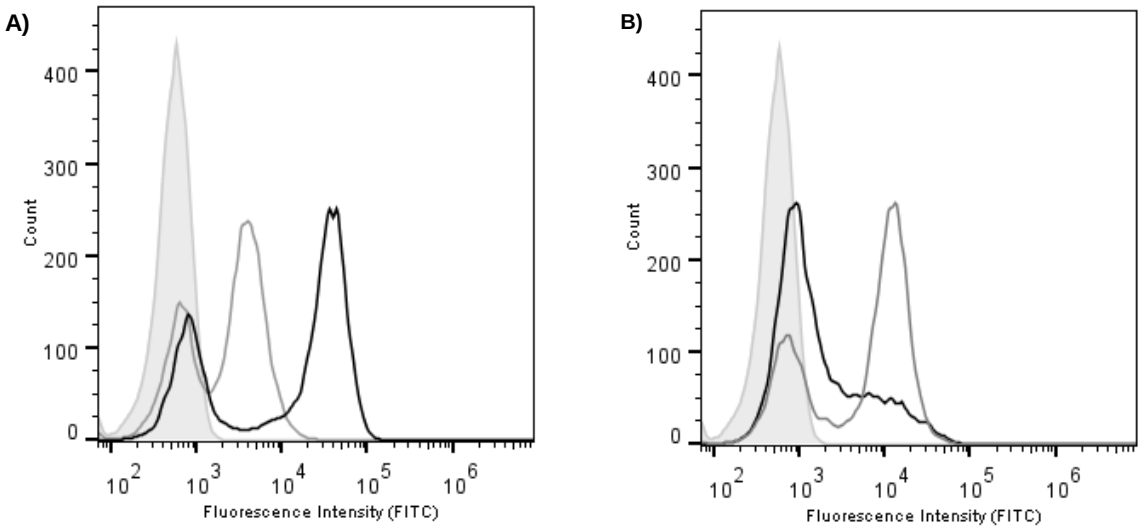

Figure 3.2 – Assessment, by flow cytometry, of the binding of the supernatant from the hybridoma clone 2C5 to the MDA-MB-231 STn cells. On the histograms, the *yy* axis represents the number of cells and the *xx* axis represents the fluorescence intensity of FITC. The grey filled histogram represents the negative control (cells stained only with the secondary antibody), the black line represents the binding to cells non-treated with sialidase and the grey line represents the binding to cells treated with sialidase. A) Positive control staining with an anti-STn antibody (3F1). B) Staining obtained when using the supernatant of the hybridoma clone 2C5.

From these results, the work proceeded to a first limiting cloning of this 2C5 hybridoma clone, from which 34 clones were obtained, with only one of them showing no reactivity towards BSM when tested by ELISA. The reactivity of these clones was also assessed using flow cytometry, and two different staining patterns were obtained. For some clones, a pattern similar to the one obtained for the initial 2C5 clone was observed (Figure 3.3, B)), and others had a similar staining when compared to the anti-STn positive control (Figure 3.3, A) and B)).

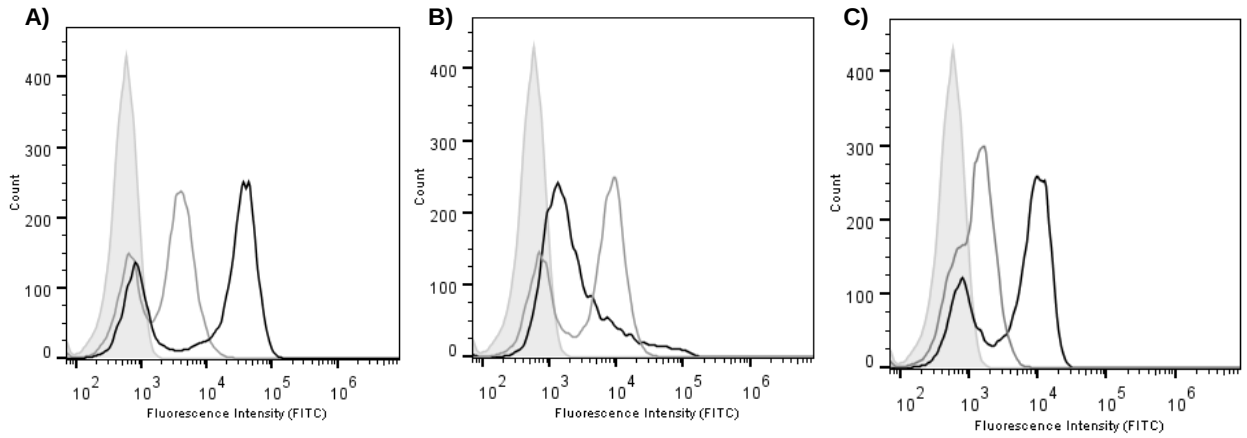

Figure 3.3 – Assessment, by flow cytometry, of the binding of the supernatants from the hybridoma clones 2C5/1D6 and 2C5/2C4 to the MDA-MB-231 STn cells. On the histograms, the *yy* axis represents the number of cells and the *xx* axis represents the fluorescence intensity of FITC. The grey filled histogram represents the negative control (cells stained only with the secondary antibody), the black line represents the binding to cells non-treated with sialidase and the grey line represents the binding to cells treated with sialidase. A) Positive control staining with an anti-STn antibody (3F1). B) Staining obtained when using the supernatant from the hybridoma clone 2C5/1D6). C) Staining obtained when using the supernatant from the hybridoma clone 2C5/2C4).

Given this, 3 clones were selected that would represent the different staining patterns obtained: 1D6 (2C5/1D6), 2B1 (2C5/2B1) and 2C4 (2C5/2C4). For these, a second limiting cloning was performed. From this, clone 2C5/1D6 originated 38 positive clones; clone 2C5/2B1 originated 58 clones (52 positive and 6 negative); and clone 2C5/2C4 originated 23 clones (1 positive and 22 negative). The reactivity of these clones was also assessed by ELISA. In order to select only the best six clones from the positive ones that resulted of the second limiting cloning, they were ordered from highest to lowest by the difference in OD obtained between sialidase-treated and non-treated results (OD at 450 nm obtained when testing the supernatant against sialidase-treated BSM and OD at 450 nm obtained when testing the supernatant against non-treated BSM). This is exemplified with one of the clones on Figure 3.4. The selected final six clones, with the exception of the only positive clone from clone 2C5/2C4, were the ones that presented the higher difference: two from clone 2C5/2B1, here named 1C3 (2C5/2B1/1C3) and 1C4 (2C5/2B1/1C4); and three from clone 2C5/1D6, here named N (2C5/1D6/N), H (2C5/1D6/H) and DD (2C5/1D6/DD).

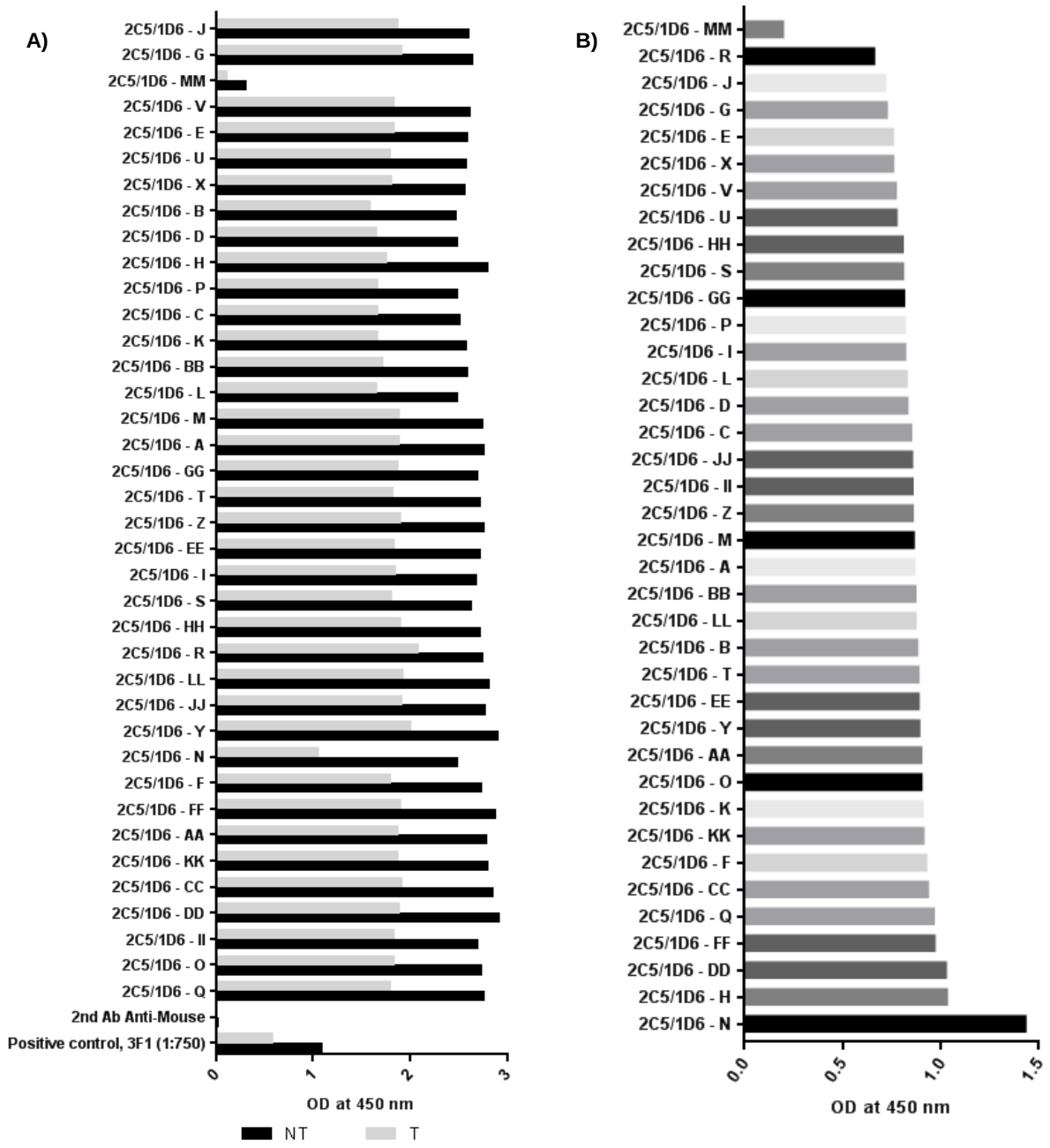

Figure 3.4 - ELISA results obtained for the clones resulting from the second limiting cloning of the 2C5/1D6 hybridoma clone. A) ODs at 450 nm obtained for all the clones, for the negative control (BSM with secondary antibody) and for the STn-positive control (3F1 bound to BSM). The ODs obtained for non-treated BSM are represented in black and the ODs obtained for sialidase-treated BSM are represented in grey. B) Method used to select the best clones: difference of the ODs obtained for non-treated and sialidase treated BSM for all the clones, ordered from highest (down) to lowest (up).

Several ELISA assays were performed to assess the titer of the selected clones. However, it was observed that after some time in culture, five of the six clones were not showing reactivity towards BSM. A difference in the OD at 450 nm between the non-treated BSM and the sialidase-treated BSM was observed only in the clone 2C5/1D6/N. On the contrary, for the other five clones similar ODs at

450 nm were observed for both non-treated and treated BSM (Figure 3.5). Also, hybridoma cells of these five clones seemed to lose the antibody production after being thawed, which led to the hypothesis that besides the genetic instability already mentioned earlier in this chapter, the freezing process could also be contributing to the loss of this ability.

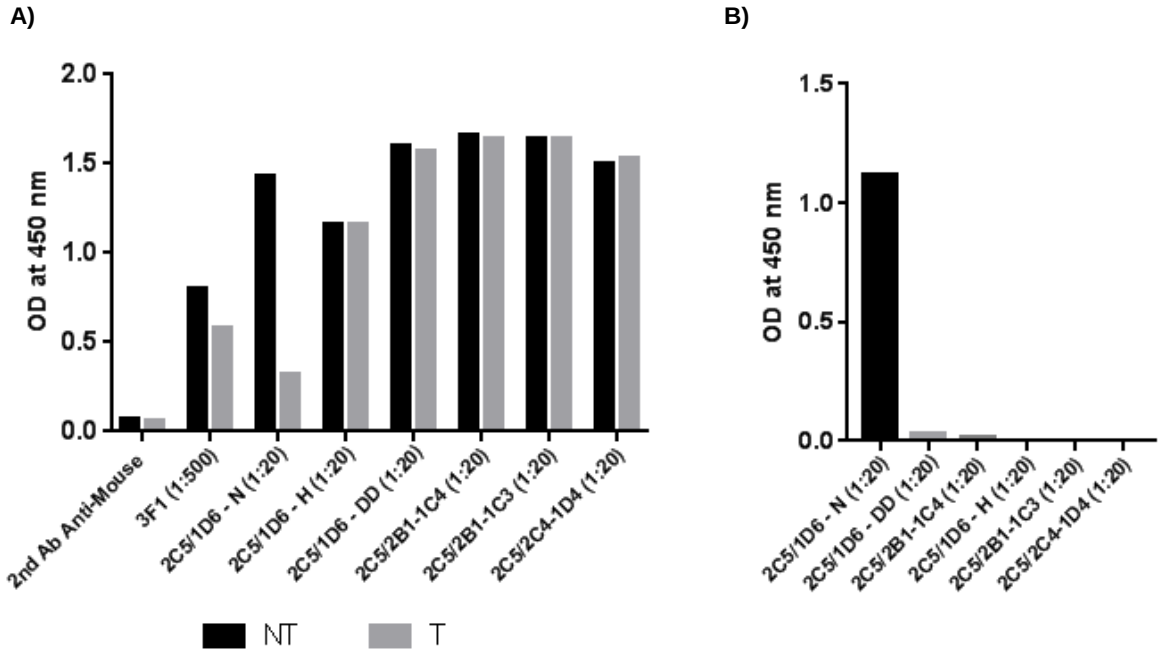

Figure 3.5 - ELISA results obtained for the six selected clones. A) ODs at 450 nm obtained for all the clones, for the negative control (secondary antibody) and for the STn-positive control (anti-STn mAb 3F1). The ODs obtained for non-treated BSM are represented in black and the ODs obtained for sialidase-treated BSM are represented in grey. B) Difference of the ODs obtained for non-treated and sialidase treated BSM for all the clones, ordered from highest to lowest.

Since at this time only one clone seemed to be stable and producing antibodies against sialylated structures, a third limiting cloning was performed, from which 41 clones were obtained: 39 with positive reactivity and 2 with no reactivity towards BSM. All the positive clones derived from the clone N bound to BSM and showed the same pattern of reduction of binding after sialidase treatment, as it had been observed for the parent clone. Some of the positive clones were also tested against STn^+ cells by flow cytometry and the reduction in the staining was confirmed after the cells were treated with sialidase (data not shown).

After three limiting cloning steps had been performed, it could be said with some confidence that the positive clones that derived from clone N were monoclonal. It could also be presumed that they had specificity towards sialylated structures, like STn, since a tendency for a decreased binding against the targets after treatment with sialidase was observed by ELISA and flow cytometry analysis against BSM and STn^+ cells, respectively (Figure 3.6). Additionally, it was observed that all the positive clones were able to maintain a high antibody titer production. Taking this into account, it was

decided to select three of the positive clones to further purify, characterize and perform functional assays. The three final clones were named final hybridoma clone 1 (FHC1), 2 (FHC2) and 3 (FHC3).

