ROLE OF SIALYLATED GLYCANS IN CANCER PROGRESSION: SIALYL-TN AND SIALYL-LEWIS X

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Tese para obtenção do grau de Doutor em Ciências da Vida,

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- Carrascal MA, Silva M, Ramalho J, Pen C, Martins M, Serrano I, Sackstein R and Videira PA. Inhibition of fucosylation abrogates E-selectin ligands expression on breast cancer primary cells and reduces ERK1/2 and p38 MAPK activation. 2016

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Abstract

Glycosylation is a posttranslational modification of proteins and lipids, able to regulate a diversity of biological processes. Changes in glycosylation result in alterations in these processes and have been associated with cancer development and progression. One of the most commonly reported change in cancer glycosylation is an increased sialylation, which have been associated with malignant features and poor prognosis. Since sialic acid is a terminal sugar, it is normally involved in numerous biological processes, such as cellular recognition, cell adhesion and cell-to-cell signaling. In cancer cells, the increase of sialylated glycans seems to affect several cell-cell mechanisms, promoting cancer survival. However, the role of sialylated glycans in cancer progression is still poorly understood. In this thesis, we aimed to clarify the role of two specific sialylated glycans, sialyl-Tn (STn) and sialyl-Lewis X/A ($sLe^{X/A}$), which are typically overexpressed in cancer. We aimed to understand whether they contribute to cancer progression, in particular in the recognition of tumor cells by immune cells and in the process of invasion and metastasis.

In the immune recognition of cancer, dendritic cells (DCs) play a unique and decisive role in tumor immunity, being capable of activating antigen-specific cytotoxic T cells. However, to efficiently prime T cells, DCs have to undergo maturation, which is usually prevented by the tumor environment through many immunosuppressive strategies. STn glycan is expressed by more than 80% of human carcinomas and is associated with many malignant features, such as invasiveness and proliferation. Clinically, STn has been used as a target for cancer therapeutic strategies; however, STn-based vaccines tested in clinical trials have not been as efficient as expected, highlighting the low immunogenicity of STn. Here, we showed that STn expression in bladder cancer tissues is associated with an immature DCs phenotype. Using bladder cancer cells overexpressing ST6GalNAc1, which leads to STn expression, we demonstrated that DCs in contact with STn+ cancer cells have a more immature profile, expressing less MHC-II antigen presenting molecules and co-stimulatory molecules (e.g. CD80 and CD86), and producing less pro-inflammatory cytokines, in comparison with DCs with STn- cancer cells. Although DCs were able to uptake more STn+ apoptotic cancer cells, it led to a reduced ability to activate T cells. These findings showed that the expression of STn glycan in cancer cells hinder the immune response by inducing a more tolerogenic profile in DCs. In addition, we also

verified that antibodies against STn, or its protein carriers (CD44 and MUC1), seem to revert this immature phenotype in DCs, suggesting that antibodies can be used to overcome the low immunogenicity of STn+ cancer cells.

Regarding the $sLe^{X/A}$ antigens, we focused in their role as E-selectin ligand in breast cancer, as an important step for cell migration. Both sLe^{X} and sLe^{A} are the most common glycan structure in the glycoconjugates (proteins or lipids) able to bind E-selectin, which is expressed upon inflammation on vascular endothelium and is crucial during the process of extravasation of cancer cells from the bloodstream to form new metastasis. In order to better understand the role of these glycans in cancer progression, in particular the mechanisms mediated by E-selectin ligands ($sLe^{X/A}$) in breast cancer, we established new primary breast cancer cell cultures from invasive ductal cancer tissues, which preliminary results had shown to have higher expression of E-selectin ligands. To understand these mechanisms, we inhibited $sLe^{X/A}$ expression by using a fucosylation inhibitor, named 2-fluorofucose (2-FF), in breast cancer cells. In the cell line showing higher expression of E-selecting ligands, namely the CF1_T cell line, we proved that 2-FF inhibitor abrogated the expression of functional E-selectin ligands, as well as reduced the proliferation index and migration capacity of the cells. We also showed that CF1_T cells treated with 2-FF inhibitor expressed lower levels of growth factors and consequently, ERK1/2 and p38 signaling pathways were less activated than in untreated cells, suggesting that the expression of E-selectin ligands, such as $sLe^{X/A}$, has a major impact on malignant features, such as cell signaling/proliferation, adhesion and migration, contributing to tumor progression.

E-selectin engagement is not only dependent on the $sLe^{X/A}$ glycan structures but also on its carrier molecules, whose nature is still poorly known in breast cancer. The identification of these molecules may reveal potential novel targets in therapy to circumvent cancer progression. Here, through the characterization of the glycoconjugates decorated with $sLe^{X/A}$ expressed by CF1_T breast cancer cell line, we revealed that they were mainly N-glycosylated proteins. Their nature was identified by mass spectrometry after immunoprecipitation with E-selectin chimera. From those, CD44 and CD13 glycoproteins were confirmed, by western blot, as carriers of $sLe^{X/A}$ glycans. CD44 had already been identified as an E-selectin ligand in cancer; however, CD13 glycoprotein, although being associated to cancer, has never before been described as being able to bind E-selectin. While further studies are still necessary to elucidate the exact contribution of

CD13, this study suggest this glycoprotein as a potential interesting target for novel anti-cancer therapy.

Within breast cancer subtypes, the triple negative breast cancer (TNBC) has a more aggressive course and increased probability of distant recurrence. So far the expression of $sLe^{X/A}$ glycans and general E-selectin ligands in TNBC is unknown. To address this, we develop a staining protocol using E-selectin chimera by immunohistochemistry. Our results showed that E-selectin ligands, such as $sLe^{X/A}$, expression is negatively correlated with the expression of CK5/6, which is a basal-like biomarker associated with better survival rate in TNBC patients. The relevance of this association of E-selectin ligands and CK5/6 expression in TNBC are still under investigation.

Overall, our findings have contributed to elucidate the role of two sialylated glycans, STn and $sLe^{X/A}$, in cancer progression. Specifically, the data reported here shed light into the putative role of STn in the immunological escape of tumor cells and explore the role of E-selectin ligands, mainly $sLe^{X/A}$, on cancer ability to growth and develop metastasis, pointing out to novel potential therapeutic targets in cancer.

Resumo

A glicosilação é uma modificação pós-traducional de proteínas e lípidos, capaz de regular uma diversidade de processos biológicos. As alterações na glicosilação resultam na alteração destes processos e têm sido associados com o desenvolvimento e progressão do cancro. Uma das alterações mais frequentemente relatadas na glicosilação em cancro é o aumento da sialilação, o qual tem sido associado a características malignas e a um mau prognóstico. Uma vez que o ácido siálico é um açúcar terminal, encontra-se normalmente envolvido em inúmeros processos biológicos, tais como o reconhecimento celular, a adesão celular e sinalização célula-a-célula. Em células cancerígenas, o aumento dos glicanos sialilados parece afetar vários mecanismos celulares, promovendo a sobrevivência do cancro. No entanto, o papel dos glicanos sialilados na progressão do cancro não é ainda totalmente compreendido. Nesta tese, pretendemos elucidar o papel de dois glicanos sialilados, o sialil-Tn (STn) e o sialil-Lewis X/A ($sLe^{X/A}$), os quais estão tipicamente sobrexpressos em cancro. O nosso objetivo consistiu em compreender se eles contribuem para a progressão do cancro, em particular no reconhecimento de células tumorais por células imunológicas e no processo de invasão e metástases.

No reconhecimento imunológico do cancro, as células dendríticas (DCs) desempenham um papel único e decisivo na imunidade tumoral, sendo capazes de ativar linfócitos T citotóxicos específicos. No entanto, de forma a ativar eficientemente os linfócitos T, as DCs têm de passar por um processo de maturação, o qual é geralmente comprometido pelo ambiente tumoral através de várias estratégicas imunossupressoras. O glicano STn é expresso em mais de 80% dos carcinomas humanos e está associado com muitas características malignas, tais como a capacidade de invasão e proliferação. Clinicamente, o STn tem sido utilizado como um alvo para estratégias terapêuticas em cancro; no entanto, vacinas com base no STn testadas em ensaios clínicos não foram tão eficientes quanto o esperado, destacando a baixa imunogenicidade do STn. Aqui mostramos que a expressão do STn em tecidos de cancro de bexiga está associada com um fenótipo de DCs imaturas. Usando células de cancro de bexiga que sobrexpressam ST6GalNAc1, o qual leva à expressão de STn, demonstrámos que as DCs em contacto com as células tumorais STn+ têm um perfil mais imaturo, expressando menos moléculas de apresentação de antigénio MHC-II e moléculas co-estimuladoras (por exemplo, CD80 e CD86), além de produzirem menos citocinas pró-inflamatórias em comparação com as DCs na presença de células tumorais STn-. Embora as DCs tenham sido capazes de internalizar mais

células tumorais STn+ apoptóticas, isto levou a uma redução da sua capacidade de ativar os linfócitos T. Estes resultados demonstraram que a expressão do glicano STn em células tumorais compromete a resposta imunológica através da indução de um perfil mais tolerogénico nas DCs. Além disso, nós também verificámos que o uso de anticorpos contra STn, ou contra as proteínas decoradas por este glicano (CD44 e MUC1), parece reverter esse fenótipo mais imaturo das DCs, sugerindo que anticorpos podem ser usados de forma a ultrapassar a baixa imunegenicidade das células de cancro STn+.

Em relação aos antigénios $sLe^{X/A}$, nós focámo-nos no seu papel como ligando de E-selectina em cancro da mama, como um importante passo na migração celular. Ambos sLe^{X} e sLe^{A} são os glicanos mais comuns que decoram glicoconjugados (proteínas ou lípidos) com capacidade de ligação à E-selectina, que é expressa no endotélio vascular inflamado e é crucial no processo de extravasão de células de cancro da corrente sanguínea para formar novas metástases. A fim de melhor compreender o papel destes glicanos na progressão do cancro, em particular os mecanismos mediados por ligandos de E-selectina ($sLe^{X/A}$) no cancro da mama, estabelecemos novas culturas celulares primárias de cancro de mama a partir de tecidos de cancro ductal invasivo, as quais resultados preliminares tinham demonstrado ter maior expressão de ligandos de E-selectina. Para melhor compreender estes mecanismos, nós inibimos a expressão de $sLe^{X/A}$ através da utilização de um inibidor de fucosilação, denominado 2-fluorofucose (2-FF), em células de cancro da mama. Na linha celular com maior expressão de ligandos de E-selectina, nomeadamente a linha celular CF1_T, provámos que o inibidor 2-FF impede a expressão de ligandos funcionais de E-selectina, bem como reduz o índice de proliferação e a capacidade de migração das células. Também mostrámos que as células CF1_T tratadas com o inibidor 2-FF expressam baixos níveis de fatores de crescimento e, consequentemente, as suas vias de sinalização ERK1/2 e p38 estão menos ativadas do que as das células não tratadas, sugerindo que a expressão de ligandos de E-selectina, tal como $sLe^{X/A}$, tem um grande impacto nas características de malignidade, tais como sinalização/proliferação celular, migração e adesão, as quais contribuem para a progressão do tumor.

A ligação a E-selectina não é só dependente do glicano $sLe^{X/A}$, mas também das moléculas que estão decoradas com este glicano, cuja natureza é pouco conhecida no cancro da mama. A identificação destas moléculas pode revelar potenciais novos alvos em terapia para contornar a progressão do cancro. Aqui, através da caracterização dos glicoconjugados decorados com $sLe^{X/A}$ que são expressos pela linha celular de cancro da

mama CF1_T, nós revelámos que são principalmente proteínas N-glicosiladas. A sua natureza foi identificada por espectrometria de massa depois de imunoprecipitação com uma molécula quimérica de E-selectina. Entre estas, as glicoproteínas CD44 e CD13 foram confirmadas, por *Western blot*, como estando decoradas com o glicano $sLe^{X/A}$. A glicoproteina CD44 já foi antes identificada como ligando de E-selectina em cancro; no entanto, a glicoproteína CD13, apesar de ter sido associada a cancro, nunca foi antes descrita como sendo capaz de se ligar a E-selectina. Embora mais estudos sejam ainda necessários para elucidar a contribuição exata de CD13, este estudo sugere esta glicoproteína como um potencial interessante alvo para novas terapias anti-cancro.

Dentro dos subtipos de cancro da mama, o cancro da mama triplo negativo (TNBC) tem um fenótipo mais agressivo e uma maior probabilidade de recorrência. Até agora, a expressão de glicanos $sLe^{X/A}$ e de todos os ligandos de E-selectina em TNBC é desconhecida. Para resolver isto, nós desenvolvemos um protocolo usando a molécula quimérica de E-selectina por imunohistoquímica. Os nossos resultados mostraram que a expressão de ligandos de E-selectina, como $sLe^{X/A}$, está negativamente correlacionada com a expressão de CK5/6, o qual é um biomarcador do subtipo *basal-like* associado a uma melhor taxa de sobrevivência em doentes com TNBC. A relevância desta associação entre a expressão de ligandos de E-selectina e CK5/6 em TNBC está ainda sob investigação.

No geral, os nossos resultados contribuíram para elucidar o papel de dois glicanos sialilados, STn e $sLe^{X/A}$, na progressão do cancro. Especificamente, os dados aqui reportados explicam o papel putativo do STn no escape imunológico das células tumorais e exploram o papel dos ligandos de E-selectina, principalmente $sLe^{X/A}$, na capacidade de crescimento do cancro e no desenvolvimento de metástases, apontando novos potenciais alvos terapêuticos em cancro.

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List of Abbreviations

Number

2-FF	2-fluorofucose

A

Ab	Antibodies
ADCC	Antibody-dependent cell-mediated cytotoxicity
APC	Allophycocyanin
APCs	Antigen presenting cells
AR	Androgen receptor
Asn	Asparagine

B

β1,3-GalT	Beta 1-3 Galactosyltransferase
β1,3-GlcNAcT	Beta 1-3 N-acetylglucosaminyltransferase
β1,4-GalT	Beta 1-4 Galactosyltransferase
BCG	*Bacillus Calmette-Guérin*
BCR	B cell receptors
BSA	Bovine serum albumin

C

CA19–9	Carbohydrate antigen 19–9
cDCs	conventional DCs
CEA	Carcinoembryonic antigen
CK	Cytokeratin
CTCs	Circulating tumor cells

CTLs	Cytotoxic T lymphocytes

D

DCs	Dendritic cells
DMEM	Dulbecco's modified Eagle medium
Dol	Dolichol

E

E-Ig	E-Selectin/CD62E Fc chimera
EGFR	Epidermal growth factor receptor
ER	Endoplasmic reticulum
ER	Estrogen receptor
ERK	Extracellular signal-regulated kinase
E-SL	E-selectin ligands
ESL-1	E-selectin ligand-1

F

FGF2	Basic fibroblast growth factor
FITC	Fluorescein isothiocyanate
FoxP3	Transcription factor forkhead box P3
Fuc	Fucose
FUTs	Fucosyltransferases

G

Gal	Galactose
GalNAc	N-acetylgalactosamine
GDP-Fuc	Guanosine diphosphate β-Lfucose
GlcNAc	N-acetylglucosamine

H

HBSS	Hank's Balanced Salt Solution
HCELL	Hematopoietic cell E/L-selectin
HER2	Human Epidermal growth factor Receptor 2
HRP	Horse radish peroxidase

I

IDCs	Invasive ductal carcinomas
IFN-γ	Interferon gamma
IL	Interleukin
ILC	Invasive lobular carcinoma
IP	Immunoprecipitate

K

KLH	Keyhole limpet hemocyanin

L

LAMP	Lysosomal membrane glycoprotein

M

Mac2-BP	Mac-2 binding protein
MAPK	Mitogen-activated protein kinase
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MHC-I	MHC class I
MHC-II	MHC class II
MIBC	Muscle invasive bladder cancer
moDCs	Monocyte-derived DCs

MUC	Mucin

N

Neu5Ac	N-Acetylneuraminic acid
NMIBC	Non-muscle invasive bladder cancer

O

OST	Oligosaccharyltransferase

P

PAMPs	Pathogen-associated molecular patterns
PBMCs	Peripheral blood mononuclear cells
PCLP	Podocalyxin-like protein
PD1	Programmed cell death protein 1
pDCs	Plasmacytoid DCs
PNGase F	Peptide-N-Glycosidase F
PODXL	Podocalyxin
ppGalNAcT	Polypeptide-N-acetyl-galactosaminyltransferase
PR	Progesterone receptor
PSGL-1	P-selectin glycoprotein ligand-1
PVDF	Polyvinylidene difluoride

S

Ser	Serine
Siglec	Sialic acid-binding immunoglobulin-type lectins
sLe^{A}	Sialyl-Lewis A
sLe^{X}	Sialyl-Lewis X
ST	Sialyltransferases

STn	Sialyl-Tn

T

TCRs	T cell receptors
TEM	Transendothelial migration
TERT	Telomerase Reverse Transcriptase
TGFβ	Transforming growth factor beta
Th cells	helper T cells
Thr	Threonine
TNBC	Triple negative breast cancer
TNF-α	Tumor-necrosis factor-α
Treg	Regulatory T cells
TURB	Transurethral resection of the bladder

V

VEGF-A	Vascular endothelial growth factor
VEGF-R	Vascular endothelial growth factor receptor
VLA4	Very late antigen 4

Chapter 1

General Introduction

1. General Introduction

1.1. Glycosylation

Glycosylation is the most frequent posttranslational modification of proteins and lipids. Glycans are present in all living organisms and regulate a diversity of biological processes, including protein folding and intracellular trafficking, cell-cell and cell-matrix interactions, cellular differentiation and immune response [1, 2]. The relevance of glycosylation is highlighted by the fact that approximately 1-2% of human genes are required for glycan biosynthesis, which is catalyzed by the enzymatic reaction of several enzymes that transfer mono or oligosaccharides, i.e. glycosyltransferases, with their strict specificity for both donor nucleotide-sugars (e.g., UDP-Gal, GDP-Fuc, or CMP-Sia) and acceptor substrates [3].

Glycan biosynthesis is not a template-based process such as DNA, RNA, or protein synthesis. In fact, the glycan expression in a given cell and glycoprotein depends on the balance achieved by the expression and activity levels of the different glycosyltransferases involved in the glycosylation process, as well as by their localization/organization in the Endoplasmic Reticulum (ER) and Golgi, and on the availability of precursor monosaccharide molecules [4].

1.1.1. Types of glycosylation

The common types of glycans found in cells are primarily defined according to the nature of the linkage to the glycoconjugate (protein or lipid). There are three main glycoconjugates: glycoproteins, proteoglycans and glycolipids (Fig. 1). A glycoprotein is a protein with one or more glycans covalently attached to a polypeptide backbone, usually via N- or O-linkages. This matter will be further detailed in the next sections. In a proteoglycan, one or more glycosaminoglycan (GAG) chains is attached to a “core protein” through a typical core region ending in a xylose residue that is linked to a serine residue. Finally, glycolipids consist of a glycan linked to a lipid. In higher organisms, most glycolipids are glycosphingolipids, with a glycan attached via glucose or galactose (Gal) to the lipid moiety ceramide. Within glycosphingolipids, the sialylated structures, such as GM3, GM1, GD3 and GD2, are called gangliosides [5].

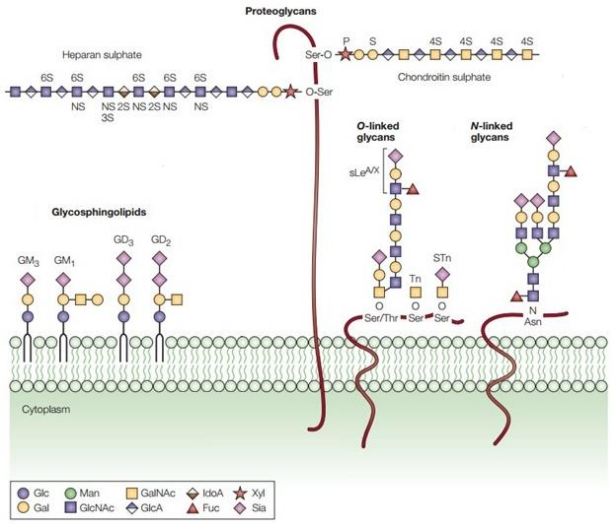

Fig. 1 – Three main classes of carbohydrates in the human plasma membrane. Glycoproteins are divided in O-linked glycans, via serine or threonine amino acid, and N-linked glycans, via asparagine amino acid. Glycolipids are mainly glycosphingolipids with different carbohydrate compositions. Sialylated glycosphingolipids are named gangliosides (GM3, GM1, GD3 and GD2). Proteoglycans have normally a part of their amino acid sequence inserted among the lipid fatty acid chains and have a glycosaminoglycan (GAG) chain attached to a xylose which is linked to a serine amino acid (adapted from [6]).

1.1.2. Glycoproteins

In humans, proteins can be glycosylated with two main types of glycans: N-glycans and O-glycans.

1.1.2.1. N-glycosylation

N-glycans are covalently attached to protein by an N-glycosidic bond at asparagine (Asn) residues within a minimal amino acid sequence that begins with Asn followed by any amino acid except proline and it ends with serine (Ser) or threonine (Thr) (Asn-X-Ser/Thr). However, due to conformational factors, not all Asn-X-Ser/Thr sequences actually have an N-glycosidic linkage. The most common N-glycan linkage is N-acetylglucosamine (GlcNAc) to Asn [7].

The biosynthesis of N-glycan begins on the cytoplasmic face of the endoplasmic reticulum (ER) membrane with the transfer of GlcNAc residue to the lipid-like precursor dolichol (Dol). Other sugar residues are sequentially added, and the Dol-linked glycan containing two GlcNAc and five mannoses is flipped inside the ER. When fourteen sugar residues (2 GlcNAc, 9 mannoses and 3 glucoses) are added to Dol, then this entire glycan is transferred in single block by the oligosaccharyltransferase (OST) complex to an Asn-X-Ser/Thr sequence in a protein

that is being synthesized into ER. The protein-bound N-glycan is subsequently modified in the ER and Golgi by a complex series of reactions catalyzed by membrane-bound glycosidases and glycosyltransferases, that remove or add sugar residues, respectively, to form the final glycan structure (Fig 2) [8].

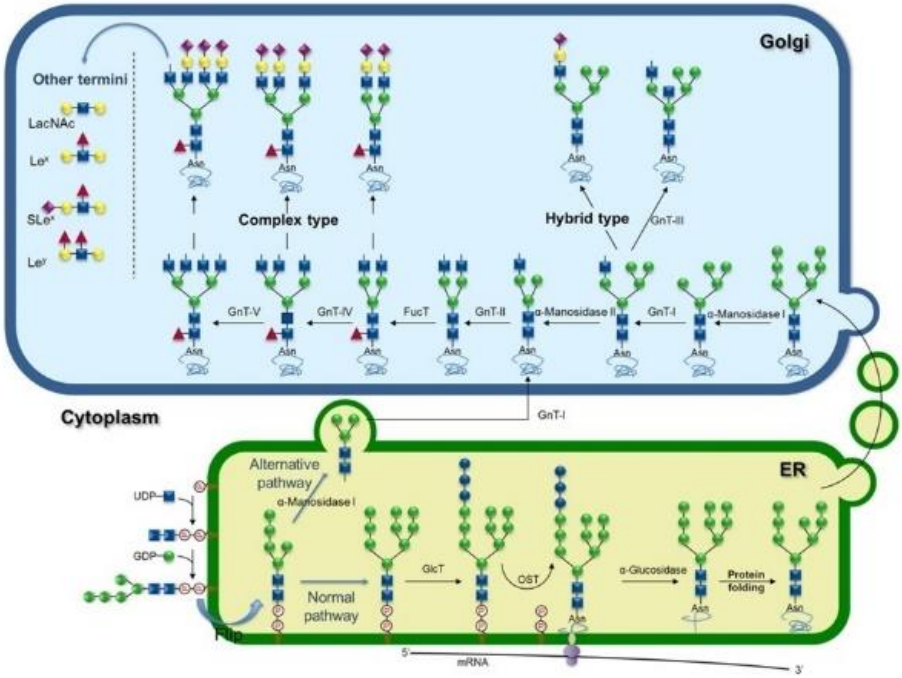

Fig. 2 - Schematic representation of biosynthesis and processing of N-linked glycans (adapted from [9]).

1.1.2.2. O-glycosylation

The most common O-glycans are linked through N-acetylgalactosamine (GalNAc). Their biosynthesis starts by the transfer of a GalNAc from UDP-GalNAc to Ser or Thr residues of a protein in the *cis* Golgi, originating the Tn antigen. This transfer is catalyzed by a specific enzyme, polypeptide-N-acetyl-galactosaminyltransferase (ppGalNAcT). Contrarily to the N-glycosylation, O-glycosylation process doesn't need a lipid-linked intermediate or any glycosidases to process their core structures. Subsequently, the simple O-GalNAc glycan can be further modified by several glycosyltransferases acting in a sequential manner in order to extend the glycan chain either branched or linearly according to substrate specificity, creating more complex O-glycan structures. Further elongation of the O-GalNAc glycans can include the addition of sialic acid, fucose, sulfate group among other residues [9, 10].

