Tiago José Alexandre da Silva Costa

Bachelor Degree in Basic Studies of Pharmaceutical Sciences

Characterization of novel antibodies and glycan binding proteins against cancer

Dissertation to obtain the Master Degree in Biochemistry

Supervisor: Professor Paula Videira,PhD,FCT-UNL
Co-Supervisor: Roberta Zoppi, MSc, FCT-UNL

Jury:

President: Prof. Doutor Carlos Alberto Gomes Salgueiro
Arguer: Doutor Hugo Ricardo Loureiro Soares
Supervisor: Professor Paula Alexandra Quintela Videira

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FCT-UNL, UNL

Acknowledgments

To my supervisor, Professor Paula Videira, for the opportunity to develop my master's thesis in her laboratory.

To my co-supervisor, Roberta Zoppi, for all the teachings about the laboratory, for all the help given at the precise times, for all the corrections of mistakes I made. Thank you also for the sharing of joy, pain, work and life.

To Zélia Silva for all the help with the hybridomas and for all the knowledge transmitted on flow cytometry.

To Rita Lóios, my laboratory partner, for the daily coexistence, for the support in the most difficult moments, for the games and the shared joy even when everything seemed to be wrong.

To all the Glycoimmunology Group for the sharing of knowledge in the laboratory, all the help they were providing whenever needed: Fanny Deschepper, Tiago Ferro, Gonçalo Mineiro and Érica Freitas.

To all the staff of the Life Sciences Department, we daily encouraged each other.

To the friends of always and forever who always hold me in the best and the worst. Luckily, I cannot list them here because I would have a thesis with only names. Thank you for being the best and for always motivating me.

To my family to support all these years of studies, for being always home, for being concern and for dealing with my bad mood when I am more stressed. Thank you for being love.

To Bruno, for being a companion always in the most difficult and the easiest. For daily encouragement and for always believing in me. Thank you for daily sharing of life, for patience, for caring and for being able to be whole with each other.

Last but not least, To the Lord who does not forget myself when I forget him. Thanks for the life.

Resumo

O cancro é a maior causa de morte no mundo e é cada vez mais urgente desenvolver novas abordagens para diagnóstico e terapia. O padrão de glicosilação das células cancerígenas difere do das células saudáveis e, portanto, a expressão aberrante de glicanos torna-se um promissor alvo terapêutico e de diagnóstico

Sialil-Tn (STn) é um glicano, cuja expressão é aumentada em células cancerígenas e quase ausente em células normais. Está envolvido em processos como o crescimento do cancro, progressão e metástase. Além do STn outros glicanos apresentam um padrão aberrante no cancro. Como é o caso de Sialil Lewis X (sLeX) que é sobreexpresso em tumores.

O objetivo deste trabalho é estudar a produção e caracterização de anticorpos monoclonais contra STn e sLeX através da tecnologia de hibridoma. A afinidade e especificidade dos anticorpos foram testadas por citometria de fluxo e ELISA.

No caso de STn, testámos diferentes lotes de um anticorpo recentemente desenvolvido, o anticorpo L2A5, e realizámos uma subclonagem para selecionar e isolar um melhor desempenho do hibridoma. Linhas celulares de cancro que expressam STn e mucinas submaxilares bovinas que também expressam STn, foram utilizadas para a análise por citometria de fluxo e ELISA, respetivamente. Ambas as técnicas mostraram que os anticorpos produzidos pelo hibridoma L2A5 tinham afinidade e especificidade para STn, embora estas fossem ligeiramente diferentes entre os lotes. Em seguida, clonamos as células de hibridoma. Os resultados permitiram selecionar o subclone 4E11 que produz o anticorpo com as características melhoradas.

No caso do sLeX, um novo anticorpo está a ser desenvolvido através da imunização de ratos com glicoproteínas que contém sLeX. O soro de ratos foi analisado por ELISA e os resultados mostraram a presença de anticorpos que reconhecem o sLeX. Em seguida, o sobrenadante dos hibridomas foi analisado por ELISA para seleccionar aqueles que produzem um candidato a anticorpo monoclonal que reconhece o sLeX.

Ambos os estudos contribuíram para o desenvolvimento de novos anticorpos monoclonais que podem ser importantes no diagnóstico e tratamento do cancro.

Palavras – chave: cancro, Sialil-Tn, Sialil Lewis X, glicosilação, anticorpos monoclonais, tecnologia de hibridoma.

Abstract

Cancer is the largest cause of death worldwide and it is increasingly urgent to develop new approaches to diagnosis and therapy. The glycosylation pattern of the cancer cells differ from that of healthy cells and therefore the aberrant expression of glycans are promising targets for diagnosis and therapy.

Sialyl-Tn (STn) is a glycan, whose expression is increased in cancer cells and almost absent in normal cells. It is involved in processes such as cancer growth, progression and metastasis. In addition to STn other glycans present an aberrant pattern in cancer. As is the case of Sialyl Lewis X (sLeX) shown overexpressed in tumors.

The objective of this work consisted in the characterization of monoclonal antibodies against STn and sLeX produced through the hybridoma technology. The antibodies' affinity and specificity were tested by flow cytometry and ELISA.

In case of STn, different batches of a L2A5 antibody were tested. To select and isolate a better performant hybridoma the original L2A5 hybridoma population was subcloned. Cancer cell lines expressing STn and bovine submaxillary mucins expressing STn, were used for screening by flow cytometry and ELISA, respectively. Both techniques showed that the antibodies produced by the L2A5 hybridoma had affinity and specificity for STn, although these were slightly different between batches. We then cloned the hybridoma cells by limiting dilution. The results allowed selecting the subclone 4E11 which produce antibody with the optimized characteristics.

In case of sLeX, a novel antibody is being developed by immunizing mice with glycoproteins carrying sLeX. Mice serum was screened by ELISA and results showed the presence of sLeX-recognizing antibodies. Then the supernatants of hybridomas were screened by ELISA to select those producing a candidate monoclonal antibody that recognize sLeX .

Both studies have contributed to the development of new monoclonal antibodies with potential use in the diagnosis and treatment of cancer.

Key words: cancer, Sialyl Tn, Sialyl Lexis X, glycosylation, monoclonal antibodies, hybridoma technology.

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Abbreviations

Abbreviation	Meaning
Abs	Antibodies
ADCC	Antibody dependent cell-mediated cytotoxicity
Asn	Asparagine
BSM	Bovine Submaxillary Mucin
CDC	Complement dependent cytotoxicity
Core1 GalT	Core 1 β1-3 galactosyltransferase
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic Acid
Dol-P	Dolichol-phosphate
ELISA	Enzyme-Linked Immunosorbent Assay
ER	Endoplasmic reticulum
Fab	Fragment antigen binding
FBS	Fetal Bovine Serum
Fc	Fragment crystalline
FSC	Forward Scatter Light
FITC	Fluorescein Isothiocyanate
GalNAc	N-acetylgalactosamine
GalNAcT	GalNAc transferase
GlcNAc	N-acetylglucosamine
H	Heavy chains
HAT	Hypoxanthine-aminopterin-thymidine
HGPRT	Hypoxanthine-guanine phosphoribosyltransferase
HRP	Horseradish peroxidase
Ig	Immunoglobulin
L	Light chains
mAb	Monoclonal antibodies
MFI	Median intensity fluorescence
MW	Molecular Weight
OD	Optical density
PBS	Phosphate buffered saline
PEG	Polyethylene glycol
PSM	Porcine Submaxillary Mucin
RPM	Rotations per minute
RT	Room Temperature
Ser	Serine

sLeX	Sialyl Lewis X
SSC	Side Scatter Light
ST	Sialyltransferase
STn	Sialyl Tn
TACAs	Tumor-associated carbohydrate antigens
TE	Trypsin-EDTA
Thr	Threonine
TK	Thymidine kinase
TMB	3,3',5,5'-Tetramethylbenzidine
UDP—GlcNAc	Uridine diphosphate N-acetylgalactosamine
WHO	World Health Organization

1 Introduction

1.1 Cancer

Cancer can be defined as a group of diseases characterized by uncontrolled growth and cell division. Due to the accumulation of somatic mutations in the progeny of normal cells, abnormal cells are generated, and the same cells can develop the ability to invade and to spread to other organs, giving rise to metastases. Depending on the capacity to invade other sites, tumors can be considered respectively, malignant or benign. The development of cancer consists of a multi-step process, where several factors are involved resulting in a high complex disease. According to World Health Organization (WHO), cancer is the leading cause of death worldwide and is responsible for the death of 8.8 million people annualy. The most deadly cancers are lung, liver, colorectal, stomach and breast [1].

Cancer results from the interaction between an individual's genetic load and external environmental factors that may be chemical, physical, or biological, and the cause of cancer is closely related to exposure to these risk factors [1].Oncogenes, genes that promote autonomous cell growth in cancer cells, and mutations in tumor suppressor genes give rise to tumorigenesis. Tumor cells can also acquire some capabilities that make them more resistant and with greater proliferation capacity. These characteristics are also known as hallmarks of cancer (Figure 1)

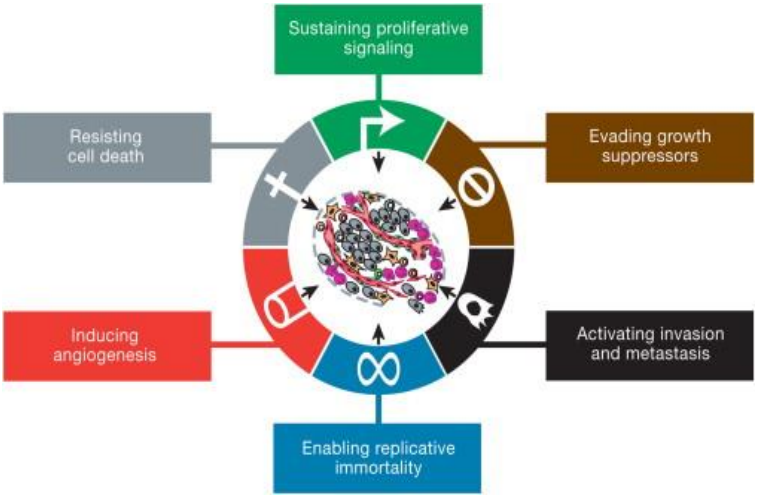

Figure 1 -The Hallmarks of Cancer are the abilities acquired by cancer cells that render them more resistant and increase their proliferation capacity. [3]

Some oncogenes, function as growth factors allowing cell proliferation. In a non-malignant cell proliferation requires the presence of a stimulatory growth factor. Malignant cells have also a mechanism that make them insensitive to growth inhibitory signals allowing cells to multiply indefinitely. Since tumor cells have accumulated mutations and are therefore abnormal cells, the apoptotic system is responsible for the defective cell elimination. However, tumor cells can escape apoptosis by modulating, increasing or decreasing the expression of anti- or pro- apoptotic genes,

respectively. Tumor cells can also replicate indefinitely due to increased telomerase activity, since telomerase has the function of increasing the size of telomeres, preventing telomere shortening by making the cells immortal. For the survival of the cells it is necessary that the correct supply of oxygen and nutrients is maintained and for this reason the angiogenesis, process of growth of new blood vessels, is increased in the tumor environment. The conjugation of all the above-mentioned capabilities leads to the growth and proliferation of cancer cells. The environment favorable to the growth of the cells may also have the capacity for invasion and metastasis, making it possible to migrate these malignant cells to other tissues [2,3].

1.2 Glycosylation

Glycosylation is the process mediated by enzymes that consists in the addition of sugars to other molecules such as proteins and lipids. In nature we can find glycoconjugates that are formed by glycosylation. Glycoconjugates, which consist of carbohydrates of varying size and complexity, attached to a non-sugar moiety as a lipid or a protein, can be found on the cell surface, inside the cell or secreted. The addition of sugars through glycosylation is considered the most common posttranslational modification. Nowadays it is known that glycosylated structures are involved in various biological functions such as immune response, inflammation, cell growth, cell-cell adhesion and signaling [4-8].

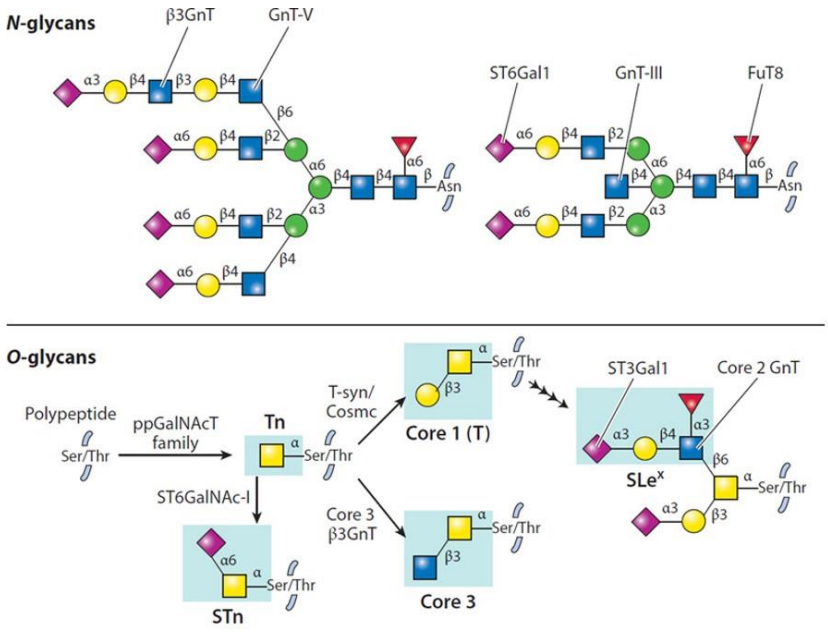

Figure 2 - N- and O-glycosylation. In N-glycosylation, the glycan is attached to an asparagine through N-acetylglucosamine, is typically branched and contains mannose. In O-glycosylation, the glycan is attached to a serine or threonine residue via N-acetylgalactosamine, and its sugars are linked one at a time by glycosyltransferases.[9]

Glycoproteins are polypeptide backbones carrying one or more glycans covalently attached via nitrogen or oxygen linkages, called as N-glycans or O-glycans, respectively. In cancer, the

most common types of glycosylation are sialylation, fucosylation, O-glycan truncation, and N- and O-linked glycan branching. Protein glycosylation occurs inside the lumen of endoplasmic reticulum (ER) and the Golgi complex [10].

The glycosylation process is carried out by glycosyltransferases that transfer specific mono- or oligosaccharides from a glycosyl donor to a glycosyl acceptor in a site-specific. Since glycosidases, which are responsible for the hydrolysis of specific glycan linkage, are also present in the ER and Golgi complex, the glycosylation is a dynamic process that depends on several factors. These factors includes the synthesis of glycosyl donors, competition of different enzymes for the same substrate, enzyme gene expression, enzyme structure and conformation, among other factors [6], [11].