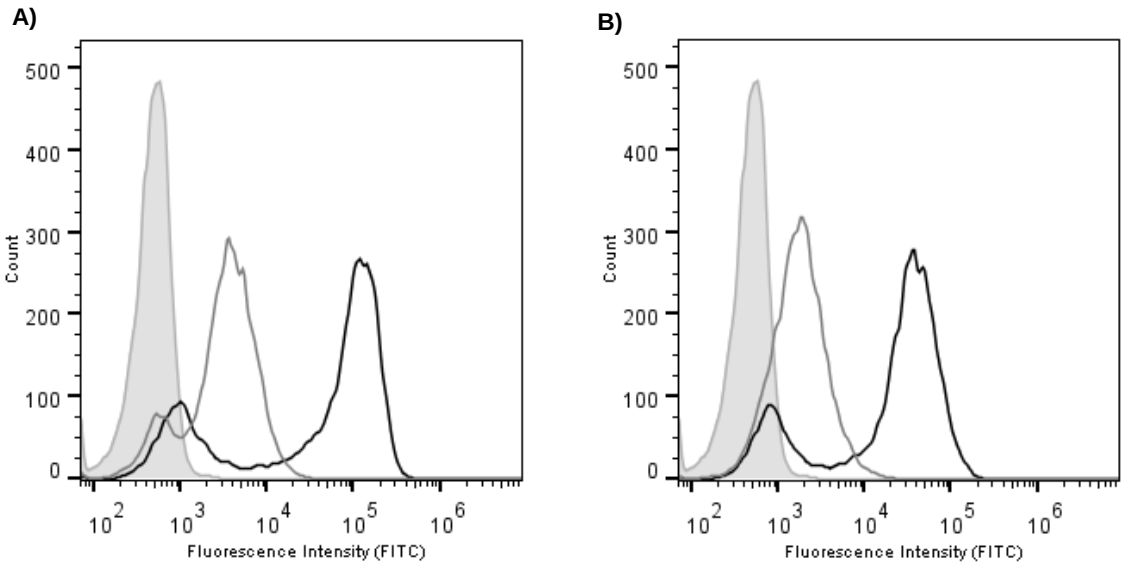

Figure 3.6 - Assessment, by flow cytometry, of the binding of the supernatant from the final hybridoma clone 2 (FHC2) to the MDA-MB-231 STn cells. The *yy* axis represents the number of cells and the *xx* axis represents the fluorescence intensity of FITC. The grey filled histogram represents the negative control (cells stained only with the secondary antibody), the black line represents the binding to cells non-treated with sialidase and the grey line represents the binding to cells treated with sialidase. A) Positive control staining with an anti-STn antibody (B72.3). B) Staining obtained when using the supernatant from the hybridoma clone FHC2.

During this screening process, the production of antibodies by the hybridoma clones that did not show, by ELISA, reactivity towards BSM, was also assessed. The goal of this assessment was to verify if these negative clones did not have the capacity of producing antibodies or if the antibodies produced were not reactive against BSM. It was observed that these clones were not producing antibodies, having probably lost the ability to produce them due to genetic instability or due to the freeze/thaw procedures, as previously mentioned.

3.2.2. Antibody purification and expansion for large scale production

The process of characterization of the antibodies requires larger amounts of supernatant, as the antibodies present in those supernatants can be characterized by several techniques. This implies the process of culturing the hybridoma clones in larger culture flasks, allowing the collection of an increased volume of supernatant, a process known as expansion. It is, however, also important to purify the antibodies before expansion and further characterization, so that, ideally, no substances interfere in those procedures.

The complete culture medium used for the culture of the hybridoma cells contains serum, which by itself contains a mixture of components like albumin, lipoproteins, hormones, other

immunoglobulins and growth factors essential for the culture of animal cells. However, the use of serum in cultures of hybridoma cells leads to difficulties in antibody purification, which is needed for further characterization steps. For this reason, the culture medium of the three final clones was changed from complete RPMI-1640 to serum-free medium (Gibco), in order to remove the main contaminants existent in the complete medium and to eliminate the need of serum supplementation, allowing an increase in the purity of the produced antibodies. Like this, the hybridoma cells had less availability of nutrients that were mainly used for cell proliferation, leading to an increase/stimulation of antibody production. During the adaptation of these clones to the new serum-free medium some changes were observed, like the loss of adhesion in the first weeks of culture, probably due to the loss of adhesion factors present in the complete medium previously used. After that time it was observed that the clones were once again starting to become adherent. The supernatants of these three final hybridoma cells, now in serum-free medium, were tested by ELISA and by flow cytometry and, though the culture medium was changed, the reactivity towards BSM and the binding to STn^+ cells, which was decreased after treatment with sialidase, was still observed (Figure 3.7). After assessing the production of antibodies with the desired specificity, the hybridoma cells were expanded to larger culture flasks, in order to collect more supernatant for further characterization steps.

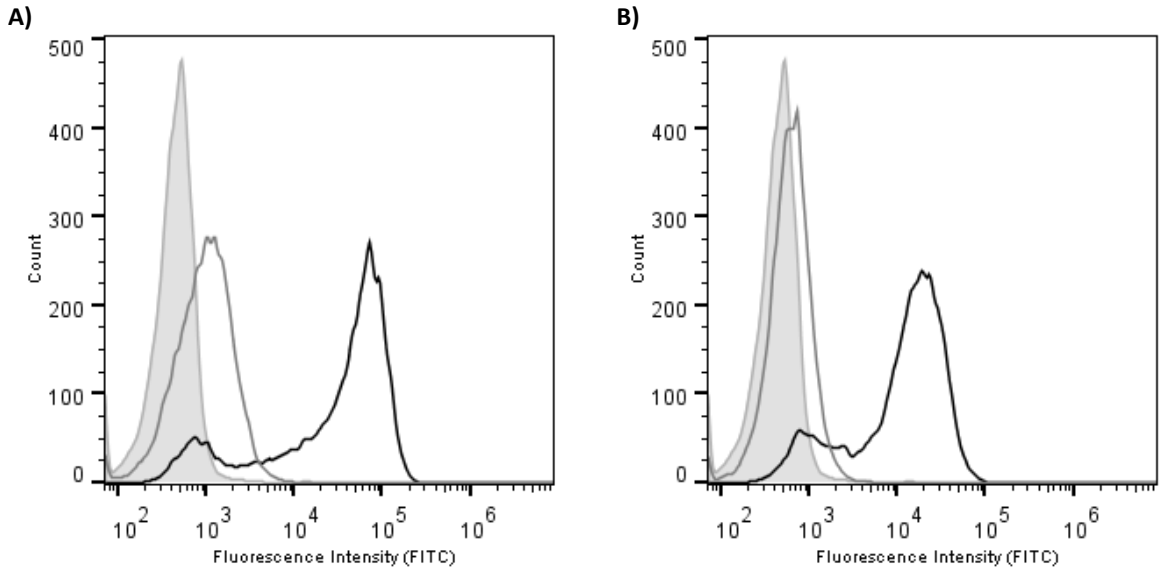

Figure 3.7- Assessment, by flow cytometry, of the binding of the supernatant from the final hybridoma clone 1 (FHC1) after culture in serum-free medium to the MDA-MB-231 STn cells. On the histograms, the *yy* axis represents the number of cells and the *xx* axis represents the fluorescence intensity of FITC. The grey filled histogram represents the negative control (cells stained only with the secondary antibody), the black line represents the binding to cells non-treated with sialidase and the grey line represents the binding to cells treated with sialidase. A) Positive control staining with an anti-STn antibody (B72.3). B) Staining obtained when using the supernatant from the hybridoma clone FHC1.

3.2.3. Isotyping of the hybridoma clones by capture ELISA

One of the initial steps of characterization performed was the determination of the isotype of the antibodies produced by the hybridoma cells. This was achieved by capture ELISA, which allowed detecting the presence of IgG_1, IgG_{2a}, IgG_{2b}, IgG_3, IgM and IgA isotypes. The candidate clones (FHC1, FHC2 and FHC3) were evaluated. All of them showed positive results for an IgM isotype, which is consistent with the knowledge that the IgM class is the first being produced, before class switching occurs. This isotyping procedure was applied throughout the limiting cloning and screening steps up to the final clones, as exemplified in Figure 3.8 with the final hybridoma clone 1. All the clones analyzed throughout the limiting cloning and screening steps presented the same isotype as the final clones, IgM.

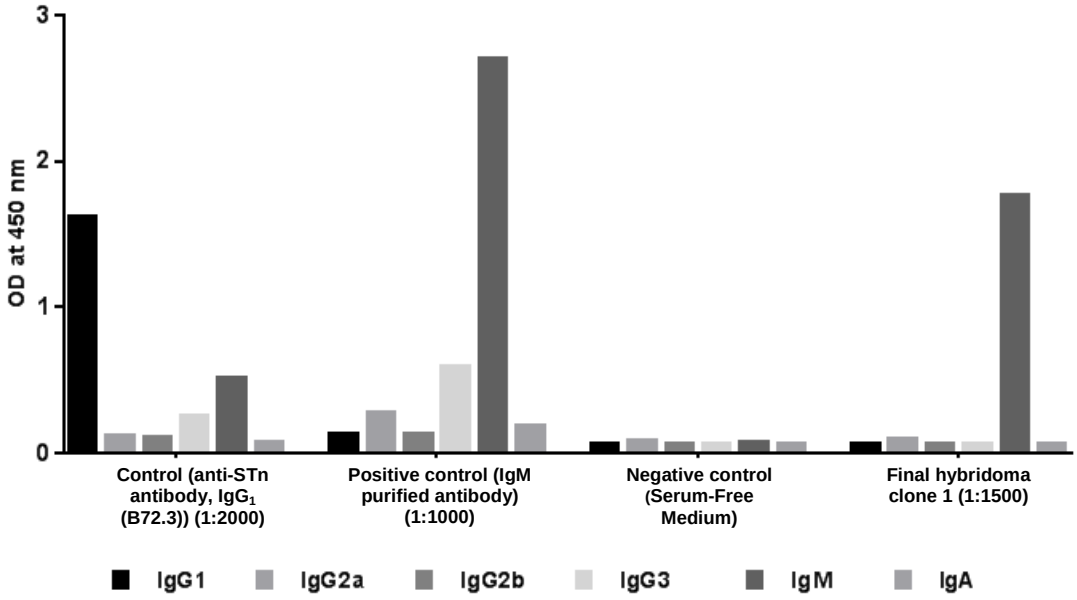

Figure 3.8 - Isotyping of the final hybridoma clone 1 supernatant by capture ELISA. An IgG_1 anti-STn antibody (B72.3) was used as control; a purified IgM antibody was used as positive control; serum-free culture medium was used as negative control.

The IgM isotype is the first to be produced in humoral immune response to antigen exposure and its activity is mostly related with the activation of the complement system and agglutination reactions, thus being an effective neutralizing agent in early stages of infection. Due to the pentamer large size, IgM molecules are mainly found in blood, not being able to diffuse to extravascular sites like IgG molecules. Thus, they are often considered not so relevant in terms of the induction and involvement on a strong cellular immune response against cancer cells, as most of the therapeutic antibodies currently used have other isotypes, mainly IgG. However, as reported by An *et al*., IgM antibodies against STn structures have already been developed and showed significant inhibitory effect on the proliferation and migration of STn^+ cells and on tumor growth by inducing apoptosis [88]. This shows that this type of antibodies has the potential to be used, not only for diagnostic purposes as frequently reported, but also for therapeutic purposes. The

most promising clone was tested by immunohistochemistry against bladder cancer tissue, and the staining obtained showed specificity towards structures containing sialic acid (data not shown). Nevertheless, more assays are going to be performed in the future in order to obtain a full characterization of the anti-STn antibodies produced by the group.

3.3. Part B: Characterization of humanized anti-STn monoclonal antibodies produced by a biotechnology company

This part of the work consisted mostly on the functional characterization of humanized anti-STn antibodies, *in vitro*, using cancer cell lines as models. Based on the results of Carrascal *et al.* [64], it was hypothesized that, by blocking the interaction of STn with DCs, the tolerogenic effect imposed on DCs by this antigen would not occur and, therefore, a normal maturation profile would be observed. Hence, based on this hypothesis, the humanized anti-STn antibodies were used to block the STn at the surface of cancer cells and these were co-cultured with DCs, in order to assess if the maturation profile could be reestablished.

3.3.1. Titration of humanized anti-STn monoclonal antibodies

As previously mentioned, three humanized antibodies with reactivity towards STn were provided to the group by collaborators. It was initially needed to determine the concentration of each antibody that would then be used for the STn blockade in the co-culture assays. To do this, a set of titrations of each of the antibodies (mAb1, mAb2 and mAb3) was performed, using flow cytometry, against MDA-MB-231 STn cells, comprising a range of concentrations between 0.0144 µg/ml and 60 µg/ml (Figure 3.9). Although all three mAbs showed similar staining of the cells up to a concentration of 1.8 µg/ml, on higher concentrations a decrease in the number of cells being stained and in the intensity of the staining was observed (Figure 3.9, A)), mainly when using mAb2 and mAb3. This was also related with an increased cellular death due to higher antibody concentrations being used, as observed in the ssc/fsc profiles (Figure 3.9, B)). Based on the dot plots of the ssc/fsc profiles, it was observed that cells stained with mAb1 and mAb3 showed a decrease in cell viability when using antibody concentrations above 1.8 µg/ml. On cells stained with mAb2, decreased cell viability was observed after staining with antibody concentrations above 0.360 µg/ml.