There are four common O-GalNAc glycan core structures, designated cores 1 through 4, being the first one (core 1 or T antigen) the most common O-GalNAc glycan and the initial core of many longer, more complex structures, such as Lewis glycans (Fig. 3) [10].

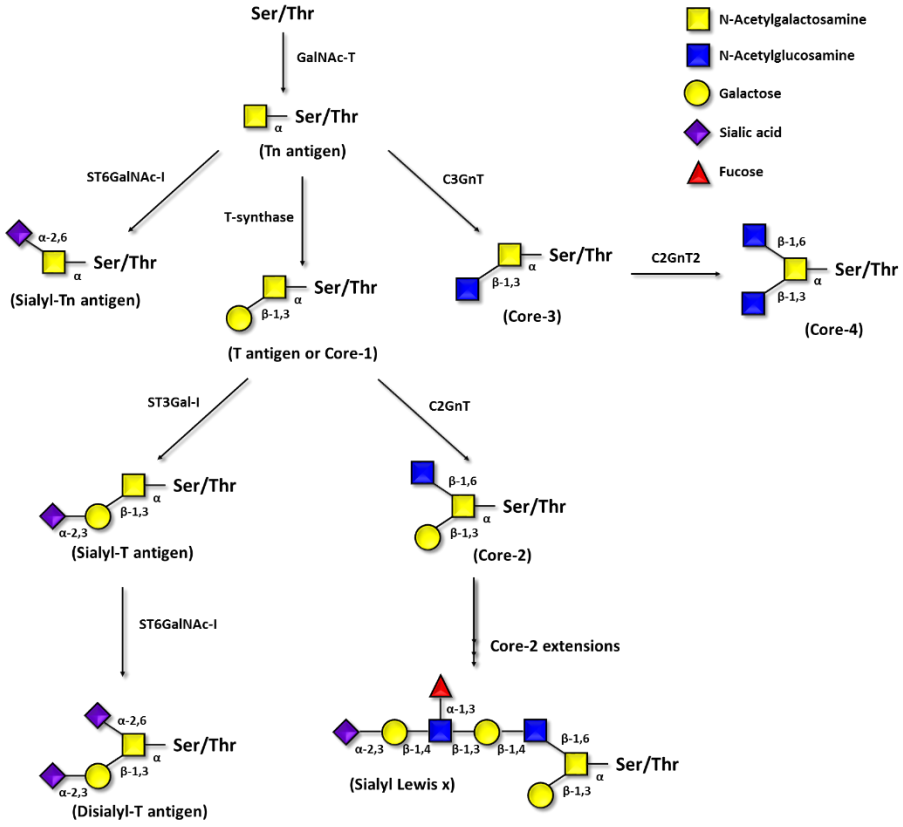

Fig. 3 – Pathways of O-glycans biosynthesis. Tn antigen is the first glycan to be created and can be sialylated (Sialyl-Tn antigen) or extended to form core 3 or T antigen (core 1). Core 3 can be extended to core 4, while core 1 can be sialylated (Sialyl-T antigen) or extended to core 2. The former can be sialylated again producing disialyl-T antigen, while the latter can be extended to originate more complex structures, such as sialyl-Lewis X structure (adapted from [10]).

1.1.3. Glycosylation in cancer

Cancer is a collection of diseases that can affect any organ of the body and is characterized as being originated from cells having an abnormally increased proliferation, loss of differentiation and a capacity to invade its surrounding tissues causing metastases in other sites in the body [11]. The main cancer feature/hallmarks, which also includes immune evasion, will be addressed in the section 1.3.

The transformation process of a normal cell into a malignant one includes changes in their cellular glycosylation profiles. It has been reported a significant correlation between certain types of altered glycosylation and cancer prognosis. In fact, the glycosylation that frequently occurs in cancer, plays a pivotal role in cancer

progression, angiogenesis and metastasis, cell-cell adhesion, signalling, motility and epithelial-mesenchymal transition (EMT) in cancer cells. These alterations of glycosylation comprise under- or over-expression of glycan structures, as well as the appearance of novel or truncated structures. However, despite the diversity of glycans at the cell surface, a few distinct structures are associated with malignant transformation and tumor progression. This suggests that specific glycosylation patterns, such as sialyl-Tn (STn) and sialyl-Lewis X/A ($sLe^{X/A}$), which are the subject of this thesis, can contribute to precise mechanisms such as gain of function, cell fitness and survival [12].

1.1.3.1. Sialylation in cancer

Sialylation is the process by which sialic acid residues are introduced onto carbohydrates attached to proteins or lipids as the terminal monosaccharide. Abnormal sialylation, specifically hypersialylation, in cancer cell is a characteristic feature associated with inferior outcome and malignant properties including invasiveness and metastatic potential, playing a key role during various stages of tumor progression [13]. Being a terminal sugar, sialic acid and their derivatives play a role in a variety of biological processes such as cellular recognition, cell adhesion, cell-matrix interaction and cell-to-cell signaling [14].

Sialic acids comprise a family of more than 50 monosaccharides that share a nine-carbon backbone (C1-9). The most common sialic acid derivate found in mammals is N-Acetylneuraminic acid (Neu5Ac), which bears an acetyl group on the fifth carbon atom (C5). In general, sialic acids are enzymatically linked to other carbohydrates by an enzymatic process that can be carried out by more than 20 distinct Golgi-resident sialyltransferases (ST) that link sialic acids via their second carbon (C2) to the carbon atom at position C3 (ST3Gal 1-6), C6 (ST6Gal 1, 2 and ST6GalNAc 1-6), or to C8 (ST8Sia 1-6) of carbohydrates, yielding α2,3-, α2,6-, or α2,8-linked sialic acids, respectively [15]. Although sialic acid can be covalently linked to Gal, GalNAc, GlcNAc, or to another sialic acid (polysialic acids), the most common linkages are the α2,3- and α2,6- to Gal residues and the α2,6- to GalNAc residues, which are carried out by ST3Gal, ST6Gal and ST6GalNAc families, respectively [16].

Up to now, three key mechanisms have been reported to cause aberrant hypersialylation in cancer cells. First, overexpression and/or altered activity of sialyltransferases results in increased sialylation of glycans and expression of specific

tumor-associated carbohydrate antigens (e.g., $sLe^{X/A}$ and STn). The second mechanism includes an enhanced metabolic flux through the sialic acid synthesis pathway in cancer cells due to increased substrate availability or overexpression of genes involved in sialic acid biosynthesis. Finally, the third mechanism consists of a decreased expression of endogenous sialidases, which can enzymatically cleave sialic acids from glycans, leading to the accumulation of sialylated glycans [15]. However, other mechanisms can also be involved in more specific glycan structures, as discussed in latter sections.

In this thesis, we will specifically focus on two main sialylated glycans: STn and $sLe^{X/A}$.

1.1.3.1.1. STn glycan

STn antigen is a disaccharide mucin-type O-glycan resulting from the sialylation of Tn antigen (Fig. 3) by an α2,6-sialyltransferase. Specifically, α2,6-sialyltransferase (ST6GalNAc 1 and 2) catalyzes the transfer of sialic acid from CMP-Neu5Ac donor to an αGalNAc O-linked to Ser or Thr residues of a protein on the C6 position. In normal tissues, STn is mostly not expressed, although in some colon tissues, STn expression was detected after de-O-acetylation, since STn was masked by O-acetyl groups which concealed its recognition by anti-STn antibodies [17]. In normal cells, Tn antigen is normally elongated to form a variety of other structures, such as T antigen (i.e, Core1, Galβ1-3GalNAcα-O-Ser/Thr), which can also be sialylated creating sialyl-T antigen (NeuAcα2-3(Galβ1-3)GalNAcα-O-Ser/Thr), or Core2 structures (GlcNAcβ1-6(Galβ1-3)GalNAcα-O-Ser/Thr), which can be further processed forming elongated structures, such as sLe^{X} [10].

In vivo, ST6GalNAc 1 enzyme has been shown to be the main sialyltransferase catalyzing the transfer of sialic acid onto the GalNAc, i.e., the Tn antigen, being its expression correlated with STn overexpression in gastric and breast tumors [10, 18, 19]. This Tn sialylation truncates further elongation of the O-glycan. Other mechanisms described as involved in the aberrant STn expression is the loss of C1GalT1 (also called T-synthase) or C3GnT enzymes, which normally extend Tn antigen into Core1 and Core3 structures, respectively. Other mechanisms are the overexpression of ppGalNAc-Ts glycosyltransferases or their re-localization from the Golgi to the ER; or even defects in Cosmc, a chaperone essential for the activity of C1GalT1, due to epigenetic silencing or mutations, which will result in an increase

expression of Tn antigen in cancer [20-23]. Specific mechanisms for STn overexpression include ST6GalNAc 1 upregulation and/or its re-localization from the Golgi to the ER and also loss of O-acetyl groups from STn, which have been demonstrated in some cancers [24].

STn antigen is highly expressed in more than 80% of human cancer types, such as gastric [25, 26], colorectal [27], ovarian [28], breast [29] and pancreatic [30] carcinomas, whereas no expression is observed in the respective normal tissues. STn expression is associated with carcinoma aggressiveness and poor prognosis. Specifically, STn expression was associated with invasiveness of liver cancer [31] and with lower survival rate in advanced gastric carcinomas [32]. In breast cancer, STn expression was associated with lymph node metastasis and high histological grade [33], as well as with resistance to adjuvant chemotherapy [34]. Studies using STn-expressing gastric and breast cancer cells reported that STn expression modulate a malignant phenotype inducing a more aggressive cell behavior, such as decreased cell-cell aggregation and increased extracellular matrix adhesion, migration and invasion [35-38].

Interestingly, the STn antigen was first discovered as a cancer marker over 30 years ago. Early studies identified the STn as a marker for diagnosis and prognosis in cancer, but later work has focused on using STn as a potential therapeutic target (see in more detail in section 1.3.1.4.1).

1.1.3.1.2. $sLe^{X/A}$ glycans

Both sLe^X and sLe^A belong to the Lewis family glycoepitopes, in which the main 8 glycan structures are represented in Fig. 4.

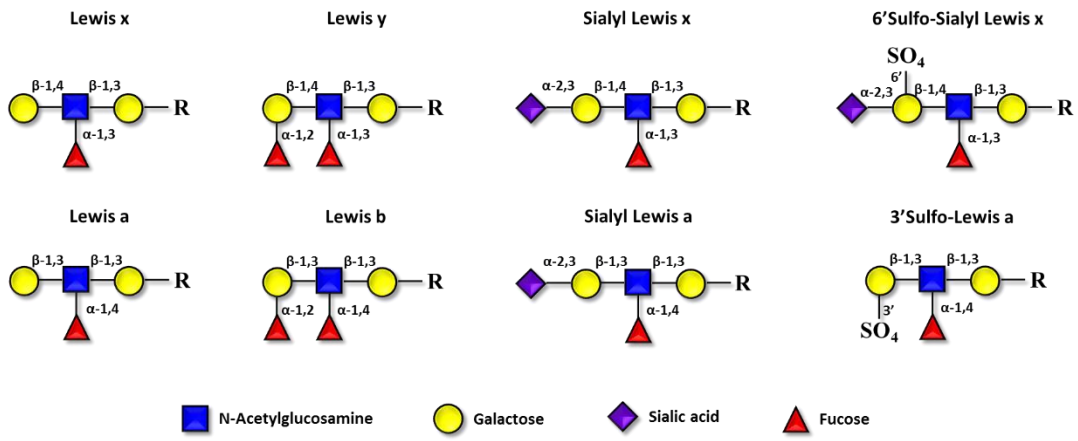

Fig. 4 – The Lewis glycoepitopes family. They are a related group of glycans that carry a fucose residue in a α1,3 (Lewis X and Y and sLe^X) or α1,4 (Lewis A and B and sLe^A) linked to GlcNAc. Here is also represented two sulfated Lewis glycans that together with sLe^X and sLe^A can bind to E-selectin expressed by the endothelium (adapted from [39]).

sLe^X is a terminal tetrasaccharide constituted by N-GlcNAc, Gal, fucose (Fuc) and sialic acid residues (NeuAcα2,3Galβ1,4(Fucα1,3)GlcNAc). Specifically, to produce sLe^X, a beta 1-3 N-acetylglucosaminyltransferase (β1,3-GlcNAcT) starts by transferring a GlcNAc residue to a Gal, being followed by the transfer of a Gal to the GlcNAc residue by beta 1-4 Galactosyltransferase (β1,4-GalT). Then a ST3Gal enzyme transfer NeuAc to the Gal residue, creating the direct precursor for sLe^X, 3'sialyllactosamine. The last step of sLe^X biosynthesis consists in the addition of Fuc to the GlcNAc residue by α1,3-fucosyltransferases (FUTs) [40, 41] (Fig. 4).

sLe^A, also termed carbohydrate antigen 19–9 (CA19–9), is an isomer of sLe^X, consisting of the same monosaccharide residues constitution but with different linkage between Gal, and Fuc, to GlcNAc (NeuAcα2,3Galβ1,3(Fucα1,4)GlcNAc) (Fig. 4), which is the result of the work of other glycosyltransferases. Differently from sLe^X, during sLe^A biosynthesis, the transfer of a Gal to the GlcNAc residue is performed by a beta 1-3 Galactosyltransferase (β1,3-GalT) and the fucosylation of the same GlcNAc residue is achieved by α1,4-FUTs [41, 42].

The two key steps in sLe^X and sLe^A biosynthesis for achieving a functional glycan are the sialylation and fucosylation. The main STs involved in sLe^A biosynthesis is ST3Gal3, while in sLe^X biosynthesis, three α2,3-STs, specifically ST3Gal3, ST3Gal4 and ST3Gal6, were described as responsible for the sialylation of sLe^X precursor [43]. Since fucosylation of sLe^X and sLe^A is the last step of their biosynthesis, it is also the most important one to guarantee the expression of these glycans. There are eight known α1,3/4-FUTs that can be involved in the biosynthesis of Lewis antigens:

FUT3, FUT4, FUT5, FUT6, FUT7, FUT9, FUT10 and FUT11. Specifically, FUT3 and FUT5 are less specific enzymes, being related not only with sLe^A and sLe^X biosynthesis, but also with their unsialylated forms, Lewis A and Lewis X, and Lewis Y and B. FUT 4 and FUT9 are more involved in the production of the unsialylated forms Lewis A and Lewis X, while FUT6 and FUT7 have been more related with sLe^X expression [44]. FUT10 and FUT11 are more recent α1,3-FUTs identified in mice and humans [45]. Recent evidences indicate that FUT10 are involved in Lewis X production and that FUT11 can be responsible for sLe^X and 6-sulfo-sLe^X expression [46, 47].

The main role of sLe^X is played during leukocyte trafficking, specifically mediating the binding of circulating leukocytes in the bloodstream and the selectins expressed by endothelium. Both sLe^X and sLe^A glycans are functional selectin ligands in cancer, facilitating the extravasation of tumor cells in the bloodstream to new metastasis sites (see in more detail in 1.3.2.1.1.1.1) [48].

1.2. Cancer

As referred, cancer can affect any organ of the body and is originated from the transformation of normal cells into malignant cells. Here, we will focus in two types of cancer: bladder cancer and breast cancer.

1.2.1. Bladder cancer

Bladder cancer is the seventh most commonly diagnosed cancer in the male population worldwide and the eleventh when both genders are considered. Tobacco smoking is the most important risk factor for bladder cancer, accounting for approximately 50% of cases. Occupational exposures to chemical carcinogens are also a well-established risk factors for bladder cancer, with about 10% of cases [49].

Bladder cancer are divided in non-muscle invasive bladder cancer (NMIBC), which is the most common representing approximately 75% of the patients, and muscle invasive bladder cancer (MIBC). NMIBC can be low grade (well-differentiated tumors), normally noninvasive papillary carcinoma (stage Ta), or high grade (poorly differentiated tumors), such as most stage T1 tumors that have penetrated the epithelial basement membrane but have not invaded the muscle, while most MIBC are high grade. NMIBCs commonly recur (50–70%) but rarely progress to invasion

(10–15%), and five-year survival is ~90% [50, 51]. In contrast, five-year survival rates in patients with MIBC are much lower, between 30% and 60%, demonstrating the lethality of the disease [52]. The most common metastatic sites in MIBC are lymph nodes, bone, lung, liver and peritoneum [53].

Treatment of stage Ta-T1 NMIBC consists in the transurethral resection of the bladder (TURB) to remove all visible lesions. Since these tumors commonly recur and may progress to MIBC, adjuvant therapy is also administered. This includes immediate postoperative single intravesical instillation of chemotherapy that will destroy circulating tumor cells after TURB, significantly reducing the recurrence rate; and intravesical instillation of *Bacillus Calmette-Guérin* (BCG) immunotherapy, which is superior in preventing the recurrence and treatment of high-risk NMIBC than TURB alone or TURB plus chemotherapy [49]. Regarding MIBC, the standard treatment is neoadjuvant cisplatin-based combination chemotherapy followed by radical cystectomy [54].

1.2.2. Breast cancer

Worldwide, breast cancer is the most frequently diagnosed cancer and the leading cause of cancer death among women. Breast cancer alone accounts for 25% of all cancer cases and 15% of all cancer deaths among women. Main risk factors include reproductive and hormonal factors such as a long menstrual history, recent use of oral contraceptives, and never having children. Normally, breast cancer is screened by mammography, which can detect early stage breast cancer enhancing the treatment efficacy and prospect of cure [55].

The histopathological classification of breast carcinoma is based on the diversity of the morphological features of the tumors. 80% of all breast cancers belong to one of two histopathological classes, namely: invasive ductal carcinomas (IDCs), which begins in the milk-carrying ducts and spreads beyond it, comprising 80% of all invasive breast cancers, or invasive lobular carcinoma (ILC), which begins in the milk-producing lobules and includes 10% of all invasive breast cancers [56].

Clinically, the molecular classification of breast cancer is more useful for treatment than the histopathological classification. The molecular subtypes are based on the expression of three biomarkers, estrogen receptor (ER), progesterone receptor (PR) and HER2 (Human Epidermal growth factor Receptor 2). Recently, proliferative rate determined by Ki67 expression is assessed in order to more precisely distinguish the

luminal group. This classification divides four subtypes: luminal A, luminal B, HER2 and “triple negative” (also known as basal-like) (Table I) [57].

Table I: Molecular subtype using four biomarkers, adapted from [57]

Molecular subtype	**Biomarker profile**
Luminal A	ER+ and/or PR+, HER2-, and low Ki67 (<14%)
Luminal B	ER+ and/or PR+ and HER2+ (luminal-HER2 group) ER+ and/or PR+, HER2-, and high Ki67 (>14%)
HER2	ER-, PR-, and HER2+
Triple Negative	ER-, PR- and HER2-

Abbreviations: ER, estrogen receptor; PR, progesterone receptor

Luminar subtype includes approximately 70% of all invasive breast cancers, while the others 30% are equally divided between HER2 and basal-like subtypes. Between luminal subtypes, luminal B tends to have higher histological grade than luminal A. However, HER2 is the one more likely to be high grade and node positive [57]. In terms of timing of relapse, there are distinct differences between the subtypes, patients with HER2 or basal-like subtypes tend to relapse within the first 5 years while the ones with luminal subtypes relapses between 5 and 15 years. Regarding the metastasis, bone is the most common metastatic site in all subtypes except basal-like tumors. Specifically, luminal A subtype have a high rate of metastasis to bone and liver, luminal B and HER2 seem to prefer bone, liver and lung and basal-like goes normally more to lung, distant nodal and then bone [58].

For therapy, luminal tumors respond to endocrine therapy and chemotherapy and HER2 tumors are treated with monoclonal antibodies, such as trastuzumab that interferes with the HER2 receptor inducing antibody-dependent cell-mediated cytotoxicity (ADCC) against tumor cells, and with anthracycline-based chemotherapy. Basal-like tumors don’t respond to endocrine therapy or trastuzumab, appearing to be sensitive to platinum-based chemotherapy and PARP inhibitors. In general, luminal A have a better prognosis than luminal B and both HER2 and basal-like tumors have poorer prognosis [57].

1.3. Cancer features

The hallmarks of cancer comprise the biological capabilities acquired during the multistep development of human tumors. Nowadays, there are eight hallmarks described, specifically sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, activating invasion and metastasis, reprogramming of energy metabolism and avoiding immune destruction. Furthermore, two enabling characteristics, genome instability and mutation and tumor-promoting inflammation, have also been included as important concepts to develop new means to treat human cancer (Fig. 5) [59].

In this thesis, two of these hallmarks, avoiding immune destruction and activating invasion and metastasis, will have a special focus, in the scope of aberrant glycosylation of cancer.

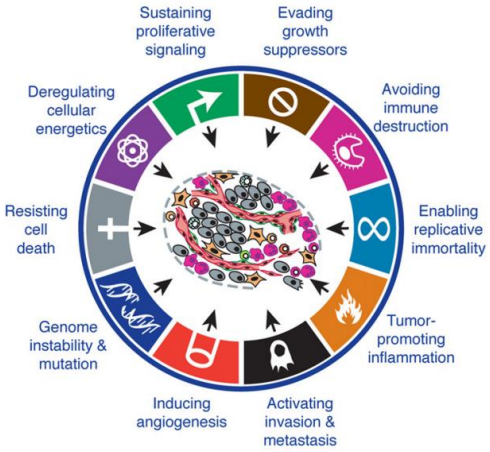

Fig. 5 – Hallmarks of Cancer. Currently, there are 8 hallmarks of cancer and 2 enabling characteristics, which are included in this illustration [59].

1.3.1. Immune responses to cancer

The immune system has evolved a powerful collection of defense mechanisms to protect the host from invading pathogenic microorganisms that would otherwise take advantage of the rich source of nutrients provided by the host. At the same time, it must be sophisticated enough to differentiate between the individual's own cells and those of harmful invading organisms and not attack the commensal flora. The immune system is able to generate an enormous variety of cells and molecules, acting together in a dynamic network, capable of specifically recognizing and eliminating an apparently limitless variety of foreign pathogenic invaders. Once a foreign organism

has been recognized, the immune system mounts an appropriate response to eliminate or neutralize the organism. This immune response is mediated by the early reactions of innate immunity and the later responses of adaptive immunity [60, 61], which will generate an effector, specific response. Later exposure to the same foreign organism induces a memory response, characterized by a more rapid and heightened immune reaction that serves to eliminate the pathogen and prevent disease.

1.3.1.1. Innate Immunity

Innate immunity provides an immediate first line of host defense and it includes physical barriers as well as cells and molecules, such as cytokines. Phagocytes are an important group of cells of innate immune response, which include dendritic cells (DCs), macrophages, monocytes and neutrophils. Their role is to recognize invading organisms using a variety of receptors that recognize pathogen-associated molecular patterns (PAMPs) and then internalize and kill the organism [62]. As mentioned below, some of these cells are antigen presenting cells (APCs) and make a crucial contribution to the activation of adaptive immunity.

1.3.1.2. Adaptive Immunity

The adaptive immunity is stimulated by exposure to infectious agents and, in contrast to innate immunity, it increases in magnitude and defensive capabilities with each successive exposure to a particular microbe. Two main characteristics of this response are the ability to distinguish different substances, called specificity, and the ability to respond more vigorously to repeated exposures to the same microbe, known as immunological memory. The adaptive immunity includes lymphocytes and molecules, such as antibodies. Naïve lymphocytes reside in and circulate between peripheral lymphoid organs, surviving for several weeks or months. They express receptors for antigens but are not able to eliminate them. To gain that capacity, they need co-stimulatory and cytokine stimulus which will lead them to differentiate, upon antigen recognition, into effector cells. After the effector phase, lymphocyte will differentiate into memory cells that survive for longer periods of time in the absence of antigen, being functionally inactive until they encounter the same antigen that induced their development, which in this case they rapidly respond to develop a secondary immune response [62].

An antigen is any molecule that can bind specifically to an antibody. Their name arises from their ability to generate antibodies. However, some antigens do not, by themselves, elicit antibody production. Antigens that can induce antibody production are called immunogens [63].

There are two types of adaptive immunity, humoral immunity and cell-mediated immunity. The humoral immunity is mediated by antibodies, which are produced by B lymphocytes and secreted into the circulation and other body fluids. They neutralize extracellular microorganism, or antigen-expressing cells, by binding to their surface. They also engage a number of other cells, such as phagocytes, providing an effector role that leads to the elimination of pathogens. However, since antibodies cannot gain access to antigens that live inside infected cells, there are another immunity response that handle the defense against intracellular antigens, the cell-mediated immunity [64].

1.3.1.2.1. Cell-mediated immunity

Cell-mediated immunity involves cell interaction and, in adaptive immune responses, the main players are T lymphocytes. The antigen receptors of T cells (TCRs) only recognize peptide fragments of protein antigens that are bound to specialized host display molecules called major histocompatibility complex (MHC) molecules, only if present on the surface of an APC.

Among T lymphocytes, $CD4^+$ T cells, also called helper T cells (Th cells), produce cytokines that activate antibody-producing B cells and phagocytes, such as macrophages, to destroy ingested antigens. Some $CD4^+$ T cells belong to a subset called regulatory T (Treg) cells, whose purpose is to prevent or limit immune responses. On the other hand, $CD8^+$ T cells or cytotoxic T lymphocytes (CTLs) have the machinery to kill infected host cells [64].