Several studies have shown that altered glycosylation patterns such as aberrant expression of sialylated structures, are present and these changes in glycosylation are related with the invasive potential of various cell types [6].

1.2.1 N-Glycosylation

N-glycosylation is when an asparagine (Asn) residue of a protein is modified by the addition of a sugar residue by a N-glycosidic bond. An N-glycan site is characterized by the amino acid sequence Asn-X-Ser/Thr. The most common N-glycan linkage is N-acetylglucosamine (GlcNAc).

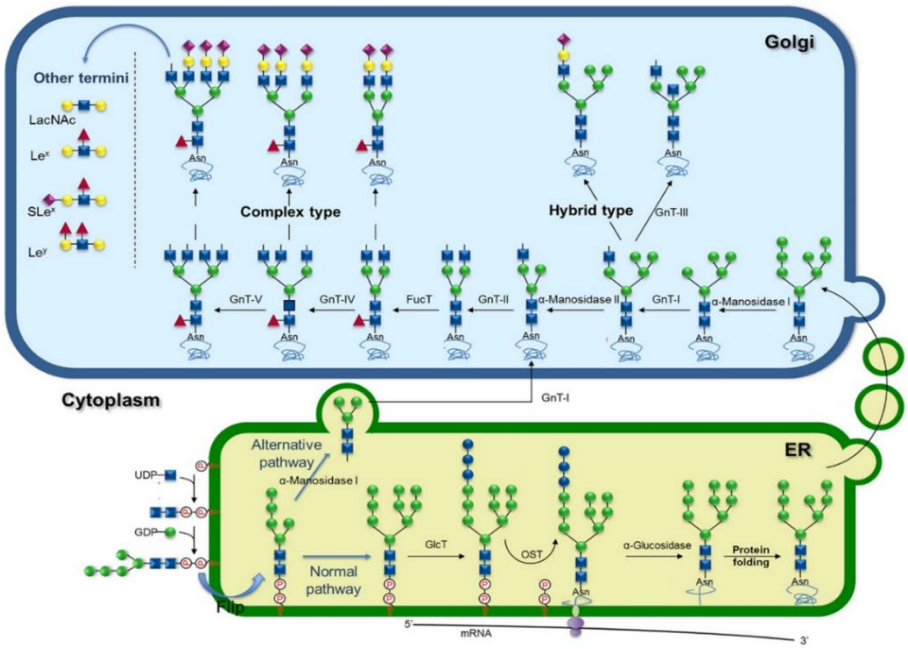

Figure 3- N-Glycosylation pathway. N glycosylation begins in the ER membrane when a GlcNAc-1-phosphotransferase transfer a GlcNAc from UDP—GlcNAc to Dol-P. The second step consists other GlcNAc and five mannoses to form Man5GlcNAc2-P-P-Dol on the cytoplasmic side of the ER membrane. Four Man and three Glc residues are transferred to the Man5GlcNAc2-P-P-Dol. After the protein is bound to N-glycan, other enzymes such as glycosidases and glycosyltransferases can act by altering the endings of the glycans present. Adapted from [12]

The biosynthesis of N-glycans occurs in two steps (figure 3). The first step develops in the ER membrane with the addition of dolichol-phosphate to an Asn residue. Dol-P is a lipid polyisoprenoid molecule that is transferred to asparagine residues after modification on oligosaccharide. This stage occurs when a GlcNAc-1-phosphotransferase transfer a GlcNAc from uridine diphosphate N-acetylgalactosamine (UDP—GlcNAc) to Dol-p forming Dol-P-P-GlcNAc. After this first addition, other GlcNAc and five mannoses (Man) are subsequently transported to form $Man_5GlcNAc_2$-P-P-Dol on the cytoplasmic side of the ER membrane. Then, four Man and three Glc residues are transferred from Dol-P-Man and Dol-P-Glc molecules to the $Man_5GlcNAc_2$-P-P-Dol. This glycan is added in single block by the oligosaccharyltransferase (OST) to the target. When the protein is linked to the N-glycan other series of reactions catalyzed by membrane-bound glycosidases and glycosyltransferases can occur thus modifying the structure of the final glycan. [12][13]

1.2.2 O-glycosylation

O-glycosylation results from a modification by addition of N-acetylgalactosamine (GalNAc) to serine or threonine residues on proteins by the action of GalNAc transferase (GalNAcT). This process is performed by a N-acetylgalactosaminyltransferase and normally is initiated in the Golgi apparatus. After this first step other specifics transferases can elongate or modify the initial structure by sialylation, sulfatation, acetylation and fucosylation [6].

After the addition of GalNAc to its target residue, different o-glycan structures are created depending on the type of reaction between different monosaccharides and GalNAc. The synthesis of most O-glycans begins with the addition of galactose in a β1-3 linkage to the GalNAc giving rise to Core 1. This step is performed by the Core 1 β1-3 galactosyltransferase (Core1 GalT). After the synthesis of Core 1 other monosaccharides can be added leading to the synthesis of other cores. The Core 2 is generated by addition of N-acetylglucosamine (GlcNAc) to the GalNAc in a β1-6 linkage. The formation of Core 2 requires the structure of Core 1 as substrate, so the structure of Core 2 also contains the Core 1 structure [14].

Some of these structures, such as Tn antigen, are cancer related and a direct relationship can be observed between their overexpression and the emergence of tumor cells.

The Tn antigen expression can occur when Core1 GalT is inactive. This inaction prevents to synthesize the Core 1, enabling the glycosyltransferase sialyltranferase (ST6GalNAc – I) to add a sialic acid to the antigen Tn to form sialyl Tn antigen (STn) thus increasing its expression.

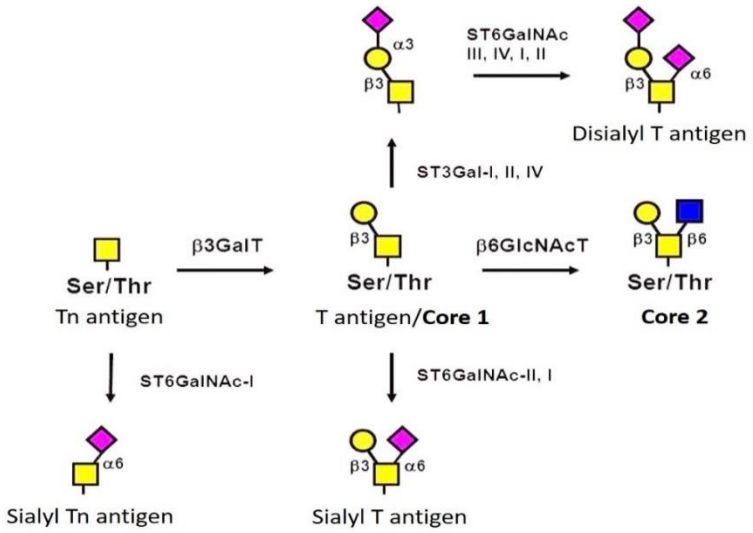

Figure 4 - Pathways of Thomsen-Friedenreich antigens biosynthesis. GalNAc is transferred to a serine or threonine residue in a polypeptide by GalNAc-T. GalNAcα1-Ser/Thr, i.e., the Tn antigen, is then converted by T-synthase to Galβ1-3GalNAcα1-Ser/Thr, i.e., the T antigen or core-1 structure. The Tn antigen can be also sialylated by ST6GalNAc-I forming the sialyl-Tn. T antigen is converted to core-2 structures by C2GnT-1, -2, and -3 or can be sialylated by ST3Gal-I, originating the sialyl-T antigen. Moreover, sialyl-T antigen can be sialylated by ST6GalNAc-I originating disialyl-T antigen. (Adapted from [15]*)*

The Core 1 is also referred as Thomsen–Friedenreich (TF) antigen or T antigen. T antigen, as with Tn, is described in 90% of carcinomas due to altered glycosylation in these tumors. The glycosyltransferases may also act on the antigenic T by adding other monosaccharides and other structures can be formed as Sialyl T or disialyl T [16] (Figure 4).

1.2.3 Glycosylation in cancer

Changes in glycosylation have been described since 60's related with oncogenic transformation. When comparing tumor with non-tumors cells, there are some differences in the glycosylation pattern. These discrepancies can interfere with cancer cell processes such as immune surveillance and inflammation and with the tumor microenvironment, as they are associated with oncogenic transformation, cancer growth, progression and metastasis. The levels and types of carbohydrates founded in cancer cells surface present several differences. This allows the distinction of a cancer cell from a healthy cell due to the glycosylated structures called tumor-associated carbohydrate antigens (TACAs).

The differences between tumor cells and healthy cells, considering the glycosylation pattern, consist of the formation of newly expressed glycans or truncated glycans. Truncated glycans appear in early stages of cancer as does STn. On the other hand, newly expressed glycans generally appear in the late stages of cancer due to the induction of the expression of certain genes, giving rise to new antigens, such as sialyl Lewis A (sLe^A) and sialyl Lewis X (sLe^X) [7] (figure 5).

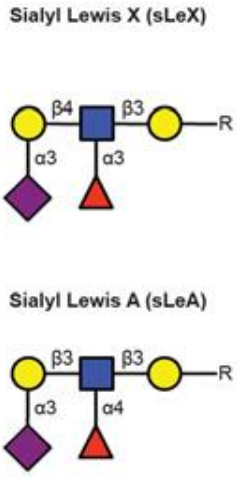

Figure 5 - Sialyl Lewis X and Sialyl Lewis A structures

1.2.3.1 Sialylation in cancer

Sialylation is a modification that occurs at the terminals of proteins, lipids and glycans when sialic acids residues are attached. This modification is important at various stages of tumor progression. Changes in sialylation may be due to alterations in sialic acid, sialoproteins, sialyltransferases (ST) activities or sialidase activities. In cancer cells abnormal sialylation is a characteristic feature related with malignant properties such as invasiveness and metastatic potential [17].

Sialic acids are a family of sugars units composed of 9 carbon backbone. The most common in mammalians is the N-acetylneuraminic acid (Neu5Ac) with a *N*-acetyl group on carbon 5 (Figure 6). These structures are linked to other carbohydrates by sialyltransferases

In tumour cells there is usually an increase in sialylation, which prevents further elongation of the O-glycans, leading to the expression of immature core glycans, such as STn.

STn (Neu5Acα2-6GalNAcα-O-Ser/Thr), also known as CD175, contains a sialic acid (N-acetylneuraminic acid, or Neu5Ac) α-2,6 linked to the Tn antigen. This antigen is rarely expressed in healthy cells; thus, it has tremendous potential both in cancer diagnosis and therapy, being described as an onco-foetal antigen. STn expression has been found in pancreatic, gastric, colorectal, lung, breast, bladder, prostate and ovary cancers, commonly associated with poor cancer prognosis [18].

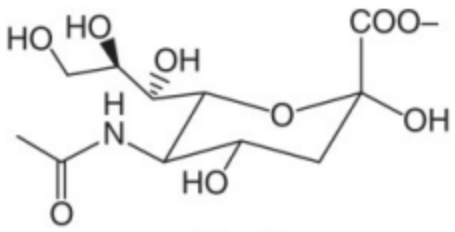

Figure 6 - Sialic Acid Conformation. N-Acetyl-Neuraminic Acid is an N-acyl derivative of neuraminic acid. N-acetylneuraminic acid occurs in many polysaccharides, glycoproteins, and glycolipids in animals and bacteria

In addition to the glycans mentioned above, there are others such as sialyl Lewis X (sLe^x) and Sialyl Lewis A (sLe^a) which are also found on the tumor cell surface. These glycans function as ligands of selectin adhesion receptors on activated endothelia, platelets, or leukocytes. They are probably involved in the formation of tumor-platelet-leukocyte emboli in the microvasculature and extravasation to distant organs during metastasis. Similar to STn, the enhanced progression of tumors and carcinogenicity with high levels of sialylated glycans is due to altered expression of sialyltransferases and fucosyltransferases [19].

Since STn neo- or over- expression has been identified in many types of epithelial cancer it can be used as a diagnostic marker and possible therapeutic target [18].

1.3 Antibodies

Antibodies (Abs) are proteins secreted by B lymphocytes with the capacity to recognize and bind to antigens with high affinity and specificity The antigen can be molecules on the surface of the non-self agents.. Antibodies play two major functions in immune defense. The first function is to recognize and bind to antigen. The second function is the elimination of the molecules recognized as non-self by the antibody. This function triggers mechanisms that aid to the elimination of the foreign agent such as activation of the complement system, phagocytosis among others [20], [21].

The antibody molecule is formed by four chains that are organized in the "Y" shape and these chains are equal two to two. The chains are called light chains (L) and heavy chains (H) and each chain has a variable region and a constant region. Each antibody has a fragment antigen binding (Fab) and a fragment crystalline (Fc) The Fab is involved in the binding of antibody to antigen while Fc is involved in binding to effector molecules (Figure 7) [20], [21].

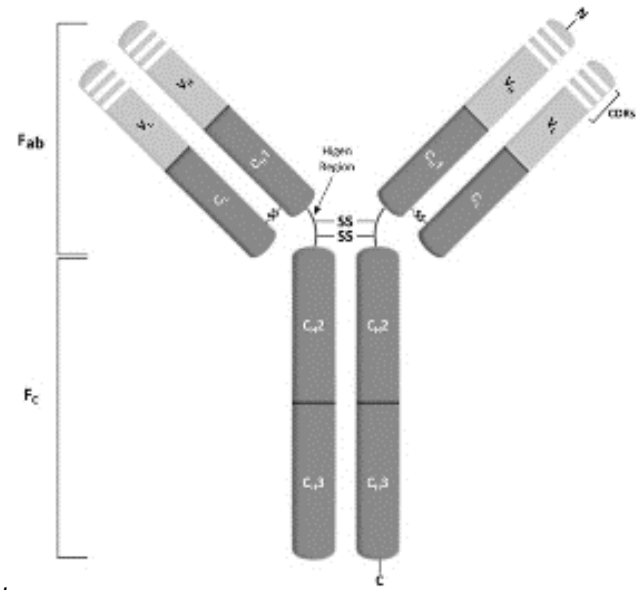

Figure 7 – ***General representation of Immunoglobulin G*** *(IgG) Abbreviations: Fab - Fragment antigen binding; Fc - Fragment crystalline; C - Constant region; V - Variable region; VL - Variable region of the light chain; VH - Variable region of one heavy chain; CDRs – Complementary determining regions.*

Antibodies are also known as immunoglobulins. There are five classes of immunoglobulins called immunoglobulin G (IgG), IgM, IgA, IgD and IgE. These classes have distinct heavy chains of the constant region that influences their functional activity. The heavy chains can be named γ, μ, α, δ and ε and corresponds to each class, respectively (Table 1). The antibody molecule has two κ light chains or two λ light chains [20], [21].