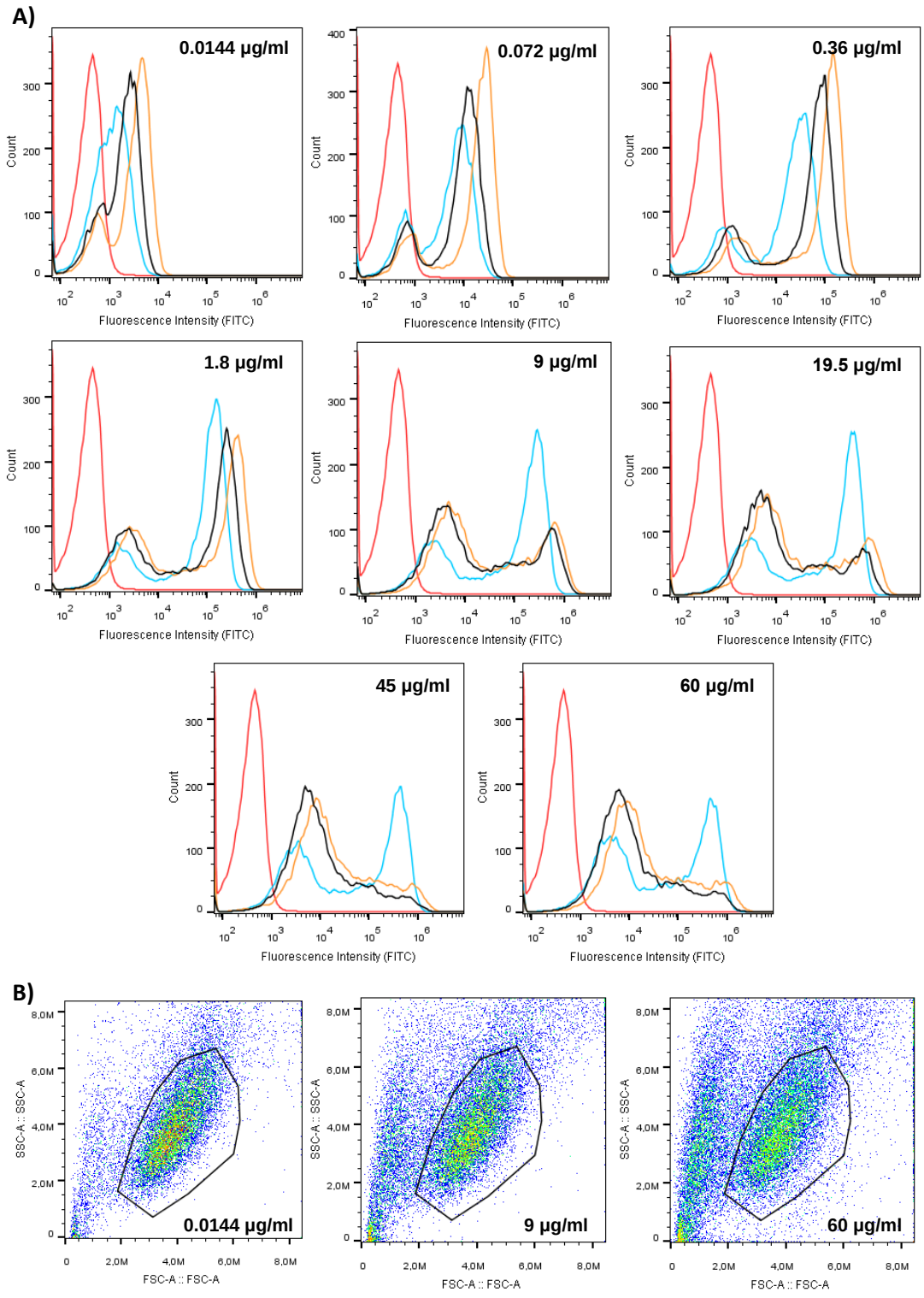

Figure 3.9 - Titration, by flow cytometry, of the humanized anti-STn mAbs against MDA-MB-231 STn cells. A) On the *yy* axis is represented the number of cells and on the *xx* axis is represented the fluorescence intensity associated with FITC. The red line represents the negative control (cells stained only with the secondary antibody), and the mAbs are represented by the blue (mAb1), the orange (mAb2) and the black (mAb3) lines. The concentrations tested are indicated on the upper right corner of the histogram representations. B) Dot plot representation of the ssc/fsc profiles obtained for three of the concentrations used. The black line represents the gate used to select the cells of interest.

Since cell death at higher concentrations in all three antibodies tested was observed, another titration experiment was performed using a set of lower concentrations, ranging from 0.8 µg/ml to 5.5 µg/ml. Once again, it was observed that only mAb1 maintained the stain on the cancer cells as the antibody concentration increased. Both mAb2 and mAb3 stained a lower number of cells when using a concentration higher than 1.2 µg/ml (Figure 3.10). However, this time, almost no cell death was observed, which was believed to be due to the presence of BSA in the adhesion buffer, thus having a stabilizing effect on the cells, maintaining their viability.

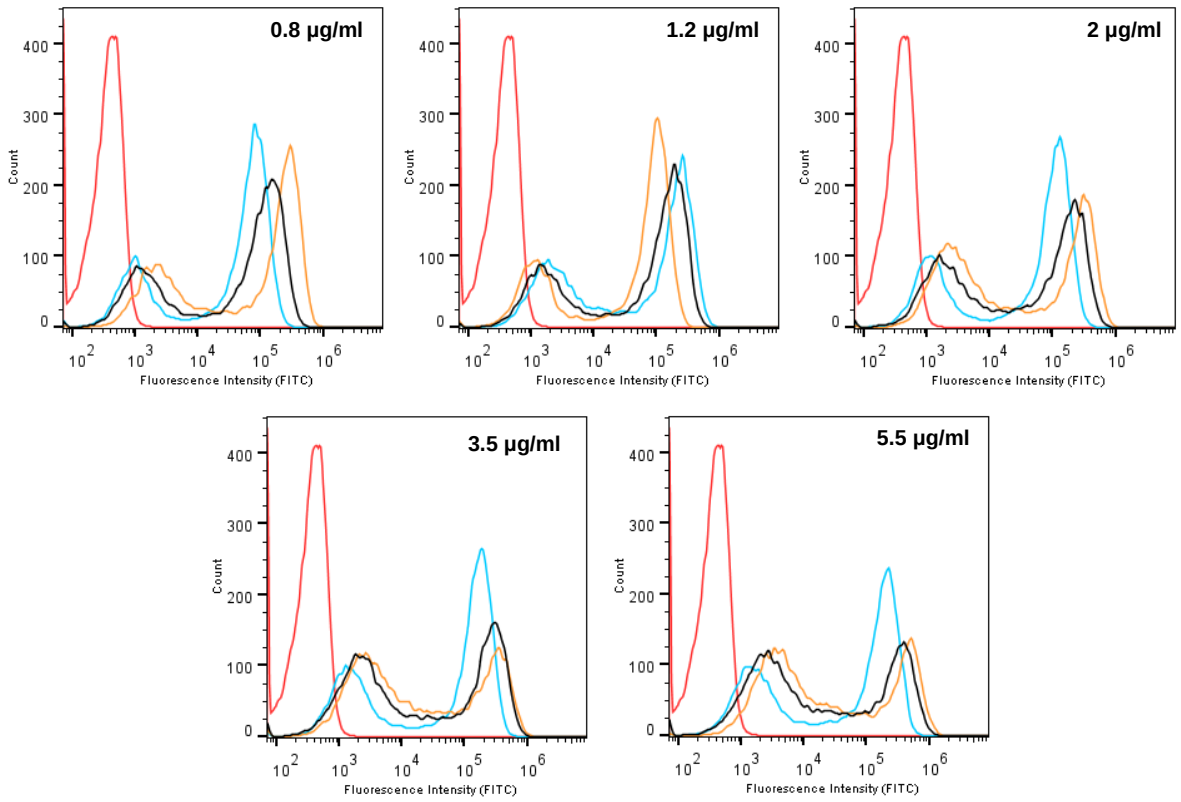

Figure 3.10 - Titration, by flow cytometry, of the humanized anti-STn mAbs against MDA-MB-231 STn cells. On the *yy* axis is represented the number of cells and on the *xx* axis is represented the fluorescence intensity associated with FITC. The red line represents the negative control (cells stained only with the secondary antibody), and the mAbs are represented by the blue (mAb1), the orange (mAb2) and the black (mAb3) lines. The concentrations tested are indicated on the upper right corner of the histogram representations.

Because in this second titration experiment it was not certain if the concentrations could or not be already inducing cellular death, and because non-specific staining (left peak on the mAbs histograms, Figure 3.10), that increased as the concentration of antibody used also increased, was observed, a third titration experiment was performed. A set of five concentrations, lower than the ones previously tested, was used. The binding of only mAb2, which was meanwhile decided to be the first

of the three humanized anti-STn antibodies to be used in the co-cultures assays, was assessed. A range of concentrations from 0.036 µg/ml to 0.8 µg/ml was used. Up to 0.1 µg/ml, almost no non-specific staining was obtained, with a MFI value of 12960 for the lowest concentration (0.036 µg/ml) and 23432 for the next concentration (0.072 µg/ml). The same was observed for the concentration of 0.1 µg/ml (MFI=33692), though with a slight increase of the non-specific staining (Figure 3.11). At a concentration of 0.36 µg/ml, this non-specific staining is increased again, and the MFI value also increased to 57286, although it can be observed in the histogram representations that the number of cells stained is lower. Lastly, for the highest concentration tested (0.8 µg/ml), a MFI value of 23536 was obtained, being lower than the previous ones probably because the number of cells being stained is also lower, and due to an increase of the non-specific staining.

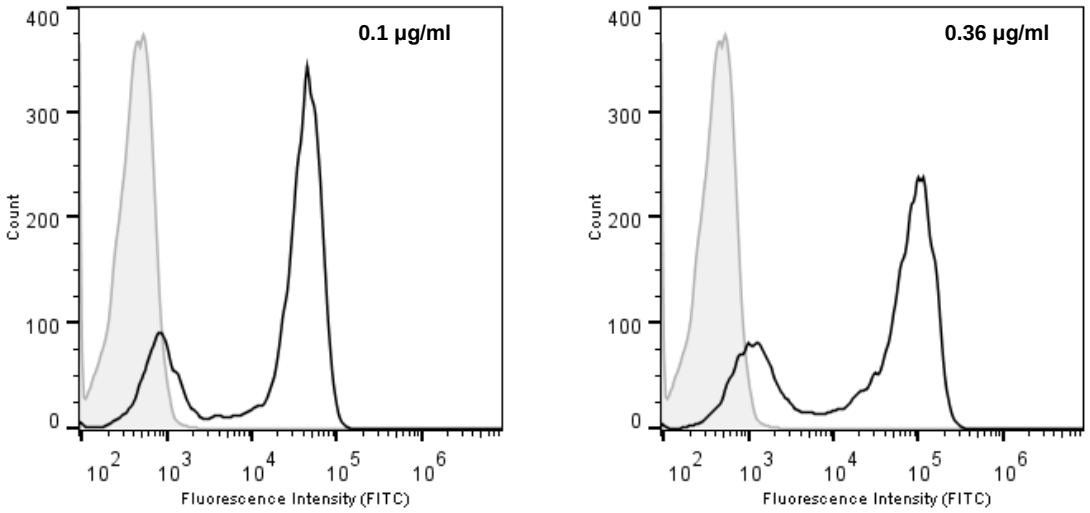

Figure 3.11 - Titration, by flow cytometry, of the humanized anti-STn mAb against MDA-MB-231 STn cells. On the *yy* axis is represented the number of cells and on the *xx* axis is represented the fluorescence intensity associated with FITC. The grey filled histogram represents the negative control (cells stained only with the secondary antibody), and the staining observed with mAb2 is represented by the black line. Two of the five concentrations tested are indicated on the upper right corner of the histogram representations.

For the reasons mentioned above, the hypothesis of using the concentrations of 0.036, 0.072 or 0.8 µg/ml was discarded. Since the goal of these titration experiments was to find the best concentration of antibody that does not compromise cell viability and that minimizes the non-specific binding to low-affinity targets, a concentration of 0.1 µg/ml was selected from the two remaining concentrations. This concentration was then going to be used for the mAb2 in the co-culture assays, as it will be mentioned further in this chapter, since that, although with an inferior MFI value, is the one that appears to promote less non-specific binding.

3.3.2. Assessment of the immunological response of DCs: evaluation of the adhesion and maturation status of moDCs after establishment of co-cultures with STn^+ and STn^- cancer cells

Before starting the co-culture assays between cancer cell lines and DCs with the anti-STn antibodies for blockade of the STn antigen, as it was the goal, the procedure of co-cultures was performed without the STn blockade. The main reason for this initial procedure without anti-STn antibodies was to reproduce the assays previously performed by the group [64,72], this time using the MDA-MB-231 cell line. This was mostly in order to assess if similar results to the previous ones obtained could be achieved, and to guarantee that all the variants in the assay (such as the cell lines, the buffers and the manipulation of monocyte-derived dendritic cells during their differentiation process) were functioning before the assays with the humanized anti-STn antibodies.

The co-cultures were performed as described in chapter 2. The co-cultured cells (moDCs adhered to cancer cells) were stained with anti-CD80, anti-CD86 and anti-HLA-DR antibodies and further analyzed by flow cytometry. Though the expression of CD80 and CD86 molecules can be used to assess the maturation state of dendritic cells, one clear marker of maturation is the molecule HLA-DR (MHC-II). The same principle can be applied to the CD80 and CD86 co-stimulatory molecules, though in this case sometimes the assessment of the maturation state of dendritic cells is not so obvious, since these molecules have a low expression when compared to HLA-DR.

MDA-MB-231 STn cells were used to assess the influence of the STn expressed on the surface of cancer cells when in contact with DCs in the co-cultures. MDA-MB-231 WT cells, which had been previously assessed by flow cytometry, and observed not to be expressing the STn antigen, were used as a negative control. A control condition of moDCs cultured alone was also used. First, the percentage of moDCs that adhered to cancer cells was assessed: this percentage was slightly lower in the co-culture with the WT variant (64.8%) when compared to the STn variant (72.8%) (Figure 3.12, A)). Secondly, the maturation status of DCs in the co-cultures was assessed. The MFI obtained for the staining of CD80 molecule was similar in all three conditions, reason why the maturation status of the DCs cannot be assessed from this specific result (Figure 3.12, B)). It was also observed that the contact with cancer cells leads to an increased expression of CD86 (induces maturation). The tolerogenic effect of STn was observed, as reported by the group, since the condition in which moDCs were co-cultured with MDA-MB-231 STn cells showed a lower MFI value (1175) than the co-culture with the WT variant (1332) (Figure 3.12, C)). Regarding the staining of HLA-DR, the results had the same tendency described for the CD86 molecule, though the MFI values obtained were higher and the difference observed between the co-culture with the WT (109723) and the STn (78565) variants was more notorious (Figure 3.12, D)).

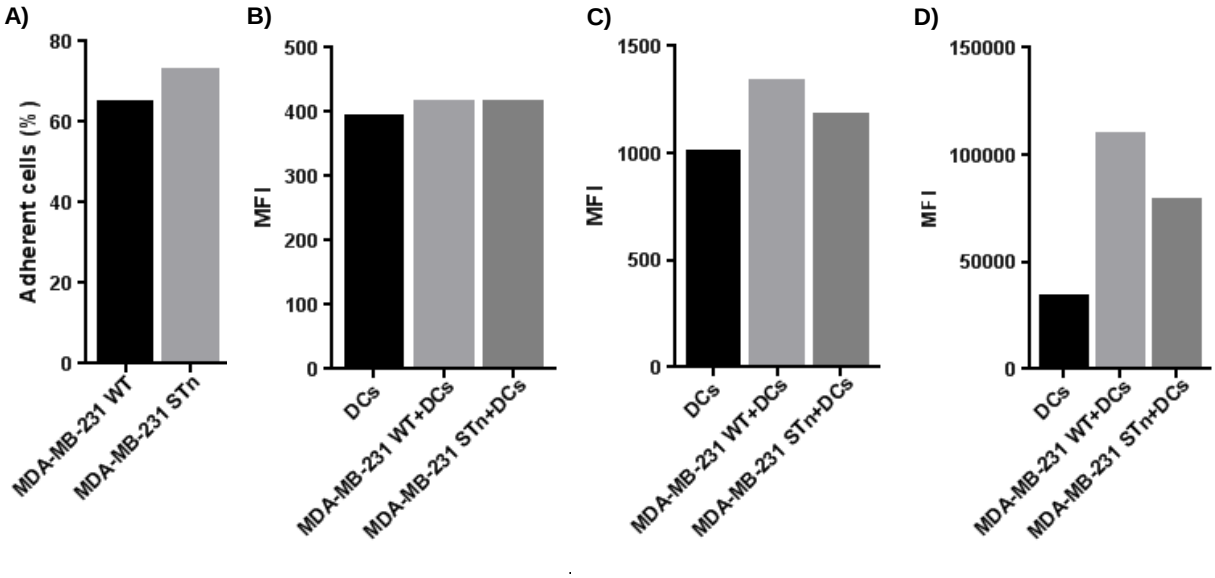

Figure 3.12 – Results, obtained by flow cytometry, for the co-culture assay without anti-STn blocking mAb (n=1). A) Percentage of dendritic cells that adhered to cancer cells. B) MFI values obtained for staining with anti-CD80 antibody. C) MFI values obtained for staining with anti-CD86 antibody. D) MFI values obtained for staining with anti-HLA-DR antibody.