1.3.1.3. APCs

APCs are cells able to capture antigens, process them and display them to T lymphocytes, providing at the same time co-stimulatory signals and cytokines that stimulate the proliferation and differentiation of lymphocytes [62].

To activate T cells, there are three main signals: i) The antigen-specific recognition, which consists in the recognition of the peptide antigen in the context of MHC molecule by the TCR; ii) The co-stimulation, which is the second signal involving the

interaction between co-stimulatory molecules expressed on the membrane of APC and the T cell. One main co-stimulatory pair is the B71/B72 (CD80/86) molecules on APCs and its recognition by CD28 molecules expressed by T cells; ii) and cytokine signals, which results in the upregulation of cytokines and their receptors that boosts the activating signals [65].

The three main types of APCs are DCs, macrophages and B cells. DCs have a major role as APCs, due to its higher ability to present antigens, co-stimulation and migration.

1.3.1.3.1. DCs

DCs are widespread throughout the organism. Although DCs were first visualized as Langerhans cells in the skin in 1868, they were really just reported by the late Nobel laureate Ralph Steinman in 1973 [66] and their role as APCs have been thoroughly described. In general, DCs are APCs with a unique ability to effectively stimulate, naïve and memory T lymphocytes, as well as being able to induce immunological tolerance [67].

1.3.1.3.1.1. DCs functions

Immature DCs constitutively patrol through the blood, peripheral tissues, lymph and secondary lymphoid organs searching for self and non-self antigens. When immature DCs encounter antigens, they internalize them, processed them into peptides, which are loaded onto MHC class I (MHC-I) and II molecules (MHC-II), starting the DCs maturation process. Maturation implies that DCs gain ability to migrate to lymphoid organs, increased display of peptide-MHC at cell surface, including co-stimulation and cytokine secretion, to activate circulating antigen-specific T lymphocytes. These activated T cells help DCs in terminal maturation, which allows lymphocyte expansion and differentiation. Activated T lymphocytes migrate and can reach the injured tissue, where Th cells secrete cytokines, which permit activation of a number of cells while CTLs ultimately lyse the infected cells. DCs, together with T cells will also activate B cells that will produce antibodies to neutralize the initial pathogen. It is believed that, after interaction with lymphocytes, DCs die by apoptosis [68].

1.3.1.3.1.1.1. Antigen Capture

Immature DCs have a high level of endocytic activity which is lost upon maturation. They capture pathogens, infected cells, apoptotic or dead cells or their derived products to use for antigen presentation. DCs exhibit three types of endocytosis: receptor-mediated endocytosis, phagocytosis and pinocytosis (macro- and micropinocytosis).

Receptor-mediated endocytosis allows the uptake of macromolecules through their recognition by specific receptors located in clathrin-coated pits in the plasma membrane. After recognition, a signal in the endocytic receptor leads to the recruitment of clathrin lattices and to the formation of clathrin-coated endocytic vesicles. Immature DCs express several endocytic receptors, such as Fc receptors; C-type lectins, for example macrophage-mannose receptor and/or Langerin; complement receptors and Scavenger receptors, like CD36 [68].

Phagocytosis is the process where a cell internalizes a particle or microorganism in a phagosome. It is generally receptor mediated and initiates by the engagement of a specific receptors, triggering a cascade of signal transduction, which is required for actin polymerization and effective engulfment of the particle in a large intracellular vacuole named phagosome.

Regarding pinocytosis, this is a constitutive process in immature DCs and consists in capturing whatever might be in the fluid phase in the vicinity of the cells, not being dependent on specific recognition of receptors [62, 68].

1.3.1.3.1.1.2. DCs maturation and migration

After antigen uptake, immature DCs undergo phenotypic and functional changes that culminate in the complete transition from antigen-capturing cell to antigen-presenting cell.

DCs maturation is a continuous process initiated in the periphery upon antigen encounter and/or inflammatory cytokines and completed during the DC–T cell interaction. Three main factors regulate DCs maturation: pathogen-related molecules, the balance between pro-inflammatory and anti-inflammatory signals in the local microenvironment and T cell–derived signals. Several coordinated events happen during DCs maturation process, including loss of endocytic/phagocytic receptors, upregulation of costimulatory molecules (such as CD80, and CD86), change in morphology and shift in lysosomal compartments and change in MHC-II

compartments. The morphological changes accompanying DCs maturation include a loss of adhesive structures, cytoskeleton reorganization, and acquisition of high cellular motility. These changes trigger DCs migration into the T cell area of lymphoid organs. This migration process involves a coordinated action of several chemokines, and expression of respective chemokine receptors by DCs, that leads maturing DCs to migrate from inflamed tissues, enter the lymph stream, and through the draining lymph nodes to encounter T cells [67].

1.3.1.3.1.1.3. Antigen Processing and presentation

As mentioned before, antigen recognition by T cells depends on the expression of a spectrum of antigen-derived peptides bound to MHC-I and MHC-II molecules, on a APC. These peptides are the products of one of the two major proteolytic systems operating in DCs. Cytosolic antigens can be degraded by the proteasome, which produces the dominant source of peptides for MHC-I binding. In sum, a targeted protein is recognized by the ubiquitylation system, resulting in the covalent attachment of polyubiquitin chains to the protein, which is recognizable by proteasomes for proteolysis. The resulting peptides will be translocated to the ER where they will bind to MHC-I [69, 70].

Antigens that were endocytosed or phagocytosed will be both degraded by lysosomal proteolysis. Specifically, endocytosed proteins enter a vesicular pathway consisting of progressively more acidic and proteolytically active compartments classically described as early endosomes, late endosomes and lysosomes. On the other end, when particles are phagocytosed, they end in phagolysosomes that are formed by the fusion of phagosomes and lysosomes. In the end, the resulting peptides generally bind MHC-II molecules in endosomes and lysosomes [70]. Although this is the most common process, it has been demonstrated, in DCs, that internalized antigens can be transported into cytoplasm and then become also presentable by MHC-I molecules in a process called cross presentation [71].

After peptides being loaded into MHC-I or -II molecules, they are transferred to the plasma membrane where they will be recognized by $CD8^+$ CTLs or $CD4^+$ Th cells, respectively, through their TCRs. The most common type of TCRs is a heterodimer of an α and β polypeptide chains. The TCRs are generated by a complex process of somatic rearrangement at the variable DNA regions of TCR, resulting in a great variety of TCRs specific for peptide–MHC-I or -II complexes. TCRs are associated

with CD3 molecules and ζ chains, forming the TCR complex, which is required for signal transduction.

$CD4^+$ T cells not only express TCRs that will recognize peptide-MHC-II complex, but also CD4 coreceptor, which recognize MHC-II molecules and is involved in signal transduction. In the case of $CD8^+$ T cells, they express CD8 coreceptor that will recognize MHC-I molecules [62]. Both cells express CD28 that will bind to the CD80 and CD86 co-stimulatory molecules expressed by DCs upon maturation, delivering the second signal for T cell activation. The combined action of these signals, plus cytokines (third signal), stimulate the proliferation of T cells (Fig. 6) and their differentiation into effector T cells and memory T cells. Proliferation is mainly induced by interleukin-2 (IL-2) cytokine that is recognized by IL-2 receptors in T cells, activating intracellular signaling pathways required for proliferation and differentiation [72].

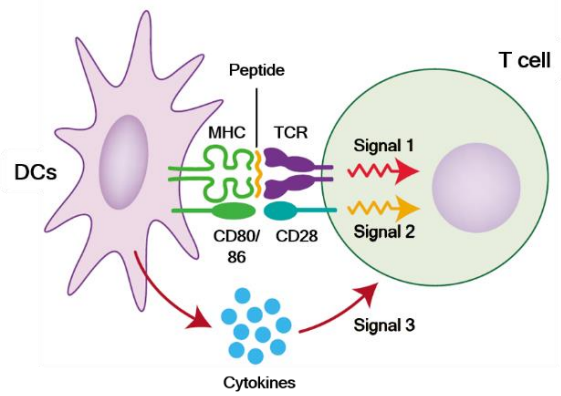

Fig. 6 – T cell activation by DCs involves three signals. The interaction of major histocompatibility complex (MHC) containing a peptide fragment with T cell receptor (TCR) on the T cell originates the signal 1. Signal 2 follows the binding of costimulatory molecules (CD80/86) on the surface of the DCs with their ligand, CD28, on the T cell surface. Finally, the secretion of cytokines by DCs represents the signal 3 (adapted from [73]).

Effector T cells are short-lived cells that actively respond to a stimulus. Effector Th cells secrete cytokines that can activate several phagocytic cells, enabling them to phagocytose and kill microorganisms more effectively. They can be divided into two main subpopulations distinguished by the different panels of cytokines they secrete, specifically Th1 subset secretes IL-2, interferon gamma (IFN-γ) and tumor-necrosis factor-α (TNF-α), being involved in the activation of CTLs, while Th2 subset secretes IL-4, IL-5, IL-6 and IL-10, helping more effectively the activation of B cells. On the other hand, effector CTLs are responsible for killing infected or tumor cells. They

have two mechanisms to accomplish this: Fas/FasL pathway and perforin/granzyme pathway, which in both will result in the apoptotic death of the target cell [61].

1.3.1.3.1.2. DCs Subsets

Multiple DCs subsets exist that vary in location, phenotype and specialized function. Two main functional subsets are the conventional DCs (cDCs) and plasmacytoid DCs (pDCs). DCs can be further divided based on their location into "lymphoid-resident" DCs, which capture antigens directly from the blood or lymph, and "migratory" DCs, that reside in the peripheral organs. In both locations, cDCs can be further segregated into multiple subsets with significant functional specialization [74].

Monocyte-derived DCs (moDCs) are another subset consisting in DCs differentiated from monocytes during inflammation or infection. moDCs differentiate rapidly from monocytes after upregulating CD11c and MHC-II molecules in response to inflammation or infection. They have the capacity to induce Th1 cell responses, crossprime antigen-specific CTLs, produce pro-inflammatory cytokines, such as TNF-α and IL-12, and regulate IgA production by B cells [75]. By exhibiting these potent functions, moDCs offer an important protective immunity role.

1.3.1.3.1.2.1. Tolerogenic DCs

While potently capable to initiate inflammatory responses, DCs also play an important role in modulating tolerance induction. Tolerogenic DCs are characterized by high antigen uptake and processing capabilities in order to present antigen to antigen-specific T cells, but fail to deliver proper cytokine and co-stimulatory signals for effector T cells activation and proliferation. This results in T cell death, T cell anergy or induction and expansion of Treg cells subsets [76]. Treg cells are characterized by the expression of CD25 and Foxp3 molecules and they act suppressing the induction and proliferation of effector T cells, maintaining tolerance to self-antigens and preventing autoimmune diseases [77].

DCs can also mediate central tolerance by negatively selecting self-antigen T cells in the thymus, inducing clonal deletion and Treg generation [78].

1.3.1.3.1.3. Tumor-infiltrating DCs

Currently, it is well accepted that the tumor microenvironment, which consists of not only tumor cells, but also fibroblasts, vascular endothelial cells and immune cells,

such as macrophages, Treg cells and regulatory DCs, is immunosuppressive and tumor-promoting. Immature and tolerogenic tumor-infiltrating DCs suppress both innate and adaptive immune effectors using a wide variety of mechanisms. Within them, is the expression of immune check points such as the PDL-1, which when engaged by PD-1 on the T cell surface suppress the immune response by inducing apoptosis and inhibiting T cell proliferation and effector function, and by promoting the differentiation of Treg cells [79, 80].

Other immunosuppressive mechanism is based on the expression of cytokines, such as PGE2, IL-10 and transforming growth factor beta (TGFβ) secreted by tumor cells, which convert activating DCs into tolerogenic DCs that suppress T cell responses [80]. Indoleamine 2,3-dioxygenase expression by tumor-infiltrating DCs also plays an important role in mediating the suppression of adaptive immune responses.

On the other hand, the presence of pDCs is generally associated with poor prognosis and tolerance in tumors. However, activated IFN-producing pDC have recently been shown to generate anti-tumor immunity *in vitro* and *in vivo* [81].

1.3.1.3.1.4. DCs in Cancer Immunotherapy

Since the discovery of DCs and the identification of their key function as mediators of T cell-mediated immune responses, there has been a major focus on the use of DCs in cancer immunotherapy. Specifically, immunotherapeutic approaches involving DCs aim to exploit the ability of these cells to direct CTLs to become potent anti-tumor effectors capable of specifically eradicate malignant cells [74, 82].

Most DCs vaccines comprises DCs loaded *ex vivo* with tumor antigen and readministered into the patient (Fig. 7). DCs are directly isolated from patients or differentiated from blood precursors, also isolated from the patients. After being loaded, DCs migrate from the injection site to the draining lymph nodes or tissues, where they are expected to prime tumor-specific T cells capable of eliminating the tumor [74].

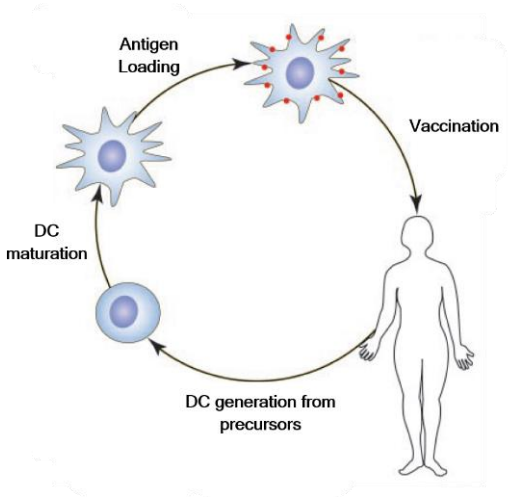

Fig. 7 - DC-based vaccines using *ex vivo* loaded DCs to induce immunity. There are many alternative approaches for the preparation and use of DCs vaccines to treat cancer. The standard protocol includes the generation of DCs from precursors, then their maturation and tumor antigen loading using a variety of techniques, and finally the readministration of these cells into the patient (vaccination) (adapted from [83]).

Most of the DCs-based immunotherapy trials have been Phase I studies on advanced cancer patients who had failed to respond to conventional therapies. These trials revealed that this approach: (1) is feasible in many different malignancies; (2) is safe and well tolerated with minimal toxicity, which allows to preserve the quality of life of cancer patients; and (3) can induce tumor-specific adaptive immune responses in many patients. In general, there was a clear evidence that this therapy can have clinical benefit by improving overall survival [74, 82]. The first and only DCs-based immunotherapy, which received FDA approval in 2010, was the Sipuleucel-T (Provenge®), which has demonstrated a significant survival benefit in metastatic prostate cancer [84]. However, despite a great enthusiasm, there were disappointing results from early studies, which raised some doubts regarding the true clinical value of DC-based vaccines [85].

The future of DCs-based immunotherapy will benefit from two developments, which are already beginning to be incorporated in clinical trials: the implementation of optimized generation of DCs, and the use of these vaccines in combination with other anti-cancer therapies which could improve their effectiveness. For example, the combination with chemotherapy has shown to be clinical beneficial, since chemotherapy depletes immunosuppressive cells, such as Tregs, thus potentiating the therapeutic efficacy of DC-based vaccines [82, 86].

Although our understanding of the complexity and subset specialization of the DCs network has increased exponentially, we still need to better understand how cancer cells alter DCs physiology in order to generate more effective cancer immunotherapies based on the powerful properties of DCs [86].

1.3.1.4. Immune response against tumor-associated carbohydrate antigens

The immune response is a key factor for not only eliciting protection against pathogens, but also to maintain immune surveillance against the development of malignant cells. From this perspective, the development of cancer can be seen as a failure of immune surveillance [87]. This idea is further supported by the immunosuppressive tumor environment, which was mentioned above.

Most tumor associated carbohydrates do not elicit strong humoral responses and, in fact, evidences have shown that their aberrant expression is one of the mechanisms developed by cancer cells to prevent effective immune responses against cancer [4].

Their low immunogenicity is related with the fact that cellular immune response is mainly formatted to fight peptide antigens, which are presented through MHC molecules, and not carbohydrates, and this may impose a limitation to develop strong immune responses against carbohydrates. While it has been reported that CTLs may recognize mono- and disaccharides attached to peptides [88-90], further investigations are necessary to better understand the relevance of such recognition in the context of antitumor immune responses.

Thus the humoral immune response plays a key role in antitumor responses against carbohydrates. In contrast to TCR, the B cell receptors (BCR) can recognize directly carbohydrate antigens with no need for presentation through MHC. Cross-linking of the antigen on BCR activates B cells, which initiate the secretion of IgM antibodies. This process is called the T cell-independent B cell activation and is able to provide immunity but with limited duration, and usually of IgM type. For the purposes of long lasting anti-tumor humoral immunity, the production of other antibodies isotypes, such as IgG, is desirable. Physiologically, this usually implies the involvement of Th cells, in a process called T cell-dependent B cell activation, which, as mentioned above, it is not easily activated in response to carbohydrates [19, 91].

The knowledge of these physiological mechanisms raised, in recent years, efforts to elicit increased immunogenicity of carbohydrate antigens, aiming to generate potent immunotherapies, such as carbohydrate-based vaccination. The approaches to

improve carbohydrate immunogenicity include the covalently coupling of carbohydrates to immunologically active protein carriers, such as keyhole limpet hemocyanin (KLH) or adjuvants or to physiological/pathological carriers, such as mucin 1 protein (MUC1), to guarantee a range of relevant epitopes [92].

1.3.1.4.1. STn glycan and immune response

Vaccination based on STn, such as Theratope (STn anchored to the KLH), have been used in pre-clinical studies and clinical trials and have demonstrated that this therapy can induce the production of antigen specific antibodies (anti-STn). STn-based vaccines were able to delay tumor growth in mice and the increased time to progression in patients [93-95]. In Theratope-treated patients, the presence of anti-STn antibodies was directly correlated with disease free survival [96]. Despite this promising results, when Theratope vaccine reached Phase III, it did not improved the overall time to progression or survival [94].

In recent years, a better understanding of the immunomodulatory role of the STn antigen has elucidated their contribution to immune tolerance, thus explaining in part the failure of STn-based vaccination protocols. For example, the overexpression of STn is associated with low infiltration of nest CD8+ lymphocytes in endometrial cancer [97], the STn present in secreted colon cancer mucins interact with CD22 and down-modulate B cell signal transduction [98]. Moreover, siglec-15 recognizes the STn antigen expressed by cancer cells and transduces a signal for enhanced TGF-β secretion in tumor-associated macrophages [99]. Additionally, mucins, in particular STn+ MUC1, released by cancer cells inhibit DCs maturation and modulate DCs towards IL-10^{high} IL-12^{low} regulatory antigen presenting cells with a limited capacity to trigger protective Th1 responses [100]. Interestingly, soluble aberrantly glycosylated MUC1 has also been described to elicit DCs maturation, yet unable to promote Th1 responses [101]. While the effect of glycoproteins secreted by tumors is becoming more elucidated, the role of the overall STn expression at tumor cell surface in immunomodulation, remains unknown.

1.3.2. Cancer Invasion and Metastasis

Metastasis is the process where the cancer cells spread from the primary tumor to colonize distant organs. Metastasis is the most fearsome aspect of cancer, since it is the cause of approximately 90% of deaths from cancer [102].

The formation of metastasis involves several complex succession of cell-biological events, whereby neoplastic cells: (1) invade locally through surrounding extracellular matrix and stromal cell layers; (2) penetrate blood vessels entering into the circulation (intravasation); (3) survive the rigors of transport through the vasculature, specifically the shear stress and the protective immune cells in the bloodstream; (4) arrest at distant organ sites by adhering to endothelial cells; (5) extravasate into the parenchyma of distant tissues, (6) survive in these foreign microenvironments in order to form micrometastases; and (7) proliferate at metastatic sites, thereby generating clinically detectable neoplastic growths (Fig. 8) [103, 104].

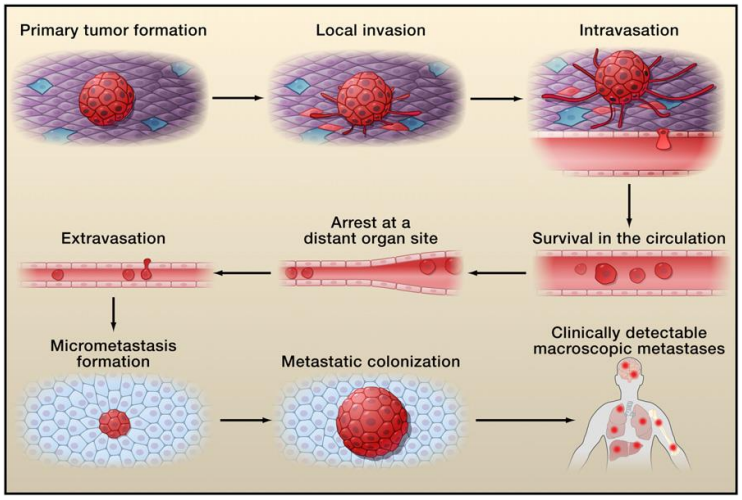

Fig. 8 – The cascade of invasion/metastasis. Tumor cells start by exiting their primary sites of growth, intravasate into blood flow, travelling systemically until they reach a distant organ site, where they colonize and growth, originating a distant metastasis [103].

1.3.2.1. Extravasation

Extravasation is generally considered the process where circulating tumor cells (CTCs) attached to endothelial cells on the microvasculature and then migrate throughout it to colonize a new tissue. Cancer cell extravasation share many similarities with leukocyte migration from the circulation across the vascular wall during inflammation. The mechanism of leukocyte recruitment from the bloodstream to diseased or inflamed tissue sites can be described as a multistep leukocyte adhesion cascade that begins with capturing events (or tethering) of leukocytes from the bloodstream and subsequent leukocyte rolling along the endothelial layer, followed by firm adhesion or arrest and ultimately transendothelial migration (TEM) into the tissue [105, 106].

Specifically, the multi-step paradigm of extravasation includes the deceleration of circulating cells on the endothelial surface (step 1 – tethering and rolling), then, when circulating cells are rolling they interact with locally produced chemotactic stimuli, resulting in integrin activation by the rolling cells and endothelial cells (step 2), which is followed by the firm adhesion of these circulating cells to endothelial cells mediated by integrins (step 3), and finally transmigration across the endothelium into the tissue allowing for the formation of new metastases (step 4) (Fig. 9) [106, 107].

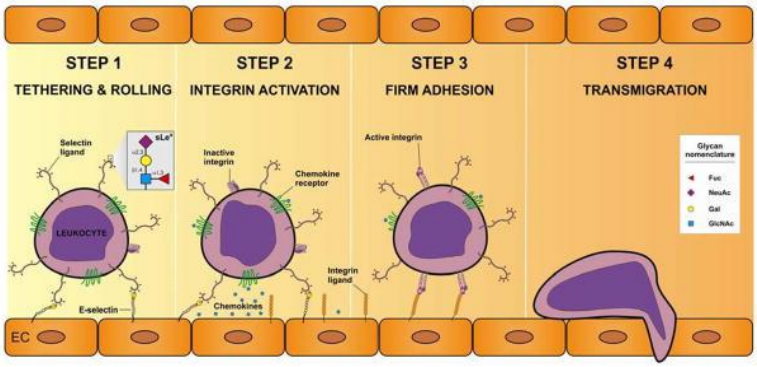

Fig. 9 - The multi-step paradigm of cellular migration through the endothelium [107].

The critical first step for extravasation is the deceleration of circulating cells on the endothelium to speed well below the local blood flow rate. Specifically, circulating cells first tether to the luminal side of the blood vessel wall, then start rolling until they are "slow rolling". The key mediators of this physiologic deceleration process are the selectins [107].

1.3.2.1.1. Selectins

Selectins are a family of three "C-type" lectins that bind to sialofucosylated proteins or lipids, within minutes, in a calcium-dependent manner - E-selectin, P-selectin and L-selectin. Selectins show a significant degree of sequence homology among themselves, especially at the lectin domain, which binds sugars, resulting that the three selectins bind similar sugar structures. Their cytoplasmic and transmembrane domains are not conserved across the selectins, being responsible for their binding to different compartments - P-selectin to secretory granules, L-selectin to the tips of microfolds on leukocytes, and E-selectin to the plasma membrane [108, 109]. P-selectin is stored in α-granules of platelets and in Weibel–Palade bodies of endothelial cells, and is translocated to the cell surface of activated endothelial cells and platelets.

L-selectin is expressed on all granulocytes and monocytes and on most lymphocytes, while E-selectin is expressed by endothelial cells. Interestingly, E-selectin is not expressed under baseline conditions, except in skin and bone marrow microvessels, but is rapidly induced by inflammatory cytokines such as TNF-α and IL-1β in endothelial cells [107, 109], thus playing an important role during inflammatory processes and metastasis.

1.3.2.1.1.1. E-selectin in cancer metastasis

E-selectin is a heavily glycosylated transmembrane protein that recognizes several glycoconjugates on numerous hematopoietic and cancer cells, mediating their rolling and initiating their extravasation at sites of inflammation [108].