Nowadays, the traditional cancer treatments have many side effects. That's why is important to develop new approaches with higher specificity and with less side effects. With the evolution

of immunotherapy and the use of antibodies in these new therapeutic approaches, it was possible to apply more specific therapies, with greater effectiveness, targeted therapy, less toxic and with less side effects [22].

Table 1- Human antibodies characteristics. Adapted from [21], [23], [24], [25]

Isotype	Subtype	Heavy Chain	Light Chain	MW (kDa)	Serum half-life (days)	General Structure	Functions
IgA	IgA1, IgA2	α1, α2	λ or κ	150-600	5-7	Dimer, monomer, 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 helminths.
IgG	IgG1 IgG2 IgG3 IgG4	γ	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

1.3.1 Cancer Immunotherapy: Therapeutics with antibodies

The conventional cancer treatment has many side effects. That is why it is important to develop new approaches more specific and with less side effects. The most common visible side effects are hair loss, anemia, nausea and vomiting and fatigue which result from toxicity of drugs.

Immunotherapy urges as a treatment capable of acting in two ways, by stimulating the patient's immune system to attack the cancerous cells or by administering to the patient components of the immune system. The main types of immunotherapies are cancer vaccines , immune checkpoints inhibitors and monoclonal antibodies[29].

With the evolution of immunotherapy and the use of antibodies in these new therapeutic approaches, it was possible to apply more specific therapies, with greater effectiveness, less toxic and with less side effects [22].

Therapeutic antibodies can induce tumor cells death by different mechanisms: the blocking of receptors or antagonist activity; induction of apoptosis or delivery of drugs or cytotoxic agents; or immune-mediated cell killing mechanisms, which involves complement dependent cytotoxicity (CDC), antibody dependent cell-mediated cytotoxicity (ADCC), and induction of phagocytosis. In addition, antibodies can improve T cells activation by antibody-mediated targeting and cross-presentation of antigens to dendritic cells. T cells targeting to the tumor is improved by antibody-

mediated blockade of T cell inhibitory check points, which are also among the strategies for tumor cell elimination [15].

Using antibodies for cancer therapy is contributing to the improvement of cancer patient's lifestyle. The improvement of therapies was possible due to serological characterization of cancer cells, development techniques for generating optimized antibodies to tumor targets, understand more and more relevant signaling pathways in cancer as well as understand the relationship between cancer cells and the immune system [31].

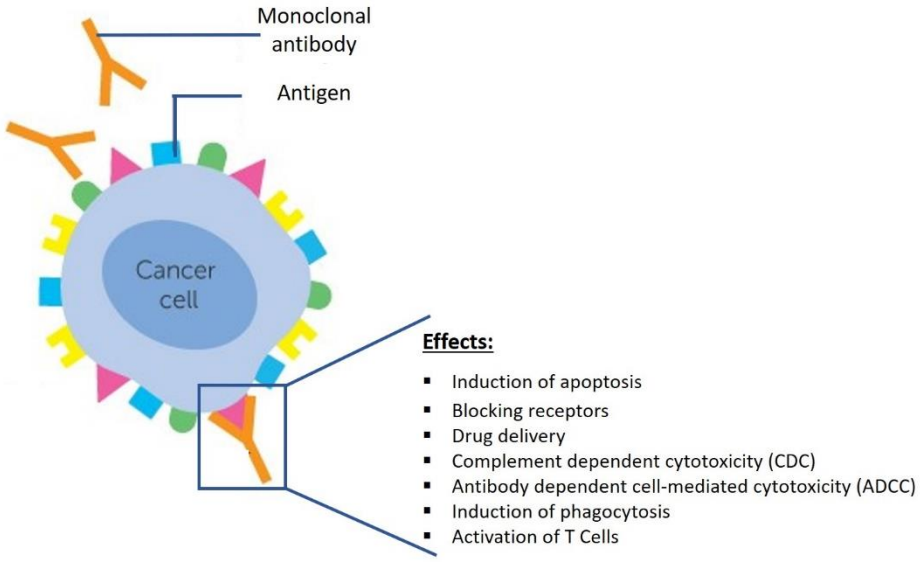

Figure 8 - Main functions of therapeutic monoclonal antibodies. mAb can induce (ADCC) by inducing the release of cytotoxic granules in effector cells (e.g., NK cells). It can also induce CDC. mAb can induce apoptosis by activating caspases. In addition, mAb can block receptor/ligand interactions preventing signaling cascade activation, as well as specifically deliver drugs into cancer cells [15].

1.3.2 Hybridoma technology

The hybridoma technology is used to produce monoclonal antibodies (mAb). A hybridoma is the result of the fusion of B lymphocytes, which are responsible for the production of antibodies, with myeloma cells that have the ability to divide indefinitely. B lymphocytes are obtained from the spleen of mice that were previously inoculated with the antigen of interest. The antigen stimulates the immune system of mice thus leading to the antibody production. To obtain the B lymphocytes producing the monoclonal antibody the mouse is sacrificed, and the spleen removed. The cells obtained from the spleen are then fused with myeloma cells with the polyethylene glycol (PEG) [26].

During this process multiple fusions can occur, not only between a B lymphocyte and a myeloma cell but also between two B lymphocytes or two myeloma cells. To ensure that only the hybrid cells grow, and the other undesired fusions are eliminated the cells are cultured in a selection medium. Under normal conditions the cells can synthesize Deoxyribonucleic Acid (DNA) nucleotides using two different pathways [26].

The *de novo* and salvage pathways. Normal cells use the *de novo* pathway but if this is blocked they can use the salvage pathway. The salvage pathway uses hypoxanthine and guanine for purine synthesis with the help of the enzyme hypoxanthine-guanine phosphoribosyltransferase (HGPRT). Similarly, this second pathway uses the substrate deoxythymidine to form thymidine monophosphate using the enzyme thymidine kinase (TK). If both pathways are blocked the cells will not survive. The medium used to select the hybrid cells is hypoxanthine-aminopterin-thymidine (HAT) medium. Aminopterin is a compound that blocks the *De Novo* pathway leading cells to use the salvage pathway. Since hypoxanthine and thymidine are converted by HGPRT and TK, respectively, if the cells are deficient in one of them, it is not possible to synthesize nucleotides. Since myeloma cells are deficient in HPGRT, they cannot synthesize nucleotides when cultured in HAT medium and therefore die. The hybrid cells survive because although the myeloma cells are HGPRT-negatives the B lymphocytes with which the myeloma cells have fused are HGPRT-positives [20], [26]–[28].

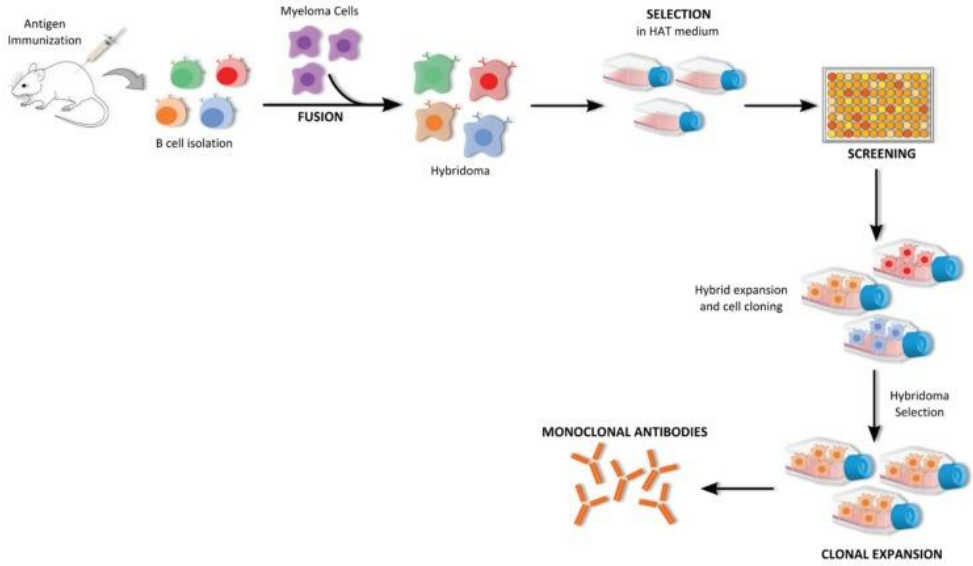

Figure 9 - Schematic representation of the hybridoma technology. This technology starts with mice immunization with the antigen of interest followed by the recovery of B cells from mice spleen and fusion with myeloma cells to form hybridoma cells. These hybridoma cells are selected in HAT medium and clones are screened according to the reactivity of their supernatants against the antigen. Finally, the selected clones are further cloned and expanded in order to obtain monoclonal antibodies with the desired specificity. (Adapted from [16])

1.4 Aim of the thesis

Since cancer is the largest cause of death worldwide, it is increasingly pressing to develop new therapeutic agents so that we can fight it more effectively depending on the specificity of each type.

Immunotherapy emerges as an alternative to conventional treatments such as chemotherapy since it presents advantages as being a targeted therapy with fewer side effects and less toxic, as mentioned above. One of the strategies used is monoclonal antibodies. These antibodies can recognize a specific antigen that is the right antigen to attack cancer cells

In this thesis the main aim is to produce and characterize novel antibodies against cancer, specifically a monoclonal antibody. L2A5, our monoclonal antibody, was obtained by hybridoma technology and its specificity and affinity were tested by flow cytometry and ELISA.

To test the specificity by flow cytometry, human breast cancer cells, MDA STn, cells were used, and these cells were treated with sialidase, an enzyme responsible for removing sialic acid, which is Neuramidase from *Clostridium perfringens,* to confirm the specificity against these glycans. On the other hand, the ability of L2A5 antibody to recognize glycoproteins or glycolipids was tested and for these assays the cells were treated with bromelain which removes the proteins from the cell surface.

Once the supernatant was collected with the antibody, ELISA was performed in which the coating of the plates was made with BSM and then treated with sialidase. On the other hand, this assay also served to analyze the antibody production titre, and several dilutions of the antibody were used.

In addition to these characterization assays, subcloning of the hybridomas was performed to select a clone that had the best antibody production so that we increased the efficiency of the production. All supernatants from the cloning were also tested by ELISA and flow cytometry.

In addition to this research work that consisted in the characterization of an antibody, a study was developed for the discovery of new antibodies. For this purpose, serum of mice immunized with CD44 and LS174T lysates were collected and analysed for the presence of antibodies recognizing the antigens for which they were immunized, CD44, Sialyl Lewis X and Sialyl Lewis A. Different ELISA techniques, such as indirect and live cells ELISA, were used for the analysis of serum. After confirmation of the presence of antibodies of interest in the serum, mice were sacrificed and hybridoma cells were generated by hybridoma technology. After obtaining the hybridomas the supernatants were collected and tested for the presence of new antibodies of interest.

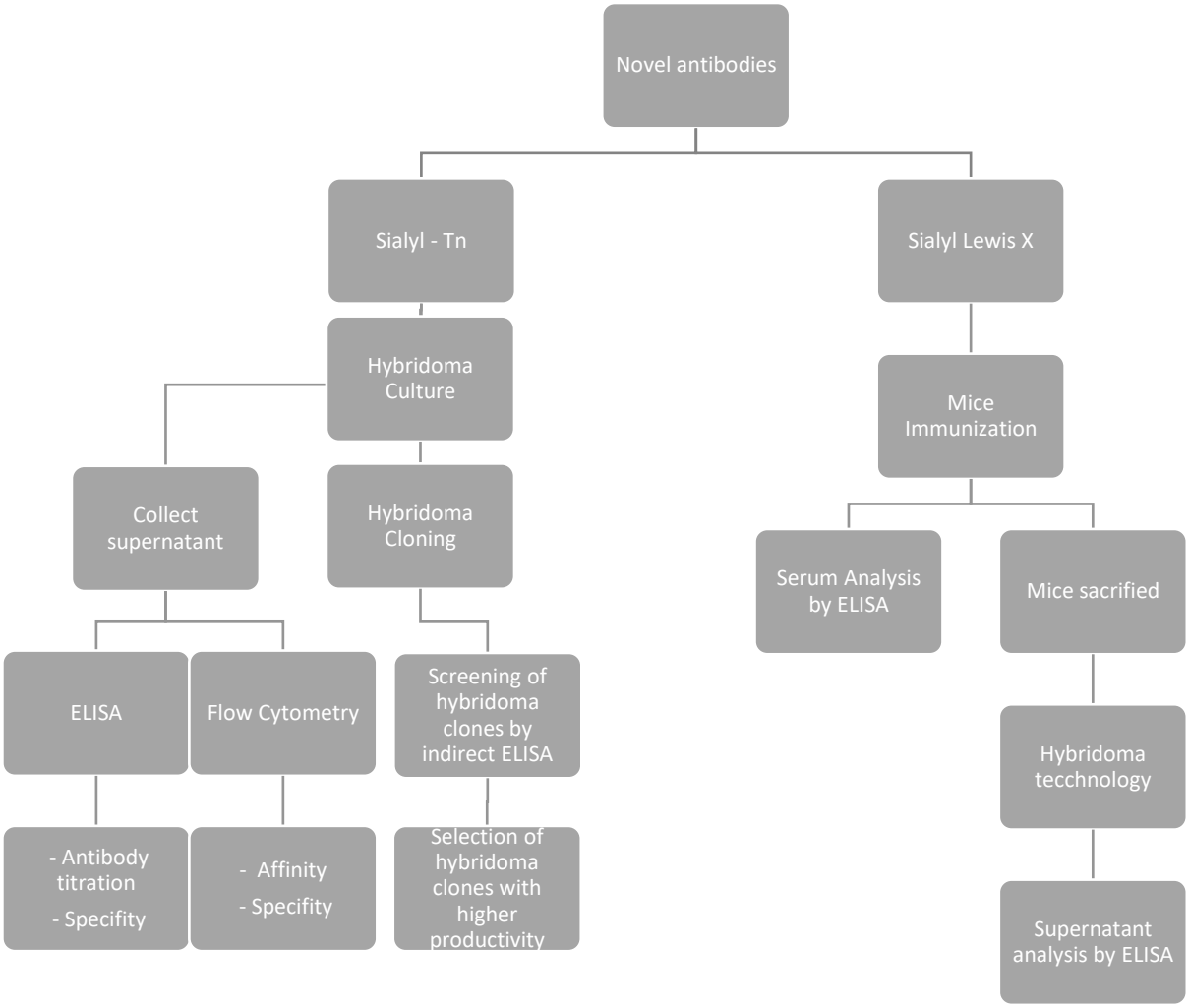

Figure 8 - Schematic representation of the work developed in this thesis

2 Materials and Methods

2.1 Cell Culture

2.1.1 Culture of breast cancer cell lines

The cell line MDA-MB-231 is a breast adenocarcinoma from a pleural effusion of a 51 years old woman [32]. This cell line was 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 line MDA-MB-231 STn.