All these results were similar to the ones previously obtained by the group [64,72]. Taking these results into account, the same experiment was performed using the anti-STn mAb2 as a STn blocker, as it will be presented in the next section.

3.3.3. Assessment of the immunological response of DCs: evaluation of the adhesion and maturation status after establishment of co-cultures of dendritic cells with cancer cells coated with the anti-STn mAb2

As previously mentioned, the anti-STn mAb2 was selected to be the first one to be tested as STn blocker in the co-cultures of cancer cells and moDCs. The selection of this anti-STn antibody over the other two available was mainly because at concentrations lower than 0.36 µg/ml it was observed an increased MFI value with an increased number of cells being stained, when comparing with anti-STn mAb1 and mAb3 (Figure 3.9). Therefore, previously to the co-culture with moDCs, the MDA-MB-231 cells (variant WT and variant STn) were incubated with humanized anti-STn mAb2 and human $IgG_1\kappa$ isotype control (IC), both at the selected concentration (0.1 µg/ml), for 30 minutes, at 37^{o}C. An isotype control is an antibody that does not have specificity to the target of interest, but matches the class and type of the primary antibody. It was used in the experiment as a negative control, to help differentiate from non-specific effects that may arise solely by the presence of any antibody and not from the specific interaction of an antibody with its target antigen. After this antibody incubation period, the immature moDCs were added to the co-culture and further incubated for two hours, in adhesion buffer, in order to assess if the blockade of the STn antigen would reestablish the mature phenotype

on DCs. The cells were stained as it was described in the previous section and in chapter 2, and analyzed by flow cytometry. The results presented are the average of two independent co-cultures performed in the same conditions. In terms of the adhesion of moDCs to cancer cells, the results observed were similar to the ones previously obtained: moDCs tend to adhere more to the STn variant in comparison to the WT variant. This was observed in all conditions tested as shown in Figure 3.13, A). The MFI values obtained for the staining with the anti-CD86 antibody were similar in all the conditions, for both WT and STn variants, being also similar to the MFI value obtained for the condition of moDCs that were not in contact with cancer cells (Figure 3.13, C)). This tendency was also observed in the staining for HLA-DR, although in this case an increase in the MFI values of the conditions of co-culture with cancer cells was observed, when compared to the condition of moDCs that were not co-cultured with cancer cells. Almost no difference was observed in the MFI values of the co-cultures with the WT variant when compared to the STn variant. An increase in the MFI on the condition with the anti-STn mAb2 was expected to be observed, if the mature phenotype was reestablished; however, neither that nor the reduction caused by the presence of the STn antigen - the tolerogenic phenotype - was observed, as the MFI values obtained were similar (Figure 3.13, D)). The results concerning the staining for the CD80 molecule were also different than what was expected: decreased MFI values in all conditions of co-culture with variant STn when comparing to the WT variant. It was also observed that the moDCs that were cultured alone had a similar MFI to the condition of the co-culture with the WT variant in all conditions (Figure 3.13, B)), a tendency that had already been observed previously (Figure 3.12, B)). An increase in the MFI was observed in the condition with the blocking anti-STn antibody, which was the expected result. This could be related to an increased expression of the co-stimulatory molecule CD80, which could mean a reestablishment of the mature phenotype on DCs. However, this result is not enough to conclude that the STn blockade with the mAb2 did indeed reestablish the mature phenotype on dendritic cells, since that result was not observed with HLA-DR, an important maturation marker. The differences and similarities observed can also be due to the fact that the moDCs used were derived from monocytes isolated from peripheral blood of two different donors, which contributes to an increase in the variability of the results obtained.

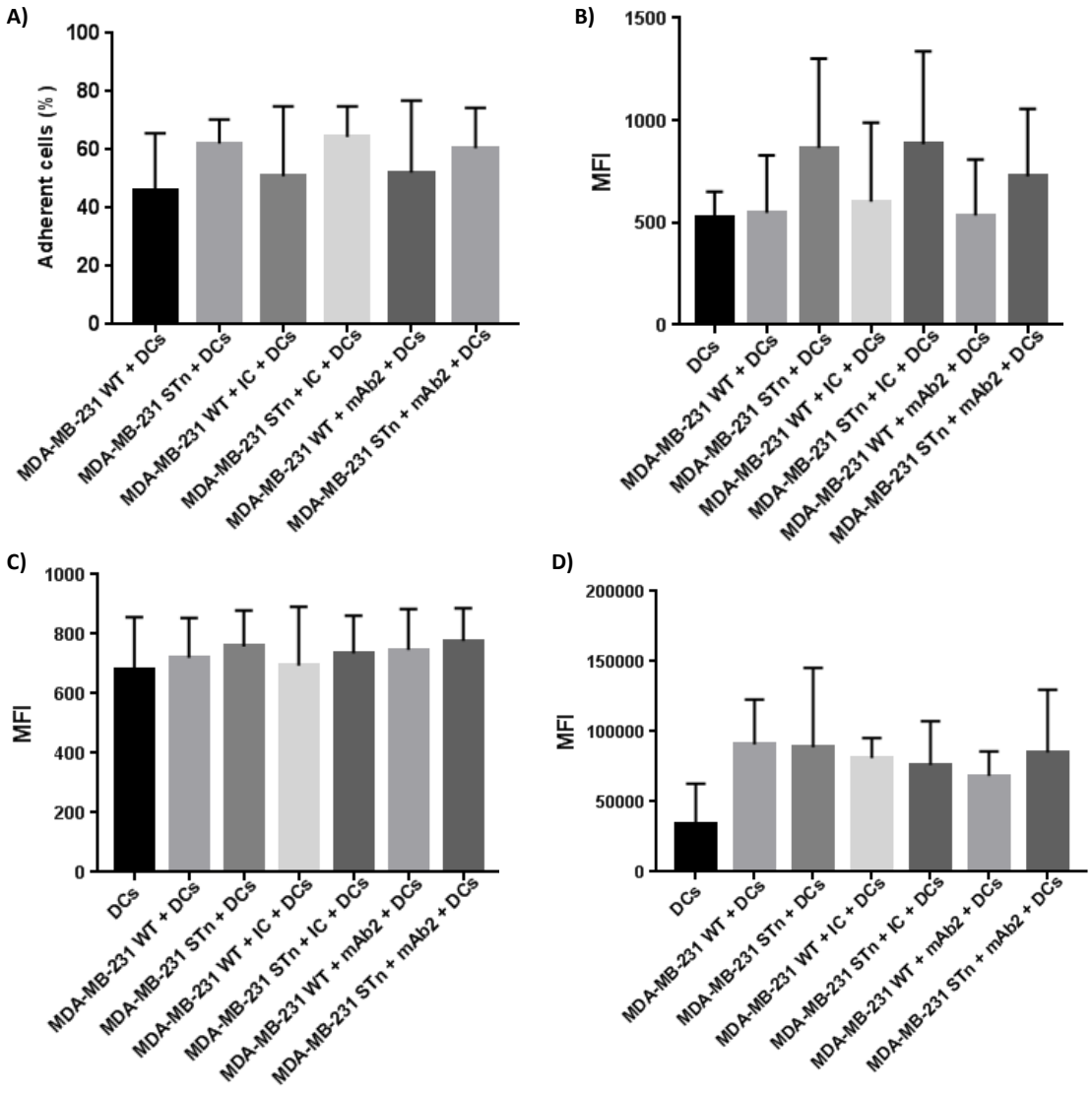

Figure 3.13 – Results, obtained by flow cytometry, for the co-culture assay with anti-STn blocking mAb2, at a concentration of 0.1 µg/ml (n=2). The error bars represent the standard deviation from the average values. A) Percentage of dendritic cells that adhered to cancer cells. B) MFI values obtained for staining with anti-CD80 antibody. C) MFI values obtained for staining with anti-CD86 antibody. D) MFI values obtained for staining with anti-HLA-DR antibody.

A capture ELISA assay was also performed in order to quantity the cytokines present in the supernatant of the co-cultures, namely IL-10 and IL-12. However, only a slight color development (low OD values) was obtained, which did not allow the cytokines in the supernatants to be quantified. One reason for this could be that the co-culture time is of two hours and that time itself may not have been enough to have the cytokines expressed and secreted to the supernatant. Because some differences might be detected in the gene expression after two hours, a RT-PCR experiment was performed to assess the expression of the genes *IL6*, *IL10*, *IL12* and *TNF*. The data obtained showed that the expression ratio of these genes (STn variant in relation to WT variant) was lower in the condition of co-culture with mAb2-coated cells, for all the four genes (Figure 3.14). Besides that, it was also observed

that the condition of co-culture with isotype control had an increased expression ratio, when comparing to the other two conditions, of *IL6* (Figure 3.14, A)), *IL10* (Figure 3.14, B)) and *IL12* (Figure 3.14, C)). The expression ratio was also approximately the same for all the genes in the condition of the co-culture with the isotype control. Regarding *TNF*, the expression ratio was similar to the condition of moDCs cultured just with cancer cells (Figure 3.14, D)). Additionally, is was noticed that the relative RNA levels of IL-6 were the highest of the four genes tested, almost 4-fold more than the levels of TNF-α (data not shown). This led to the hypothesis that the cancer cells could also be producing IL-6, hence the relatively high levels of expression of this cytokine when compared to the others.

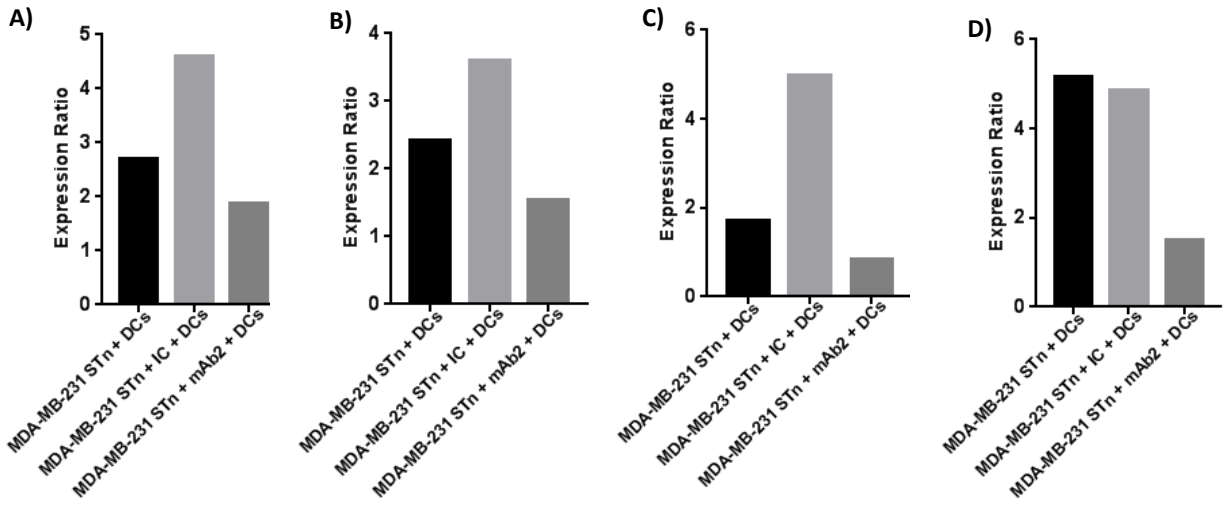

Figure 3.14 – Ratio between the gene expression of moDCs incubated with MDA-MB-231 STn (cells only, and in the presence of isotype control and blocking mAb2), and the gene expression of moDCs incubated with MDA-MB-231 WT in the same conditions (n=1). A) *IL6*; B) *IL10*; C) *IL12*; D) *TNF*.

Taking into account the results obtained in the assays with the anti-STn mAb2, and since no evidence was observed that using this antibody to block STn was decreasing the tolerogenic phenotype and/or reestablishing the mature phenotype on moDCs, it was hypothesized if the cancer cells could be internalizing the antibodies, hence decreasing the blocking of the STn antigen. In order to address this question, an internalization assay was performed, as described in the next section and in chapter 2. Moreover, the increased expression of the genes in the condition of co-culture with isotype control led to the hypothesis that the isotype control (or the mAb2) could be having some effect on the moDCs alone, which was not expected. Thus, a culture of moDCs with the isotype control and anti-STn antibody was performed to assess if some interaction was possibly affecting the results of the co-cultures, as it will also be mentioned further in this chapter.

3.3.4. Assessment of the internalization of the humanized anti-STn mAbs by the cancer cells

In order to test the hypothesis that the anti-STn mAb2 was being internalized by the cancer cells, an internalization assay was performed by flow cytometry, as described in chapter 2. The cancer cells (MDA-MB-231, WT and STn variants) were incubated with mAb2 in the same concentration used in the co-culture experiment (0.1 µg/ml). The WT variant was used as a control, as it was already mentioned that these cells do not express the STn antigen. The MFI values obtained for each of the five time points tested, from 15 minutes (the usual minimum required for flow cytometry staining) up to 150 minutes (the total time of incubation with antibody in the co-culture experiments), were analyzed. The staining with FITC-conjugated secondary antibody was performed at 4°C to minimize the internalization of this antibody, so that it could be assessed if the anti-STn antibody had been internalized, via a decrease in the fluorescence intensity observed with time. As shown in Figure 3.15, it was observed that, for MDA-MB-231 WT cells (Figure 3.15, A)) there was a slight decrease in the MFI between 120 and 150 minutes; in MDA-MB-231 STn cells (Figure 3.15, B)) this decrease occurred earlier, between 30 and 60 minutes. However, taking into account that these time points comprise the two hours of the co-culture experiment, plus the 30 minutes of pre-incubation with the anti-STn antibody, it was considered that the decrease in the MFI corresponded only to a slight decrease in surface bound antibody but was not strong enough to show that internalization had occurred. Therefore, from this result, it was excluded that the co-culture results were affected by internalization of the humanized antibodies.