Several studies have pointed to a key role played by E-selectin-mediated endothelial adhesion in metastasis [110, 111]. In fact, the binding efficiency of colon cancer cell lines to E-selectin is directly proportional to their respective metastatic potential [112]. Furthermore, metastatic prostate cancer cells were able to roll over E selectin-expressing bone marrow endothelial cells *in vitro* and home to the bone marrow *in vivo* [113]. However, when cancer cells metastasize to non-inflammatory sites, excepting bone marrow which constitutively express E-selectin, it is still not clear if E-selectin is involved in the initial step of cancer cell attachment, which occurs in minutes. Since it takes 1-4h for E-selectin to be expressed on endothelial cell surface upon cytokine stimulation, which in this case is provided by the cancer cells, it is probable that, E-selectin would contribute more to a later strengthening of the interaction of cancer cells with endothelial cells and not in the initial contact in the non-inflammatory sites [107, 114].

1.3.2.1.1.1.1. E-selectin ligands

Selectin ligands are generally transmembrane glycoproteins that require fucosylation to be properly functional. sLe^X and sLe^A tetrasaccharides, which were described in section 1.1.3.1.2, are two of the minimum carbohydrate motifs displayed on protein or lipid scaffolds that are critical components of functional selectin ligands [48]. In fact, it has been demonstrated that sLe^X expressed on leukocytes and sLe^X and sLe^A on CTCs promote better recognition and high-affinity binding to selectins. Crystal structures of sLe^X bound to the lectin domains of E-selectin reveal multiple interactions between the fucose, Ca^{2+} ion, and several amino acids, including those

that coordinate the Ca^{2+} ion, which explains the Ca^{2+}-dependent binding of selectins to fucosylated glycans [115].

In several cancer types, high levels of sLe^X and sLe^A have been associated with poor prognosis and extravasation to generate metastasis [116-122]. In colon cancer, the expression of sLe^A and sLe^X was also associated with tumor growth, enhanced angiogenesis and epithelial-mesenchymal transition [123, 124]. Using antibodies against these glycans, it was possible to determine slightly different physiological roles for both glycans, specifically, sLe^A seems to be more involved in tethering while sLe^X seems to be more focus on the arrest formation [125].

Besides sLe^X and sLe^A, other minor glycans have been demonstrated to be able to bind to E-selectin and be potentially associated with metastasis, such as 3'-sulfo-Le^A, which was correlated with invasion and metastasis in gastric carcinoma [126] and 6-sulfo sLe^X, which seems to be involved in E-selectin-mediated bladder urothelial carcinoma cell adhesion to vascular endothelial cells [127].

As mentioned within section 1.1.3.1, several glycosyltransferases are involved in the synthesis of these Lewis glycans. However, since fucose is the critical component to have a functional E-selectin ligand, α1,3-FUTs expression is particularly important to form E-selectin ligands. Specifically, FUT3, FUT6 and FUT7 have been associated with sLe^X and sLe^A expression and therefore with metastasis in cancer [128-132].

Since metastasis is the main cause of death in cancer, it is essential to find treatments that can abrogate this process. Several potential new therapeutic agents have been described in the last years, being some of them directed to interrupt or prevent E-selectin engagement with cancer cells. For example, the use of FUTs inhibitors, such as 2-fluorofucose, or nanoscale liposomes conjugated with E-selectin protein and Apo2L/TRAIL apoptosis ligand, which attach to the surface of leukocytes and rapidly clear viable cancer cells from circulating blood, or molecules that block the interaction between selectin and its ligands, like E-selectin specific DNA Aptamer, have been shown to be effective tools in preventing metastasis [133-135]. Cimetidine is a histamine H_2-receptor antagonist used in the clinic, that have anti-cancer properties, including anti-proliferative action on cancer cells, immunomodulatory effects, effects on cell adhesion and anti-angiogenic action. In terms of cell adhesion, cimetidine seems to exert its anti-metastatic effects in several cancer types by inhibiting E-selectin/sLe^X-mediated adherence of cancer cells to endothelial cells in the metastasis

target organs [110, 136, 137], resulting in an increased survival rate in cancer patients [138].

1.3.2.1.1.1.1.1. Glycoproteins as E-selectin ligands in cancer

Since not only the glycan structure but also the protein carrier is important for E-selectin engagement, several studies have reported glycoproteins that play a role as E-selectin ligands, being decorated with sLe^X and/or sLe^A glycan structures.

In colon cancer, hematopoietic cell E/L-selectin (HCELL) glycoform of CD44 is a major E-selectin ligand, being expressed on O-glycans instead of N-glycans like in hematopoietic cell and being responsible for approximately 50% of the LS174T colon cancer cells adhesion to endothelial cells in physiological shear stress [139, 140]. On the same cell line, colon cancer LS147T, it was also identified two other glycoproteins, the carcinoembryonic antigen (CEA) and the podocalyxin-like protein (PCLP), which both can act as an alternative acceptor for E-selectin binding [141, 142]. Furthermore, in other human colon cancer cell line, Colo-205, two other glycoproteins, LAMP-1 (lysosomal membrane glycoprotein-1) and LAMP-2, were identified as E-selectin ligands [143].

In pancreatic cancer, the main two glycoproteins identified as functional E-selectin ligands under flow conditions are podocalyxin (PODXL) and mucin 16 (MUC16) [144, 145].

Regarding prostate cancer, P-selectin glycoprotein ligand-1 (PSGL-1) and E-selectin ligand-1 (ESL-1) were identified as E-selectin ligands, being involved in the rolling capacity that contributes to metastasis. Specifically, PSGL-1 was notably detected on the surfaces of bone-metastatic prostate tumor cells, implicating a functional role of PSGL-1 in the bone tropism of prostate tumor cells [146, 147]. In leukemia cells, several glycoproteins have been identified as E-selectin ligands, including CD44, PSGL-1, CD43 and CD65 [148-151].

E-selectin ligands in breast cancer

Focusing on breast cancer, several studies have shown that sLe^X and sLe^A expression was associated with generation of metastasis. Specifically, expression of sLe^X and sLe^A was higher in metastatic lesions compared with primary tissues [122]. Similarly, sLe^X in serum was also elevated in patients with advanced and recurrent breast cancer, especially in those with distant metastases [152]. Comparing invasive micropapillary

carcinoma and invasive ductal carcinoma, the former has higher expression of sLe^X than the latter, which was also associated with lymph node metastasis [116]. When comparing estrogen receptor (ER) positive and negative tumors, glycosyltransferases involved in sLe^X synthesis, such as FUT3, FUT4 and ST3Gal6, were significantly overexpressed in ER-negative tumors than in ER-positive. However, it's the high expression of sLe^X in ER-positive tumors that is correlated with bone metastasis [153]. Among FUT3, FUT5, FUT6 and FUT7, only FUT6 expression was correlated with sLe^X concentration in breast cancer, suggesting its involvement in sLe^X synthesis on breast cancer cells [154].

Several glycoproteins in breast cancer cells have been identified as being able to bind to E-selectin, such as CD44v4, which mediated cell adhesion to and migration across endothelial cells and its expression is correlated with high levels of FUT3 and FUT6 [155, 156]. Mucin 1 (MUC1) was also found to be implicated in binding to E-selectin as well as to ICAM-1, which is expressed by endothelial cells and is normally involved in the arrest of circulating cells through integrins engagement, demonstrating the synergistic adhesion capabilities of MUC1 in the metastatic adhesion cascade [157]. Furthermore, CD24 and Mac-2 binding protein (Mac2-BP) were identified E-selectin ligands [158, 159]. Recently, it was found that the expression of two E-selectin ligands, BST-2 and LGALS3BP (Mac2-BP), was associated with distant metastasis risk and poorer survival. In addition, concomitant high expression of BST-2 with sLe^X defined a sub-group of patients with ER-negative tumors presenting higher risk of liver and brain metastasis and a reduced survival rate [160].

Nonetheless, E-selectin ligands are not only glycoproteins, but can also be glycolipids, as is shown in BT-20 and MDA-MB-468 breast cancer cells that have a high level of gangliosides (sialylated glycosphingolipids) playing a role as E-selectin ligands [161].

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Chapter 2

Rationale and specific aims

2. Rationale and specific aims

Cancer is a disease that can affect any organ of the body and is characterized by the transformation of a normal cell into a malignant cell, which present several changed features that leads to its survival, including an altered/aberrant glycosylation. The expression of aberrant glycans in cancer has been associated with several cancer features, such as cell proliferation, adhesion, migration, angiogenesis and metastasis. In sum, changes in glycosylation promote cancer cell fitness and survival.

One of the most common changes in glycosylation in cancer is the increased expression of sialylated structures, which is associated with malignant features and poor prognosis. Since sialic acid is a terminal sugar, it is involved in numerous biological processes such as cellular recognition, cell adhesion and cell-to-cell signaling. In cancer cells, the increase of sialylated structures affects several cell-cell mechanisms, promoting cancer progression.

Two cancer mechanisms that seem to be affected by sialylated structures expressed on cancer cells are the recognition of tumor by immune cells and the process of tumor invasion and metastasis.

Our main aim is to characterize the role of two specific sialylated glycans, sialyl-Tn (STn) and sialyl-Lewis X (sLe^X), during cancer progression, in particular in the recognition of tumor cells by immune cells, specifically dendritic cells, and in the process of invasion and metastasis, respectively.

Our specific aims are:

2.1. To evaluate the impact of the STn glycan expressed by bladder cancer in the function of the key orchestrators of the immune response, the dendritic cells (DCs)

The STn antigen is one of the most common tumor-associated carbohydrate antigen, expressed by more than 80% of human carcinomas but rarely observed in normal tissues. Its expression in cancer cells is associated with many malignant features such as invasiveness, proliferation and recurrence/progression. STn is clinically relevant, not only as a marker for diagnosis and prognosis in cancer, but also as target for therapeutic strategies. STn-based vaccines were developed; however, their efficacy was inferior to what was expected, highlighting the low immunogenicity of STn. Thus, the elucidation of the immune mechanisms affected by the expression of STn by cancer cells will

contribute to improve anti-STn immunotherapy approaches. DCs play a unique and decisive role in tumor immunity, being capable of activating antigen-specific T cells against cancer cells. However, to efficiently prime T cells, DCs have to undergo maturation, which includes the downregulation of the antigen-uptake machinery, the upregulation of antigen presenting molecules, class I and class II MHC (MHC-I and -II); costimulatory molecules, such as CD80 and CD86 and the synthesis of immune-enhancing cytokines. The degree of DCs maturation is dependent on the type of stimulus and cancer cells usually prevent maturation, through many immunosuppressive strategies. As the role of STn expression at cancer cell surface in immunomodulation remains unknown, in **Chapter 3**, we evaluate the influence of STn expression by bladder cancer cells on human DCs immune potency and functions, such as phagocytic capacity, expression of antigen presenting molecules MHC-II, costimulatory molecules and cytokines, as well as ability to activate T cells.

2.2. To characterize the role of fucosylated structures, such as sLe^X glycan, in breast cancer features by the inhibition of fucosylation

sLe^X glycan is the main terminal structure recognized by E-selectin. E-selectin is expressed upon inflammation on vascular endothelium and is crucial during the process of extravasation of cancer cells from the bloodstream to form new metastasis. The adhesion of cancer cells to endothelium mediated by E-selectin ligands, mainly sLe^X, engagement with E-selectin, leads to the slowing down of the circulating cells, resisting the disruptive shear exerted by blood flow and initiating tethering and rolling process (first step of extravasation). sLe^X is a sialofucosylated glycan determinant associated with cancer invasion and metastasis; however, the mechanistic insights are still unclear. In order to inhibit the expression of sLe^X, we used a compound, a fucosylated fucose (2-fluorofucose) described as being able to inhibit the fucosylation process. In **Chapter 4**, we evaluate the biological role of sLe^X in malignant features, such as cell adhesion to E-selectin, proliferation and migration, by comparing breast cancer cells treated with the fucosylation compound (2-fluorofucose) versus untreated cells.

2.3. To identify glycoproteins decorated with sLe^X, which are able to bind E-selectin, in breast cancer cells

Breast cancer is the most common cancer in women and their second leading cause of death, mainly due to bone metastasis. During the formation of metastasis, cancer cells

have to spread from the primary tumor, enter into the blood vessels and get out of them (extravasation) to colonize new organs. The first step of extravasation involves the binding of circulating cancer cells to E-selectin, reducing the speed of this cells initiating the tethering and rolling step. Since E-selectin is constitutively expressed in bone marrow microvessels and bone is the main organ affected by breast metastasis, the molecules that bind to it (E-selectin ligands) expressed on breast cancer cells should be the main targets if we want to avoid bone metastasis. Although sLe^X is the most common terminal glycan structure able to bind E-selectin, the protein scaffold that carries sLe^X is also important for the engagement with E-selectin. There have been some E-selectin ligand glycoproteins identified in cancer; however, the ones expressed by breast cancer cells still need to be elucidated and are the focus of the work in **Chapter 5**. In the present work, we separate the glycoproteins able to bind E-selectin by immunoprecipitation and identified them by mass spectrometry. The most probable glycoproteins were verified by western blot regarding their decoration with $sLe^{X/A}$ glycans.

2.4. To disclose the expression of E-selectin ligands in triple negative breast cancer by immunohistochemistry

Approximately 15% of all types of breast cancer are triple negative breast cancer (TNBC), which is characterized by the negative expression of estrogen receptor, progesterone receptor and HER2. TNBC has been associated with high grade tumors and worse prognosis, being mostly basal-like tumors, which express basal-like markers such as CK5/6 and EGFR. The expression of this E-selectin ligands have never been evaluated before in TNBC. $sLe^{X/A}$ are the main glycans involved in the engagement with E-selectin, being recognize by HECA-452 antibody, however there were some other glycan structures identified as binding E-selectin but were not recognized by this antibody. In order to detect all E-selectin ligands by immunohistochemistry, we needed to establish a new approach/protocol using the E-selectin chimeric molecule (E-Ig), which is the aim of **Chapter 6**. With the new protocol established, we aimed in **Chapter 7** to study the significance of E-selectin ligands expression in TNBC by immunohistochemistry, comparing it with the expression of TNBC biomarkers and clinical features.

Chapter 3

Sialyl Tn-expressing bladder cancer cells induce a tolerogenic phenotype in innate and adaptive immune cells

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Sialyl Tn-expressing bladder cancer cells induce a tolerogenic phenotype in innate and adaptive immune cells

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Running title: Immunological potency of dendritic cells is affected by sialyl Tn

Key words: dendritic cells, sialyl-Tn, immunological potency, T cells, CD44, mucins

Abstract

Despite the wide acceptance that glycans are centrally implicated in immunity, exactly how they contribute to the tilt immune response remains poorly defined. In this study, we sought to evaluate the impact of the malignant phenotype-associated glycan, sialyl-Tn (STn) in the function of the key orchestrators of the immune response, the dendritic cells (DCs). In high grade bladder cancer tissue, the STn antigen is significantly overexpressed and correlated with the increased expression of ST6GALNAC1 sialyltransferase. Bladder cancer tissue presenting elevated expression of ST6GALNAC1 showed a correlation with increased expression of CD1a, a marker for bladder immature DCs and showed concomitant low levels of Th1-inducing cytokines IL-12 and TNF-α. *In vitro*, human DCs

co-incubated with STn^+ bladder cancer cells, had an immature phenotype ($MHC\text{-}II^{low}$, $CD80^{low}$ and $CD86^{low}$) and were unresponsive to further maturation stimuli. When contacting with STn^+ cancer cells, DCs expressed significantly less IL-12 and TNF-α. Consistent with a tolerogenic DC profile, T cells that were primed by DCs pulsed with antigens derived from STn^+ cancer cells were not activated and showed a $FoxP3^{high}$ IFN-γ^{low} phenotype. Blockade of STn antigens and of STn^+ glycoprotein, CD44 and MUC1, in STn^+ cancer cells was able to lower the induction of tolerance and DCs become more mature.

Overall, our data suggest that STn-expressing cancer cells impair DC maturation and endow DCs with a tolerogenic function, limiting their capacity to trigger protective anti-tumour T cell responses. STn antigens and, in particular, STn^+ glycoproteins are potential targets for circumventing tumour-induced tolerogenic mechanisms.

1. Introduction

The sialyl-Tn (STn) antigen is one of the most common tumour-associated carbohydrate antigen, expressed by more than 80% of human carcinomas but rarely observed in normal tissues (Cao et al., 1996). STn is a posttranslational modification of cell surface glycoproteins commonly resulting from the overexpression of the ST6GALNAC1 sialyltransferase. This enzyme transfers a sialic acid to the *O*-6 position of *N*-acetylgalactosamine residue linked to a serine or a threonine (GalNAcα-*O*-Ser/Thr) on a given protein, blocking the typical elongation of *O*-glycosidic chains in glycoproteins. STn expression in cancer is associated with adverse outcome and decreased overall survival of the patients (Itzkowitz et al., 1990; Werther et al., 1996). It has been reported that STn expression by cancer cells is associated with many malignant features such as invasiveness (Julien et al., 2012; Pinho et al., 2007), and with epithelial to mesenchymal transition (Lin et al., 2009), a loss of cell differentiation that is an important milestone towards cancer metastasis.

STn is highly expressed in high-grade bladder tumours, which present elevated proliferation rates and high risk of recurrence/progression. In bladder cancer, STn enhances motility and invasive capacity of the cancer cells, thus being associated with malignancy (Ferreira et al., 2013).

The expression of STn is clinically relevant, not only as a marker for diagnosis and prognosis in cancer (the CA72-4 serological test), but also as target for therapeutic strategies. One example is a vaccine consisting of STn-clustered epitopes, Theratope, that has been meaningfully used in clinical trials to immunize breast cancer patients (Adis International, 2003; Holmberg and Sandmaier, 2004; Julien et al., 2012). Other STn-based vaccines have also been developed for application in clinical trial, which includes multi-epitope vaccines containing STn and other tumour-associated carbohydrates and STn-expressing glycopeptides (Heimburg-Molinaro et al., 2011; Madsen et al., 2013; Niederhafner et al., 2008; Slovin et al., 2007). Nevertheless, STn-based vaccines have had limited success (Miles et al., 2011) probably because the pathophysiological role of STn-epitopes in cancer cells remains unclear. One of the factors that has been highlighted by many authors is the low immunogenicity of STn-based vaccines (Julien et al., 2012; Lakshminarayanan et al., 2012; Monti et al., 2004). Thus, the elucidation of the immune mechanisms affected by the expression of STn by cancer cells will contribute to improve anti-STn immunotherapy approaches.

Glycans are involved in multiple biological functions, controlling many features of the immune response. In fact the diversity of glycans and of glycan-recognizing receptors, known as lectins, is highly regulated by the immune cells. One of the examples is the modulation of dendritic cell (DC) functions by changes of their glycan phenotype during differentiation and maturation (Crespo et al., 2009; Videira et al., 2008).

DCs play a unique and decisive role in tumour immunity, being capable of activating antigen-specific T cells against cancer cells (Banchereau and Steinman, 1998). To efficiently prime T cells, DCs undergo maturation, which includes the downregulation of the antigen-uptake machinery, the upregulation of antigen presenting molecules, class I and class II MHC; costimulatory molecules, such as CD80 and CD86 and the synthesis of immune-enhancing cytokines (Langenkamp et al., 2000). However, the degree of DC maturation is dependent on the type of stimulus and tumour cells usually prevent maturation, through many immunosuppressive strategies employed *in situ*. Tumours render DC tolerogenic and bias the immune response in favour of their own progression (Almand et al., 2001; Vicari et al., 2002). The presence of immature DCs at tumour sites is consistent with DC involvement in the tumour progression, most likely by inducing immune unresponsiveness, i.e. tolerance against cancer (Almand et al., 2001). We have previously reported, in bladder cancer tissue, that the lower expression of markers of DC maturation, such as MHC-II, was correlated with risk for recurrence (Videira et al.,

2009a). Tumour-residing DCs showing limited maturation or anergy, hold back strategies to induce immune responses and create one important obstacle to the efficacy of immune-based-therapies. Concordantly, in bladder cancer tissue, lower levels of mature DCs are associated with low responses to the *Bacillus Calmette-Guerin* (BCG) immunotherapy, the gold standard treatment for the prophylaxis and management of non-muscle invasive bladder cancer (Beatty et al., 2004; Videira et al., 2009a).

It has been described that STn epitope may confer, to different cancer cell, protection from immune defence thus contributing to malignant phenotype and cancer progression (Monti et al., 2004; Ozaki et al., 2012). Mucins, and in particular STn^{+} MUC1 mucins released by cancer cells inhibited DC maturation and modulate DCs towards IL-10^{high}IL-12^{low} regulatory antigen presenting cells with a limited capacity to trigger protective T helper type 1 (Th1) responses (Monti et al., 2004). Interestingly, soluble aberrantly glycosylated MUC1 has also been described to elicit maturation, yet unable to promote Th1 responses (Carlos et al., 2005). While the effect of glycoproteins secreted by tumours is becoming more elucidated, the role of the overall STn expression at tumour cell surface in immunomodulation, remains unknown. Therefore, in this study, we further investigated the influence of STn expression by bladder cancer cells on the immune potency and functionality of human DCs.

2. Material and methods

2.1. Reagents

Fluorescently-conjugated or unlabeled anti-CD14 (M5E2), anti-CD80 (2D10), anti-CD86 (IT2.2) and anti-CD45 (HI30) monoclonal antibodies (mAbs) were purchased from BD Biosciences (San Jose, CA). Anti-MHC-II (L243) and anti-CD1a mAb (HI149) were from Miltenyi Biotec (Bergisch Gladbach, Germany). As anti-STn, we used the HB-STn1 mAb from Dako (Dako Cytomation, Denmark) or clone TKH2 (Kjeldsen et al., 1988). Clone HMFG-2 was used as anti-MUC1 (Griffiths et al., 1986). Interleukin (IL)-4 and Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF) were purchased from R&D Systems (Minneapolis, MN). Sialidase from *Clostridium perfringens* was from Roche Diagnostics (Basel, Switzerland) and carboxi-fluorescein diacetate succinimidyl ester (CFSE) from Molecular Probes (Leiden, The Netherlands). All other reagents were from Sigma (St. Louis, MO, USA) unless otherwise stated.

2.2. Patient and tissue specimens

This study involved 49 patients, from Hospital São José in Lisbon, who underwent transurethral resection of the bladder tumours. Matched pairs of histologically verified bladder tumours and normal appearing mucosa remote from the tumour were collected and analysed individually. Based on urothelial carcinoma grading and staging criteria of the World Health Organization 132 (WHO), three different groups were considered (Table I), low-grade (LG, n=22) and high-grade HG non muscle-invasive (NMIBC, n=21) and muscle-invasive (MIBC, n=6) bladder cancers. None of these patients had received prior adjuvant therapy. Patients with *carcinoma in situ* (CIS) were not included, as well as patients with presence of upper tract malignancy, other malignancies, and chronic infections, women expectant or lactating and patients with congenital or acquired immunodeficiency. Prior patient consent and approval from the institute research ethics committee were obtained.

Table I: Information of patients included in this study

	Number of cancer patients	Age, years Median (range)
Total Number of cases	49	
Muscle invasive (MIBC)	6	65.1 (56 to 79)
Non muscle invasive (NMIBC)		
High Grade	21	68.7 (47 to 84)
Low Grade	22	69.9 (55 to 90)

2.3. Histological analysis

The immunohistochemical analysis was performed in automated equipment (Ventana BenchMark®ULTRA). All reagents were from Ventana, USA, unless otherwise stated. Briefly, the slides were heated at 72ºC, deparaffinized with EZ Prep and antigenic recovery at 97°C. Endogenous peroxidase was blocked with 3% of hydrogen peroxide and the slides were incubated with TKH2 mAb (1:10). This was followed by amplification with HRP UltraView Universal Multimer, revelation with UltraView Universal DAB Chromogen and DAB H202 and the intensification was achieved with UltraView Universal DAB Copper. The nuclear contrast was performed with

hematoxylin and bluing. After the immunohistochemical technique, the slides were washed, dehydrated, treated with increasing concentrations of alcohol (75%, 90% and 99%) for 1 minute each, cleared in xylene and mounted with synthetic mounting medium (Quick-D-M-Klinipath). A semi-quantitative approach was established to score STn expression based on the percentage of tumour that stained positively in comparison to the tumour bulk. The STn expression was assessed double-blindly by two independent observers and validated by an experienced pathologist. Whenever there was a disagreement, the slides were reviewed, until a consensus was reached.

2.4. Cell isolation and culture

Monocytes were isolated by positive selection using anti-CD14 coated magnetic beads (Miltenyi Biotech, Germany) from peripheral blood mononuclear cells (PBMCs) of healthy volunteers, provided and ethically approved by the Portuguese Blood Institute. Monocytes were differentiated into immature mo-DCs (mo-DCs) as described (Videira et al., 2008). Whenever needed, maturation of mo-DCs (mmo-DCs) was induced, at day 5, with 1μg/ml of lipopolysaccharide (LPS), for 24 h.