MDA-MB-231 are adherent cell lines and were cultured on T25 and/or T75 culture flasks (Sarstedt) at 37ºC on an incubator (Panasonic), with a humidified atmosphere and 5% CO_2. The culture medium used for its growth was Dulbecco's Modified Eagle Medium (DMEM) (Gibco) supplemented with 10% de Fetal Bovine Serum (FBS) (Gibco), 2mM of L-glutamine (Sigma) and 100 µg/ml Penicillin/Streptomycin (Sigma). 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) by centrifugation at 1200 rpm for 5 minutes and subcultured according to the desired dilutions for subsequent uses. Before the subculture, the cells were also stored at -80ºC by resuspending the pellet in DMEM with 10% (v/v) of dimethyl sulfoxide (DMSO).

2.1.2 Culture of hybridomas cells

The hybridoma cells that were previously obtained as described on [16], were cultured to produce antibody against Sialyl Tn (STn). The hybridoma cells were mainly adherent and were maintained on T25 and/or T75 culture flasks (Sarstedt) on an incubator at 37ºC, with a humidified atmosphere and 5% CO_2, and cultivated in Dulbecco's Modified Eagle Medium (DMEM) (Gibco) supplemented with 10% de Fetal Bovine Serum (FBS) (Gibco), 2mM of L-glutamine (Sigma) and 100 µg/ml Penicillin/Streptomycin (Sigma). The hybridoma cells were detached from the culture flasks with a scraper whenever their confluence was approximately of 80%. After centrifugation at 1200 RPM 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 Colorectal Cancer Cell Lines

Two colorectal cancer cell lines were culture to test serum of mouse immunized against Sialyl Lexis X. LS174T (ATCC®CL-188™) cell line was established from Dukes' type B

adenocarcinoma of colon from a 58-year-old Caucasian female. SW948 (ATCC®CCL-237™) was established from Dukes' type C, grade III, *adenocarcinoma* of colon from an 81-year-old Caucasian female.

Both cell lines were adherent and were culture in a T25 or T75 flasks at 37ºC with a humidified atmosphere and 5% CO_2

LS174T were cultured in MEM (Gibco) supplemented with 10% FBS (Gibco), 2Mm L-glutamine (Gibco), 100 µg/mL Penicillin/Streptomycin (Gibco), Non-Essential Aminoacids (Gibco) and Sodium Pyruvate (Gibco);

SW948 were cultured in Leibowitz's medium (Biowest) supplemented with 10% of Fetal Bovine Serum (FBS) (Gibco), 2mM of L-glutamine (Gibco) and 100 µg/mL Penicillin/Streptomycin (Gibco);

The medium was changed every three days. For passages, at 80 / 90% of confluency, the culture was washed with 1X phosphate buffered saline (PBS) and 1X Trypsin-EDTA (TE) (Gibco) was used for cell detachment from the flask, followed by a centrifugation step at 200x*g* for 5 minutes and cultured according to the desired dilution. All cell lines were criopreserved at -80°C resuspended in culture medium and 10% (v/v) o dimethyl sulfoxide (DMSO) (Sigma).

2.2 Flow Cytometry

Flow cytometry is a technique that allows analyzing different cell parameters from distinct populations. This tool requires a cell suspension, or other particles, in a fluid system, causing one cell at a time pass in front of a laser. When a laser reaches out a cell two phenomena occur: light scattering and fluorescence. Light scattering is measured at different angles and is related with morphological cell characteristics. Light at small angles, forward scatter light, (FSC) is collected in the axis of the laser beam and is related with relative size of the cells while light at large angles, side scatter light, (SSC) is collected at 90^{o} of the laser beam and is related with structural complexity. The second phenomenon is fluorescence. Fluorescence occurs when a molecule absorbs energy and then the energy is emitted as photons with lower energy. It can happen naturally or by using fluorescent compounds that allow to detect molecules that do not have this property by themselves. The cytometer has filters and detectors allowing the determination of median intensity fluorescence (MFI). The fluorescence compounds can be chemicals or other probes, such as antibodies conjugated with one of these compounds. These compounds, regardless of their type, allow to label the cells and detect some specific characteristic. With MFI it's possible perform functional assays and phenotypical analysis of the cells, once MFI is proportional to the fluorescence which is in turn proportional to the amount of antibody that is binding to the cell due to the presence of antigen molecules on cell surface [33], [34].

2.2.1 Assessment of the binding of antibodies produced by hybridoma technology

2.2.1.1 General protocol - Cell preparation and Staining

The cells were harvested and washed with PBS by centrifugation 1200 rotations per minute (rpm) for 5 minutes. After centrifugation, the pellet was resuspended in 1mL of PBS and the cells were counted. The cells were divided in different Eppendorf's tubes to be stained and analyzed by flow cytometry ($2x10^5$ to $3x10^5$ cells/tube). Depending on the assay the cells were pretreated with sialidase or bromelain or directly stained with the antibody. At this point, 500 µL of PBS were added and the samples were centrifuge at 1200 RPM for 5 minutes. The supernatant was discarded, and the previous wash was repeated. The pellet was resuspended in 100 µL of PBS. Hybridoma supernatants was added, and the samples were incubated at 4^{o}C for 30 minutes. As positive control was used 3 µL of 3F1 hybridoma supernatant. The incubation with L2A5 was made resuspending the pellet in 150 µL of L2A5 supernatant. After the incubation, the cells were washed with 500 µL of PBS by centrifugation at 1200 rpm for 5 minutes. The supernatant was discarded, and the pellet was resuspended in 100 µL of PBS. The secondary antibody (polyclonal Goat Anti-Mouse Immunoglobulins/Fluorescein Isothiocyanate (FITC) - Goat F(ab')2 0,40 g/L) (Dako) was diluted 1:40 in PBS and 5 µL of this dilution 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 1200 rpm for 2 minutes. The pellets were then resuspended in 1 ml of PBS and the cells were analyzed in the flow cytometer.

2.2.1.2 Sialidase treatment to test specificity against STn

The sialidase treatment was performed to remove the sialic acids from the cell surface. After the first wash the supernatant was discarded, and the cells were resuspended in 400 µL of PBS. The cell suspension was divided into two tubes, one marked as non-treated and the other marked as treated. For the treated one, sialidase was added to a final concentration of 250 mU/mL. The samples were incubated for 90 minutes at 37ºC. After that the protocol proceeds as previously mentioned.

2.2.1.3 Bromelain treatment to test specificity against glycoproteins

The bromelain treatment was performed to remove proteins form the cell surface. After the first wash the supernatant was discarded, and the cells were resuspended in 360 µL of DMEM. The cell suspension was divided into two tubes, one marked as non-treated and the other marked as treated. To control if the bromelain works anti - CD44 APC was used. For the treated one, bromelain was added to a final concentration of 1µg/µL. The samples were incubated for 60 minutes at 37ºC. After that the protocol proceeds as previously mentioned.

2.3 Enzyme-Linked Immunosorbent Assay (ELISA)

The Enzyme-Linked Immunosorbent Assay (ELISA) is a technique used to detect and quantify immobilized proteins. This immunological tool is based on antigen/antibody binding properties. ELISA works with enzyme-labeled antibodies to detect molecules such as hormones, peptides and proteins. ELISA can be performed in different protocols, considering small variations to the protocol: direct, indirect, sandwich and competitive. The difference between the indirect ELISA and the direct ELISA is that the second one uses only one antibody to detect the antigen and this antibody is already coupled to an enzyme necessary for the revelation. On this project was performed indirect ELISA where the antigen is immobilized in a 96-well microplate and after that a specific antibody is added. This specific antibody is recognized by a second antibody that is covalently bound to an enzyme. The most common enzymes are horseradish peroxidase (HRP) and alkaline phosphatase. In this case HRP labeled antibodies are used and enzyme will catalyze the conversion of a substrate added, for example 3,3',5,5'-tetramethylbenzidine (TMB), into a product that can be colored, fluorescent or chemiluminescence allowing its detection [35], [36], [37].

2.3.1 Antibody titration by indirect ELISA

To determine the antibody titre the wells of 96-well plates were coated with 50 µL of bovine submaxillary mucin (BSM) at 5 µg/ml and the plate was incubated overnight at 4ºC. 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 37ºC for 90 minutes. The content of the plate was discarded, 100 µl of blocking buffer (5% of slim milk in PBS-T 0.1%) were added to the wells and the plate was incubated for 1 hour at RT. The blocking buffer was discarded, and the wells were washed four times. The washing steps were always performed using 150 µl of washing buffer (PBS - 0.1% Tween 20). Then, 100 µl of the samples to analyze – hybridoma supernatants – were added to the corresponding wells with different dilutions: 1:5, 1:20, 1:50, 1:70, 1:100, 1:150, 1:200. These dilutions were performed in PBS-T 0,1%. As positive control, an anti-STn antibody, 3F1, was used also with different dilutions: 1:250, 1:500, 1:700, 1:1000. To the secondary antibody binding control wells were added 100 µl of washing buffer. 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 1 hour. After the incubation with the secondary antibody, the content of the plate was discarded, and the wells were washed four times with washing buffer; 50 µl of TMB were added to all the wells and the plate was incubated at RT, in the dark for 5 to 10 minutes. 50 µl of HCl at 1M were added to stop the reaction. The absorbance was read at 450 nm.

2.3.2 Screening of hybridoma clones by indirect ELISA – assessment of antibody production and reactivity against STn

To select the hybridoma clone that was producing antibodies against STn, 100 hybridoma cells diluted in 60 mL of DMEM with the same composition described in 2.1.1 were distributed by five 96-well plates with 200 µL each well. When the cells were confluent the supernatant was collected, and the ELISA assay was performed as described in 2.3.1 but without sialidase treatment. The positive hybridomas for STn were cultured on T25/75 as described in (8.1.2).

2.4 Analysis of the antibodies against sLeX present in the serum of mice and of hybridomas obtained thereof.

To detect the presence of antibodies in the serum of the mice an indirect ELISA was performed. The difference between these experiments and the experiences described above is the blocking step. Since this experiment served to discover yet unknown and uncharacterized antibodies, the blocking step was not performed because it aims to decrease non-specific interactions between proteins, it could mask the antibody binding signal to the proteins present.

2.4.1 Screening of the antibodies by cell ELISA

To analyze the supernatants collected from the hybridomas the cell ELISA technique was used to test the ability of the ELISA to recognize the antigen in living cells. This technique consisted of culturing two cell lines, LS174T and SW948 WT, in a 96-well plate until reaching confluency. When the cells reached the confluency the washing steps were performed using 150 µl of washing buffer (PBS - 0.1% Tween 20). Then, 100 µl of the samples to analyze, were added to the corresponding wells. 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. The time and temperature of incubation with the primary and secondary antibody were differents from the indirect ELISA described above being these 1h at 25ºC. The blocking step was not performed because, since its function was to decrease non-specific interactions between proteins, it could mask the antibody binding signal to the proteins present [38].

3 Results and Discussion

The main objective of this thesis comprised the production and characterization of anti-STn-monoclonal antibodies. The hybridoma cells responsible for producing the antibody of interest had already been obtained by other members of the group. This thesis focusses these hybridomas and the characterization of the antibodies produced by them.

The characterization was performed by indirect ELISA as well as flow cytometry to test the affinity and specificity. To evaluate the antibody production, supernatant titrations were also made.

In addition, and to improve the production characteristics of the hybridomas, a cloning was performed to select a clone with the best characteristics such as specificity, affinity and productivity.

3.1 L2A5: Monoclonal antibodies against STn

3.1.1 Antibody titration by indirect ELISA

To perform the antibody titration and to test the specificity of the antibody produced by the hybridoma cell cultures, BSM was used as target for the screening by ELISA, since it has a STn content of approximately 50%. BSM was either in its native form or treated with sialidase. The enzyme used cleaves α2-3- and α2-6-linked sialic acids. Thus, after treatment BSM loses the sialic acid that contains, and it is possible to assess the presence of antibodies with sialic acid-dependent binding. When supernatants tested show dependent bindings, a reduction in the optical density is observed in the samples of BSM treated with sialidase. This protocol allowed to determine if the antibodies present in the supernatant bind to STn residues.

During the experimental study several batches of supernatants were collected and pooled. These pools of supernatants were used for titration of the antibody. The different batches were all produced by the L2A5 clone. The nomenclature used, L2A5 followed by a number, corresponds to the name of the pool and the number is merely indicative of the production order, the number 1 being the first supernatants collected and analyzed together.

With the increase of the dilution of the supernatants, a decrease of the optical density (OD) can be observed as represented in the figure 11. In this way it is possible to determine the highest dilution where it is still possible to have a signal and thus to discover the antibody titre. As can be seen in the figure 10, all the different batches, L2A5 1, L2A5 2, L2A5 3 and L2A5 4 have a titre of 1:150 since it is the highest dilution where signal still exists. These results allow to conclude that the L2A5 clone produce the antibody of interest since there is a signal which indicates binding of the antibody to BSM.