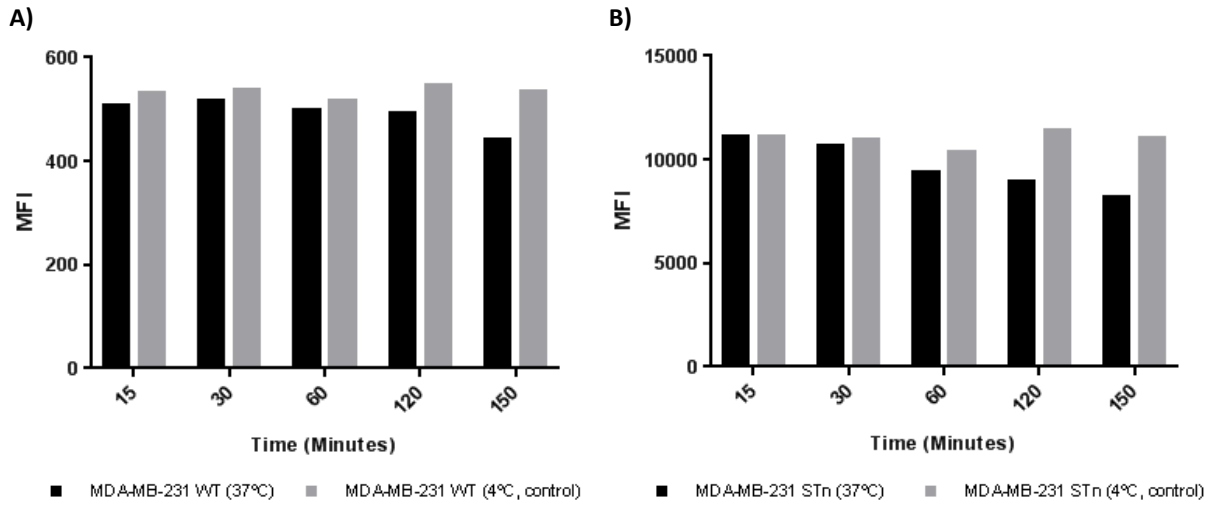

Figure 3.15 – MFI values associated with FITC, obtained by flow cytometry, on the internalization assay performed with cells MDA-MB-231 WT (A) and STn (B) variants and mAb2 (n=1). The black bars represent the MFI obtained after incubation at 37°C and the grey bars represent the MFI obtained after the control incubation at 4°C.

3.3.5. Assessment of the effect of humanized anti-STn antibody (mAb1) and isotype control (IC) on immature moDCs

Considering the results obtained up to this point, some alterations to the protocol being performed were applied and will be addressed further in this chapter. Since the desired effect of the reduction of the tolerogenic phenotype through the blockade of the STn antigen with mAb2 was not being observed with a two-hour co-culture, the time of the co-culture experiment was increased to 6 and 24 hours. This increase in the co-culture time raised an awareness about the viability of the cells being used, reason why the adhesion buffer was replaced with complete RPMI-1640 medium, with only 5% of FBS and supplemented with GM-CSF and IL-4 cytokines, to guarantee the viability of moDCs. Lastly, in addition to the anti-STn mAb2, mAb1 was also tested. The concentration used in the experiment was also changed, increasing to 1.8 µg/ml. This concentration was almost twenty times higher than the previous one tested (0.1 µg/ml) and is also the concentration above which increased cell death was observed in the titration experiments mentioned earlier in this chapter (see section 3.3.1). These alterations on the anti-STn antibody used and on the concentration were based on the results obtained and also on suggestions from the collaborators.

The hypothesis of the anti-STn antibody mAb1 and the isotype control being, in some way, interacting with the moDCs, leading to an unwanted effect on the results of the co-culture experiments, was assessed. To do this, immature moDCs were cultured alone, with isotype control and with mAb1 (both at 1.8 µg/ml), for 6 and 24 hours. After the incubation, the cells were stained with anti-CD86 and anti-HLA-DR antibodies and analyzed by flow cytometry. Considering the results regarding the CD86 molecule, the MFI values obtained for the moDCs cultured with isotype control and mAb1 were similar to the value obtained for the moDCs cultured alone, after 6 hours and 24 of incubation (Figure 3.16, A) and B), respectively). Regarding HLA-DR, the results were also similar between the three conditions, though a slight increase in the MFI value of the culture with mAb1 after 6 hours (Figure 3.16, C)) and a decrease in all values after 24 hours (Figure 3.16, D)) were observed.

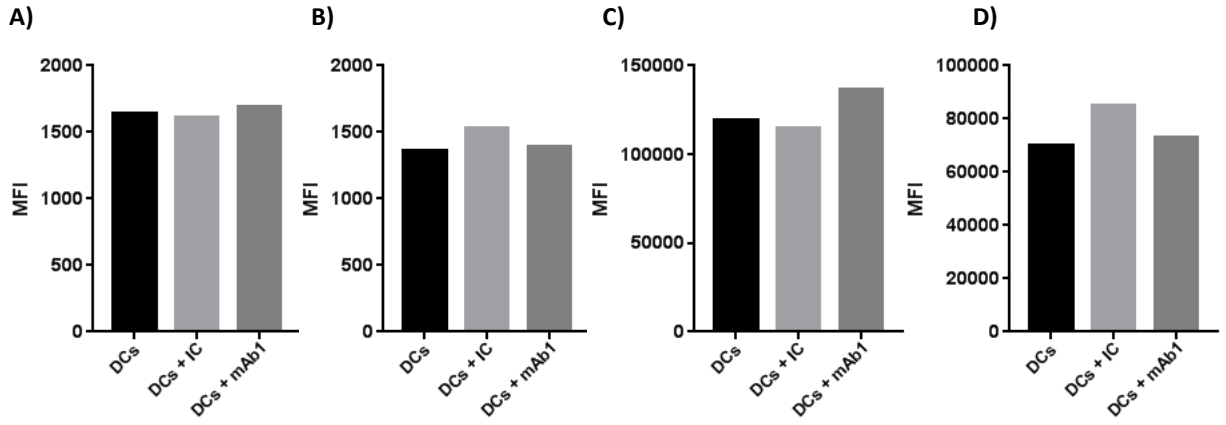

Figure 3.16 – Results, obtained by flow citometry, for the culture of moDCs and isotype control and anti-STn blocking mAb1, at a concentration of 1.8 µg/ml (n=1). A) MFI values obtained for staining with anti-CD86 antibody, after incubation of 6 hours. B) MFI values obtained for staining with anti-CD86 antibody, after incubation of 24 hours. C) MFI values obtained for staining with anti-HLA-DR antibody, after incubation of 6 hours. D) MFI values obtained for staining with anti-HLA-DR antibody, after incubation of 24 hours.

These results did not seem to show that the interaction between the isotype control/mAb1 and the moDCs was having an effect on their maturation status, so a new co-culture experiment with the new conditions was performed to assess if different results could be obtained from the ones obtained with mAb2.

3.3.6. Assessment of the immunological response of DCs: evaluation of the adhesion and maturation status after establishment of co-cultures of dendritic cells with cancer cells coated with the anti-STn mAb1

Besides the alterations mentioned in the previous section, further modifications were performed, namely the use of other control conditions. Another condition of moDCs cultured alone was added, but in this case the cells were stimulated with 5 µg/ml of LPS, being then used as a positive control of maturation and as a way to assess if the immature moDCs were responsive to stimuli. Also, a condition with just cancer cells cultured alone was added, in order to later assess the expression of cytokines by the cancer cells, since the previously obtained results originated the hypothesis that the cancer cells could be expressing IL-6. In the co-culture experiments with these conditions, however, only the MDA-MB-231 STn cell line was used, mainly because at this point the idea was to focus on the main goal of the application of these antibodies: the STn blockade and decrease of the tolerogenic phenotype. This could be assessed by comparing the condition of the co-culture of STn^+ cancer cells and moDCs to the same condition but with mAb1-coated cancer cells, so it was decided not use the WT variant, as a comparative model, at this point. This does not exclude that a STn^- variant could be used in future experiments, once all the conditions are optimized and the desired effect is obtained.

After an incubation of 6 hours, the MFI values obtained for CD86 were similar between all conditions, thus reinforcing the tolerogenic phenotype caused by STn. The condition in which the moDCs were stimulated with LPS had a MFI value similar to the other conditions, though slightly increased (Figure 3.17, A)). This created the hypothesis that either the moDCs were not responsive to stimuli or this incubation time could not be enough to see a difference between this condition and the others, this is, that 6 hours could not be enough to have a stronger stimulation of moDCs. However, after 24 hours the expected increase in the MFI value in this condition was observed. This increase was approximately seven times higher in comparison to the other conditions, showing that the moDCs used were indeed responsive to stimuli. The moDCs that were co-cultured with STn^+ cells showed a decrease in the MFI values when comparing to the condition of DCs cultured alone, which reflected the tolerogenic phenotype driven by STn. Furthermore, no increase in the MFI values obtained in the staining of this molecule was observed in the condition with mAb1-coated cancer cells, maintaining the hypothesis that the reestablishment of the mature phenotype had not been achieved (Figure 3.17, B)). The same tendency that had been observed with CD86 after 6 hours was observed when regarding HLA-DR. The moDCs stimulated with LPS presented almost the same MFI values as the other conditions, with the exception of the moDCs cultured alone, which had lower MFI values, showing again that the contact with cancer cells tends to maturate the moDCs (Figure 3.17, C)). After 24 hours the values for the stimulation with LPS had almost doubled, thus confirming that the moDCs used were responsive to stimuli. This was not observed in the other conditions (Figure 3.17, D)), as these

values almost did not differ from the ones obtained at 6 hours. These results, together with the ones obtained for CD86, showed that once again the goal of reestablishing the mature phenotype via blockade of the STn antigen was not achieved. Besides that, it was also observed that, after incubation of 24 hours, the percentage of moDCs that adhered to cancer cells was reduced to almost half of the percentage obtained in the incubation of 6 hours (Figure 3.17, E)). This may be due to loss of cell viability, since after 24 hours some cancer cells may have died and therefore detached from the bottom of the well; and also some cancer cells with moDCs adhered to them may have been lost in the washing steps before trypsinization of the co-cultures and collection of the cells. Moreover, the variability observed in the three experiments performed was probably promoted by the usage of moDCs differentiated from monocytes of different donors and by the manipulation of the monocytes during their differentiation into DCs.

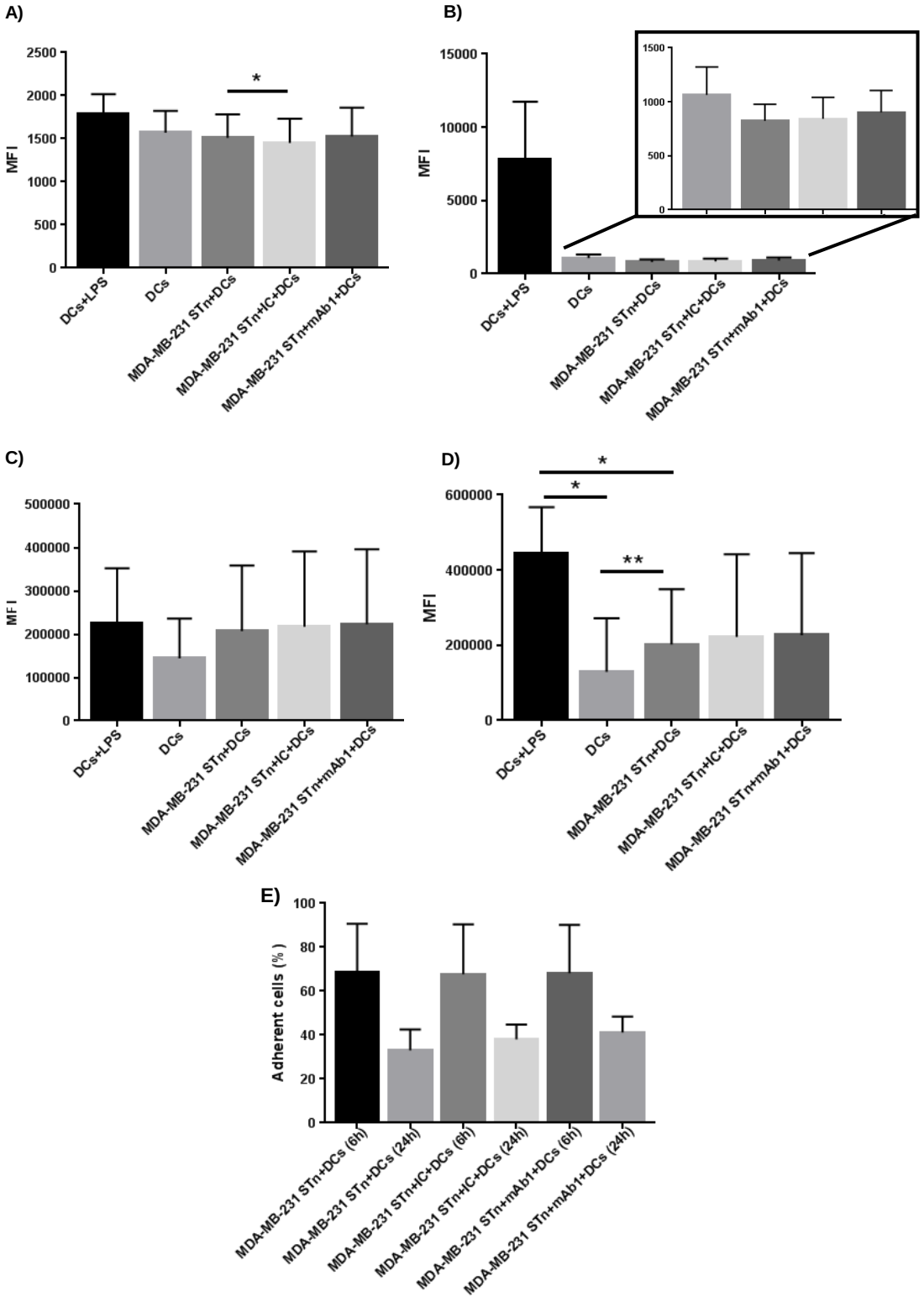

Figure 3.17 – Results, obtained by flow cytometry, for the co-culture assay with anti-STn blocking mAb1, at a concentration of 1.8 µg/ml (n=3). The error bars represent the standard deviation from the average values. A) MFI values obtained for staining with anti-CD86 antibody, after incubation of 6 hours. B) MFI values obtained for staining with anti-CD86 antibody, after incubation of 24 hours, and zoom to some conditions. C) MFI values obtained for staining with anti-HLA-DR antibody, after incubation of 6 hours. D) MFI values obtained for staining with anti-HLA-DR antibody, after incubation of 24 hours. E) Percentage of dendritic cells that adhered to cancer cells (6 and 24 hours).

The presence of cytokines in the supernatant of the co-cultures was also assessed by ELISA. It had been previously observed (section 3.3.3) that the two-hour co-culture did not seem to be enough for cytokines to be expressed and secreted to the supernatant. Taking into account that the experiments with mAb1 were performed on two time-points, 6 and 24 hours, it was hypothesized that maybe at these time-points the presence of cytokines in the supernatant could already be detected and that these cytokines could be quantified. However, as explained in chapter 2, only the presence of IL-10 and IL-12 could be assessed. The results are presented on Table 3.1. As expected, the highest concentrations of both cytokines were observed on the condition in which the moDCs had been stimulated with LPS, leading to an increased maturation status. Similarly, it was observed that the control condition of moDCs cultured alone had an increased concentration of these cytokines when comparing to the conditions of the co-cultures with STn^+ cancer cells. Considering that the results obtained by flow cytometry showed no evidence of the reestablishment of the mature phenotype, this result also reinforced that idea. The values obtained for IL-12 are higher than the ones obtained for IL-10, which was expected, since that IL-10 is a cytokine with an anti-inflammatory and immunosuppressive action, mostly regulatory, and IL-12 is a pro-inflammatory cytokine responsible for the induction of the differentiation of $CD4^+$ T lymphocytes into a Th1 phenotype [89]. Despite that, the values obtained for IL-12 were not considered relevant to indicate an increase in the maturation state after STn blockade, as they are around fourteen times lower than the ones obtained in the LPS-stimulated moDCs after 24 hours of co-culture, which reflects the production of this cytokine by activated dendritic cells.