2.5. Flow cytometry

Cell purity, differentiation and maturation of mo-DCs was assessed by staining with fluorescein isothiocyanate (FITC)-labeled mAbs against CD14, BDCA-1, CD80 and CD86 or Allophycocyanin (APC)-labeled anti-HLA-DR. Unlabeled mouse anti-STn, -CD44 or –MUC1, followed by anti-mouse Ig-FITC were used to characterize MCR cells. Flow cytometry acquisition was performed using a FacsCalibur Flow Cytometer. File data was analysed using the CELL QUEST (BD Biosciences) and FLOWING (Turku, Finland) software to discriminate specific populations, and to determine the mean fluorescence intensity (MFI) of the cells.

2.6. Cell lines

The human bladder cancer cell line variants, MCRcont and MCRSTn, were generated as described (Ferreira et al., 2013) and were grown in Dulbecco's modified Eagle medium (DMEM) (Sigma), supplemented with foetal bovine serum, glutamine, penicillin and streptomycin.

2.7. Confocal laser scanning microscopy

Cells were cultured in cover glasses, fixed with 3.7% paraformaldehyde and permeabilized with 0.1% TritonX-100. After blocking with 1% bovine serum albumin (BSA), cells were stained using anti-STn, clone TKH2, or anti-ST6GALNAC1, clone 2C3 (Marcos et al., 2011), followed by fluorescent polyclonal anti-Ig antibody. The cell nuclei were stained with 1μM TO-PRO-3 dye (Molecular Probes, Leiden, Netherlands). Images were acquired with a Leica TCS SP2 AOBS confocal microscope (Leica Microsystem, Mannheim, GmbH). Representative confocal cross-section images were selected after Z-stacking.

2.8. Establishment of DC: bladder cancer cell lines cocultures

Cancer cell lines were incubated at $0.2x10^6$ cells/ml in a 6-well plate, at 37ºC. After 24 hours, mo-DCs were added in the proportion of 1:5 (cancer cell : mo-DC) in adhesion buffer (20mmol/l of trizma hydrochloride, 150mmol/l of sodium chloride, 1mmol/l calcium chloride, 1mmol/l magnesium chloride and 0.5% BSA, pH 8.0) at 37ºC. After 2 hours incubation, the non-adherent mo-DCs were washed and the coculture of MCR with adherent mo-DCs was stained with mAbs against MHC-II, which is expressed by DCs, but not by MCR cell lines, thus allowing to assess the percentage of adhering mo-DCs in the coculture. The cell viability was measured by annexin-V staining. Some experiments were performed in adhesion buffer without calcium or magnesium, to assess the impact of divalent ions. In other experiments, mo-DCs were incubated with 24 hour supernatants obtained from MCR cell cultures to assess the requirement for cell: cell interactions. When testing function blocking mAb, to avoid unspecific Fc receptor-mediated mAb binding to mo-DCs, the Fc receptors from mo-DCs were blocked with 10% human serum. In addition, the blocking antibodies were added to the MCR cells and not to mo-DCs. We used 20 μg/ml of anti-CD44 mAb (clone IM7), 1:100 of anti-STn mAb (clone HB-STn1), 1:20 of anti-MUC1 (clone HMFG-2) or isotype control for 30 min, washed and then cocultured the mAb coated MCR cells with mo-DCs, as described above.

2.9. Gene expression analysis by real-time PCR

RNA extraction from formalin-fixed, paraffin embedded sections was performed after deparaffinization of tissues, using Absolutely RNA FFPE kit (Agilent technologies) while RNA from the cocultures was isolated using the GenElute Mammalian Total RNA Purification kit (Sigma), according to the manufacturer's instructions. After and DNAase

treatment, 1µg of total RNA was reverse transcribed with random primers and the real-time PCR was performed with Master Mix, TaqMan probes and primers from Applied Biosystems. The assay ID provided by the manufacturer were: Hs00300842_m1 (*ST6GALNAC1*); Hs00168405_m1 (*IL-12α*); Hs00174128_m1 (*TNF-α*); Hs00174086_m1 (*IL-10*); Hs00372324_m1 (*IL-23*), Hs00203958_m1 (*FoxP3*) and Hs00174143_m1 (*IFN-γ*). The relative mRNA levels were normalized against the arithmetic mean of the *β-actin* and GAPDH expression and calculated by adapted formula $2^{-\Delta Ct} \times 1000$ which infers the number of mRNA molecules of the gene of interest per 1000 molecules of the endogenous controls (Videira et al., 2009b). ΔCt stands for the difference between the cycle threshold of the target gene and that of the endogenous control genes. The efficiency for each primer/probe was above 95% (as determined by the manufacturer). When analysing the gene expression of mo-DCs in the coculture, the contamination with MCR cells was disregarded since the analysis of cytokine gene expression in both MCR cell line variants showed a completely absence of *IL-12α*, *TNF-α, IL-10*, *IL-23*, *FoxP3* and *IFN-γ* gene expression.

2.10. Phagocytosis assay

Cell lines were labeled with CFSE according to the manufacturer's instructions and then induced to apoptosis with 10µM of camptothecin. After 48 hours, cells were incubated with mo-DCs in the proportion of 1:2, in a 48-well plate, for 6 hours at 37ºC or 4ºC. After incubation, cells were stained with anti-MHC-II mAb and the percentage of MHC-$II^+/CFSE^+$ cells (mo-DCs that phagocytosed MCR cells) was calculated by flow cytometry and confirmed by confocal microscopy. The values obtained at 4ºC were subtracted from the 37ºC values.

2.11. T cell activation

Human T cells were obtained during monocyte isolation procedure ($CD14^-$ PBMC fraction) and maintained in complete RPMI medium until complete mo-DC differentiation. Autologous T cells were then incubated with mo-DCs following phagocytosis of MCR cells, as described above, in the proportion of 8:1, in a 96-well round bottom plate, throughout 11 days. As controls, similar experiments were performed with unstimulated mo-DC or with the MCR cell lines (negative controls) or with phytohaemagglutinin (positive control). T cell activation was assessed by the percentage

of $CD69^+$ cells within the $CD3^+$ population (T cells) and their MFI values were evaluated by flow cytometry.

2.12. Protein extraction, immunoprecipitation and Western blotting

Proteins were isolated from cell lines using RIPA buffer (Sigma-Aldrich) and bladder tumour proteins were extracted from formalin-fixed paraffin embedded tissues using the Qproteome FFPE tissue kit (Qiagen). The amount of protein was estimated with RC protein assay kit (BioRad). CD44 protein was immunoprecipitated from total protein extracts (IP) with anti-CD44 monoclonal antibody (2C5 clone; R&D Systems) using Pierce Direct IP Kit (Thermo Scientific). Protein samples were separated in reducing SDS-PAGE gels, using 20μg per lane, transferred to 0.45 μm nitrocellulose membrane (GE Healthcare Life Sciences) and blocked with 1% Carbo-Free Blocking Solution (Vector Laboratories). TKH2 and goat anti-mouse IgG1 heavy chain horseradish peroxidase conjugate (Abcam) were used as primary and secondary antibodies, respectively. The Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare Life Sciences) was used as developing reagent. Protein extracts treated with sialidase were used as controls.

2.13. Statistical analysis

Statistical analyses were conducted using GraphPad Prism software, version 5.0 (GraphPad Software, La Jolla, CA). Paired student's t-test was used when data was normally distributed, and the results expressed as the mean values ± SDs. Alternatively, Wilcoxon signed-rank test was used in the case of non-normal distribution, and the results were plotted individually or as box and whiskers plot. The correlations were analyzed using Spearman and Pearson methods. Tests were considered statistically significant when $p< 0.05$ (*), $p< 0.01$ (**) and $p< 0.005$ (***) and marginal significance was considered for $p< 0.1$.

3. Results

3.1. The STn antigen expression and DCs are increased in bladder cancer

We have previously reported that STn is highly overexpressed in bladder cancer tissues, showing a markedly high incidence and intensity only in malignant tissue and was barely detectable in normal mucosa (Ferreira et al., 2013) (Figure 1A). Considering the tumour

bulk the maximum STn expression was 30%. The STn expression is highly correlated with the gene expression of the sialyltransferase ST6GALNAC1 (Figure 1B) either in low and high grade tumours. To assess whether STn expression was correlated with altered immune function, we quantified the expression of ST6GALNAC1 and CD1a, a marker for immature DC subtype predominant in bladder carcinoma (Figure 1S) (Troy et al., 1999). We also investigated the expression of interleukin (IL)-12, and tumour necrosis factor (TNF)-α, cytokines naturally expressed by DCs and involved in inducing Th1-immune responses. We have analysed tumour tissue and adjacent normal urothelium, and our data showed that both CD1a and ST6GALNAC1 are upregulated in tumour tissue, as compared with corresponding normal urothelium. Interestingly, the upregulation was more pronounced and significantly correlated in muscle invasive and high grade non muscle invasive tumours rather than low grade tumours (Figure 1C). By contrast IL-12 and TNF-α expression, which is associated with DC maturation and Th1-inducing, showed an inverse correspondence with significantly lower expression in high grade tumour tissue than low grade tumour tissue. A correlation was observable between *CD1a* and *ST6GALNAC1* (r= 0.41 and p= 0.003) expression, pointing out for an association between STn expression by cancer cells and the presence of immature DCs in tumour tissue.

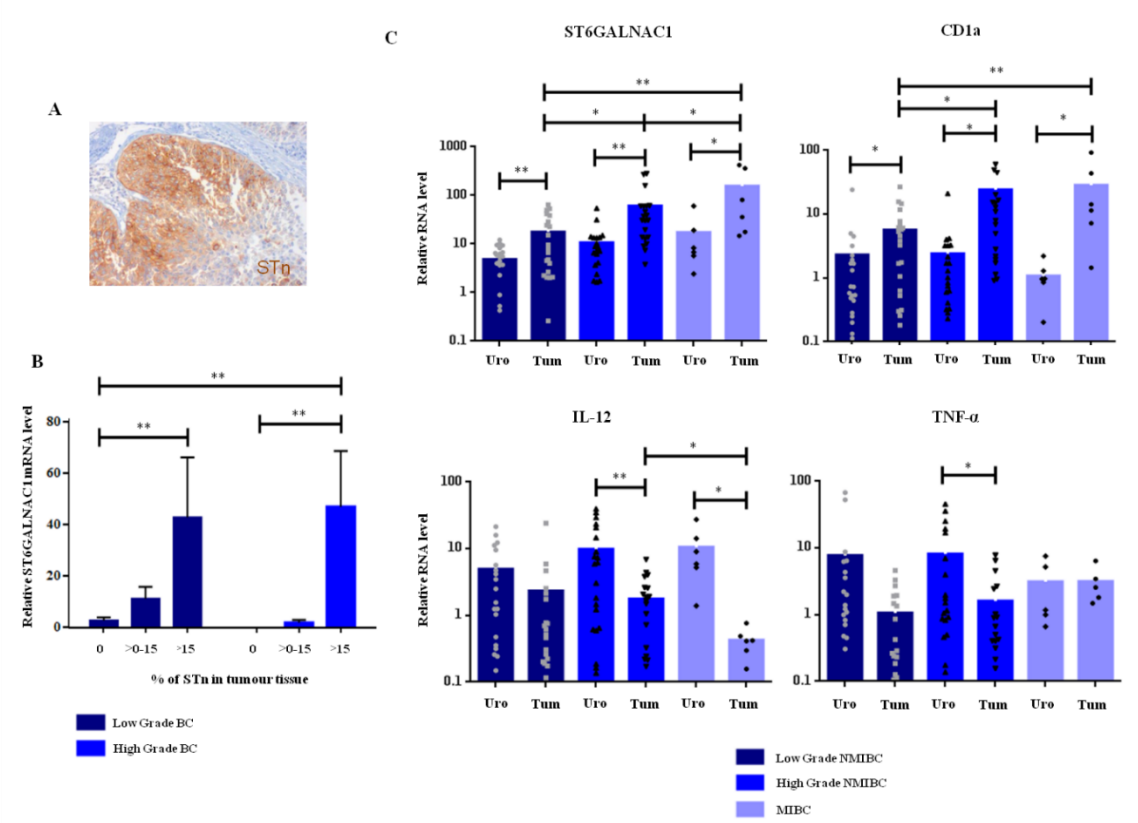

Figure 1. Bladder cancer tissues show differential expression of STn antigen, CD1a, IL-12 and TNF-α cytokines. A: Analysis of STn expression in high grade tumour bladder cancer tissue. Representative image of a paraffin embedded section, processed for immunohistochemical staining with anti-STn mAb. STn expression was detected in tumour tissue. **B:** Association between ST6GALNAC1 and STn expression in bladder tumours. STn expression was determined in low and high grade bladder tumours, by immunohistochemistry, using TKH2 mAbs. Specimens were then selected and distributed into three groups, according to their expression of STn related with the tumour bulk: 0%, 0 to 15% and more than 15% of tissue expressing STn. The relative mRNA levels of *ST6GALNAC1* gene, in the paraffin-embedded sections, was analysed individually, by real time PCR and paired compared with data regarding STn expression. Values infer the number of mRNA molecules of a *ST6GALNAC1* gene, per 1000 molecules of the average of the endogenous controls. **C:** Gene expression analysis in bladder tissue. The relative mRNA levels of *CD1a*, *IL-12* and *TNF-α* cytokines and *ST6GALNAC1* genes were analysed by real time RT-PCR in specimens from low and high grade non-muscle invasive bladder cancer (NMIBC) (n=22 and n=21, respectively) and muscle invasive bladder cancer (MIBC) (n=6) tissues and also from matched normal urothelium (n= 49). Values infer the number of mRNA molecules of each gene per 1000 molecules of the average of the endogenous controls. *CD1a* and *ST6GALNAC1* expression was significantly increased in all tumour tissue, as compared with matched urothelium; while *IL-12* and *TNF-α* cytokines were decreased ($p< 0.05$ (*) and $p< 0.01$ (**)). In general, the differences were more evident in high grade NMIBC.

3.2. Mo-DCs tend to adhere to STn$^+$ bladder cancer cell lines and show significant less mature phenotype

To further investigate the functional implications of STn-expressing cancer interaction on DC functionality, we established *in vitro* coculture models with STn$^+$ bladder cancer cell

lines and human mo-DCs. The transduction of *ST6GALNAC1* sialyltransferase cDNA in MCR bladder cancer cell lines, that natively do not express STn, induced a dramatic expression of STn (Figure 2A), corroborating the role of ST6GALNAC1 in STn biosynthesis by bladder cancer cells (Ferreira et al., 2013). The obtained STn^+ (MCRSTn) and mock transduced STn^- (MCRcont) cell lines were used throughout this study. Co-incubation of human mo-DCs together with MCR cells showed that a significant number of mo-DCs adhered to the cancer cells. The mean percentage of adhering mo-DCs was significantly higher in the coculture with MCRSTn (1.27-fold more) than with MCRcont (Figure 2B). We also investigate whether the observed cellular interactions were dependent on divalent ions, a characteristic of many intercellular interactions. In the absence of divalent ions, mo-DC adhesion to bladder cancer cells was reduced to an average of 10% adherent cells and it was not statistically different between the cocultures with each of two cell line variants (data not shown), suggesting that the differences in adhesion of mo-DCs to MCR cells were dependent on divalent cations.

To characterize the maturation phenotype of the mo-DCs adhering to the bladder cancer cells we assessed the expression of the MHC-II antigen presenting molecule, and co-stimulatory molecules, CD80 and CD86. While the mo-DCs co-incubated with MCRcont cells showed increased expression of MHC-II, CD80 and CD86, mo-DCs adhering to MCRSTn presented an expression level similar to the non-stimulated mo-DCs, suggesting that no maturation was induced (Figure 2C, 2D and 2E). To verify whether the immature phenotype of mo-DCs incubated with MCRSTn was dependent on cell contact, parallel experiences were performed where mo-DCs were incubated in the presence of conditioned media, obtained from MCRcont or MCRSTn cell cultures. However, in these conditions the phenotype of mo-DCs incubated with the conditioned medium from either MCR variants was similar (data not shown). The viability of the mo-DCs in culture was approximately 97%, indicating that the observed differences in adhesion and maturation were not due to loss of mo-DC viability. Effects on the MCR cells was also observed, but only when the cocultures were prolonged until 24 hours. Specifically, we observed that the proliferative capacity of MCRSTn cells increased 26% (p= 0.007), as compared with the proliferative capacity of this cell line in the absence of mo-DCs (Figure 2S).

In order to confirm that the effects on maturation and adhesion to mo-DCs were specifically caused by the enhanced expression of STn, the adhesion assays were performed with a different cell line, namely the breast cancer cell line, MDA-MB-

231STn, overexpressing the STn antigen (Julien et al., 2001). Similarly to MCRSTn, mo-DCs adhered significantly more to MDA-MB-231STn cells when compared to control cells, and these mo-DCs expressed significantly less MHC-II on their surface, showing a less mature phenotype (Figure 3S). Therefore, the same negative effects is obtained after co-incubation of mo-DCs with STn^+ cancer cells, irrespective of the cancer type, corroborating the immunomodulatory role specifically for STn.

We then investigated the effect of STn^+ cancer cells in semi-matured mo-DCs. We stimulated mo-DCs with LPS, a canonical inducer of mo-DC maturation, which resulted in a remarkable increase of MHC-II expression by DCs (Figure 4SA). In our conditions, LPS-matured DCs could still respond to secondary maturation stimuli and were therefore referred semi-mature. We observed that semi-matured mo-DCs adhere significantly less to both MCRcont and MCRSTn, when compared with immature mo-DCs (55% and 61% less, respectively, data not shown). After co-incubation of semi-matured mo-DCs with MCRcont, a significant increased expression of MHC-II was observed (p= 0.043), while no significant effect was obtained by a parallel incubation with MCRSTn cells (Figure 4SA).

To assess whether the contact with MCR cancer cells influenced further mo-DC responses to maturation stimulus, we stimulated mo-DCs with LPS, after coculture with MCR cells. In the presence of the cancer cells, mo-DCs showed defective LPS-induced maturation, while in their absence mo-DCs underwent maturation (Figure 4SB). The resistance to maturation was more evident when mo-DCs were previously incubated with MCRSTn, as compared with MCRcont cancer cells. Thus, the data suggest that the contact with STn^+ bladder cancer cells not only hinders maturation, but also prevents further mo-DC maturation.

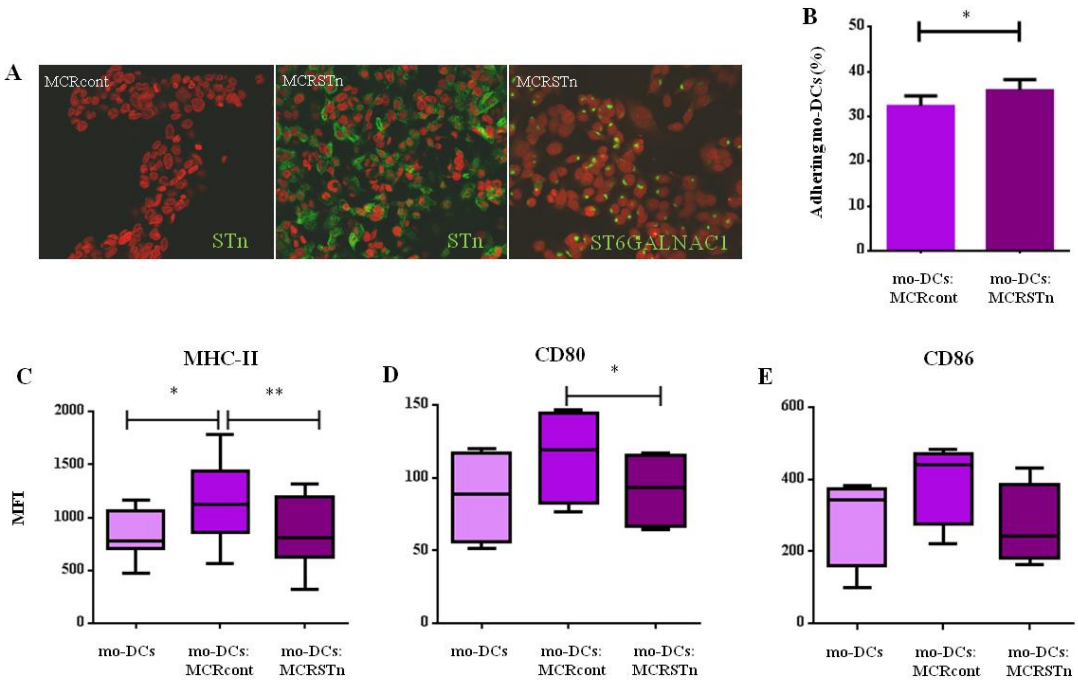

Figure 2. Mo-DCs adhere preferentially to STn$^+$ MCR cell line and show a less mature phenotype. A: Overexpression of ST6GALNAC1 in MCR bladder cancer cells. MCR cells were transduced or not with a retroviral vector expressing the whole coding region of human *ST6GALNAC1*. Both negative control (MCRcont) and ST6GALNAC1-transduced (MCRSTn) cell lines were stained with anti-STn or anti-ST6GALNAC1 mAbs and then analysed by confocal microscopy. The MCRSTn cell line, but not MCRcont cells expressed the STn antigen and ST6GALNAC1. **B:** Characteristics of mo-DCs adherent to MCR cell lines. Mo-DCs were cocultured with MCR cell lines and after 2 hours incubation, non-adherent mo-DCs were washed and the percentage of mo-DCs adherent to MCR cell lines was estimated by flow cytometry, as the total of MHC-II$^+$ cells in the coculture, following staining with APC-labeled anti-MHC-II mAb. The mean number of mo-DCs adhering to MCR cells was significantly higher in the co-incubation with MCRSTn than with MCRcont (p= 0.045 (*), n= 14). **C-E:** Analysis of mo-DC maturation and co-stimulatory profile. Adherent mo-DCs were stained with anti-MHC-II (C), anti-CD80 (D) or CD86 (E) mAbs and then analysed by flow cytometry. The expression level of the antigens was inferred from the mean fluorescence intensity (MFI) of the cells. Values are displayed as box-and-whisker plot with mean and quartiles±maximum/minimum of 5 independent assays. The expression of MHC-II and CD80 was significantly different in mo-DCs co-incubated with MCRSTn, as compared with MCRcont (p= 0.0043 (**) and p= 0.0438 (*), respectively). MFI values in mo-DCs alone were comparable to mo-DCs co-incubated with MCRSTn, while mo-DCs co-incubated with MCRcont showed a significant more mature phenotype.

3.3. Contact with STn$^+$ cancer cells compromises mo-DC cytokine expression and phagocytosis

Next, we investigated the function of mo-DCs following contact with STn$^+$ cancer cells. We analysed the expression of several cytokine genes and found that the expression of IL-12, TNF-α, IL-23 and IL-10 was significantly decreased in mo-DCs co-incubated with

MCRSTn, as compared with controls (Figure 3A). Analysis of the level of phosphorylated extracellular signal-regulated kinase (ERK), which is involved in cytokine expression, was reduced by 21% in mo-DCs following contact with MCRSTn cells (data not shown). Since mo-DCs physiologically phagocyte apoptotic cancer cells, we then compared the capacity of mo-DCs to phagocyte both MCR cancer cell line variants. As shown in Figure 4A and 4B, the percentage of mo-DCs which phagocytosed MCRSTn is significantly higher (1.25-fold more) than those that phagocytosed MCRcont. Confocal microscopy analysis confirmed the internalization of MCRSTn cells by mo-DCs (Figure 4B).

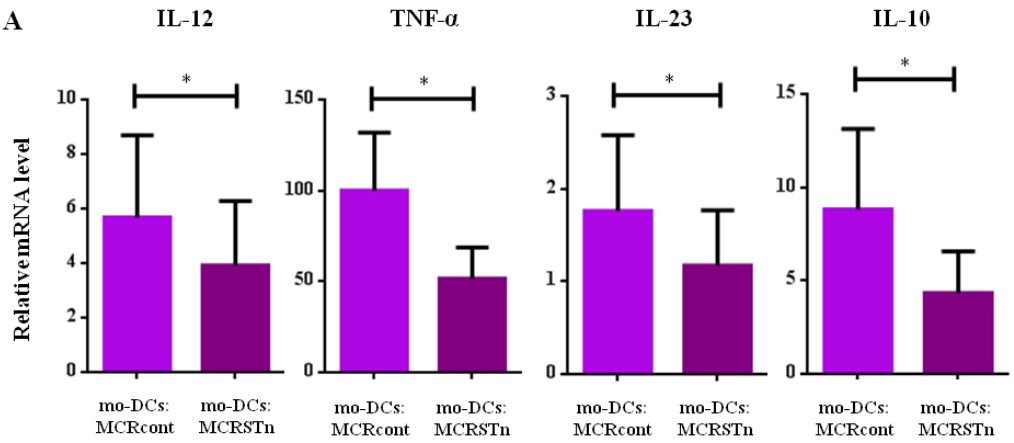

Figure 3. Co-incubation with STn+ MCR cell line downregulates cytokine expression levels. **A:** The expression of TNF-α, IL-23, IL-12 and IL-10 cytokine genes was evaluated by quantitative real-time PCR. The relative mRNA levels for each cytokine are expressed as the permillage (‰) of the expression of the endogenous positive controls. Values represent the means of at least 5 independent assays. The expression of TNF-α, IL-12, IL-23 and IL-10 were significantly decreased (p= 0.015 (*), p= 0.031 (*), p= 0.034 (*) and p= 0.015 (*) respectively) in mo-DCs co-incubated with MCRSTn as compared with those incubated with MCRcont.