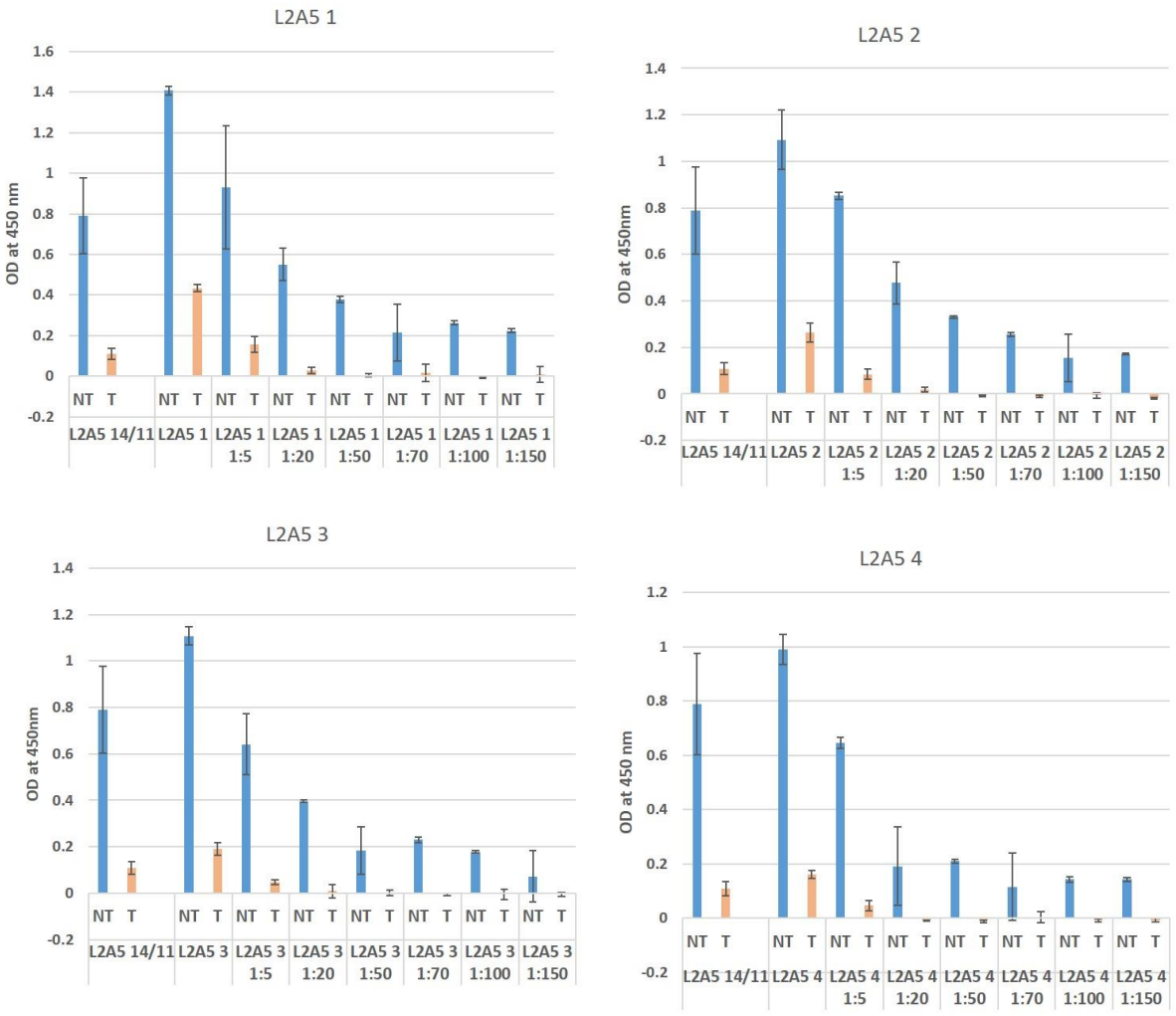

Figure 9 - ***Antibody titration and specificity*** *- An indirect ELISA was performed using BSM at 5 µg/mL as the antigen. L2A5 14/11 was used as a positive control it had previously been tested. As negative control was used the wells when only secondary antibody was present, and these values were subtracted from the values presented here. The absorvance was read at 450 nm. L2A5 hybridoma supernatants were used at 1:5, 1:20, 1:50, 1:70, 1:100 and 1:150. All supernatants dilutions were tested in BSM untreated (NT) and treated (T) with sialidase. Secondary antibody used to detect the reaction was goat α-mouse Ig HRP at 1:1000 dilution. All the values are the average of triplicates determination.*

In addition, specificity was tested and a decrease in optical density was observed when BSM was treated with sialidase. (Figure 11)

Although the decrease is accentuated, there is still some signal at the lowest dilutions even when treatment with sialidase is performed. This signal is due to the presence of antibodies produced recognizing other molecules, but not specific against STn. The existence of this signal or even the decrease of the signal between different pools is due to the characteristic instability of the hybridomas. Often this instability is due to the loss of chromosomes containing genes encoding the immunoglobulin and to the growth of subpopulations of non-producing cells. Among the four different batches a decrease can be noted from L2A5 1 to L2A5 4 with L2A5 1 having an OD of approximately 1,4 and L2A5 4 of 1,0.

3.1.2 Assessment of the binding of antibodies produced by hybridoma technology

To assess the ability of L2A5 to bind to STn, flow cytometry assays were performed. MDA-MB-231 and MDA-MB-231 STn, also referred as MDA WT and MDA STn respectively, were used. Since the MDA WT do not overexpress STn, the antigen of interest, were used as a negative control in all experiments. Both cell lines were incubated with L2A5 and 3F1,which is an anti-STn monoclonal antibody from the mouse hybridoma 3F1 clone, used as a positive control.

The results of flow cytometry (figure 12) shows the binding capacity of the L2A5 antibody. As a negative control, the cells were labeled with the secondary antibody only, in order to determine aspecific or background signals.

The results demonstrate the ability of L2A5 to bind to the antigen of interest since when MDA WT is used, which do not express the antigen of interest, there is labeling and when MDA STn is used, which overexpress the antigen of interest, there is a strong positive signal. In fact, in figure 12A shows the absence of binding to MDA WT cells, negative control. Here, the L2A5 and 3F1 antibodies binding is similar to the negative control peak. In figure 12B, about 40% of the cells MDA STn are labeled with the L2A5 antibody represented by gate R1.

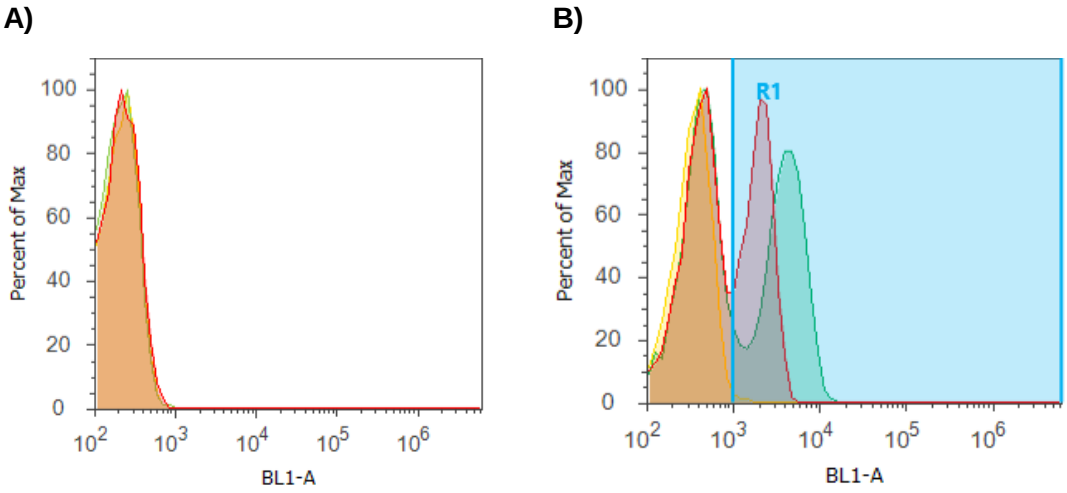

Figure 10 - Flow cytometry was performed with MDA WT and MDA STn. In this experiment the ability of binding of the L2A5 antibody to the antigen of interest in living cells was evaluated. In panel A) are presented the results of the experiment with the MDA WT cells and in panel B) the results with the MDA STn cells. The positive control used was 3F1 whereas the negative control was the labeling of the cells with the secondary antibody. The yellow peak corresponds to the negative control, the greenpeak corresponds to the positive control and the red peak corresponds to the L2A5.

3.1.3 Sialidase treatment to test specificity against STn

To ensure that the produced antibodies are specific for STn, MDA STn were used in two different conditions. One of them was treated with sialidase and the other untreated. As previously

mentioned, treatment with sialidase leads to the removal of the sialic acid molecules present on the surface of the cell.

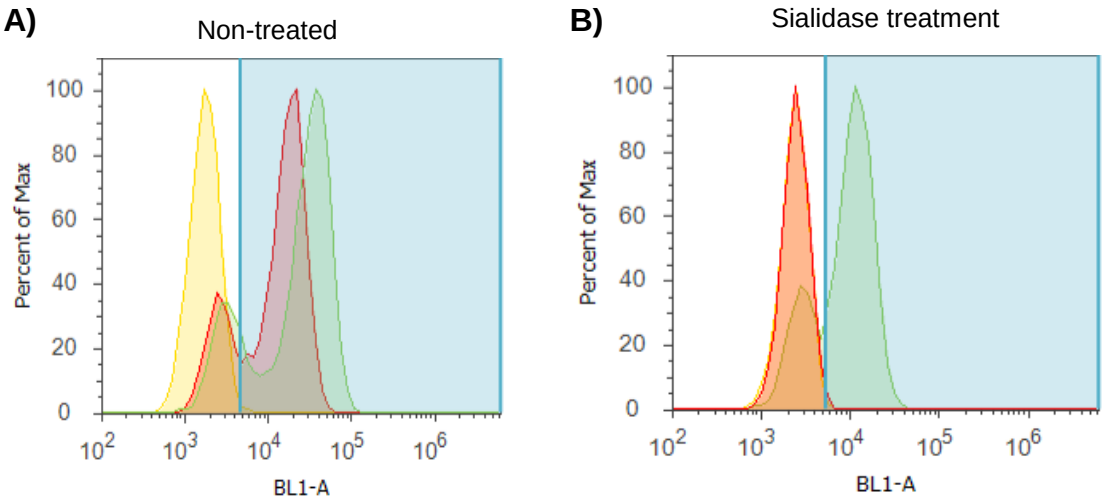

Figure 11 - Flow cytometry was performed with MDA STn cell line that overexpress STn. In this experiment MDA STn was treated with sialidase. In panels A) and B) the yellow peak corresponds to reading only with MDA STn labeled with the secondary antibody used as the negative control and the greenpeak corresponds to the positive control, 3F1. The red peak corresponds to the L2A5 labeling, panel A) with MDA STn without sialidase treatment and panel B with MDA STn with sialidase treatment.

In figure 13 the positive control used was 3F1, represented by the green peak, the yellow peak represents the negative control and the red peak represents the L2A5 labeling. In figure 13A where MDA STn cells were not treated with sialidase, the L2A5 labeling was about 48.83% represented by the blue color gate. The labeling with the same antibody but with the sialidase-treated cells was about 48.29%. A shift in the peak in figure 12B resulting from the decrease of the signal can be observed. Still the signal decrease is relatively small which shows that 3F1 has little specificity against STn.

On the other hand, the labeling with L2A5 in figure 13A) indicates a positive labeling in 48.38% of untreated cells and in figure 13B) overlapping with negative control is observed showing that after treatment with sialidase and consequent removal of sialic acids, L2A5 loses the ability to bind to cells. By comparing the produced L2A5 antibody with the positive control it can be understood that L2A5, in addition to recognizing STn, exhibits a higher specificity against this antigen since the signal overlapping the negative control shows that it does not recognize other structures present in the cells after removal of the sialic acid.

3.1.4 Bromelain treatment to test specificity against glycoproteins

In addition to testing the specificity against STn it was also tested whether this specificity depends on the carrier to which the glycan is bound as these may be conjugated to proteins or lipids. Bromelain treatment proteolytically removes proteins that are found on the cell surface[41].

MDA STn were divided into two fractions, one of them being treated with bromelain. After bromelain treatment, incubation was performed with L2A5 and anti-CD44 APC, which is an antibody that recognizes CD44 that is present in cell membranes and CD44 is degraded by bromelain.

Incubation with anti-CD44 served as a control to evaluate the efficacy of bromelain treatment in the removal of proteins from the cell surface. When using anti-CD44, cells which have not undergone treatment with bromelain have a label shown in R2 of approximately 99.5% (Figure14). On the other hand, when cells are subjected to bromelain treatment and when incubated with anti-CD44 the signal is lost, the peak being superimposed with the peak signal of the cells without any type of incubation which is serve as a negative control. This peak shift when cells are labeled with anti-CD44 and are treated is due to the removal of CD44 present on the cell surface by the action of bromelain. This action had already been described previously by other authors thus validating efficacy of treatment [41].

To understand if L2A5 recognize glycoproteins or glycolipids labeling with this antibody was performed on cells treated and not treated with bromelain. The incubation with L2A5 it was intended to verify the existence of antibody binding after the removal of the proteins from the cell surface. In figure 14A about 99.50% of the cells are labeled with the anti-CD44 antibody used as represented in the respective figure as R2. In figure 14B, with cells treated with bromelain, a peak overlap with the negative control can be seen. This overlap reveals the efficacy of bromelain since, with the removal of CD44, the signal overlaps the negative control. Thus, it has been confirmed that bromelain removes the proteins on the surface of cells. In figure 14C about 37% of the cells are labeled with either the 3F1 positive control or the L2A5 antibody. The labeling with these antibodies is represented in this figure with the gate represented as R3. When the cells are treated with bromelain the results are shown in figure 14D and the level of labeling plotted on gate R4 is about 1.7%.

Thus, the bromelain action of cell surface proteins removal infers that L2A5 binds mostly to glycoproteins since when the proteins are removed the signal is greatly reduced. The small percentage of binding of L2A5 to cells observed even after bromelain treatment may be due to the fact that there are glycolipids at the cell surface that are recognized by the antibody or because some proteins have not been completely removed [42].

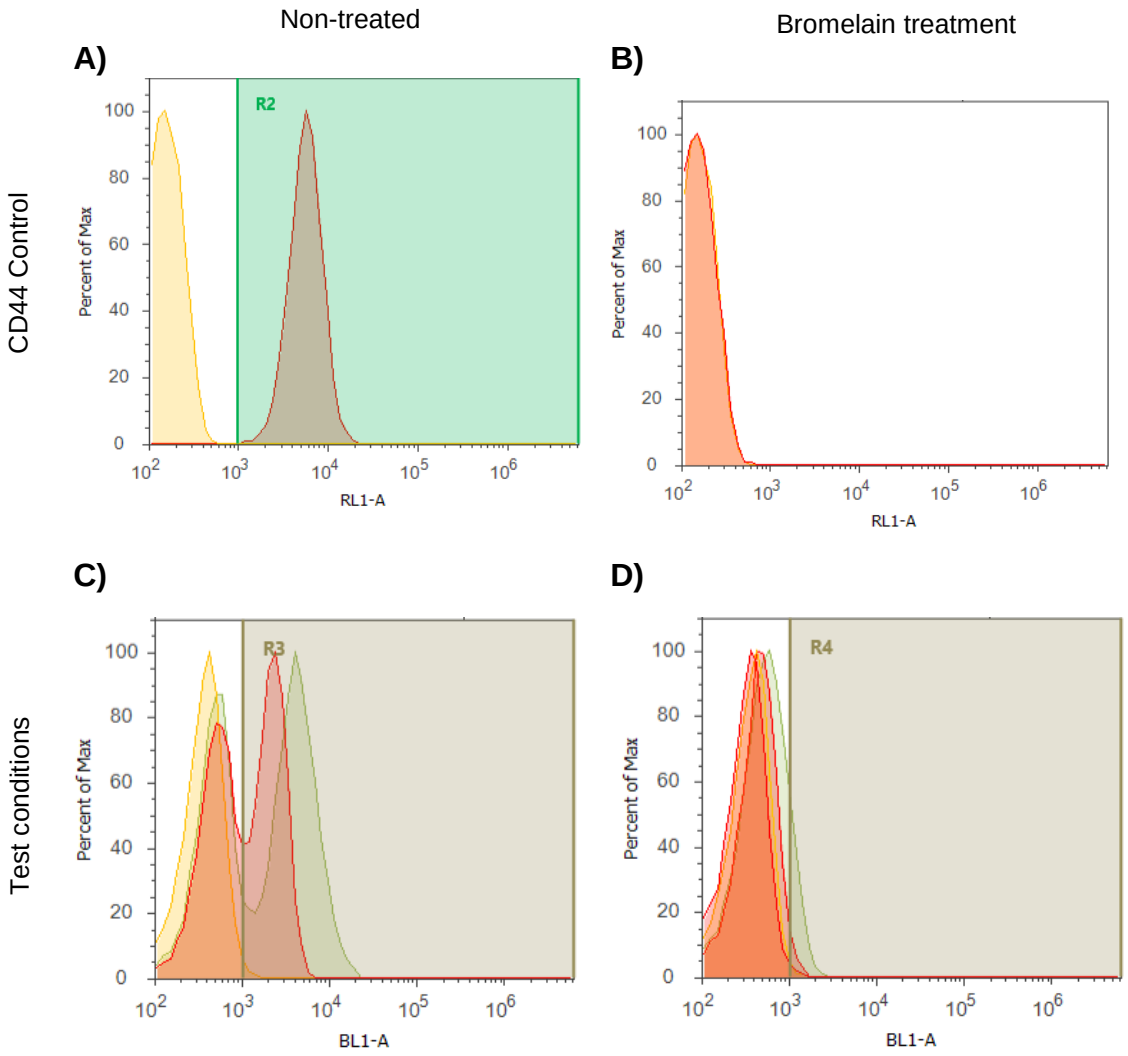

Figure 12 - Flow cytometry was performed with MDA STn cell line that overexpress STn. In this experiment MDA STn was treated with bromelain. In panels A) and B) the yellow peak corresponds to reading only with MDA STn without any marking and is used as negative control. The red peak corresponds to the anti-cd44 labeling, panel A) with MDA STn without bromelain treatment and Figure B with MDA STn with bromelain treatment. In panels C) and D) the yellow peak corresponds to the MDA STn cells labeled with the secondary antibody used as the negative control. Panels C) and D) correspond to the untreated cells and bromelain treated cells, respectively. The green peak shows the labeling with the positive control used, 3F1, while the red peak shows the labeling with L2A5.