Table 3.1 – Average concentrations (± standard deviation) obtained, by ELISA, for cytokines IL-10 and IL-12 for all the conditions of the co-culture experiment with mAb1, after analysis of the supernatants collected after 6 and 24 hours (n=3).

	[IL-10] (pg/ml)		**[IL-12] (pg/ml)**	
	6h	**24h**	**6h**	**24h**
DCs + LPS	879.8±614.1	2230±1705	2647±556.7	3773±206.2
DCs	0	65.58±75.94	14.58±17	445.1±252.7
MDA-MB-231 STn	0	0	0	3.583±6.207
MDA-MB-231 STn + DCs	0	24.42±22.26	8.833±15,3	277.9±259
MDA-MB-231 STn + IC + DCs	0	26.75±32.49	10.83±18.76	269.3±208.1
MDA-MB-231 STn + mAb1 + DCs	2.083±3.608	33.92±30.41	13.67±23.24	256.3±240.1

As in the co-culture experiments with mAb2, a RT-PCR analysis of the genes coding these cytokines was performed. In the results regarding the relative RNA levels of each cytokine (data not shown), the expression of all four genes was upregulated on the condition of moDCs stimulated with LPS, as expected. This result was consistent with the maturation process. This was observed mostly after 6 hours, as a decrease in the expression was observed after 24 hours. It was also observed that the MDA-MB-231 STn cells did not seem to be expressing IL-10, IL-12 and TNF-α, therefore not

contributing in this way to the co-cultures results. However, the expression of IL-6 was similar to the other conditions of co-culture with cancer cells, which again created the hypothesis that the levels of IL-6 observed in the co-cultures are mostly produced by cancer cells and not by moDCs, as it had already been considered in the experiment with mAb2. Concerning IL-10, with the exception of the condition with LPS stimulation, almost no upregulation was observed after 6 hours. After 24 hours, the RNA levels were lower and mostly only observed in the conditions in which MDA-MB-231 STn cells were not present, this is, on the condition of moDCs cultured alone and on the condition of moDCs cultured alone in the presence of LPS. The same was observed for IL-12 at 6 hours: almost no upregulation on all conditions with the exception of the one with LPS stimulation. This was also observed at 24 hours, though the LPS-maturated moDCs showed a much inferior RNA level than at 6 hours. The results observed on IL-12 equally apply to TNF-α, especially at 6 hours, since that at 24 hours a decrease on the condition with LPS but a slight increase in the condition of moDCs cultured alone were observed.

The analysis of the expression ratios obtained in the co-culture of STn^+ cancer cells, in relation to the condition with isotype control, showed that IL-6 and TNF-α maintained a similar expression between 6 and 24 hours of co-culture (Figure 3.18, A) and D), respectively). In IL-10 and IL-12 there was a decrease in the expression at 24 hours, more noticeable in IL-12 (Figure 3.18, B) and C), respectively).

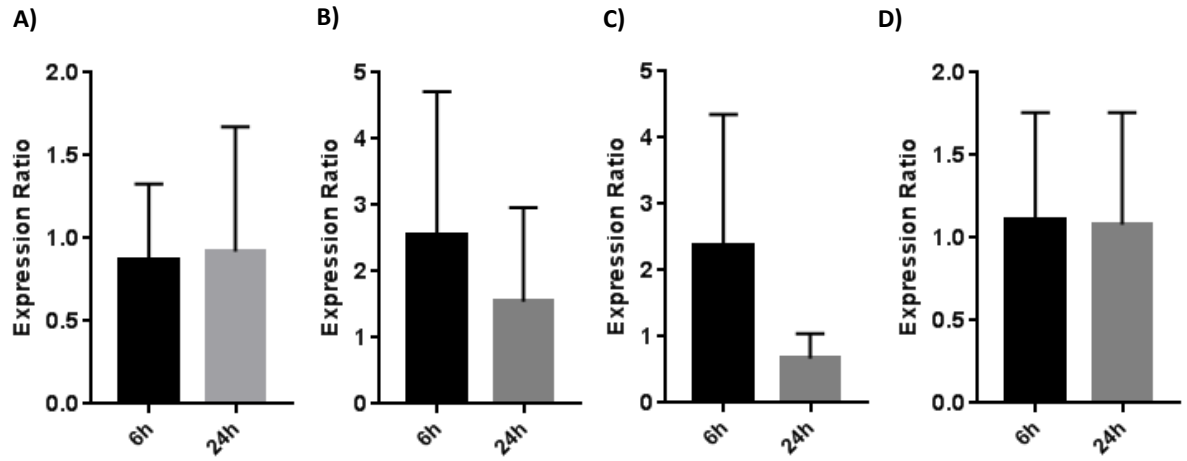

Figure 3.18 – Ratio between the gene expression of moDCs incubated with MDA-MB-231 STn in the presence of blocking mAb2 and the gene expression of moDCs incubated with MDA-MB-231 STn in the presence of isotype control, after 6 (black bars) and 24h (grey bars) (n=3). The error bars represent the standard deviation from the average values. A) *IL6*; B) *IL10*; C) *IL12*; D) *TNF*.

From these results, and considering the total set of results previously obtained by flow cytometry and ELISA, it was concluded that the reduction of the tolerogenic phenotype and reestablishment of the mature phenotype was not achieved. It was observed, in all experiments performed, a strong variability between experiments, due to the differences in the blood collected from different donors, from which the monocytes were isolated to be differentiated into moDCs. Additionally, the manipulation of these cells during the differentiation process could have also contributed to these differences. Despite the reestablishment of the mature phenotype not being observed in the results

obtained, the work developed contributed to understanding that further improvements and assays will be necessary, mostly relying on optimization of the co-culture procedures and anti-STn antibody concentrations to be used.

4. GENERAL CONCLUSIONS AND FUTURE PERSPECTIVES

Sialyl-Tn (STn) is an interesting tumor-associated carbohydrate antigen and potential target for anti-cancer therapies due to its expression by over 80% of the carcinomas and absence in the respective normal healthy cells. The group has reported that the presence of STn on the surface of cancer cells has an influence on dendritic cells (DCs) by inducing a tolerogenic phenotype. This can be regarded as a strategy developed by cancer cells to evade the recognition and destruction by the immune system, since DCs with a tolerogenic phenotype are less prone to activate T cells and to promote a specific and directed response against STn. Thus, a successful therapeutic approach against STn has to take in consideration the activation of the immune response. At present, a number of immunotherapies are being developed in order to boost the patient's immune system and direct its response towards the tumor. These therapies offer advantage over conventional anti-cancer therapies by reducing side-effects and preventing more efficiently recurrence and metastasis. Considering the relevance of STn as a potential target and its influence on the immune system, the development of targeted immunotherapies against STn is an attractive approach to treat patients with STn-expressing tumors. Antibodies are currently being broadly used as therapeutic strategies, namely targeted immunotherapies against cancer, due to their high specificity and possibility of activating the immune system through recognition by immune cells.

The main goal of this thesis was to assess if the use of anti-STn antibodies to block the STn antigen could improve anti-tumor immune responses by reestablishing the mature phenotype on DCs. The work developed was divided in two parts, each with specific goals.

The first part of the work comprised the development and characterization of anti-STn antibodies by hybridoma technology. To this purpose, mice were immunized with seven distinct antigens containing STn. When the serum recovered from the mice seemed to be positive for the presence of anti-STn antibodies, mice were sacrificed and the B cells collected from their spleen and fused with myeloma cells, generating hybridoma cells. Out of seven antigens used, only OSM showed to be immunogenic enough to promote a strong humoral response and the development of antibodies against STn. The reactivity of the antibodies in the supernatant of the fused hybridoma clones was assessed by ELISA and flow cytometry. The hybridoma clones showed to produce antibodies that reacted against sialylated structures and also against other structures, exposed after sialidase treatment. Throughout the screening process, the hybridoma clones were selected according to the production of antibodies and their reactivity towards sialylated structures. Six hybridoma clones were selected but due to genetic instability and/or freezing/thawing processes, five of those clones lost the ability to produce antibodies. The remaining one was further cloned and the three best clones were selected and considered as the final clones to be further characterized. All the clones tested were of IgM isotype, including the final ones. The IgM isotype is the first to be produced by B cells, and is more easily obtained towards glycan antigens through direct presentation by APCs, as carbohydrates alone, without a peptide carrier, cannot be presented by MHC molecules and then recognized by T cells. High-affinity IgG isotype antibodies have the capacity to bind to the target cancer cells and mark them for destruction by either antibody-dependent cell-mediated cytotoxicity or by complement

activation, thus being efficient for immunotherapy applications. However, the class switching from IgM to IgG only occurs through T cell-dependent B cell activation, this is, when B cells receive stimulatory signals from T helper cells. Also, throughout the screening process, the ELISA technique used and the dilutions adopted for the anti-STn positive controls were optimized, and the same occurred with flow cytometry, with the addition of the optimization of the sialidase treatment conditions. The main conclusion for this part is that mouse anti-STn antibodies were successfully produced through hybridoma technology, with the producing hybridoma clones maintaining a stable antibody production in culture. Further characterization steps will be performed by several techniques, such as western blot, glycoarrays and immunohistochemistry, also including flow cytometry testing with different cell lines. Possible interesting improvements for future attempts of antibody production through hybridoma technology could include an initial targeting towards IgG-producing clones, since this isotype is mainly involved in opsonization and antibody-dependent cell-mediated cytotoxicity processes, thus being the most common isotype found in therapeutic antibodies.

The second part of the work comprised a functional *in vitro* characterization of humanized anti-STn antibodies through the assessment of the immune response of DCs against STn-expressing cancer cells, with and without STn blockade by those antibodies. This implied, besides the culture of cancer cell lines, the use of DCs generated from monocytes that were isolated from peripheral blood mononuclear cells. Co-cultures between cancer cells and DCs were established and several assays were performed, in order to assess the effect of the blockade of the STn antigen in the maturation process of DCs. Throughout the co-culture process, two humanized anti-STn antibodies were tested, in two different concentrations. Moreover, the conditions of the assay, in terms of controls, reagents and incubation times were also optimized. However, it was not possible to observe the reestablishment of the mature phenotype in the assays performed. The blockade of the STn antigen with these antibodies did not show relevant differences in the expression of MHC-II (HLA-DR) molecules, co-stimulatory molecules (CD80/CD86) and cytokines (IL-6, IL-10, IL-12 and TNF-α). Besides staining by flow cytometry, ELISA and RT-PCR experiments were also performed and the results obtained were consistent within the three techniques. Furthermore, some experiments were repeated, in the same conditions, by our collaborators, namely the co-cultures with mAb1 as blocking antibody and the similar results were obtained (data not shown). Therefore, further optimization steps might be applied to the procedure. In the future, different co-culture conditions may be tested, such as the co-culture times, the cell lines used, and the antibody and its concentration. Concentrations of antibody higher than 1.8 μg/ml will be tested, though viability studies should be performed in parallel as it was observed that higher concentrations seemed to induce cell death. Other STn-expressing cell lines can also be tested. Once that the reestablishment of the mature phonotype is observed, the DCs will be loaded with tumor antigens obtained in the presence and absence of the blocking antibodies. The effect of that blockade will be studied by the capacity of antigen-loaded DCs to activate T cells, an important step needed for the induction of tumor-specific immune responses.

The work developed in both parts implied the use of cancer cell lines expressing STn. Three cell lines were initially used (MDA-MB-231 STn, MCR STn and MCF-7 STn), although two of them rapidly lost the expression of the STn antigen, possibly due to genomic instability, as previously

observed by the group. Another future procedure will be the performance of a new transduction or transfection of these and other cell lines, so that the enzyme ST6GalNAc-I will be overexpressed, allowing the expression of STn, which will also contribute for a new set of STn-expressing cell lines to be tested and used in assays like the ones performed in the second part of this thesis.

The work developed contributed to understanding that further improvements and assays are necessary. It also contributed to the understanding that the production of antibodies, though achieved by a simple process through hybridoma technology, comprises many variants that can be modified during the screening processes, as well as during the characterization procedures, thus being a long process and needing many optimizations along the way before success can be achieved.

Hopefully, in the future, new immunotherapies will be developed targeting specifically the STn antigen, and will contribute for a better and earlier diagnosis and treatment, promoting a reduction in the number of cancer-related deaths.

5. REFERENCES

[1] World Health Organization, Cancer Key Facts, Fact Sheet n°297. (2015). http://www.who.int/mediacentre/factsheets/fs297/en/ (accessed September 6, 2016).

[2] US National Center For Health Statistics, Leading Causes of Death, Heal. United States, 2015 With Spec. Featur. Racial Ethn. Heal. Disparities. (2016). http://www.cdc.gov/nchs/fastats/leading-causes-of-death.htm (accessed September 6, 2016).

[3] G.M. Cooper, "The Development and Causes of Cancer" in The Cell: A Molecular Approach. 2nd edition., (2000). http://www.ncbi.nlm.nih.gov/books/NBK9963/ (accessed September 7, 2016).

[4] C. Blanpain, Tracing the cellular origin of cancer., Nat. Cell Biol. 15 (2013) 126–134. doi:10.1038/ncb2657.

[5] D. Hanahan, R.A. Weinberg, The Hallmarks of Cancer, Cell. 100 (2000) 57–70. doi:10.1016/S0092-8674(00)81683-9.

[6] D. Hanahan, R.A. Weinberg, Hallmarks of cancer: The next generation, Cell. 144 (2011) 646–674. doi:10.1016/j.cell.2011.02.013.

[7] S.S. Pinho, C.A. Reis, Glycosylation in cancer: mechanisms and clinical implications, Nat. Rev. Cancer. 15 (2015) 540–555. doi:10.1038/nrc3982.

[8] Bertozzi CR, Rabuka D, Structural Basis of Glycan Diversity, in: A. Varki, R.D. Cummings, J.D. Esko, H.H. Freeze, P. Stanley, C.R. Bertozzi, G.W. Hart, M.E. Etzler (Eds.), Essentials Glycobiol., 2nd ed., Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press. Available from: http://www.ncbi.nlm.nih.gov/books/NBK1955/, 2009.

[9] Consortium for Functional Glycomics, Symbol and Text Nomenclature for Representation of Glycan Structure, (2012). http://www.functionalglycomics.org/static/consortium/Nomenclature.shtml (accessed September 10, 2016).

[10] A. Varki, J. Esko, K. Colley, Cellular Organization of Glycosylation, in: A. Varki, R.D. Cummings, J.D. Esko, H.H. Freeze, P. Stanley, C.R. Bertozzi, G.W. Hart, M.E. Etzler (Eds.), Essentials Glycobiol., 2nd ed., Cold Spring Harbor (NY): Cold Spring Harbor Laboratory Press. Available from: http://www.ncbi.nlm.nih.gov/books/NBK1926/, 2009.

[11] A. Varki, Biological Roles of Glycans, Glycobiology. (2016). doi:10.1093/glycob/cww086.