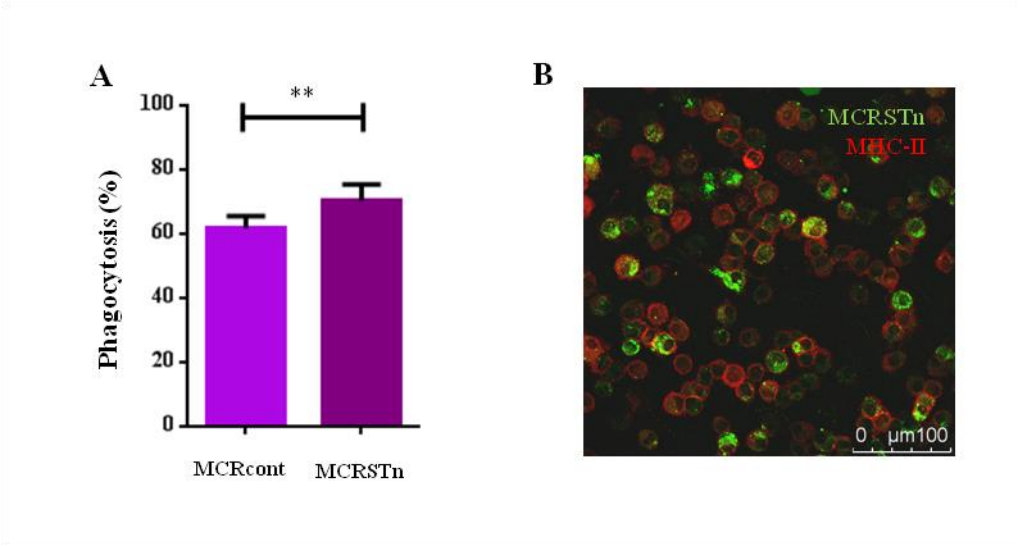

Figure 4. STn+ MCR cell lines are better phagocytosed by mo-DCs. Both MCRcont and MCRSTn cell lines were labeled with CFSE and then induced to apoptosis. Cells were then incubated with mo-DCs to allow phagocytsosis, in the proportion of 1:2 for 6 hours at 37ºC or 4ºC and then stained with anti-MHC-II mAb. **A:** Flow cytometric analysis of percentage of mo-DCs that phagocytosed MCR cells lines. The percentage of mo-DCs that phagocytosed MCR cells was calculated based on the positivity for both MHC-II and CFSE stains. The values obtained at 4ºC were subtracted from the 37ºC values. MCRSTn cells were significantly more phagocytosed than MCRcont (p= 0.001 (**), n= 4). **B:** Microscopic analysis of mo-DCs that phagocytosed MCR cells lines. Representative confocal microscopy image showing mo-DCs that phagocytosed MCRSTn cells [MHC-II+ (red)/CFSE+ (green)]. The image is representative of a confocal cross-section image, selected from Z-stack images.

3.4. T cells activated with mo-DC loaded with STn$^+$ cancer cells show defective activation profiles

We next analysed the capacity of mo-DCs to activate autologous T cells by measuring the expression of the T cell activation marker CD69. We observed that the mo-DCs that phagocytosed MCRSTn tended to activate significantly less number of T cells, and more weakly, than mo-DCs phagocytosing MCRcont (Figure 5A and 5B). T cell activation was also lower in T cells primed by mo-DCs that adhered to STn$^+$ cancer cells (Figure 5S), as compared to control MCR cells. T cells alone and T cells incubated with each apoptotic MCR cell lines (with no mo-DCs) were not significantly activated and lost viability (less than 5% viable cells after 11 days). By contrast, after 11 days, more than 35% of the T cells cocultured with mo-DCs remained viable, suggesting that mo-DCs stimulus is necessary to maintain T cell viability. Interestingly, in the T cell: mo-DC (MCRSTn) coculture, the expression of interferon (IFN)-γ and the transcription factor forkhead box P3 (FoxP3) was significantly affected. Namely, IFN-γ was decreased by 54%, while FoxP3 was increased 39% compared with control (Figure 5C and 5D). While

the differences were marginally significant ($p< 0.1$), they indicated a clear tendency for less Th1-skewed T cell response. These data are in agreement with the above mentioned defective maturation profile of mo-DCs, following contact with STn^+ cancer cells.

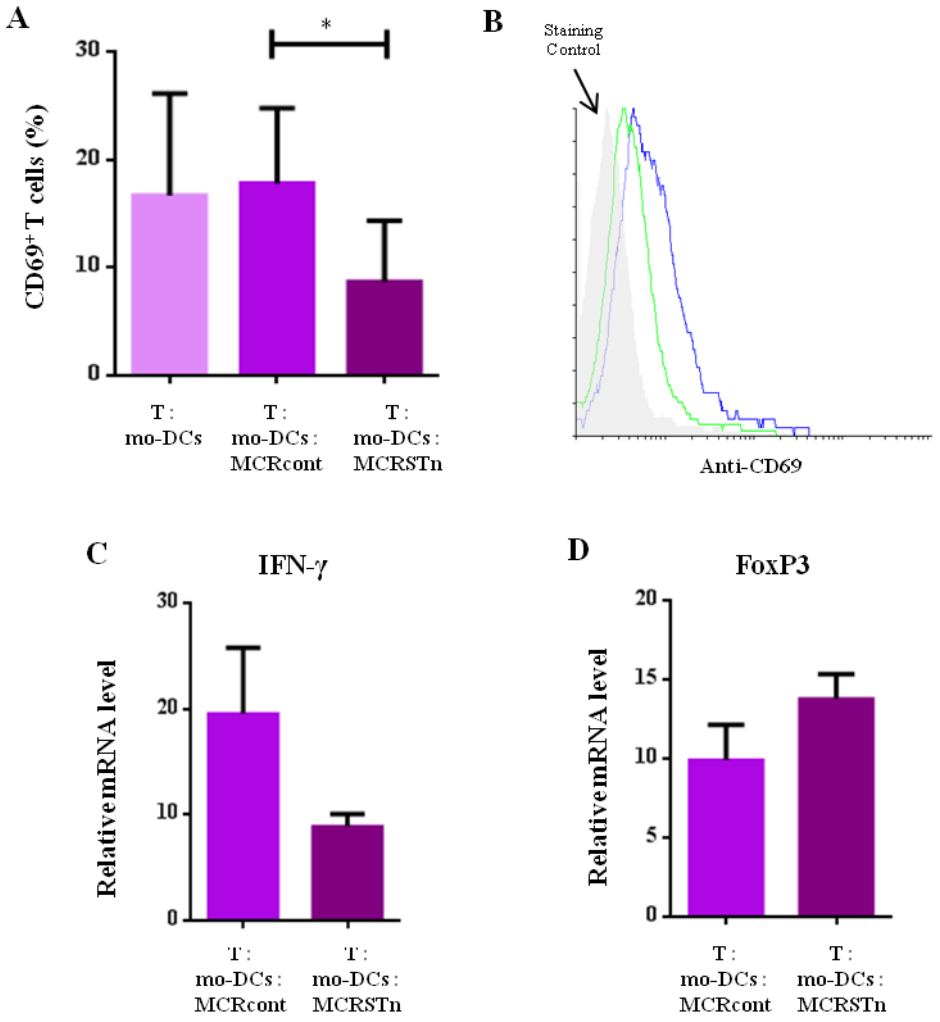

Figure 5. T cell activation is reduced when primed with mo-DCs that phagocytosed STn^+ cancer cells. Mo-DCs were allowed to phagocytose MCR cells and then incubated with autologous T cells (1:8 proportion). **A-B:** The coculture was analysed by flow cytometry for the expression of the T cell early activation marker CD69. **A:** Graphical representation of the percentage of $CD69^+$ T cells. Data was determined by the percentage of $CD69^+$, within the CD3 population (n=3). Mo-DCs that phagocytosed MCRSTn cells induce significantly less activation in T cells than mo-DCs that phagocytosed MCRcont cells (p=0.026 (*)). **B:** A representative CD69 histogram for T cells following priming with mo-DCs that phagocytosed MCRcont (solid line) or MCRSTn (dashed line) cell line. Expression of CD69 by resting T cells is shown as staining control (grey filled peak). **C-D:** The coculture was analysed by quantitative real-time PCR regarding the expression of *IFN-γ* and *FoxP3* genes. The relative mRNA levels for each gene are expressed as the permillage (‰) of the expression of the endogenous positive controls. **C:** The expression of IFN-γ was reduced by 54% (p= 0.074) in T cells co-incubated with mo-DCs that phagocytosed MCRSTn as compared to controls (n=4). **D:** T cells co-incubated with mo-DCs that phagocytosed MCRSTn expressed 39% (p= 0.081) more FoxP3 than control mo-DCs (n=4).

3.5. STn^+ glycoproteins blockade restores DC maturation

MUC1 and CD44 are glycoproteins equally expressed by both MCR cells (Figure 6A and 6B) and described as the most likely candidates for being modified by STn by human cancer cells (Julien et al., 2006). The Western blot analysis of MCRSTn cell lysates, using anti-STn mAb, identified one prominent band of approximately 75KDa, and two weak bands of 150KDa and 260KDa each. In all cases, the blot staining was completely abolished after sialidase treatment, proving the STn staining specificity. Immunoprecipitation assays led us to identify the most prominent band (75KDa) as CD44. Bladder tumours also showed STn in CD44 proteins, as shown by the detection of a STn^+ ~75KDa band in the Western blot analysis (Figure 6C). In order to confirm the role of the STn^+ protein scaffold, we blocked STn^+ glycoproteins in MCRSTn cells and conducted coculture assays with mo-DCs similar to those described above. In the cocultures, whenever the CD44 was blocked in the MCRSTn cells, they gained the capacity to significantly increase, in mo-DCs, the expression of MHC-II and cytokines, in particular of IL-12 and TNF-α (Figure 6D). Blocking of MUC1, as well as STn antigen in MCRSTn cells also upregulated MHC-II and cytokine expression, indicating a tendency, although not statistically significant (Figure 6D).

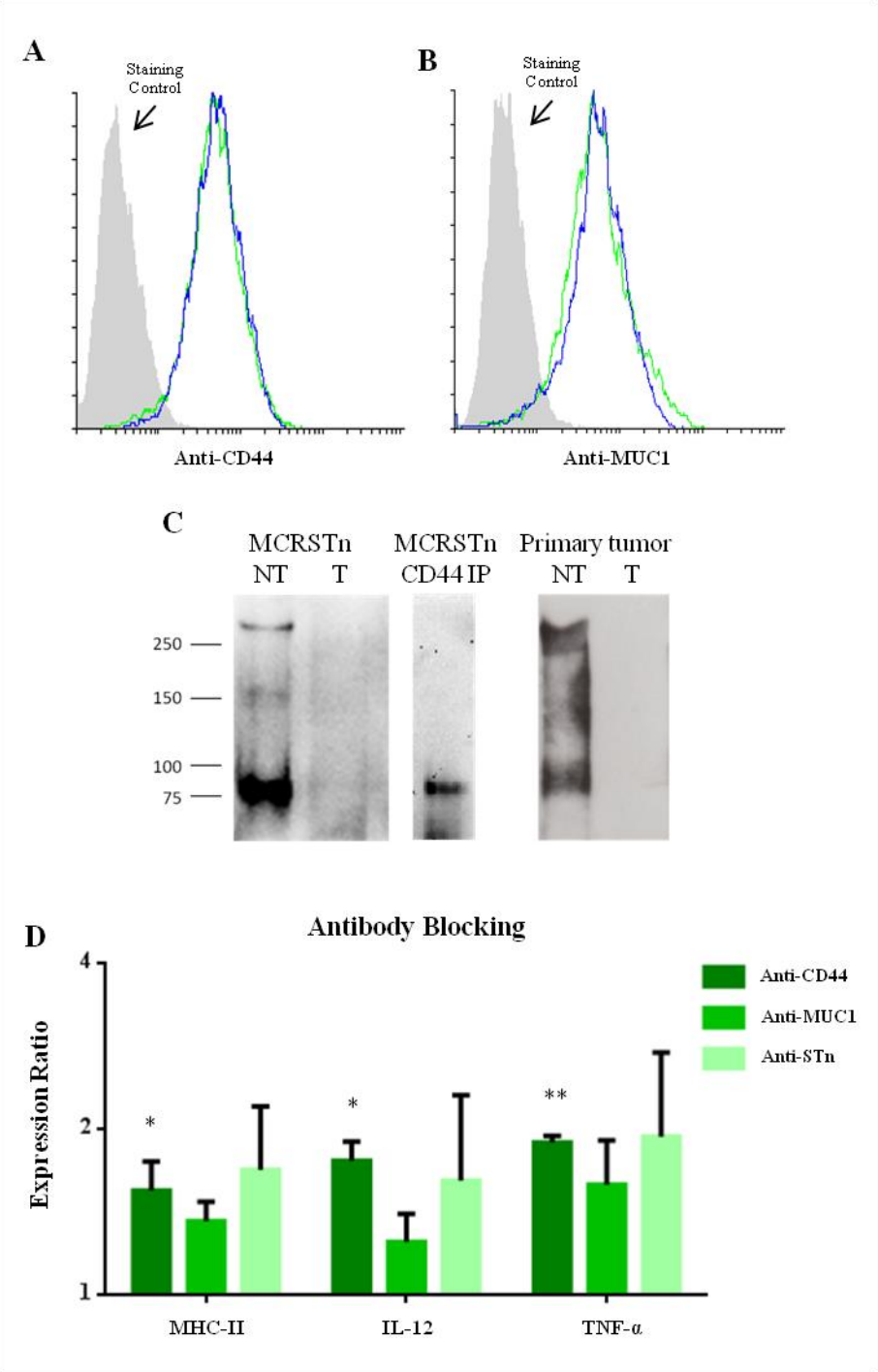

Figure 6. STn$^+$ glycoproteins blockade restores mo-DCs maturation. A-B: MCR cell lines were analysed by flow cytometry regarding the expression of possible scaffolds of STn, CD44 and MUC1 glycoproteins. MCRcont (dashed line) and MCRSTn (solid line) cell lines were stained with anti-CD44 **(A)** or anti-MUC1 **(B)** mAb (not filled peaks) or only with secondary mAb (grey filled peak) as staining control. Both MCR cell lines express similarly CD44 and MUC1 glycoproteins. **C:** Analysis of STn$^+$ proteins in cancer cells. Total protein lysates from MCRSTn cell line (left image) and primary bladder tumour samples (right image) were treated (T) or not (NT) with sialidase. CD44 immunoprecipitation (IP) from MCRSTn total proteins was performed using Pierce Direct IP Kit (middle image). Cell lysates and CD44 IP were separated and the proteins were transferred to nitrocellulose membrane and stained with anti-STn mAb (clone TKH2). MCRSTn cells showed three proteins (≈ 75KDa, 150KDa and 260KDa) decorated with STn and the most prominent STn+ protein showed a molecular weight of ≈ 75KDa. As

control, when the lysate was treated with sialidase, staining with anti-STn was completely abrogated. The CD44 IP analysis showed that CD44 protein is decorated with STn in MCRSTn cells. **D:** The expression of *MHC-II*, *IL-12* and *TNF-α* is restored when blocking STn and STn^+ glycoproteins. Gene expression was evaluated by quantitative real-time PCR in mo-DCs incubated with MCRSTn cell line in presence of anti-CD44, -MUC1 or -STn blocking mAbs. Mo-DC Fc receptors were previously blocked to avoid non-specific Fc receptor-mediated antibody binding. The expression values were calculated as described in the Material and Method section and correspond to the ratio between the expression of mo-DCs incubated with MCRSTn cell line in presence of blocking mAbs and the expression of mo-DCs incubated with MCRSTn in presence of isotypic control. CD44 blockade was able to increase the expression of MHC-II, IL-12 and TNF-α (p= 0.0294 (*), p= 0.0357 (*), p= 0.0035 (**), n=3). Similarly, the expression of MHC-II, IL-12 and TNF-α was also increased by MUC1 and STn blockage.

4. Discussion

We have recently reported that the majority of high-grade bladder tumours, presenting elevated proliferation indexes and high risk of recurrence/progression and invasion, expressed STn (Ferreira et al., 2013). This glycan is not expressed by normal epithelium and it has been associated with a poor prognosis, and the invasive capacity of the tumours (Ferreira et al., 2013). Similar observations were made for breast and gastric cancers and other solid tumours, supporting the ubiquitous association of STn with malignancy.

The expression of the STn antigen is also accepted to be implicated in the immunogenicity of cancer cells and this has been explored in STn-based vaccines to induce protective immune responses against STn^+ cancers. Humoral immune responses against STn have been reported in vaccinated patients and mouse models (Holmberg and Sandmaier, 2004; Julien et al., 2009), demonstrating that the immune system reacts against STn antigens. However, the potency of the immune response is not clinical relevant to provide patients with robust anti-tumour protection.

While, glycans are known to be implicated in several immune responses, the involved biological processes are diverse and complex and thus still poorly understood. Lectin-glycan ligand interactions are implicated not only in mechanisms that establish immune protection, but also in those that establish immunological tolerance. Given the clinical relevance of STn antigens, a deeper investigation on the interaction of STn expressing cancer cells with the immune system is warranted to improve and develop novel STn-based therapeutics.

Here we have studied the influence of STn antigen in the modulation of DCs, which are recognized by their pivotal role in the definition of immune responses. In the tumour tissue, we observed a significant correlation between STn expression and an immature

profile of DCs. Indeed, the expression of ST6GALNAC1, which is correlated with the expression of the STn epitopes, in bladder cancer, is correlated with CD1a, a marker of immature DCs. Interestingly, high grade bladder tumours presented not only higher levels of ST6GALNAC1 and CD1a, but also lower levels of the pro-inflammatory, Th1-inducing cytokines, IL-12 and TNF-α, thus supporting the association between STn and DC immature profile.

Maturation is a crucial process that enables DCs to effectively prime T cells to mount responses against malignant cells (Steinman et al., 2003). To confirm a possible inverse association between STn^+ cancer cells and DC maturation and to investigate if STn antigen played a role in DC maturation, we used *in vitro* models of STn^+ cancer cells cocultured with human DCs. We have found that DCs tend to adhere more to STn^+ cancer cells and that this contact inhibits DC maturation and co-stimulation, when compared with DCs cocultured with STn^- control cells. The higher cell adhesion to STn^+ cancer cells, which could be due to either stronger or longer cell contacts, was responsible for the defective maturation of mo-DCs. After coculture with STn^+ cancer cells, the lower expression of MHC-II and of co-stimulatory molecules could not be rescued by LPS stimulation, suggesting that mo-DC become resistant to further maturation stimuli.

A hallmark of immature DCs is a high phagocytic capacity, which is lowered upon maturation. In agreement with the fact that STn-expressing cancer cells prevents the maturation of mo-DCs, mo-DCs showed better phagocytic capacity for STn^+ cancer cells than for control cells. Nevertheless, the effects seen might not only be caused by STn but also some general feature of the MCR cells, since MCRcont cells also induced small maturation arrest.

Mo-DCs incubated with STn^+ cancer cells showed defective expression of TNF-α and IL-12, which is consistent not only with DC immature phenotype but also with data observed in bladder tumour tissues. These observations were also in agreement with other reports showing that tumour environment lowers the expression of inflammatory cytokines by DCs (Ishida et al., 2008). We have not detected significantly altered expression of anti-inflammatory cytokines, with the exception of IL-10. IL-10 downregulation may be a balanced consequence of the downregulation of pro-inflammatory cytokines, playing a dual proliferative and inhibitory effect (Ogden et al., 2005).

The negative effect of STn^+ cancer cells on the maturation and Th1-inducing profile of mo-DCs were observable in less than 2 hours of coculture. On the other hand, STn^+ cancer cells could themselves be influenced by mo-DCs. This was evidenced by the significant

increase proliferation of MCRSTn cells in the presence of mo-DCs that was not observable in MCRcont cells. Yet these differences were only observable after 24 hours coculture, and the mechanism behind the altered proliferation may be dissociated from the ones implied on the altered mo-DC phenotype. However, in the future, the effect of mo-DCs in tumour cell proliferation should be further clarified and it might be associated with the fact that STn^+ bladder tumours present elevated proliferation indexes *in situ*.

The fact that using other STn^+ cell line, i.e., the MDA-MB-231STn breast cancer cell line, also rendered mo-DCs with an immature phenotype, with an extent similarly to the phenotype induced by MCRSTn bladder cancer line, strongly suggested that the specific STn overexpression in cancer cell was responsible for the induction of tolerogenic DCs reported here. Nevertheless, we cannot disregard the fact that ST6GALNAC1 overexpression in cell lines also alters other glycan structures beyond the STn (Table IS).

To further confirm the role of STn expression by cancer cells in DC maturation, we used different strategies to abrogate the MCRSTn cancer cell interaction with DCs. Blocking of STn antigen in MCR cells, by means of the HB-STn antibody, described to block STn interactions (Pinho et al., 2007), resulted in tendency of MCRSTn cells to induce DC maturation. This suggested that blocking STn antigen may reverse the propensity of STn^+ cancer cells to induce tolerogenic DCs. The fact that we were not able to obtain statistical significant differences may have to do with the role of the protein scaffolds that also participate in the recognition or interaction of glycans (Padler-Karavani et al., 2008). Therefore, anti-glycan mAbs may not have efficient function-blocking properties, as anti-scaffolds have.

We have observed that the major protein scaffold in the MCRSTn cells and bladder tumours is CD44. Moreover CD44 is also decorated with STn in MDA-MB-231STn breast cancer cells (Julien et al., 2006), which in this work induced the same DC immunomodulation as MCRSTn cells. These findings suggested us a possible involvement of STn^+ CD44 in the acquisition of the immature profile by DCs. Concordantly, blockade of MCR CD44 by means of functional blocking antibodies was able to restore the capacity of the mo-DCs to become matured.

CD44 is a rather ubiquitously expressed adhesion molecule, involved in several processes including migration and cell signalling and recognition. The role of CD44 in governing the progression of tumours, including bladder carcinoma (Golshani et al., 2008), is well documented and its expression by leukocytes also plays a role in modulating immune responses (Hegde et al., 2008; Jacobs and Sackstein, 2011; Weiss et al., 1997). However

the role of its recognition by immune cells, such as DCs, is unclear. In our model, it is possible that DC receptor for CD44 may recognize differently the protein due to substitution of normal glycosylation by STn in cancer cells. CD44 is a receptor for hyaluronic acid and other extracellular proteins, such as osteopontin and collagens. Very recently it has been reported that the mannose receptor interacts with CD44 (Salmi et al., 2013). Although, mannose receptor primarily binds mannose and fucose, it is possible that in our model, the STn expression affected the CD44 recognition by mannose receptor, leading to the described immunological implications. Nevertheless, other receptors expressed by DCs may also be involved, such as the Sialic acid-binding immunoglobulin-type lectins (Siglec)-9 that have also been reported to recognize STn antigen (Ohta et al., 2010). In addition, the participation of other STn scaffolds such as MUC1 (Monti et al., 2004) should not be excluded and as matter of fact, we also observed that blockade of MUC1 results in a slight induction of DC maturation. Further studies are therefore necessary to better characterize the mechanisms of STn antigen recognition by DCs.

As mentioned above, previous studies were controversial in demonstrating that STn mucins were implicated in inducing the immature DC phenotype (Carlos et al., 2005; Monti et al., 2004). While this controversy may be due to the complexity of the involved mechanisms, these studies were in agreement, when showing that STn expression has a negative influence on the capacity of DCs to activate T cells, resulting in DCs that do not support T cell commitment to Th1 phenotype, which is important for tumour rejection (Carlos et al., 2005; Monti et al., 2004).

DCs are currently used as anti-tumour cell-based vaccines, where one of the most common strategies is the upload of patient's DCs *ex vivo* with the tumour cell antigens (lysates, cells fusion or apoptotic bodies). Tumour antigens are phagocytosed by DCs and these cells are then used to induce anti-tumour T cells responses and further tumour rejection (Palucka and Banchereau, 2013). It has been reported that DCs pulsed with apoptotic cancer cells are able to activate cytotoxic T cells against poorly immunogenic cancer cells (Goldszmid et al., 2003) and in bladder cancer patients increases survival and cure rate (Nair et al., 1997). In the present study, DCs pulsed with immunosuppressive STn^{+} cancer cells showed a tolerogenic/regulatory profile, resistant to further maturation stimuli and limited capacity to activate T cells. In these conditions, mo-DCs express lower levels of Th1-inducing cytokines and preferentially polarize T cells towards a $FOXP3^{high}IFN\text{-}\gamma^{low}$ phenotype, typical of regulatory T cells. On the other hand, it has been recently reported by us that STn expression in bladder cancer is associated with better responses to complementary

immunotherapy with *Bacillus Calmette-Guérin*, a potent inducer of Th1 responses (Lima et al., 2013). Therefore, the combination of Th1-inducing therapies with DC-based therapy against STn-bearing cancer cells could be a promising strategy to induce anti-tumour protective responses. Nevertheless, a better understanding of the mechanisms behind the tolerogenic/regulatory profile of DCs induced by STn^+ cancer cells is still necessary.