3.1.5 Screening of hybridoma clones by indirect ELISA – assessment of antibody production and reactivity against STn

In order to select a subclone with higher productivity, specificity and affinity for STn, a cloning was performed (Figure 15). The work started from the hybridoma clone L2A5, with 100 cells of this clone diluted in 60 mL of DMEM so that when they were distributed in the wells, there was 0,5 cell/well, resulting in that some wells would have only one cell and others would have none. When confluence was reached in a 96 well the supernatant was collected for ELISA analysis and the cells were expanded to a 24-well plate. The names of the clones were assigned according to the plate, line and column where they were located. The first clones reaching the confluence were 1C11, 2B6, 2C10, 2D10, 2E9, 2F3, 2G10, 3B8, 3E4, 3E9, 3F8, 3F10, 3G7, 4D5, 4D6, 4E11, 4F10, 5C4, 5C6, 5C7 and 5C8.

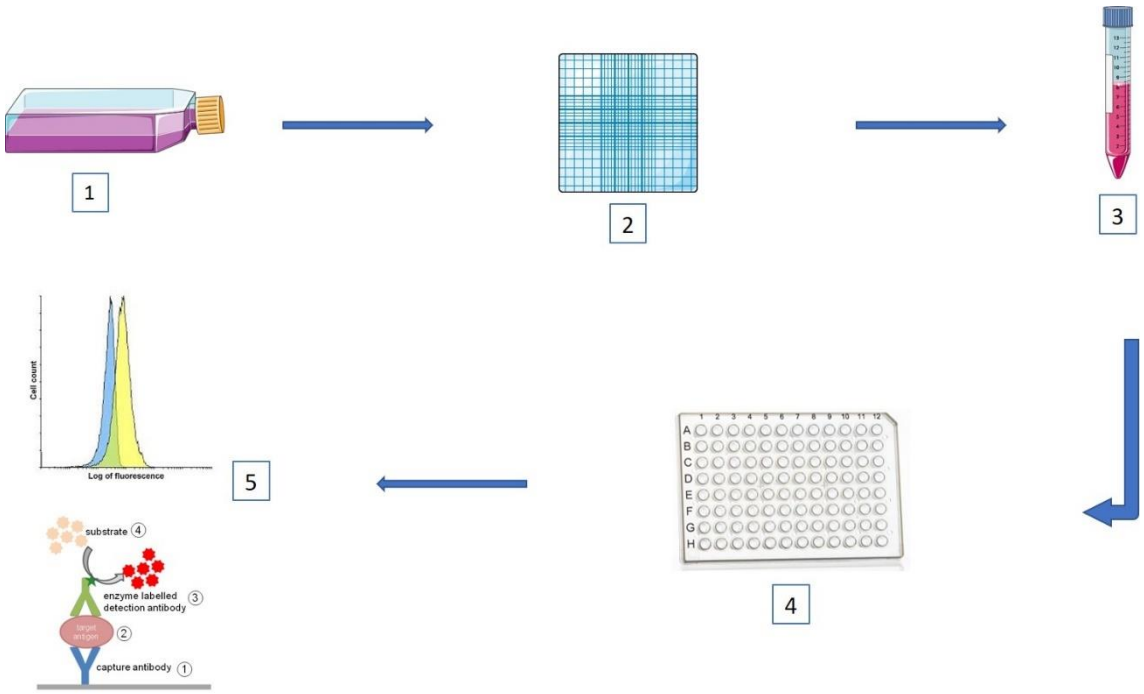

Figure 13 - Cloning strategy - 1. ***Hybridoma Culture*** *– Remove the cells with the scrapper and centrifuge 5 minutes 1200RPM; 2* ***Counting cells*** *– Resuspend the pellet in 1mL of DMEM 10%FBS and count the cells; 3* ***Cell dilution –*** *Take the amount of volume where is 100 cells inside and add 9 mL of DMEM 10% FBS; 4* ***Cloning –*** *Put 200µL in each well. In the outer columns put medium without FBS; 5* ***Screening*** *– Test the supernatants by flow cytometry and ELISA to select the clone that are producing the desired monoclonal antibody*

The results of the ELISA are shown in figure 16 and most of the clones presented an absorbance value similar to or greater than the positive controls used, L245 14/11 and 3F1.

Except clone 4D5 which showed no absorbance. In order to have sufficient hybridoma supernatant to obtain triplicates of the values, all clones were cultured in a 24-well plate.

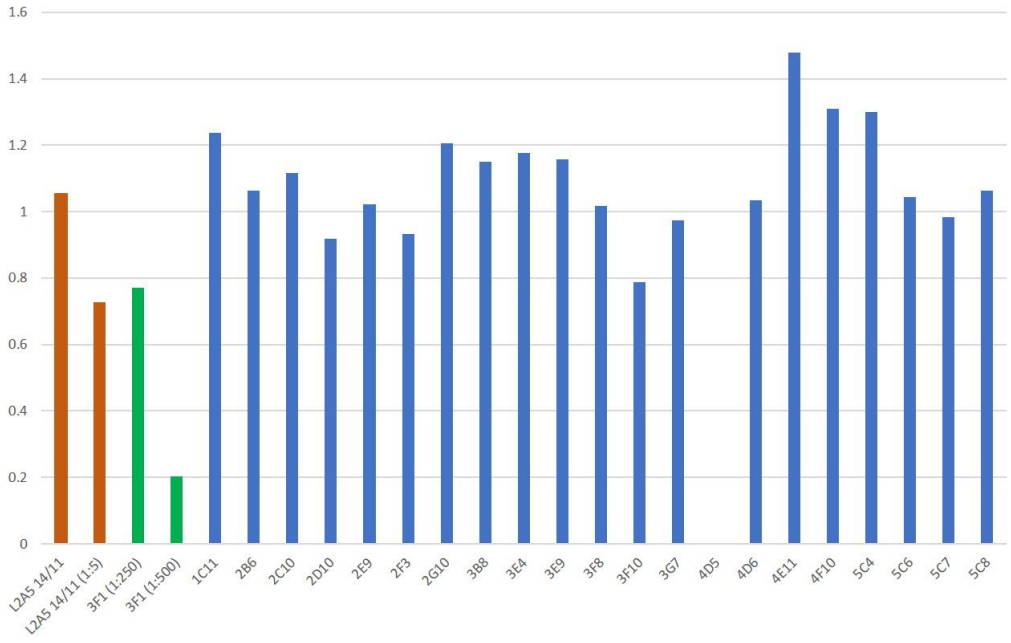

Figure 14 –An indirect ELISA was performed using BSM at 5 µg/mL as the antigen. L2A5 14/11 and 3F1 were used as positive controls. As negative control was used the wells when only secondary antibody was present, and these values were subtracted from the values presented here. The absorvance was read at 450 nm. Secondary antibody used to detect the reaction was goat α-mouse Ig HRP at 1:1000 dilution.

When the above-mentioned clones were again confluent in the 24-well plate, the supernatants were collected and analyzed by ELISA using BSM at 5 µg/mL as the antigen. L2A5 14/11 and 3F1 were used as positive controls. As negative control was used the wells when only secondary antibody was present, and the specificity was also analyzed after sialidase treatment of BSM.

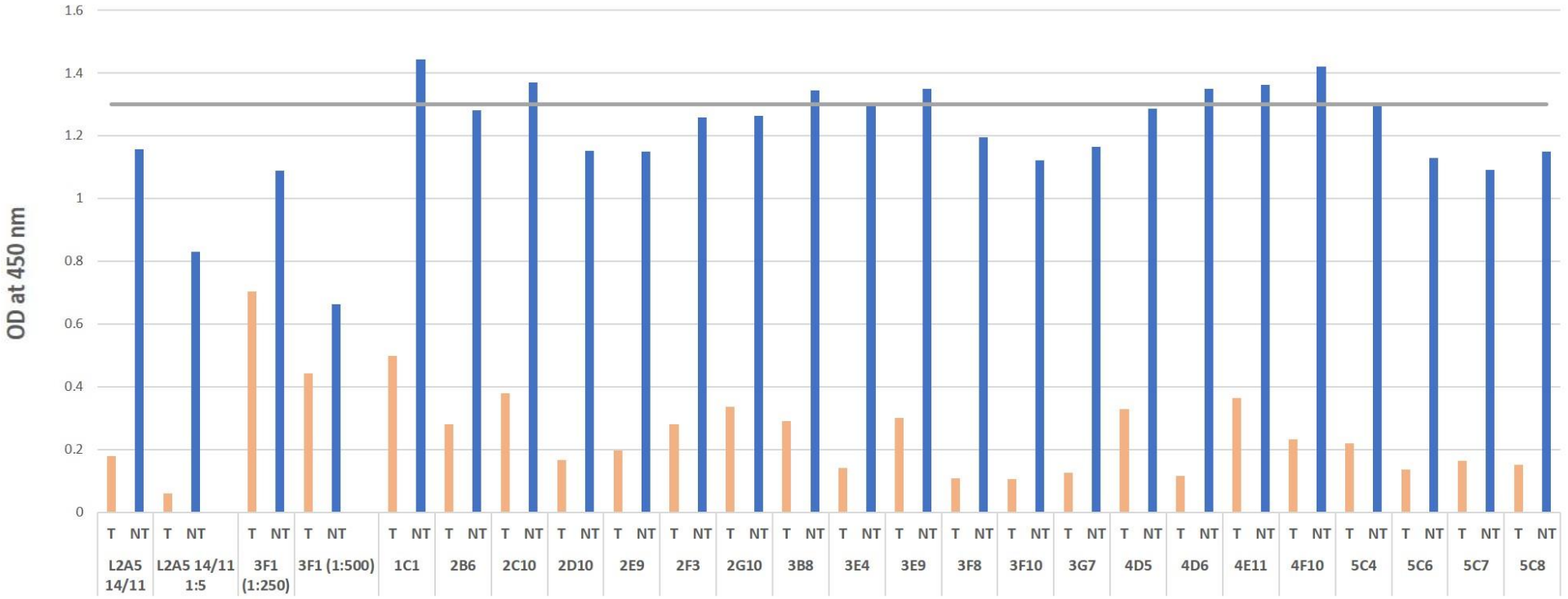

Figure 15 - An indirect ELISA was performed using BSM at 5 µg/mL as the antigen. L2A5 14/11 and 3F1 were used as positive controls. As negative control was used the wells when only secondary antibody was present, and these values were subtracted from the values presented here. The absorvance was read at 450 nm. All supernatants were tested in BSM untreated (NT) and Treated (T) with sialidase. Secondary antibody used to detect the reaction was goat α-mouse Ig HRP at 1:1000 dilution.

The results are shown in figure 17. Clones with higher absorbance were selected, more specifically all those with an absorbance greater than 1.3: 1C1, 2C10, 3B8, 3E9, 4D6, 4E11 and 4F10.

In addition to the selected clones mentioned above, other clones reached confluency in the 96-well plates, other clones reached confluency in the 96-well plates but with a difference of 6 days. The experimental protocol was repeated for these clones as for the previous ones, the supernatants being tested by indirect ELISA and re-cultured on a 24-well plate. The results of the first screening of the supernatants can be seen in figure 18 and the screening results after reaching the confluence in the 24 well plate can be seen in figure 19.