[12] A. Varki, J. Lowe, Biological Roles of Glycans, in: A. Varki, R.D. Cummings, J.D. Esko, H.H. Freeze, P. Stanley, C.R. Bertozzi, G.W. Hart, M.E. Etzler (Eds.), Essentials Glycobiol., 2nd ed., Cold Spring Harbor (NY): Cold Spring Harbor Laboratory Press. Available from: http://www.ncbi.nlm.nih.gov/books/NBK1897, 2009.

[13] C.A. Reis, H. Osorio, L. Silva, C. Gomes, L. David, Alterations in glycosylation as biomarkers for cancer detection, J. Clin. Pathol. 63 (2010) 322–329. doi:10.1136/jcp.2009.071035.

[14] P. Van Den Steen, P.M. Rudd, R.A. Dwek, G. Opdenakker, Concepts and Principles of O-Linked Glycosylation, Crit. Rev. Biochem. Mol. Biol. 33 (1998) 151–208. doi:10.1080/10409239891204198.

[15] J. Munkley, The role of sialyl-Tn in cancer, Int. J. Mol. Sci. 17 (2016). doi:10.3390/ijms17030275.

[16] A. Cazet, S. Julien, M. Bobowski, J. Burchell, P. Delannoy, Tumour-associated carbohydrate antigens in breast cancer., Breast Cancer Res. 12 (2010) 204. doi:10.1186/bcr2577.

[17] I. Brockhausen, H. Schachter, P. Stanley, O-GalNAc Glycans, in: A. Varki, R.D. Cummings, J.D. Esko, H.H. Freeze, P. Stanley, C.R. Bertozzi, G.W. Hart, M.E. Etzler (Eds.), Essentials Glycobiol., 2nd ed., Cold Spring Harbor (NY): Cold Spring Harbor Laboratory Press. Available from: http://www.ncbi.nlm.nih.gov/books/NBK1896/, 2009.

[18] M.J. Schultz, A.F. Swindall, S.L. Bellis, Regulation of the metastatic cell phenotype by sialylated glycans, Cancer Metastasis Rev. 31 (2012) 501–518. doi:10.1007/s10555-012-9359-7.

[19] A. Varki, R. Schauer, Sialic Acids, in: A. Varki, R.D. Cummings, J.D. Esko, H.H. Freeze, P. Stanley, C.R. Bertozzi, G.W. Hart, M.E. Etzler (Eds.), Essentials Glycobiol., 2nd ed., Available from: http://www.ncbi.nlm.nih.gov/books/NBK1920/, 2009.

[20] R. Schauer, Achievements and challenges of sialic acid research., Glycoconj. J. 17 (2000) 485–99. doi:10.1023/A:1011062223612.

[21] D. Feng, A.S. Shaikh, F. Wang, Recent Advance in Tumor-associated Carbohydrate Antigens (TACAs)-based Antitumor Vaccines, ACS Chem. Biol. 11 (2016) 850–863. doi:10.1021/acschembio.6b00084.

[22] S. Julien, E. Adriaenssens, K. Ottenberg, A. Furlan, G. Courtand, A.S. Vercoutter-Edouart, F.G. Hanisch, P. Delannoy, X. Le Bourhis, ST6GalNAc I expression in MDA-MB-231 breast cancer cells greatly modifies their O-glycosylation pattern and enhances their tumourigenicity, Glycobiology. 16 (2006) 54–64. doi:10.1093/glycob/cwj033.

[23] R. Kannagi, J. Yin, K. Miyazaki, M. Izawa, Current relevance of incomplete synthesis and neo-synthesis for cancer-associated alteration of carbohydrate determinants-Hakomori's concepts revisited, Biochim. Biophys. Acta - Gen. Subj. 1780 (2008) 525–531. doi:10.1016/j.bbagen.2007.10.007.

[24] A. Varki, R. Kannagi, B.P. Toole, Glycosylation Changes in Cancer, in: A. Varki, R.D. Cummings, J.D. Esko, H.H. Freeze, P. Stanley, C.R. Bertozzi, G.W. Hart, M.E. Etzler (Eds.), Essentials Glycobiol., 2nd ed., Cold Spring Harbor (NY): Cold Spring Harbor Laboratory Press. Available from: http://www.ncbi.nlm.nih.gov/books/NBK1955/, 2009.

[25] N.T. Marcos, E.P. Bennett, J. Gomes, A. Magalhaes, C. Gomes, L. David, I. Dar, C. Jeanneau, S. DeFrees, D. Krustrup, L.K. Vogel, E.H. Kure, J. Burchell, J. Taylor-Papadimitriou, H. Clausen, U. Mandel, C.A. Reis, ST6GalNAc-I controls expression of sialyl-Tn antigen in gastrointestinal tissues, Front Biosci (Elite Ed). 3 (2011) 1443–1455. doi:345 [pii].

[26] M.R. Kudelka, T. Ju, J. Heimburg-Molinaro, R.D. Cummings, Simple sugars to complex disease-mucin-type O-glycans in cancer, in: Adv. Cancer Res. - Glycosylation Cancer, 1st ed., Elsevier Inc., 2015: pp. 53–135. doi:10.1016/bs.acr.2014.11.002.

[27] L.G. Yu, The oncofetal Thomsen-Friedenreich carbohydrate antigen in cancer progression, Glycoconj. J. 24 (2007) 411–420. doi:10.1007/s10719-007-9034-3.

[28] G. Springer, T and Tn, general carcinoma autoantigens, Science (80-.). 224 (1984) 1198–1206. doi:10.1126/science.6729450.

[29] M.F. Wolf, A. Ludwig, P. Fritz, K. Schumacher, Increased Expression of Thomsen-Friedenreich Antigens during Tumor Progression in Breast Cancer Patients, Tumor Biol. 9 (1988) 190–194. doi:10.1159/000217561.

[30] M. Osako, S. Yonezawa, B. Siddiki, J. Huang, J.J.L. Ho, Y.S. Kim, E. Sato, Immunohistochemical study of mucin carbohydrates and core proteins in human pancreatic tumors, Cancer. 71 (1993) 2191–2199. doi:10.1002/1097-0142(19930401)71:7<2191::AID-CNCR2820710705>3.0.CO;2-X.

[31] M.R. Kudelka, T. Ju, J. Heimburg-Molinaro, R.D. Cummings, Simple Sugars to Complex Disease—Mucin-Type O-Glycans in Cancer, in: Adv. Cancer Res., 1st ed., Elsevier, 2015: pp. 53–135. doi:10.1016/bs.acr.2014.11.002.

[32] S.H. Itzkowitz, E.J. Bloom, T.S. Lau, Y.S. Kim, Mucin associated Tn and sialosyl-Tn antigen expression in colorectal polyps., Gut. 33 (1992) 518–523. doi:10.1136/gut.33.4.518.

[33] S. Julien, P.A. Videira, P. Delannoy, Sialyl-tn in cancer: (how) did we miss the target?, Biomolecules. 2 (2012) 435–66. doi:10.3390/biom2040435.

[34] T. Ju, G.S. Lanneau, T. Gautam, Y. Wang, B. Xia, S.R. Stowell, M.T. Willard, W. Wang, J.Y. Xia, R.E. Zuna, Z. Laszik, D.M. Benbrook, M.H. Hanigan, R.D. Cummings, Human tumor antigens Tn and sialyl Tn arise from mutations in Cosmc, Cancer Res. 68 (2008) 1636–1646. doi:10.1158/0008-5472.CAN-07-2345.

[35] J.A. Ferreira, P.A. Videira, L. Lima, S. Pereira, M. Silva, M. Carrascal, P.F. Severino, E. Fernandes, A. Almeida, C. Costa, R. Vitorino, T. Amaro, M.J. Oliveira, C.A. Reis, F. Dall'Olio, F. Amado, L.L. Santos, Overexpression of tumour-associated carbohydrate antigen sialyl-Tn in advanced bladder tumours, Mol. Oncol. 7 (2013) 719–731. doi:10.1016/j.molonc.2013.03.001.

[36] S. Julien, M.A. Krzewinski-Recchi, A. Harduin-Lepers, V. Gouyer, G. Huet, X. Le Bourhis, P. Delannoy, Expression of Sialyl-Tn antigen in breast cancer cells transfected with the human CMP-Neu5Ac: GalNAc α2,6-sialyltransferase (ST6GalNAc I) cDNA, Glycoconj. J. 18 (2001) 883–893. doi:10.1023/A:1022200525695.

[37] C. Vázquez-Martín, E. Cuevas, E. Gil-Martín, A. Fernández-Briera, Correlation Analysis between Tumorous Associated Antigen Sialyl-Tn Expression and ST6GalNAc I Activity in Human Colon Adenocarcinoma, Oncology. 67 (2004) 159–165. doi:10.1159/000081003.

[38] S. Pinho, N.T. Marcos, B. Ferreira, A.S. Carvalho, M.J. Oliveira, F. Santos-Silva, A. Harduin-Lepers, C.A. Reis, Biological significance of cancer-associated sialyl-Tn antigen: Modulation of malignant phenotype in gastric carcinoma cells, Cancer Lett. 249 (2007) 157–170. doi:10.1016/j.canlet.2006.08.010.

[39] S. Julien, C. Lagadec, M.A. Krzewinski-Recchi, G. Courtand, X. Le Bourhis, P. Delannoy, Stable expression of sialyl-Tn antigen in T47-D cells induces a decrease of cell adhesion and an increase of cell migration, Breast Cancer Res. Treat. 90 (2005) 77–84. doi:10.1007/s10549-004-3137-3.

[40] A.K. Abbas, A.H. Lichtman, S. Pillai, Introduction to the Immune System, in: A.K. Abbas, A.H. Lichtman, S. Pillai (Eds.), Cell. Mol. Immunol., 6th ed., Saunders, Elsevier, 2007: pp. 1–71.

[41] P.M. Lydyard, A. Whelan, M.W. Fanger, Instant Notes: Immunology, 2nd ed., BIOS Scientific Publishers, 2004.

[42] J. Banchereau, F. Briere, C. Caux, J. Davoust, S. Lebecque, Y.-J. Liu, B. Pulendran, K. Palucka, Immunobiology of Dendritic Cells, Annu. Rev. Immunol. 18 (2000) 767–811. doi:10.1146/annurev.immunol.18.1.767.

[43] E.M. Cardoso, Visão Global do Sistema Imunológico, in: F.A. Arosa, E.M. Cardoso, F.C. Pacheco (Eds.), Fundam. Imunol., 2nd ed., Lidel, 2012: pp. 32–56.

[44] C. Reis E Sousa, Activation of dendritic cells: Translating innate into adaptive immunity, Curr. Opin. Immunol. 16 (2004) 21–25. doi:10.1016/j.coi.2003.11.007.

[45] M.T. Cruz, M.C. Lopes, Células Dendríticas, in: F.A. Arosa, E.M. Cardoso, F.C. Pacheco (Eds.), Fundam. Imunol., 2nd ed., Lidel, 2012: pp. 171–194.

[46] M. Celia, F. Sallusto, A. Lanzavecchia, Origin, maturation and antigen presenting function of dendritic cells Marina Celia, Federica Sallusto and Antonio Lanzavecchia, Curr Biol. 9 (1997) 10–16. doi:10.1016/S0952-7915(97)80153-7.

[47] F. Sallusto, Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor alpha, J. Exp. Med. 179 (1994) 1109–1118. doi:10.1084/jem.179.4.1109.

[48] A.K. Abbas, A.H. Lichtman, S. Pillai, Antigen Processing and Presentation to T lymphocytes, in: A.K. Abbas, A.H. Lichtman, S. Pillai (Eds.), Cell. Mol. Immunol., 6th ed., Saunders, Elsevier, 2007: pp. 113–136.

[49] K. Palucka, J. Banchereau, Cancer immunotherapy via dendritic cells, Nat. Rev. Cancer. 12 (2012) 265–277. doi:10.1038/nrc3258.

[50] J. Banchereau, R.M. Steinman, Dendritic cells and the control of immunity., Nature. 392 (1998) 245–52. doi:10.1038/32588.

[51] F. Granucci, E. Ferrero, M. Foti, D. Aggujaro, K. Vettoretto, P. Ricciardi-Castagnoli, Early events in dendritic cell maturation induced by LPS, Microbes Infect. 1 (1999) 1079–1084. doi:10.1016/S1286-4579(99)00209-9.

[52] R.M. Steinman, Dendritic cells and vaccines., Proc. (Bayl. Univ. Med. Cent). 21 (2008) 3–8.

[53] E.M. Cardoso, Immunoglobulinas, in: F.A. Arosa, E.M. Cardoso, F.C. Pacheco (Eds.), Fundam. Imunol., 2nd ed., Lisboa, 2012: pp. 195–216.

[54] A.K. Abbas, A.H. Lichtman, S. Pillai, Antibodies and Antigens, in: A.K. Abbas, A.H. Lichtman, S. Pillai (Eds.), Cell. Mol. Immunol., 6th ed., Saunders, Elsevier, 2007: pp. 75–96.

[55] abcam, Antibody Structure and Isotypes, (n.d.). http://www.abcam.com/protocols/antibody-structure-and-isotypes (accessed September 18, 2016).

[56] A.K. Abbas, A.H. Lichtman, Cellular and Molecular Immunology, 5th ed., Saunders, 2003.

[57] O.H.S. Brekke, Therapeutic antibodies for human diseases at the dawn of the twenty-first century, Nat. Rev. Drug disco (2003) 52–62. doi:10.1038/nrd984.

[58] P. Carter, Improving the efficacy of antibody-based cancer therapies, Nat. Rev. Cancer. 1 (2001) 118–129. doi:10.1038/35101072.

[59] G. Köhler, C. Milstein, Continuous cultures of fused cells secreting antibody of predefined specificity, Nature. 256 (1975) 495–497. doi:10.1038/256495a0.

[60] T. Masahiro, K. Tsumoto, Hybridoma Technologies for antibody production, Immunotherapy. 3 (2011) 371–380. doi:http://dx.doi.org/10.1108/17506200710779521.

[61] L.R. Loureiro, M.A. Carrascal, A. Barbas, J.S. Ramalho, C. Novo, P. Delannoy, P.A. Videira, Challenges in Antibody Development against Tn and Sialyl-Tn Antigens., Biomolecules. 5 (2015) 1783–809. doi:10.3390/biom5031783.

[62] B. Monzavi-Karbassi, A. Pashov, T. Kieber-Emmons, Tumor-Associated Glycans and Immune Surveillance., Vaccines. 1 (2013) 174–203. doi:10.3390/vaccines1020174.

[63] S. Maia, Â.A. Cardoso, Imunologia Tumoral, in: F.A. Arosa, E.M. Cardoso, F.C. Pacheco (Eds.), Fundam. Imunol., 2nd ed., Lidel, 2012: pp. 363–389.

[64] M.A. Carrascal, P.F. Severino, M. Guadalupe Cabral, M. Silva, J.A. Ferreira, F. Calais, H. Quinto, C. Pen, D. Ligeiro, L.L. Santos, F. Dall'Olio, P.A. Videira, Sialyl Tn-expressing bladder cancer cells induce a tolerogenic phenotype in innate and adaptive immune cells, Mol. Oncol. 8 (2014) 753–765. doi:10.1016/j.molonc.2014.02.008.

[65] A.M. Scott, J.D. Wolchok, L.J. Old, Antibody therapy of cancer, Nat Rev Cancer. 12 (2012) 278–287. doi:10.1038/nrc3236.