Conclusions

This study shows that STn, a cancer-associated carbohydrate antigen expressed in high-grade bladder cancers, has the ability to down-regulate the anti-cancer immune-response through different mechanisms. First, it hinders the expression of MHC-II and co-stimulatory molecules by DCs, resulting in impaired ability to present cancer-associated antigens to T cells and making DCs unresponsive to successive activation stimuli. Second, it hinders the expression of inflammatory, Th1-inducing cytokines in DCs, which may result in an attenuation of the Th1 microenvironment. Third, DCs pulsed with STn-expressing cancer cells show a reduced ability to activate and polarize T cells towards the Th1 phenotype, resulting in impaired ability to mount an effective anti-tumour response. Finally, blockade of STn antigens and STn protein may reverse tolerance induced by cancer cells. Altogether, these results highlight the expression of STn by cancer cells as a crucial event in the establishment of the tolerogenic microenvironment which allows cancers to escape from the attack of innate and adaptive immunity.

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Supplementary data

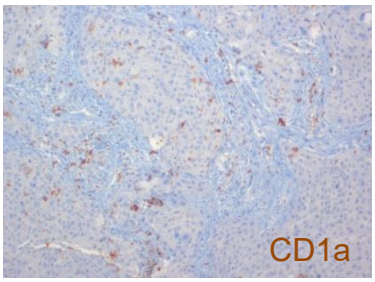

Figure 1S. Immature DCs present in bladder cancer tissue. CD1a staining in tumour tissue was used to identify DCs. Representative image of serial paraffin embedded sections that were processed for CD1a immunohistochemical staining. Tumour tissue showed moderate presence of $CD1a^{+}$ DCs.

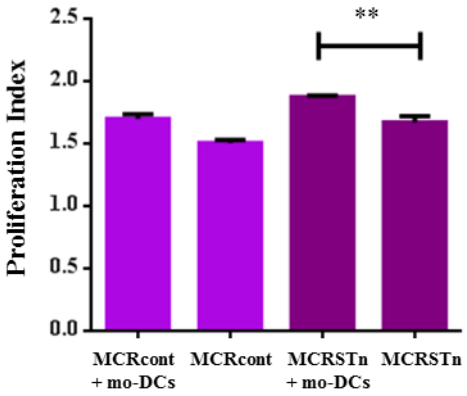

Figure 2S. Incubation with mo-DCs increase the proliferation capacity of bladder cancer cell lines. The MCRcont and MCRSTn cells were labelled with CFSE and cultured 24 hours in the presence or absence of mo-DCs. The cells were analysed by flow cytometry with Modfit software, allowing the calculation of the proliferation index, which represents the average number of cells that was originated by a single cell of the parent generation. Mo-DC co-incubation significantly increases the proliferation index of MCRSTn cells (p=0.007 (**)). The data are shown as a mean of 3 independent studies.

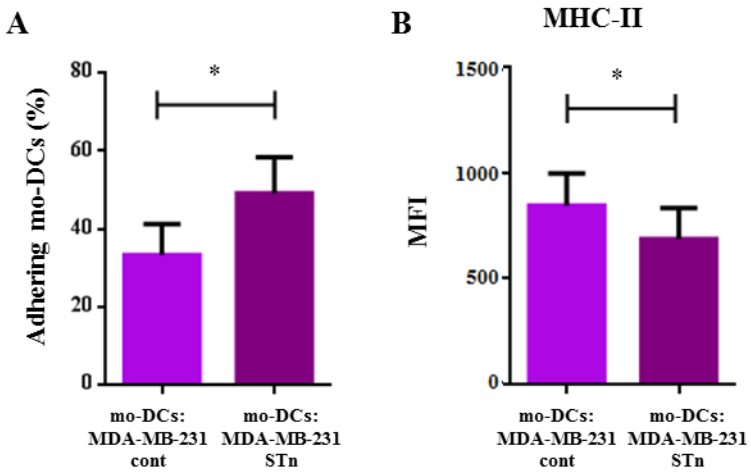

Figure 3S. Mo-DCs adhere preferentially to STn$^+$ MDA-MB-231 breast cancer cell line and show a less mature phenotype. A: Adhesion of mo-DCs to MDA-MB-231 cell lines. Mo-DCs were co-cultured with MDA-MB-231 cell line variants for 2 hours. Non-adherent mo-DCs were washed and the percentage of mo-DCs adherent to MDA-MB-231 cell lines was estimated by flow cytometry, as the total of MHC-II$^+$ cells in the co-culture, following staining with anti-MHC-II. The mean number of adhering mo-DCs was significantly higher in the co-incubation with MDA-MB-231STn than with MDA-MB-231Cont (p= 0.026 (*), n= 5). **B:** Analysis of MHC-II expression in mo-DC. Adherent mo-DCs were stained with anti-MHC-II mAb and then analysed by flow cytometry. The expression level was inferred from the mean fluorescence intensity (MFI) of the cells, which was significantly lower in mo-DCs co-incubated with MDA-MB-231 STn than those with MDA-MB-231cont (p= 0.039 (*), n= 5).

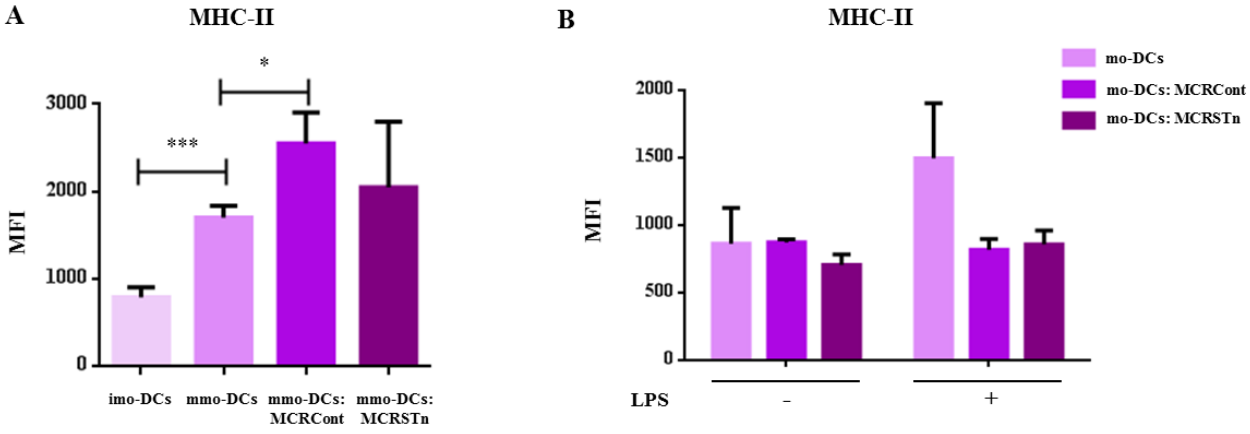

Figure 4S. Maturation phenotype of mo-DCs remains unchanged in the presence of STn$^+$ cancer cells. A: Immature mo-DCs (imo-DCs) and semi-mature mo-DCs (mmo-DCs, obtained after incubation with LPS) were co-cultured or not with MCR bladder cancer cell lines, stained with anti-MHC-II mAb and then analysed by flow cytometry. The expression level of MHC-II was inferred from the mean fluorescence intensity (MFI) of the cells. As expected, MFI values were significantly lower in imo-DCs than mmo-DCs (p= 0.0007 (***)). Compared with mmo-DCs alone, the expression of MHC-II was increased in mmo-DCs co-incubated with MCRcont (p= 0.0341 (*)), while it was unchanged after co-incubation with MCRSTn (n=6). **B:** Immature mo-DCs were co-cultured or not for 2 hours with MCR bladder cancer cell lines and then incubated with LPS for an additional 24 hours. The co-culture was stained with anti-MHC-II mAb and analysed by flow cytometry. MHC-II expression in the adherent mo-DCs incubated with

MCR cells was not significantly changed by LPS stimulation, and was comparable to the expression in the absence of LPS.

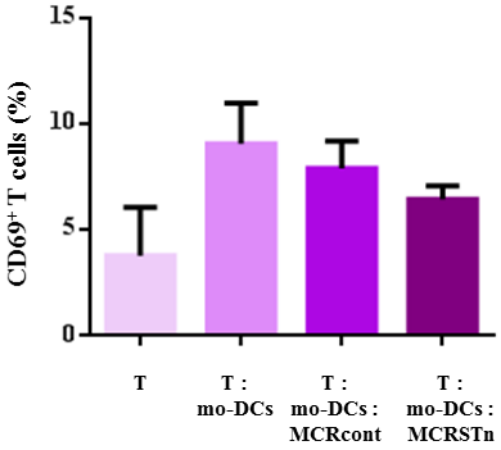

Figure 5S. The activation of T cells is also compromised when primed with mo-DCs that adhered to STn+ cancer cells. After adhesion assay, mo-DCs were incubated with a culture enriched in autologous T cells, in the proportion of 1:8. The co-culture was analysed by flow cytometry for the expression of the T cells early activation marker CD69. T cell activation was determined by the percentage of $CD69^{+}$, within the $CD3^{+}$ cell population. Mo-DCs co-incubated with MCRSTn cells tended to induce less T cell activation than those with co-incubated with MCRcont cells (n=3).

Table IS: Glycan phenotype of MCR cell lines.

	MCRcont		MCRSTn	
	Intensity	% positive cells	Intensity	% positive cells
Sialyl-Tn antigen	-	-	+++	>95
T antigen	++	>95	+	>85
Neu α2-6Gal	+	50-75	++	>95
Neu α2-3Gal	++	>95	++	>95
Sialyl-Lewis^{x}	+	10-20	+	<10
Sialyl-Lewis^{a}	-	-	+	<10
Lewis^{a}	-	-	-	-

The expression of short *O*-glycans (sialyl-Tn, T, sialyl-T antigens) and Lewis antigens, as representative of elongated glycan structures were analysed at the cell surface of MCRcont and MCRSTn cell lines, by flow cytometry. MCRSTn expressed significant higher levels of sialyl-Tn. Moderate lower levels of T, sialyl-T and sialyl-Lewis^{x} antigens were observed when

compared with MCRcont. MCR cells were stained with antibodies against sialyl-Tn (TKH2), sialyl-Lewis^{x} (SH1), sialyl-Lewis^{a} (HB80) and Lewis^{a} (CA3F4) followed by staining with FITC-labeled anti-mouse Ig. T antigen, Neu α2-6Gal and Neu α2-3Gal expression was obtained by FITC-labeled lectins staining, specifically *Peanut agglutinin lectin*, *Sambucus nigra agglutinin* and *Maackia amurensis agglutinin*, respectively. The mean fluorescence intensity was semi-quantified as negative (-), weakly positive (+), positive (++) and strongly positive (+++).

Chapter 4

Inhibition of fucosylation abrogates E-selectin ligands expression on breast cancer primary cells and reduces ERK1/2 and p38 MAPK activation

(Paper II)

Inhibition of fucosylation abrogates E-selectin ligands expression on breast cancer primary cells and reduces ERK1/2 and p38 MAPK activation

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Abstract:

Sialofucosylated structures, such as sialyl-Lewis X (sLe^X), are the main glycans responsible for binding E-selectin during extravasation. E-selectin ligands expressed by cancer cells are crucial for the first step of transendothelial migration. In breast cancer, sLe^X expression is highly associated with metastasis formation and invasion. Here, we aimed to clarify the role of fucosylated glycans, such as sLe^X, in malignant features, such as cell proliferation, migration and adhesion. Preliminary results have shown that poorly-differentiated invasive ductal carcinomas tend to express more E-selectin ligands. Then, breast cancer cell cultures were established from invasive ductal carcinoma tissues and we verified that all cultures expressed E-selectin ligands, such as sLe^X, and FUT4, FUT5, FUT10 and FUT11 α-1,3/4 fucosyltransferases. Focusing in one primary cell culture, which were immortalized by overexpression of *hTERT* gene, creating CF1_T cell line, we tested the effect of 2-fluorofucose (2-FF) compound on this new cell line. Since 2-FF is a fucosylation inhibitor, we assessed the relevance of 2-FF treatment of CF1_T cell line in its adhesion to E-selectin, verifying that this treatment completely impaired the expression of functional E-selectin ligands, such as sLe^X. In addition, 2-FF treated CF1_T cells have also shown a reduced migratory ability, as well as a decreased cell proliferation

rate. The latter was probably a result of lower growth factors expression by CF1_T cells treated with 2-FF, which negatively affected the activation of ERK1/2 and p38 MAPK pathways. Our data shows that fucosylated structures, such as functional E-selectin ligands decorated with sLe^X glycans, have a major impact on malignant features, such as cell proliferation, adhesion and migration, contributing to tumor progression.

1. Introduction

Worldwide, breast cancer is the main cancer in women, accounting for 29% of all new cases of diagnosed cancer in women. Specifically, invasive ductal carcinoma is the most common type of breast cancer, making up nearly 70-80% of all breast cancer diagnoses. The five-year survival rate for breast cancer patients is almost 99% if the disease is detected in early stages. However, if the tumor has metastasized, the survival rate drastically decreases to 25% [1]. These statistics show the need for new therapies that can prevent metastization and the importance of better understanding its underlying mechanisms. Metastatic invasion to distant organs is a multistep process requiring detachment of malignant cells from the primary tumor, their circulation in the blood vessels and emigration from the vasculature to colonize distant organs [2]. Each step in this process requires different types of cell-cell interactions between cancer cells and the host microenvironment. Of particular interest is the elucidation of the molecular mechanisms by which metastatic cells adhere to the vascular endothelium while resisting the disruptive shear exerted by blood flow.

Such adhesive interactions are initiated by the vascular selectins, of which E-selectin is especially important due to its induced expression inflamed endothelium and constitutively expression on bone marrow microvasculature, that represents a relevant site of breast cancer metastasis [3]. E-selectin engages its ligands expressed on circulating cells, which not only captures and slows down these cells in the bloodstream but may also activate other mechanisms that promote tissue homing [4-6]. The implication of E-selectin-mediated interactions in cancer metastasis is clear in several *in vivo* studies, wherein metastasis in mice was reduced when E-selectin and/or E-selectin ligand activity were blocked, compared to control conditions [7, 8]. Therefore, blocking or removal of E-selectin ligands expressed on cancer cells may be a potent therapeutic strategy for breast cancer patients against cancer metastasis. The major ligands of E-selectin on the tumor cell surface have been identified as the sialofucosylated glycan determinants, sLe^X

and sialyl-Lewis A (sLe^A). A positive correlation between expression of sLe^X and sLe^A in breast cancer cells and tumor cell metastasis or invasion has been reported [9-11]. However, the mechanistic insights are still unclear.

Biosynthesis of sLe^X and sLe^A requires the addition of L-fucose α-1,3 or α-1,4-linked respectively, to N-acetylglucosamine by enzymatic catalysis [12]. Fucosyltransferases (FUTs) are the biosynthetic enzymes responsible for catalyzing the L-fucose transfer from the donor substrate guanosine diphosphate β-Lfucose (GDP-Fuc) to various sugar acceptor substrates [13]. α-1,3/4 fucosyltransferases, such as FUT3, FUT4, FUT6 and FUT7 have been suggested as responsible for sLe^X and sLe^A synthesis in breast cancer [14-16]. FUT7 was associated to lymph node metastasis and poor disease-free survival in breast cancer. Recently, FUT4 was suggested as a novel biomarker for breast cancer, correlated with the conventional cancer biomarkers cancer antigen CA15.3 [17]. Furthermore, FUT4 was also identified as a novel regulator of epithelial-mesenchymal transition, facilitating the acquisition of a mesenchymal phenotype [18].

Recently, a fluorinated fucose, named 2-FF, was shown to be able to inhibit the fucosyltransferase activity, thus reducing cell surface fucose content, as well as the expression of Lewis antigens in colon carcinoma cells. This compound also diminished the adhesion of colon carcinoma cells to E-selectin. In the same study, *in vivo* experiments showed that oral treatment of tumor-bearing mice with 2-FF resulted in inhibition of tumor outgrowth, as well as inhibition of xenograft tumor cell surface fucosylation [19].

Here, using the same 2-FF compound to inhibit fucosylation, we verified that it similarly inhibited the adhesion of a human primary breast cancer cell line, CF1_T, to E-selectin. The treatment of CF1_T cells with 2-FF also affected negatively their migration and proliferation, dramatically reducing their expression of growth factors and cytokines and diminishing the activation of ERK1/2 and p38 mitogen-activated protein kinase (MAPK) cell signaling pathways.

2. Material and methods

2.1. Reagents

Recombinant mouse E-Selectin/CD62E Fc chimera (E-Ig) was purchased from R&D Systems (Minneapolis, MN). Anti-CLA monoclonal antibodies (mAb), clone HECA-452, was from Biolegend (San Diego, CA). Anti-human CD29 mAb was from Immunotools (Germany). Rat anti-mouse CD62E, anti-mouse Ig conjugated with horse

radish peroxidase (HRP) and anti-rat Ig conjugated with allophycocyanin (APC) mAbs were from BD Biosciences (San Jose, CA). Anti-rat IgG – HRP, anti-rat IgM – HRP and anti-β-tubulin mAbs were from Santa Cruz Biotechnology (Dallas, Texas). Anti-human Ig – fluorescein (FITC) mAb was from Sigma-Aldrich (St. Louis, MO). CellTrace™ CFSE Cell Proliferation Kit was purchased from Molecular Probes, Thermo Fisher Scientific (Waltham, MA). Anti-human cytokeratin, clone AE1/AE3, was from Dako. p44/42 MAPK (Erk1/2), phospho-Erk1/2, p38 MAPK and phospho-p38 MAPK mAbs were from Cell Signaling Technology (Danvers, MA).

2.2. Patients

This study involved patients from Hospital São José in Lisbon and from Hospital de Cascais in Cascais, who underwent a mastectomy with gross resection of the primary tumor. This study was approved by the institutional research ethical committees.

2.3. Histological analysis

The immunohistochemical analysis was performed using reagents from Ventana, USA, unless otherwise stated. Briefly, the slides were heated at 72 °C, deparaffinized and antigenic recovery at 98 °C in citrate buffer pH8. Endogenous peroxidase was blocked with 3% of hydrogen peroxide and the slides were incubated with E-Ig (5ug/mL), followed by incubation with anti-mouse CD62E mAb (1:50) and anti-rat IgG – HRP mAb (1:100). The revelation was performed using UltraView Universal DAB Chromogen and DAB H202 and the intensification was achieved with UltraView Universal DAB Copper. The nuclear contrast was performed with hematoxylin and bluing. After the immunohistochemical technique, the slides were washed, dehydrated, treated with increasing concentrations of alcohol (75%, 90% and 99%), cleared in xylene and mounted with synthetic mounting medium (Quick-D-M-Klinipath). The E-Ig staining was assessed by an experienced pathologist.

2.4. Primary cell culture

A portion of the primary tumor tissue collected from patients was placed in growth medium (Dulbecco's modified Eagle medium (DMEM) with 20% fetal bovine serum, glutamine and antibiotics) immediately after surgical removal. Tumor tissue was minced and incubated in collagenase overnight to release tumor cells. Then, cells were cultured in T-25 flasks at 37°C with 5% CO_2. Medium was changed weekly, and cultures were

observed for cell growth. Cultures were trypsinized and passaged when sufficient colonies of epithelial cell growth were noted. When cultures had been established (5 to 10 passages), the serum concentration was reduced to 10%, and the cells were then fed 2 to 3 times/week by replacement of the medium.

2.5. Immortalization of CF1 breast cancer primary cells

Cell immortalization was performed by viral transduction of the *hTERT* gene in the CF1 primary cells. Human Telomerase Reverse Transcriptase (hTERT) is a catalytic subunit of the enzyme telomerase, which, together with the telomerase RNA component, comprises the most important unit of the telomerase complex. With hTERT overexpression, active telomerase can be formed in primary cells to allow continuous cell proliferation in culture. Briefly, lentivirus production was achieved through the integration of three different plasmids into HEK293 cell line: two of them coding for the viral capsid and the packaging proteins, integrase and reverse transcriptase, and the other plasmid having an inserted cloned *hTERT* gene being expressed through a CMV promotor. This last plasmid would also contain a gene responsible for resistance to blasticidin S, an antibiotic that inhibits protein synthesis. As a result, *hTERT* gene is packed inside a lentiviral particle that exit the cell line by exocytosis, into the cell culture medium. CF1 cells were cultured at 30% confluency and lentiviral medium was added with the final concentration of approximately 10^6 viral particles/mL as well as 6 mg/mL of polybrene (Sigma-Aldrich). After 72h of incubation in a 37ºC 5% CO_2 atmosphere, blasticidin, was added to kill cells that were not transduced. In the end, we obtained a CF1_T cell line that express hTERT.

2.6. Flow cytometry

The membrane expression of $sLe^{X/A}$ glycans (HECA-452 mAb) on breast cancer cells were analyzed by flow cytometry. Intracellularly, the expression of cytokeratins as well as Erk1/2, phospho-Erk1/2, p38 and phospho-p38 was also analyzed using Fixation/Permeabilization Solution Kit (BD Bioscience), previous to antibody staining.
Cell suspensions were incubated with E-Ig chimera plus anti-human Ig FITC in PBS-Ca^{2+} (Sigma-Aldrich) or, as negative control, in PBS with 2mM EDTA (Merck) for 1 h, at 4°C to detect E-selectin ligands. Antibodies staining were performed for 30 min, at 4°C followed by incubation with fluorescent-labeled secondary antibodies for 30 min, at 4°C.

Background levels were determined by incubating cell suspensions with only fluorescent-labeled secondary antibodies.

2.7. Confocal laser scanning microscopy

Cells were cultured in cover glasses overnight and then fixed with 3.7% paraformaldehyde. After blocking with 1% bovine serum albumin (BSA), cells were stained using E-Ig (1 µg) plus anti-human Ig FITC (1,5 µL) in PBS-Ca^{2+}. Then, cells were permeabilized with 0.1% TritonX-100 and F-actin was stained with Alexa Fluor 568 phalloidin (Molecular Probes, Leiden, Netherlands). Images were acquired with a Leica TCS SP2 AOBS confocal microscope. Representative cross-section confocal images were selected after Z-stacking.

2.8. 2-FF treatment

2-FF was kindly given by Professor Peter Senter from Seattle Genetics (Bothell, WA). CF1_T cell line was treated or not with 1mM of 2-FF inhibitor in DMEM medium with 10% fetal bovine serum, glutamine and antibiotics for 5, 10 or 14 days.

2.9. SDS-PAGE and Western blot

The cells were lysed in lysis buffer consisting of 150 mM NaCl, 2 mM $CaCl_2$, 50 mM Tris (pH 7.4), 1 mM phenylmethylsulfonyl fluoride, 2% NP-40 and 1 EDTA-free protease inhibitor cocktail tablet (Roche, Indianapolis, IN). Lysates were incubated overnight in agitation at 4°C and centrifuged for 10 min at 10,000 g. Supernatants were collected and stored at -80°C until use. Protein concentrations were determined using Pierce BCA protein assay kit (Thermo scientific). Protein samples were diluted with SDS-PAGE sample loading buffer (NZYTech, Portugal) and electrophoresed through 8% polyacrylamide SDS-PAGE gels. Prestained molecular mass standard (NZYTech, Portugal) were included in adjacent lanes in all experiments. SDS-PAGE separated proteins were transferred to nitrocellulose membranes (Bio-Rad) and blocked with TBS/0.1% Tween 20/10% dry milk (anti-$sLe^{X/A}$ staining) or TBS/0.1% Tween 20/4% BSA (ERK and p38 staining) for 1 hour. Immunoblots were stained with primary mAbs overnight at 4°C and then rinsed with TBS/0.1% Tween 20. Subsequently, blots were incubated with appropriate HRP-conjugated secondary antibodies for 1h at room temperature. After rinsed with TBS/0.1% Tween 20, Lumi-light western blotting substrate (Roche) was used as developing reagent.