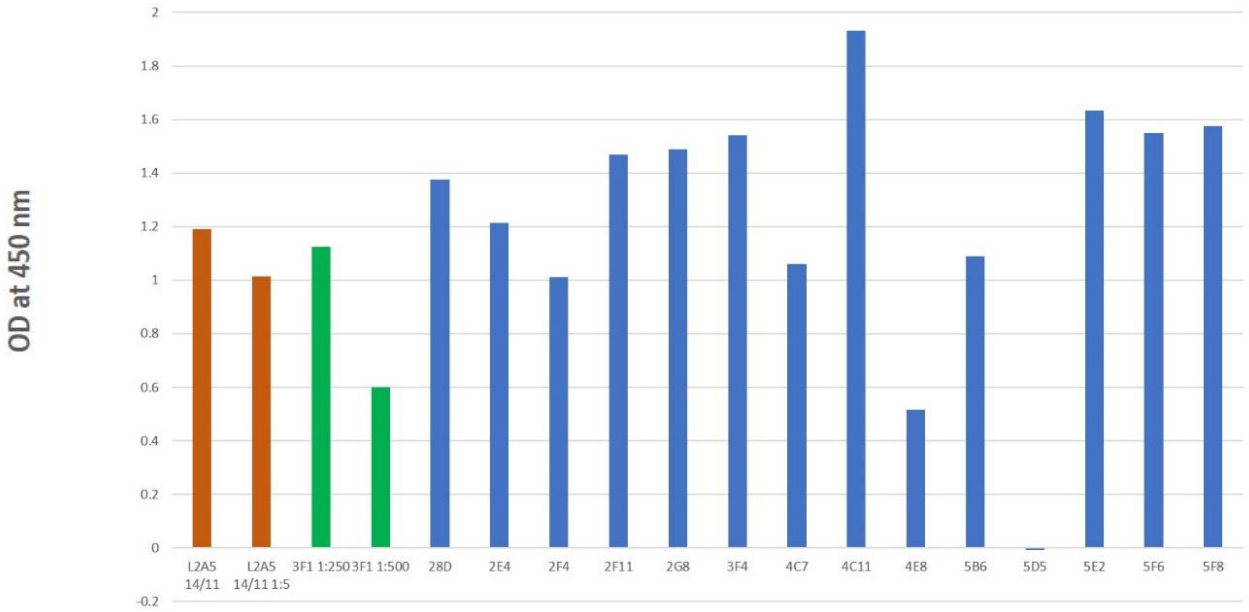

Figure 16 - An indirect ELISA was performed using BSM at 5 µg/mL as the antigen. L2A5 14/11 and 3F1 were used as positive controls. As negative control was used the wells when only secondary antibody was present, and these values were subtracted from the values presented here. The absorbance was read at 450 nm. Secondary antibody used to detect the reaction was goat α-mouse Ig HRP at 1:1000 dilution

In figure 18 the results of the first screening can be observed. Clones reaching the confluence showed a considerable absorbance value other than 5D5 and 4E8. All clones were cultured in a 24-well plate. When these clones reached confluence in the new plate the supernatants were again tested but this time the specificity was tested by treatment of BSM with sialidase. Clone 4E8 upon passage from the 96-well plate to the 24-well plate did not re-grow having been discarded. The results presented in figure 19 allowed to select again the clones that had higher absorbance, above 1.3, so only clones 3F4 and 4C11 were selected. The clones selected in the first analysis and in this second analysis were maintained in culture in a T25 flask. The specificity analyzed in these experiments was not considered in this selection being only the absorbance above 1.3 the factor taken into account.

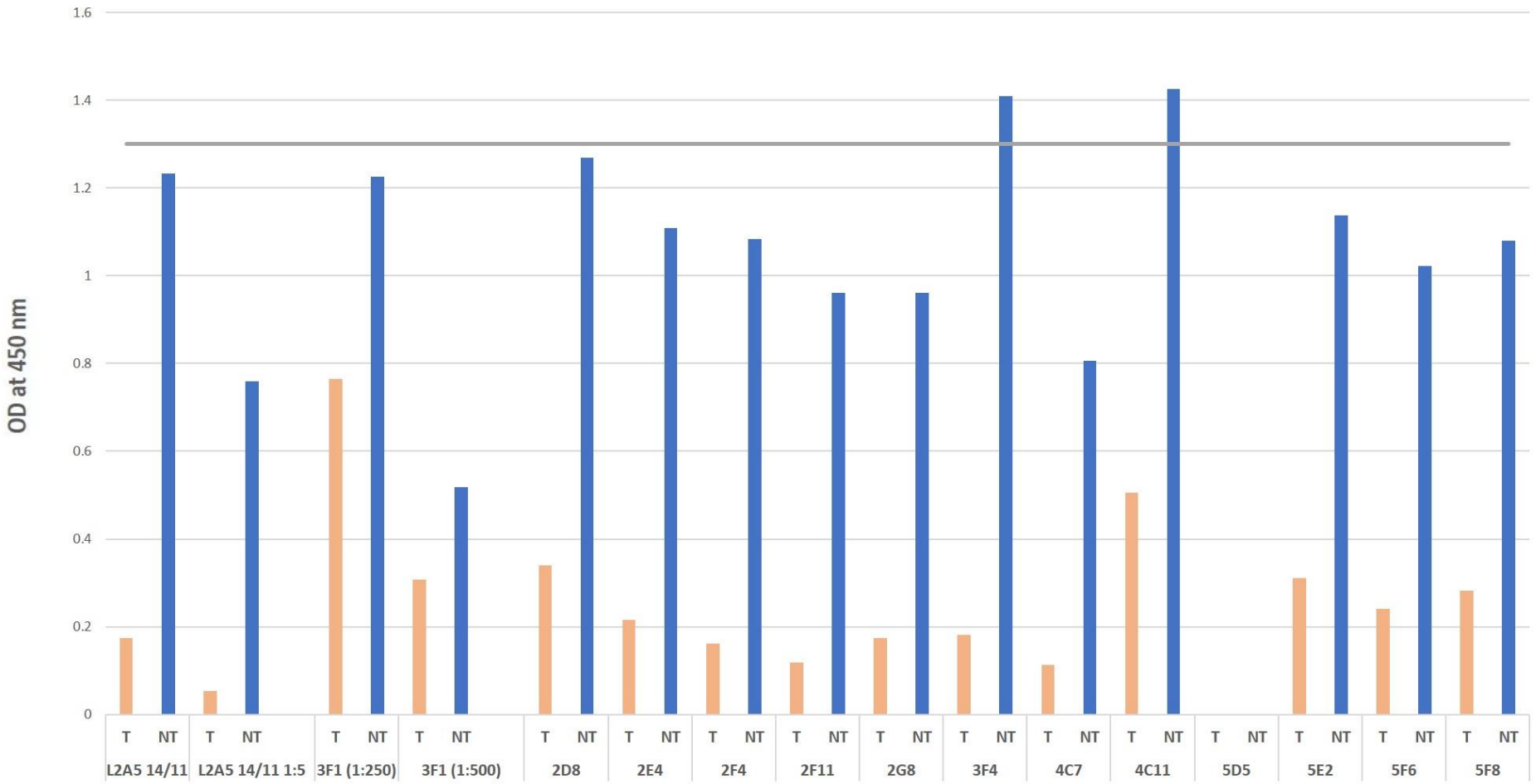

Figure 17 - An indirect ELISA was performed using BSM at 5 µg/mL as the antigen. L2A5 14/11 and 3F1 were used as positive controls. As negative control was used the wells when only secondary antibody was present, and these values were subtracted from the values presented here. The absorbance was read at 450 nm. All supernatants were tested in BSM untreated (NT) and Treated (T) with sialidase. Secondary antibody used to detect the reaction was goat α-mouse Ig HRP at 1:1000 dilution.

After the screening, 9 clones were selected and expanded in culture in T25 flasks: 1C1, 2C10, 3B8, 3E9, 3F4, 4C11, 4D6, 4E11 and 4F10. Although these clones exhibited considerable absorbance values most part died when they were cultured in the T25 flasks having survived only clone 4E11. The death of the other hybridomas may have occurred due to some undetected contamination being common in this type of cell line contamination by mycoplasma.

This clone was expanded, and the supernatants were collected over the growth and passages of this clone. The supernatants were collected for testing of the production stability of the selected clone.

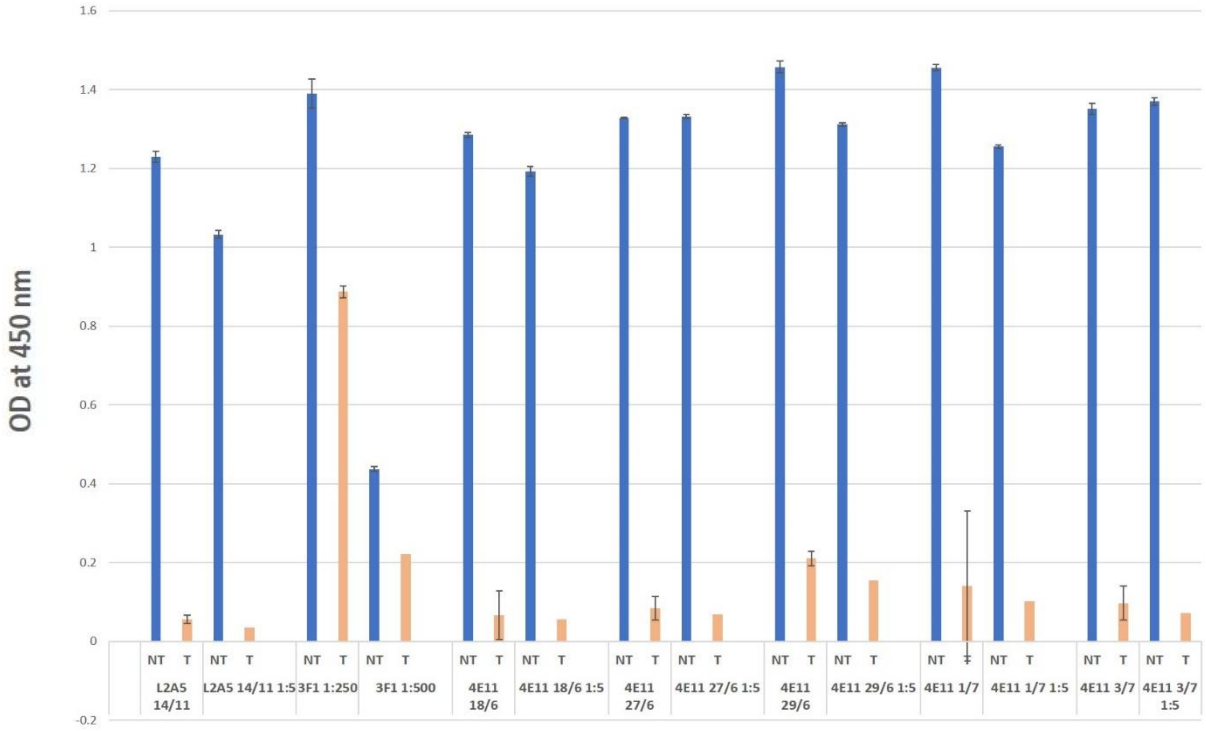

Figure 18 - An indirect ELISA was performed using BSM at 5 µg/mL as the antigen. L2A5 14/11 and 3F1 were used as positive controls. As negative control was used the wells when only secondary antibody was present, and these values were subtracted from the values presented here. The absorbance was read at 450 nm. All supernatants were tested in BSM untreated (NT) and Treated (T) with sialidase. Secondary antibody used to detect the reaction was goat α-mouse Ig HRP at 1:1000 dilution.

In figure 20 the analysis of supernatants collected over several dates can be observed. The supernatants were analyzed without being diluted and diluted 1:5. The above-mentioned dilutions were used so that the antibody could be diluted if it was too concentrated and to test whether there was a relationship between the antibody concentration and the obtained signal. As with the positive controls all the supernatants under test have an absorbance between 1.2 and 1.4. When diluted, a decrease in absorbance would be expected. The fact that the absorbance when the supernatants are diluted 1:5 to remain similar may be due to not being a significant decrease in antibody concentration. In order to observe a decrease should have carried out more dilutions with higher dilution factors to optimize the experiment

Regarding treatment with sialidase a significant decrease of the signal is observed, this being never greater than 0.2 absorbance. The sialidase removed the sialic acids present in the STn however the existence of a signal may be due to the sialidase not having removed all sialic acids present or by the presence of antibodies which also recognize another antigen present in the BSM as Tn.

3.2 Novel antibodies against Sialyl Lewis X

3.2.1 Analysis of the antibodies present in the serum of the mice and of the hybridomas obtained therefrom.

In the second part of this thesis the aim of the project was select specific antibodies against Sialyl Lewis antigens formed from hybridomas. The hybridomas were obtained from mouse immunized with CD44. Sialyl Lewis X and Sialyl Lewis A are sialofucosylated glycans that are overexpressed in cancer and decorate proteins on the surface of the cell such as CD44. In addition to the mouse immunized with CD44, a second mouse was immunized with cell lysates. The serum of mouse immunized with lysates from LS174T that is hypersialylated was analyzed. The mouse serum was analyzed so that the presence of antibodies of interest could be detected if the mouse had produced them after the immunization. After analysis of the mouse serum and after confirming the presence of antibodies of interest therein the same was sacrificed and hybridomas were obtained by hybridoma technology. To test the sensitivity of the hybridoma supernatants and mouse serum to sialylated structures an ELISA experiment was conducted in which the coating was made with BSM and Porcine Submaxillary Mucin (PSM). The PSM was chosen has negative control having low sialylation, bound sialic acid between 0.5-1.5%. In addition, both BSM and PSM were treated with sialidase. The positive control used was the Heca-452 antibody which recognizes sialofucosylated glycans, sialyl Lewis X and sialyl Lewis A. In order to compare the signals obtained when the serum from immunized mouse was used, the non-immunized mouse serum was also used as the control sample (Crtl Mouse). As it is shown in figure 21 the positive control has a higher signal when BSM is used as antigen and untreated. This signal can demonstrate that Heca-452 recognizes sialylated structures although when using sialidase-treated BSM and PSM in which the sialic acid content is reduced still some signal is observed.

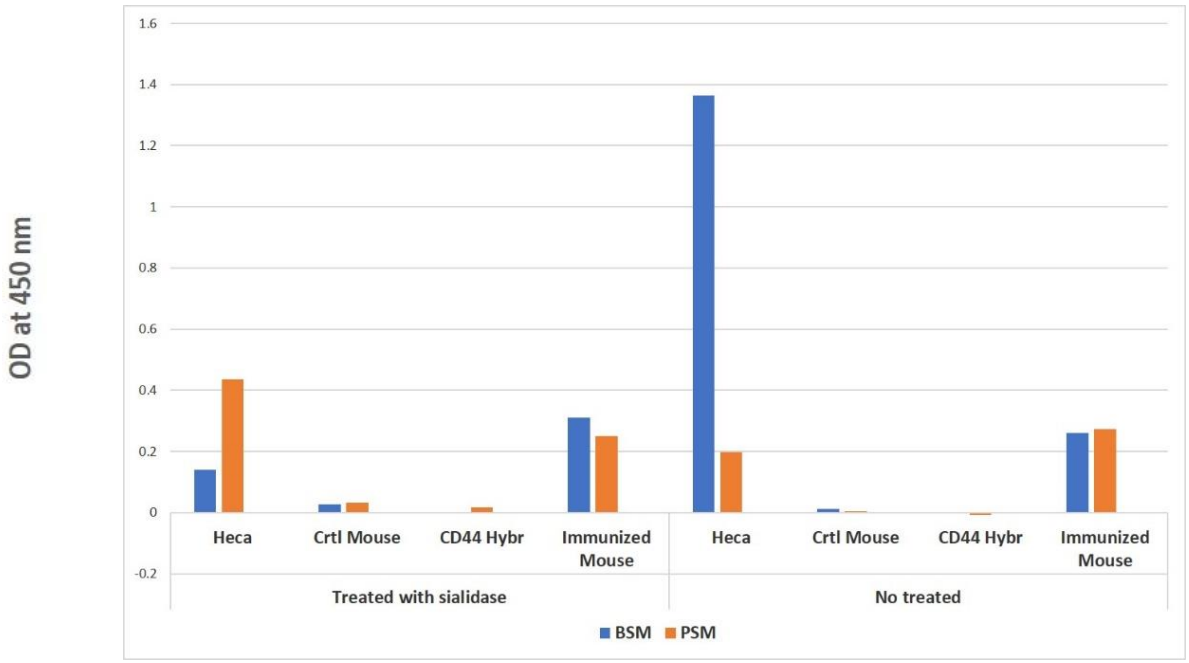

Figure 19 – An indirect ELISA was performed using BSM at 5 µg/mL and PSM at 3 µg/mL as antigens. Crtl Mouse was used as negative control. As positive control Heca-425 was used. Secondary antibody used to detect the reaction was goat α-mouse Ig HRP at 1:1000 dilution with the exception of HECA-452 in which Anti-Rat IgM HRP was used. The absorbance was read at 450 nm

As for the serum of the immunized mice, when compared to the control mouse which does not have a significant signal, a more evident signal is observed. This signal is similar for both treated and untreated BSM and PSM. The fact that the serum signal is similar for both antigens used may be due to the interference of all components present in the mouse serum not demonstrating specificity and affinity for neither of them. In relation to the supernatant of one of the obtained hybridomas does not present signal in any of the conditions. This may be due to the instability of the hybridomas as previously mentioned or the need for performing a cloning to select the clone expressing the antibody of interest and test others hybridomas.