[66] R. Cailleau, R. Young, M. Olivé, W.J.J. Reeves, Breast Tumor Cell Lines From Pleural Effusions, Jounal Natl. Cancer Inst. 53 (1974) 661–674. doi:10.1017/CBO9781107415324.004.

[67] H.D. Soule, J. Vazquez, A. Long, S. Albert, M. Brennan, A Human Cell Line From a Pleural Effusion Derived From a Breast Carcinoma, J. Natl. Cancer Inst. 51 (1973) 1409–1416. doi:10.1093/jnci/51.5.1409.

[68] F. Fabbri, G. Brigliadori, P. Ulivi, A. Tesei, I. Vannini, M. Rosetti, S. Bravaccini, D. Amadori, M. Bolla, W. Zoli, Pro-apoptotic effect of a nitric oxide-donating NSAID, NCX 4040, on bladder carcinoma cells, Apoptosis. 10 (2005) 1095–1103. doi:10.1007/s10495-005-0619-7.

[69] M.G. Ormerod, Flow Cytometry - A Basic Introduction, (2008). http://flowbook.denovosoftware.com/ (accessed August 16, 2016).

[70] J. Picot, C.L. Guerin, C. Le Van Kim, C.M. Boulanger, Flow cytometry: Retrospective, fundamentals and recent instrumentation, Cytotechnology. 64 (2012) 109–130. doi:10.1007/s10616-011-9415-0.

[71] Life Technologies Corporation, Attune® Acoustic Focusing Cytometer: User Guide, (2012).

[72] M.A. Carrascal, Estudo da resposta imunológica desencadeada por células de cancro de bexiga que expressam antigénios sialil-Tn, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa, 2010.

[73] S.D. Gan, K.R. Patel, Enzyme immunoassay and enzyme-linked immunosorbent assay., J. Invest. Dermatol. 133 (2013) e12. doi:10.1038/jid.2013.287.

[74] R.M. Lequin, Enzyme immunoassay (EIA)/enzyme-linked immunosorbent assay (ELISA), Clin. Chem. 51 (2005) 2415–2418. doi:10.1373/clinchem.2005.051532.

[75] A.H.W.M. Schuurs, B.K. Van Weemen, Enzyme-Immunoassay: A Powerful Analytical Tool, J. Immunoassay. 1 (1980) 229–249. doi:10.1080/01971528008055786.

[76] Thermo Fisher Scientific, Horseradish Peroxidase Substrates, in: Thermo Sci. Pierce Assay Dev. Tech. Handbook, Version 2, 2011: p. 59.

[77] M.L. Wong, J.F. Medrano, Real-time PCR for mRNA quantitation, Biotechniques. 39 (2005) 75–85. doi:10.2144/05391RV01.

[78] Life Technologies Corporation, Introduction to Gene Expression: Getting Started Guide, (2010) 1–36.

[79] K.J. Livak, T.D. Schmittgen, Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the $2^{-\Delta\Delta CT}$ Method, Methods. 25 (2001) 402–408. doi:10.1006/meth.2001.1262.

[80] T.D. Schmittgen, K.J. Livak, Analyzing real-time PCR data by the comparative CT method, Nat. Protoc. 3 (2008) 1101–1108. doi:10.1038/nprot.2008.73.

[81] M.A.G. Reis, Produção de anticorpos para tratamento do cancro da mama, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa, 2014.

[82] P.F. Severino, Thomsen-Friedenreich Antigens In Bladder Cancer: Evaluation Of Their Prognostic Value, Faculdade de Ciências Médicas, Universidade Nova de Lisboa, 2014.

[83] S. Sasawatari, T. Tadaki, M. Isogai, M. Takahara, M. Nieda, K. Kakimi, Efficient priming and expansion of antigen-specific CD8 + T cells by a novel cell-based artificial APC, Immunol. Cell Biol. 84 (2006) 512–521. doi:10.1111/j.1440-1711.2006.01462.x.

[84] R. Hulspas, M.R.G.G. O'Gorman, B.L. Wood, J.W. Gratama, D.R. Sutherland, D. Robert Sutherland, Considerations for the control of background fluorescence in clinical flow cytometry, Cytom. Part B Clin. Cytom. 76B (2009) 355–364. doi:10.1002/cyto.b.20485.

[85] S.J. Golubović, Ž.T. Bojić-Trobjević, Human carcinoma-associated and salivary mucins detected by anti-bovine submaxillary mucin antibodies, Biochem. 71 (2006) S9–S17. doi:10.1134/S0006297906130025.

[86] K.P. O'Boyle, R. Zamore, S. Adluri, A. Cohen, N. Kemeny, S. Welt, K.O. Lloyd, H.F. Oettgen, L.J. Old, P.O. Livingston, Immunization of Colorectal Cancer Patients with Modified Ovine Submaxillary Gland Mucin and Adjuvants Induces IgM and IgG Antibodies to Sialylated Tn,

Cancer Res. 52 (1992) 5663–5667.

[87] R.T. Taggart, Culture Methods for the Selection and Isolation of Stable Antibody-Producing Murine Hybridomas, in: A.H. Bartal, Y. Hirshaut (Eds.), Methods Hybrid. Form., Humana Press, Totowa, NJ, 1987: pp. 181–194. doi:10.1007/978-1-4612-4826-2_8.

[88] Y. An, W. Han, X. Chen, X. Zhao, D. Lu, J. Feng, D. Yang, L. Song, X. Yan, A Novel Anti-sTn Monoclonal Antibody 3P9 Inhibits Human Xenografted Colorectal Carcinomas, J. Immunother. 36 (2013) 20–28. doi:10.1097/CJI.0b013e31827810d1.

[89] G.E. Kaiko, J.C. Horvat, K.W. Beagley, P.M. Hansbro, Immunological decision-making: how does the immune system decide to mount a helper T-cell response?, Immunology. 123 (2008) 326–338. doi:10.1111/j.1365-2567.2007.02719.x.

[90] Cancer Research UK, Worldwide cancer statistics, (2014). http://www.cancerresearchuk.org/health-professional/cancer-statistics/worldwide-cancer#heading-Two (accessed August 13, 2016).

[91] Cancer Research UK, Worldwide cancer incidence statistics, (2014). http://www.cancerresearchuk.org/health-professional/cancer-statistics/worldwide-cancer/incidence (accessed August 13, 2016).

[92] R. Nordén, Leukocyte Migration over the Endothelial Wall, Herpesvirus-Induced Glycans. (n.d.). http://www.glycopedia.eu/e-chapters/herpesvirus-induced-glycans/Leukocyte-Migration-over-the (accessed September 16, 2016).

6. APPENDIXES

Appendix I: Symbol and Text Nomenclature for Representation of Glycan Structure [9]

(as selected by the Nomenclature Committee Consortium of Functional Glycomics, based on a version originally put forth by Stuart Kornfeld and later adapted by the editors of the textbook *Essentials of Glycobiology* (Cold Spring Harbor Laboratory Press)

Symbol Nomenclature (Figure 6.1):

- The symbol nomenclature must be convenient for the annotation of mass spectra. To this end, it was decided that:
  - Each sugar type (i.e. sugars of the same mass: hexose, hexosamine and N-acetylhexosamine), should have the same symbol shape;
  - Isomers of each sugar type (e.g. mannose/galactose/glucose) should be differentiated by color or by black/white/shading;
  - Where possible, the same color or shading should be used for derivatives of hexose (e.g. the corresponding N-acetylhexosamine and hexosamine);
  - Representing different sugars using the same shape but different orientation should be avoided so that structures can be represented either horizontally or vertically.
- Colored symbols should still appear distinguishable when copied or printed in black and white;
- Considering 10% of the population is color blind, the use of both red and green for the same shaped symbols should be avoided;
- When desired, linkage information can be represented in text next to a line connecting the symbols (e.g. alpha4, beta4).

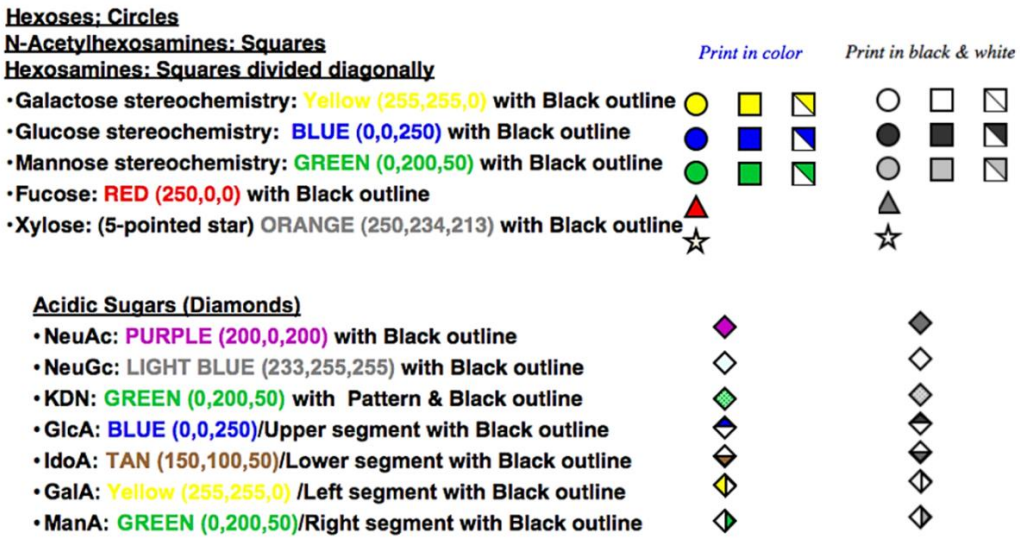

Figure 6.1 – Symbol nomenclature adopted for glycan structures.

Text Nomenclature (Figure 6.2):

A 'modified International Union of Pure and Applied Chemistry (IUPAC) condensed' text nomenclature, which includes the anomeric carbon but not the parentheses, and which can be written in either a linear or 2D version is recommended:

- Including the anomeric carbon is important, and is likely to become increasingly more so in the future as more complicated structures are discovered;
- The presence of parentheses (which then necessitates the use of brackets to indicate branching structures) is unnecessarily cumbersome, particularly when representing the structure in 2D form.

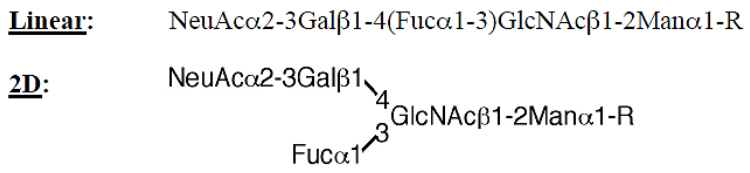

Figure 6.2 – Text nomenclature adopted for glycan structures, both linear and 2D [9].

Appendix II: Composition of the solutions and reagents used in the work developed

Adhesion buffer: Solution containing 20 mM of Tris-HCl (pH 8.0), 150 mM of NaCl, 1 mM of $CaCl_2$, 1 mM of $MgCl_2$ and 0.5% of BSA, in distilled water

Beads buffer: Solution containing 0.5% of BSA (w/v) and 2 mM of EDTA, in PBS 1x

Blocking buffer (indirect ELISA): Solution containing 5% low-fat milk (w/v) in washing buffer (indirect ELISA, see below)

Blocking buffer/reagent diluent (cytokines capture ELISA): Solution containing 2% of BSA (w/v) in washing buffer (capture ELISA, see below)

Complete DMEM culture medium: Simple DMEM supplemented with 10% (v/v) FBS, 2 mM L-glutamine, 100 U/ml of penicillin and 100 µg/ml of streptomycin

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

PBS 1x: Solution containing 1.47 mM of KH_2PO_4, 4.29 mM of $Na_2HPO_4.7H_2O$, 137 mM of NaCl and 2.68 mM of KCl, in distilled water (pH=7.4)

Sialidase buffer (1x): 10 mM Na_2HPO_4 in MiliQ water (pH=6.0)

Washing buffer (capture ELISA): Solution containing 0.05% (v/v) of Tween 20 in PBS 1x

Washing buffer (indirect ELISA): Solution containing 0.1% (v/v) of Tween 20 in PBS 1x

Appendix III: Schematic representation of the hybridoma technology procedure adopted

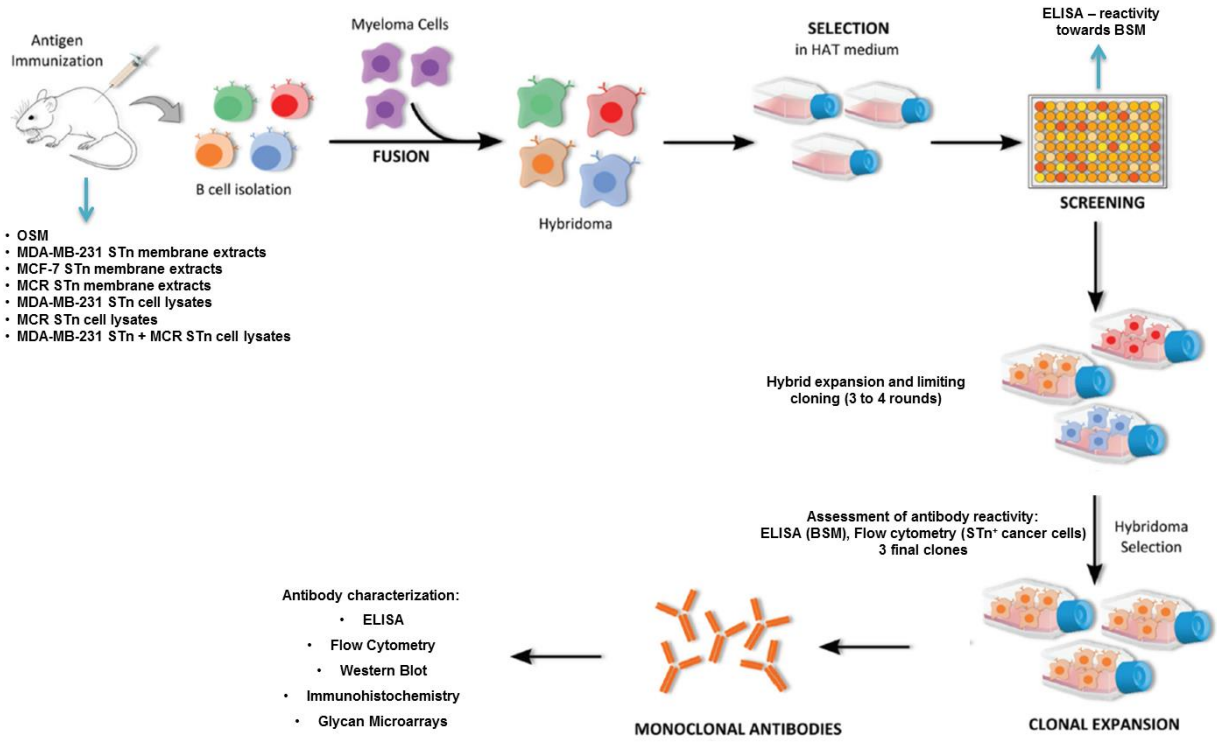

Figure 6.3 - Schematic representation of the hybridoma technology procedure adopted by the group for the development of anti-STn monoclonal antibodies. Adapted from [61].