The second mouse immunized with cell lysates from LS174T, which is a cell line hypersialylated, the would be expected to produce antibodies against SLex after immunization. For ELISA analysis, mouse serum immunized with cell lysates from LS174T was used. In figure 22 it can be seen that the immunized mouse serum represented as Immunized Mouse shows a higher absorbance when compared to the control mouse represented with Crtl Mouse but only when analyzed against lysates from LS174T. The observed signal may indicate that the immunized mouse was producing antibodies with the ability to recognize SLe^x.

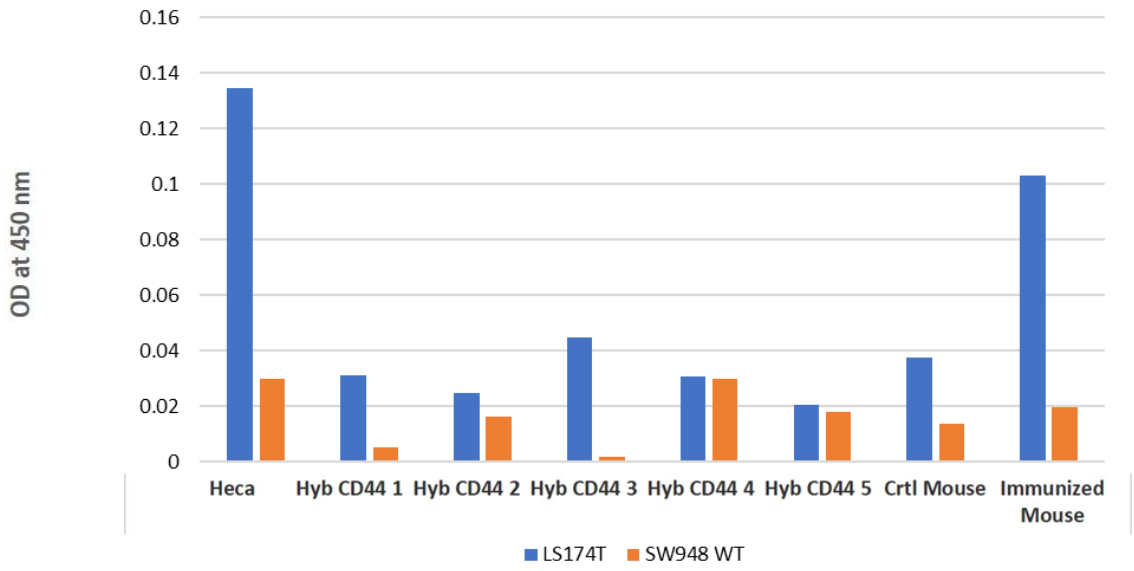

Figure 20 - Lysate ELISA was performed using lysates from LS174T and from SW948WT. Crtl Mouse was used as negative control. As positive control Heca-425 was used. Secondary antibody used to detect the reaction was goat α-mouse Ig HRP at 1:1000 dilution

In addition to this analysis a cell ELISA was performed in which the cells grow directly on the ELISA plate to test the binding capacity of the novel antibodies to living cells. Two cell lines, LS174T and SW948 WT, were used that were previously characterized by other co-workers in the group. The above-mentioned cell lines have been used because they are colorectal cancer lines which have an increased sialylation pattern. 5 supernatants from the hybridomas obtained from the CD44 immunized mouse was analyzed as well as the serum from the mouse immunized with cell lysate. The controls used were the same as those described in previous experience.

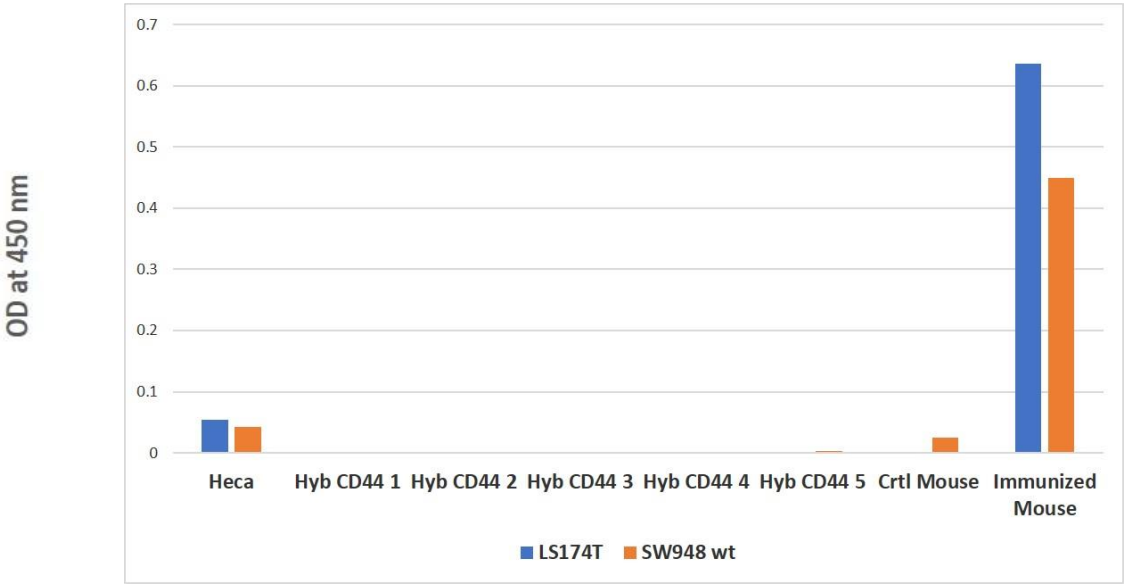

Figure 21 – A cell ELISA was performed using LS174T and SW948 WT. Crtl Mouse was used as negative control. As positive control Heca-425 was used. Secondary antibody used to detect the reaction was goat α-mouse Ig HRP at 1:1000 dilution except for HECA-452 in which Anti-Rat IgM HRP was used.

The absorbance was read at 450 nm. Immunized mouse. In this experiment five hybridoma supernatants were tested as well as the immunized mouse serum.

In figure 23 we can observe that the Crtl Mouse shows no signal, as well as the supernatants tested. This indicates that none of the supernatants tested from mouse immunized CD44 have antibodies that recognize the proteins and their glycosylation on the surface of these cells. On the other hand, the serum of the mouse immunized with lysates shows an absorbance of 0.64 for the LS174 T and 0.45 for the SW948 WT. Comparing the results obtained in Figures 22 and 23, the serum of mouse immunized with lysate from LS174T shows a higher signal in the cell ELISA. Since the cell ELISA uses live cells and, having a larger signal when testing the mouse serum in the cells from which the lysates with which it was immunized came from, it can be concluded that the mouse produced antibodies with the ability to recognize glycans with SLex in an intact membrane protein such as those present in the cells used for the cell ELISA. These results demonstrate the presence of antibodies in the serum of the immunized mouse recognizing antigens in the cells used. The difference between the serum of the lysate immunized mouse and the hybridomas from CD44 immunized mouse may be due to the lack or loss of glycans in CD44 or because these hybridomas recognize other antigens and not the antigens for which the mouse was immunized. Another hypothesis is that there is some antibody that recognizes the protein and not the glycan attached to it. These 5 hybridomas obtained were the first to be stable in culture whereby other hybridomas were still growing. new screening is ongoing, in order to perform cloning useful to select specific and high titer of the antibody.

4 Conclusions and future perspectives

Cancer is one of the most prevalent diseases in the world as well as one of the biggest causes of death. The glycosylation pattern of the cell surface is altered in cancer by aberrantly expressing glycans and this pattern is associated with cancer malignancy, tumor progression and migration, producing tumor associated carbohydrates (TACA). These $TACA_s$ then become candidates for biomarkers and possible therapeutic targets.

Since STn is a glycan associated with various cancers and is absent in healthy cells, it makes it an antigen of interest for diagnosis and therapy. For this reason, studies have been developed to potentiate the development of novel anti-cancer immunotherapies including monoclonal antibodies.

Monoclonal antibodies are increasingly being used as a therapeutic approach because of their high specificity and their different modes of action such as the ability to interact with the immune system thus stimulating an immune response against cancer.

The main goal of this thesis was the characterization of the monoclonal antibodies produced by hybridoma technology. Hybridoma cells were cultured and the supernatants were collected. The specificity of the antibodies in the supernatant of the hybridoma cells was assessed by ELISA and flow cytometry. The supernatants of the L2A5 clone showed production of antibodies recognizing sialylated structures since in the ELISA results the signal was high although after the sialidase treatment there was still a reduced signal showing that the antibody could recognize other structures. The same supernatants were tested by flow cytometry using MDA STn cells overexpressing STn. These cells were also treated with bromelain to test for glycoproteins. With the action of this enzyme the glycoproteins on the surface of the cells were removed and the results showed that the binding of L2A5 to the cells decreases almost to zero and it can be concluded that the antibody can recognize STn and it has specificity for glycans attached to proteins.

Of the 34 L2A5 subclones that showed reactivity against STn when analyzed by ELISA only 9 were selected because they had the highest absorbance signal above 1.4. The selected clone 4E11, showed absorbance when analyzed by ELISA showing an efficacy in STn binding although there is a signal after treatment with sialidase.

The main conclusion is anti-STn monoclonal antibodies were successfully produced by hybridoma technology, with the producing hybridoma clones maintaining a stable antibody production in culture. Further assays can be performed as western blot, glycoarrays and immunohistochemistry.

MDA WT and MDA STn cells were used for flow cytometry assays. MDA STn cells demonstrated a loss of expression along passages already described by other collaborators in the group. A new transduction or transfection of this cell line should be performed so that the enzyme ST6GalNAc-I is again overexpressed, allowing the stable expression of STn, so that this cell line can be used in future assays with a higher STn expression.

The second part of the work led to the conclusion that the mouse immunization produced antibodies against cells, which probably have SLe^x and SLe^a on their surface, as a significant signal is observed in the ELISAs performed both in the evaluation of the sensitivity to sialylation and in the cell ELISA with live cells. As for the hybridomas none of them produced a monoclonal antibody that recognizes the antigens of interest, SLe^x and SLe^a . The analysis of other hybridomas in culture will need to be performed to be able to confirm that one of them produces the desired antibody.

The work developed allowed to understand the production of monoclonal antibodies by the hybridoma technology and to understand the assays necessary for the characterization thereof. Complementary tests to this work should be carried out as previously mentioned.

In the near future, new therapeutic approaches will be developed using the knowledge of glycosylation which will contribute to new immunotherapies improving the diagnosis and therapy of cancer by increasing success in the fight against cancer.

5 References

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6 Appendixes

6.1 Appendix 1: Symbol and Text Nomenclature for Representation of Glycan Structures

(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 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).

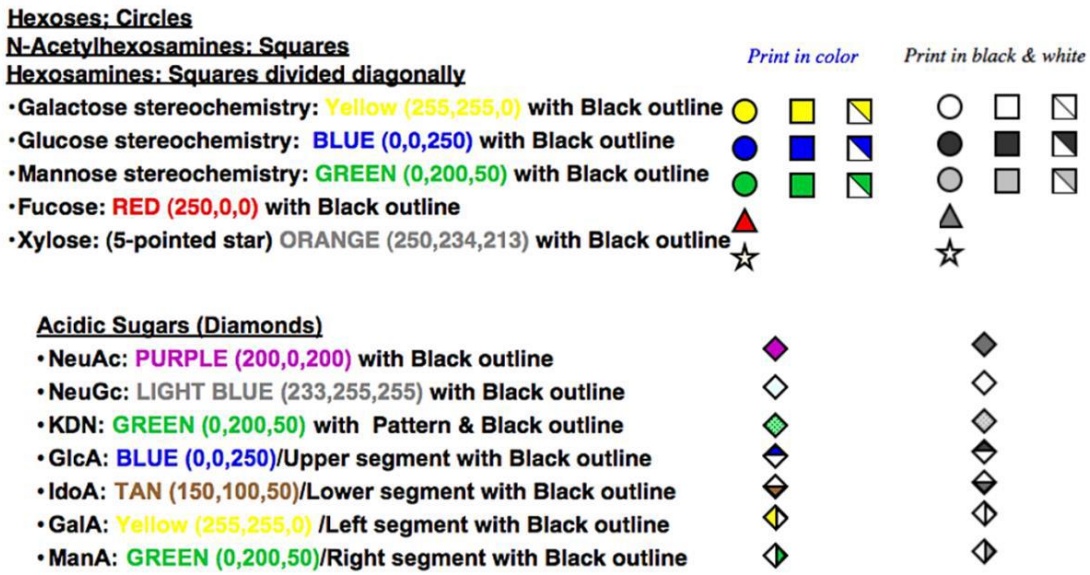

Figure 22 - Symbol nomenclature adopted for glycan structures.

Text nomenclature:

The Nomenclature Committee recommends a 'modified 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:

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.

Linear: NeuAcα2-3Galβ1-4(Fucα1-3)GlcNAcβ1-2Manα1-R

2D:

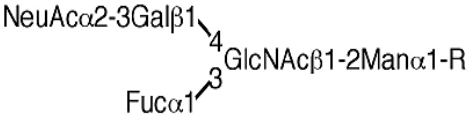

Figure 23 - Text nomenclature adopted for glycan stuctures, both linear and 2D. Adapted from Nomenclature Committee Consortium of Functional Glycomics

6.2 Appendix 2: Composition of the solutions and reagents used in the work developed

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

PBS 1x: Solution containing 1.47 mM of KH2PO4, 4.29 mM of Na2HPO4.7H2O, 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)

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

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