2.10. Gene expression analysis by real-time PCR

RNA extraction was performed using the GenElute Mammalian Total RNA Purification kit (Sigma-Aldrich), according to the manufacturer's instructions. After DNAase (NZYTech, Portugal) treatment, 1µg of total RNA was reverse transcribed using the random-primers-based High Capacity cDNA Archive Kit (Applied Biosystems) and the real-time PCR was performed in a 7500 Fast Real-Time PCR System with Master Mix, and TaqMan assays from Applied Biosystems). The assay ID provided by the manufacturer were: Hs00356857_m1 (*FUT3*); Hs01106466_s1 (*FUT4*); Hs00704908_s1 (*FUT5*); Hs00173404_m1 (*FUT6*); Hs00237083_m1 (*FUT7*); Hs00276003_m1 (*FUT9*); Hs00327091_m1 (*FUT10*); Hs00543033_m1 (*FUT11*); Hs00174097_m1 (*IL-1β*); Hs00174131_m1 (*IL-6*); HS00174103_m1 (*IL-8*); Hs00171257_m1 (*TGF-β1*); Hs00170433_m1 (*ErbB2* (*HER2*)); Hs00900055_m1 (*VEGFA*); Hs01052937_m1 (*FLT1 (VEGFR1*)); Hs00911700_m1 (*KDR* (*VEGFR2*)) and Hs00960934_m1 (*FGF2*). ΔCt stands for the difference between the cycle threshold of the target gene and that of the endogenous control genes (*β-actin* and *GAPDH*). The relative mRNA levels were normalized against the arithmetic mean of the β-actin and GAPDH expression and calculated by adapted formula $2^{-\Delta Ct}\times 1000$ which infers the number of mRNA molecules of the gene of interest per 1000 molecules of the endogenous controls [20]. Relative quantification (RQ) measures the relative change in mRNA expression levels from a given sample (2-FF treated cells) relative to another reference sample (untreated cells) ($RQ=2^{-(\Delta Ct_{sample}-\Delta Ct_{control})}$). The efficiency for each primer/probe was above 95% (as determined by the manufacturer).

2.11. Cell proliferation measurement

To study their proliferative capacity, cells were labelled with CellTrace™ CFSE Cell Proliferation Kit (Molecular Probes). The CF1_T cells previously treated or not with 1mM of 2-FF for 5 days, were resuspended into medium at final concentration of $1x10^6$ cells/mL and incubated with 5 µM CFSE, following the manufacturer's instructions. Subsequently, the CFSE-labelled cells were seeded into T-25 flasks, incubated in a 5% CO_2 incubator at 37 °C and harvested after 5 and 9 days (total of 10 and 14 days of treatment, respectively). The cell fluorescence was collected using Attune Flow cytometer and the data were analyzed with ModFit LT 3.2 software (Verity Software House, Topsham, ME), allowing to assess the cell proliferation index (PI). The PI

represents the average number of cells that were originated from a single cell of the parental generation. The parental generation was set based on the analysis of data obtained from the cells corresponding to the 24 h of culture.

2.12. Analysis of cell motility using a wound-healing assay

Cell motility was tested in a wound-healing migration assay. CF1_T cells were seed into 12-well microplates and grown to confluency in the presence or not of 1mM of 2-FF inhibitor (10 days of treatment). A scratch was made in the monolayer with a sterile 200µL pipette tip. After wounding, the suspended cells and debris were washed away and fresh medium was added. At 0 and 16 h after wounding, scratched regions were photographed with an inverted microscope equipped with a digital camera. The wounded area in the photographs was measured using ImageJ software and the percentage of closed area was calculated using the formula: ((wounded area (24h) / wounded area (0h)) *100) – 100)).

2.13. Cell adhesion to E-selectin using alternative Stamper-Woodruff assay

For analysis of CF1_T cells adherence to E-selectin, shear-dependent Stamper-Woodruff assays were performed as previously described [21]. Briefly, recombinant E-Ig was placed on glass slides and let dry. The adhesion area was blocked with 1% BSA and then let dry. CF1_T cells were treated or not with 1mM 2-FF and then resuspended in Hank's Balanced Salt Solution (HBSS) containing 2mM $CaCl_2$ (HBSS-Ca). The cells were overlaid onto E-Ig spot in the glass slides and incubated on an orbital shaker at 80 rpm for 30 minutes at 4°C. The slides were placed in HBSS-Ca to remove the non-adherent cells and adherent cells were fixed with 3% glutaraldehyde. Negative control assays were performed with HBSS containing 5mM EDTA. CF1_T cell adherence to E-selectin was examined under light microscopy at ×100 magnification, and representative photomicrographs were taken for analysis. The number of adherent cells in each photomicrographs was counted using ImageJ software.

2.14. Statistical analysis

Statistical analysis was performed using the Student’s T-test for paired samples. Differences were considered to be statistically significant when $p< 0.05$ (*), $p< 0.01$ (**) and $p< 0.005$ (***).

3. Results

3.1. Poorly differentiated ductal carcinoma expresses more E-selectin ligands

Firstly, we analyze paraffin-embedded breast cancer tissues, specifically 3 poorly differentiated (high grade) ductal carcinomas, 3 well differentiated (low grade) ductal carcinomas and 3 poorly differentiated (high grade) lobular carcinomas, regarding their expression of E-selectin ligands using E-Ig chimera staining by immunohistochemistry. Comparing these 2 most common subtypes of breast cancer, we were able to observe that poorly differentiated invasive ductal carcinomas presented a higher expression of E-selectin ligands on both their cytoplasm and plasma membrane than the well differentiated (low grade) ones or the high grade lobular carcinomas (Fig. 1). These results suggested that E-selectin ligands may play a role in high grade invasive ductal carcinomas.

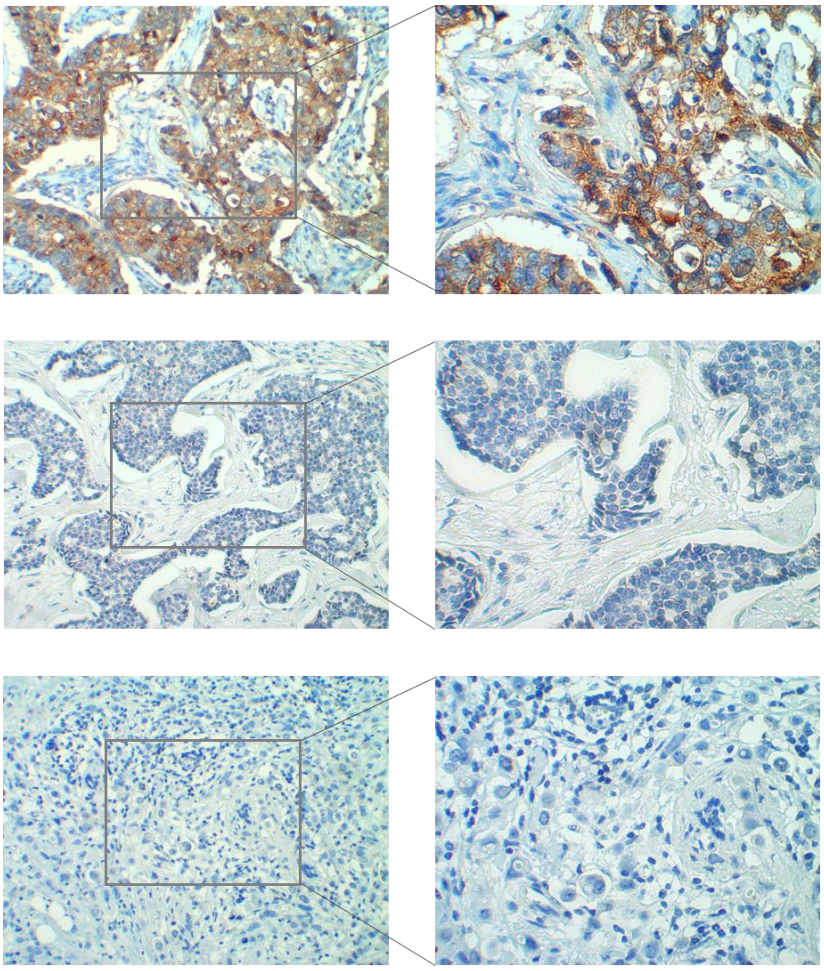

Fig 1: E-selectin ligands are highly expressed on poorly-differentiated ductal carcinoma. E-selectin ligands were stained using E-selectin chimera by immunohistochemistry. Representative images of well-differentiated ductal carcinoma (top), poorly-differentiated ductal carcinoma

(middle) and poorly differentiated lobular carcinoma (bottom) are shown at 200x and 400x magnification (n=3).

3.2. Establishment and characterization of primary cell lines from invasive ductal breast carcinoma patients

To better understand the role of E-selectin ligand expression in invasive ductal carcinomas, we established primary cells from invasive ductal carcinoma tissues. We obtained a diverse group of primary cells with a broad expression of breast cancer standard markers, such as Her2, progesterone receptor, cytokeratin 5 (Table I). After establishment, the epithelial origin of the cells was confirmed by the expression of cytokeratins. Only epithelial cells were considerate in the following studies. These primary cells duplicate between 3 and 15 days and all cultures grew until passage 10, except CF1 cells which grew until passage 30. All primary cell cultures were tested regarding the expression of E-selectin ligands using anti-CLA (HECA-452 clone) mAb to detect specifically sLe^X and sLe^A by flow cytometry and western blot. All primary cells expressed E-selectin ligands, being the CF1 cells the ones with the highest expression of $sLe^{X/A}$ (Table II). By western blot, we were able to verify that all primary cells have a similar pattern of glycoproteins decorated with $sLe^{X/A}$, specifically all the primary cells have at least two high weight glycofroms (at approximately 150 kDa and 200 kDa) that play a role as E-selectin ligands (Fig. 2). Regarding the expression of alpha(1,3)-fucosyltransferases, all primary cancer cells express FUT-4, -5, -10 and -11, not expressing FUT-3, -6, -7 and -9 (Table III). Taking all these results in account, we selected the CF1 cells to continue this study since these cells have the highest expression of E-selectin ligands.

Table I - Clinical information about breast cancer features from tissues used to establish primary cell cultures

Cancer cell culture	Patient's Age	Histologic Type	Clinical Stage	Tumor Markers				
				Estrogen Receptor	Progesterone Receptor	HER2	Ki.67	Cytokeratin 5
CF1	31	Invasive Ductal Carcinoma	Stage IIA (T2, N0, M0)	25%	15%	+	5%	Negative
TB2	41	Invasive Ductal Carcinoma	Stage IIA (T1c, N1, M0)	100%	90%	-	20%	na
MC5	61	Invasive Ductal Carcinoma	Stage IA (pT1c, pN0 (sn)(i-))	80%	40%	+	10%	Negative
MS6	74	Invasive Ductal Carcinoma	Stage IA (pT1c, pN0 (sn)(i-))	100%	60%	-	10%	Negative
MC8	62	Invasive Ductal Carcinoma	Stage IIB (pT2, pN1a (1/16))	100%	50%	-	10%	Negative
KB10	62	Invasive Ductal Carcinoma	Stage IA (pT1c, pN0 (sn)(i-))	100%	20%	-	5%	Negative
MG11	47	Invasive Ductal Carcinoma	Stage IA (pT1c, pN0 (sn)(i-))	90%	80%	-	20%	Negative
MP13	73	Invasive Ductal Carcinoma	Stage IA (pT1b, pN0 (sn)(i-))	90%	0%	-	10%	Negative

Table II – E-selectin ligands expression on primary breast cancer cells analyzed by flow cytometry

	CF1	TB2	MC5	MS6	MC8	KB10	MG11	MP13
HECA-452 staining	++++	++	+	+++	++	++	+	+++

+ → MFI < 1000; ++ → 1000 < MFI < 5000; +++ → 5000 < MFI < 7500; ++++ → MFI > 7500

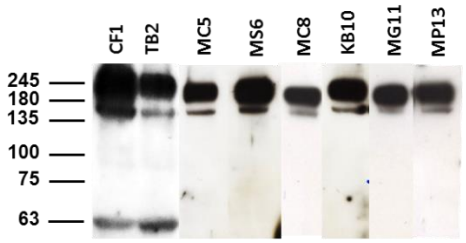

Fig 2: Similar profile of glycoproteins decorated with sialofucosylated glycans between primary cell cultures. Total lysate proteins from the 8 established primary breast cancer cell cultures were stained with HECA-452 mAb and analyzed by western blot.

Table III - Gene expression of alpha(1,3/4)-fucosyltransferases in primary breast cancer cells analyzed by RT-PCR (Values correspond to the amount of RNA copies of each FUT per each 1000 copies of housekeeping genes (β-actin and GAPDH)

Fucosyltransferases (FUTs)	Primary breast cancer cells							
	CF1	TB2	MC5	MS6	MC8	KB10	MG11	MP13
FUT3	ND	ND	ND	ND	ND	ND	ND	ND
FUT4	0,351	0,399	1,190	0,482	0,011	0,873	2,940	1,524
FUT5	0,050	0,059	0,364	0,177	1,065	0,129	0,927	0,378
FUT6	ND	ND	ND	ND	ND	ND	ND	ND
FUT7	ND	ND	ND	ND	ND	ND	ND	ND
FUT9	ND	ND	ND	ND	ND	ND	ND	ND
FUT10	0,241	0,698	0,903	0,585	0,439	0,353	0,459	0,176
FUT11	0,550	0,995	0,509	0,585	0,020	1,341	1,137	0,985

3.3. Immortalization process of CF1 cells did not significantly decrease E-selectin ligands and fucosyltransferases and sialyltransferases expression

To perform further studies, we immortalized CF1 primary cells by transducing them with *hTERT* cDNA, which codify for TERT that lengthens telomeres in DNA strands, thereby allowing senescent cells that would otherwise become postmitotic and undergo apoptosis to become immortal. Although the expression of E-selectin ligands was downregulated by this cell immortalization process, CF1_T immortalized cells still express $sLe^{X/A}$ (Fig. 3A). The pattern of glycoproteins decorated with $sLe^{X/A}$ analyzed by western blot was still the same as the primary cells (Fig. 3B). As CF1 primary cells expresses FUT4, FUT5, FUT10 and FUT11, we also confirmed that, after the immortalization process, the CF1_T cells maintained the expression of the originally expressed fucosyltransferases and did not start expressing other alpha(1,3)-fucosyltransferases (Table IV). In general, the immortalization process undergone by CF1_T cells did not alter their expression of E-selectin ligands, allowing us to use them in representative of the CF1 primary cells.

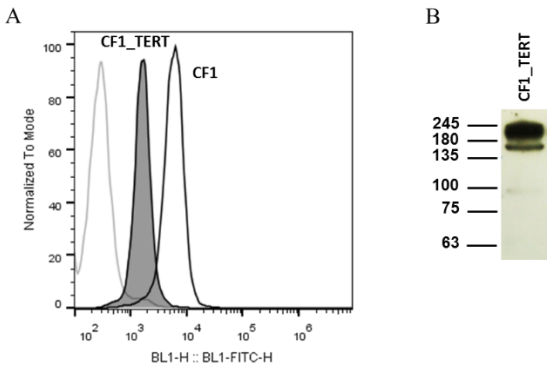

Table IV - Gene expression of alpha(1,3/4)-fucosyltransferases in CF1 primary cells and CF1_TERT cell line analyzed by RT-PCR

Fucosyltransferases	Cancer cells	
	CF1	CF1_TERT
FUT3	ND	ND
FUT4	0.351	0.594
FUT5	0.050	0.004
FUT6	ND	ND
FUT7	ND	ND
FUT9	ND	ND
FUT10	0.241	1.309
FUT11	0.550	1.234

Fig 3: CF1 cells continue to express E-selectin ligands and α1,3/4-fucosyltransferases after immortalization process. A: Both CF1 primary cells (black line) and CF1_T cell line (filled pick) were stained with E-selectin chimera plus fluorescent secondary antibody in PBS with calcium and analyzed by flow cytometry. Cells stained in PBS with EDTA or with just fluorescent secondary antibody were used as negative control (grey line). **B:** CF1_T cell lysate was run by SDS-PAGE, blotted in PVDF membrane and stained using HECA-452 mAb.

3.4. 2-FF treatment in CF1_T cells abrogates their capacity to bind E-selectin

2-FF was described as an inhibitor capable of blocking fucosylation of a cancer cell line *in vitro*, and as consequence, decreasing its ability to bind E-selectin [19]. Here, we verify if 2-FF inhibitor have the same effect on the CF1_T cell line ability to bind E-selectin. By western blot, we observed that 2-FF treated CF1_T cells expressed significantly less E-selectin ligands in comparison with non-treated cells (Fig. 4A). In flow conditions, we were able to see that 2-FF treated CF1_T cells completely lose their ability to bind to E-selectin (Fig. 4B). These data show the importance of fucosylated structures expressed by CF1_T cells in binding E-selectin, being this the first step of transendothelial migration.

3.5. 2-FF treated CF1_T cells have lower migration capacity than non-treated cells

Previous experiments have shown that E-selectin ligands in metastatic breast cancer cells are co-distributed with F-actin, including in the ‘‘spiking’’ cellular protrusions [22], which suggested that the E-selectin binding protein(s) play a role in cell migration. We obtained similar results when using CF1_T cells. Co-staining of E-selectin ligands, using E-Ig chimera, and F-actin (phalloidin staining) in these cells show a co-localization of both, mainly in the leading edge of CF1_T cells (Fig. 4C), suggesting that E-selectin ligands play a role in cell motility. In order to better understand this role, we used 2-FF inhibitor to remove E-selectin ligands expression in CF1_T cells for 10 days and then evaluate their cell migration capacity by a wound-healing assay. Therefore, uniform

scratches were made in confluent monolayers of 2-FF treated and non-treated CF1_T cells and their capability to migrate and fill the scratches was monitored after 16h. We verified that 2-FF treated cells just closed 49% of the opened "gap" while non-treated cells were able to fill 57% (Fig. 4D). These data demonstrated the statistically significant lower capability to closure the wound of CF1_T cells when previously treated with 2-FF, which results in a lack of functional E-selectin ligands expression. Our results evidence the role of E-selectin ligands in CF1_T cell migration.

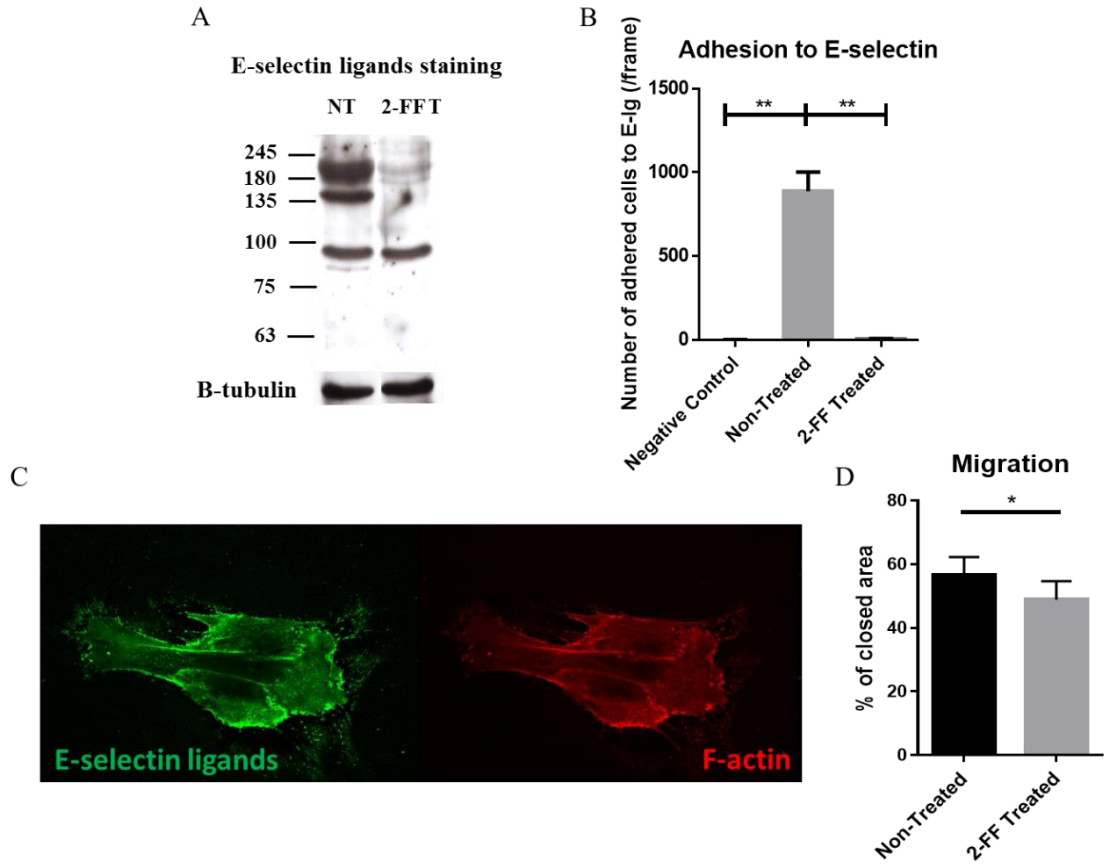

Fig 4: CF1_T cell line treated with 2-FF compound lose functional E-selectin ligands, leading to a reduced cell migration capacity. A: CF1_T cell line was treated with 2-FF inhibitor for 5 days. Total lysate proteins were stained with HECA-452 mAb and analyzed by western blot. B-tubulin expression was analyzed as a loading control. **B:** The capacity of CF1_T cells treated with 2-FF for 5 days, or non-treated, adhere to E-Ig chimera was analyzed in flow conditions by an alternative Stamper-Woodruff assay. Breast cancer cells were added in calcium buffer over an E-Ig spot in a shaker. Assay performed in EDTA buffer was used as negative control. (n=4; $p< 0.01$ (**)) **C:** CF1_T cells stained with E-Ig chimera plus anti-human IgG-FITC (left image) and Alexa Fluor 568 Phalloidin (right image) were analyzed by confocal microscopy. **D:** The migratory ability of CF1_T cell line after 2-FF treatment for 10 days was analyzed by scratch wound healing assay (n=5; $p< 0.05$ (*)).

3.6. Treatment with 2-FF reduces cell proliferation and expression of growth factors

Previous studies have shown that the suppression of fucosyltransferases expression in cancer can attenuate cell proliferation [23]. To assess the influence of E-selectin ligands removal in proliferation, CF1_T cells were treated with 2-FF inhibitor for 5 days and then cells were stained with CFSE, being the CFSE-labelled 2-FF treated and non-treated CF1_T cells analyzed by flow cytometry after 5 and 9 days (10 and 14 days of treatment, respectively). The comparison of both conditions showed that the proliferation index of non-treated CF1_T cells was statistically higher than the index of 2-FF treated cells after 14 days of treatment (Fig. 5A), thus demonstrating the influence of fucosylated structures, such as E-selectin ligands, in cell proliferation. The expression of several growth factors and pro-inflammatory cytokines have been associated with increased proliferation, migration, invasion and survival in cancer, such as basic fibroblast growth factor (FGF2), vascular endothelial growth factor (VEGF-A), VEGFR-1 (Flt-1), VEGFR-2 (KDR), human epidermal growth factor receptor 2 (Her2), transforming growth factor beta (TGF-β), interleukin 1 beta (IL-1β), interleukin 6 (IL-6) and interleukin 8 (IL-8) [24-31]. We verified that 2-FF treated CF1_T cells express statistically less FGF2 (RQ= 0,9002), VEGF-A (RQ=0,6859), VEGFR-1 (RQ=0,7756), VEGFR-2 (RQ=0,7228), TGF-β (RQ=0,7871) and IL-8 (RQ=0,8412) than non-treated CF1_T cells by real-time PCR (Fig. 5B). These results are in agreement with the effect of 2-FF treatment on cell proliferation and migration reported here, thus demonstrating the importance of fucosylated structures, such as E-selectin ligands, on cancer progression.

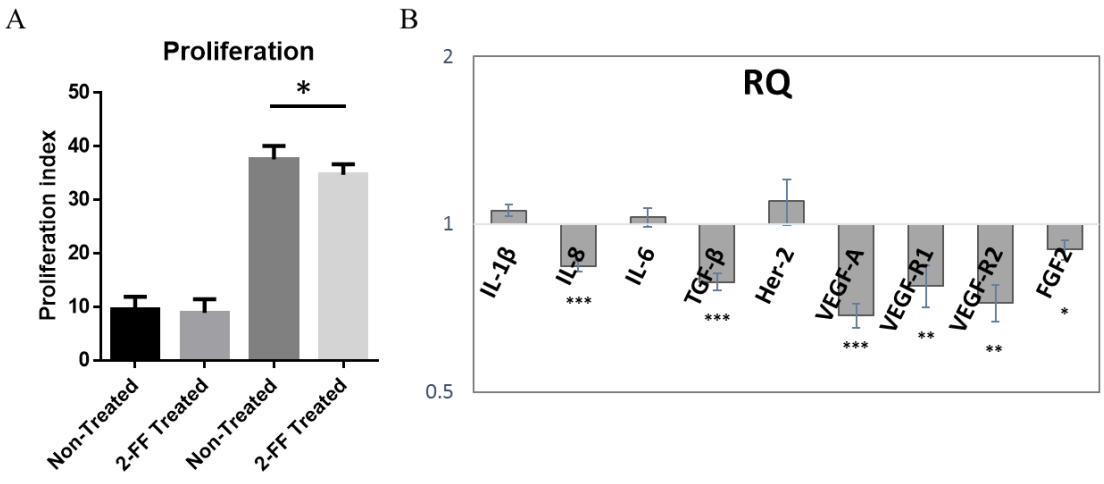

Fig 5: 2-FF treatment on CF1_T induces lower expression of growth factors and reduced cell proliferation rate. A: Proliferation índex of CF1_T cell line treated with 2-FF, or not, for 10

and 14 days (left and right side of the graph, respectively) was analyzed by flow cytometry. Specifically, CF1_T cells were pretreated with 2-FF for 5 days, or not, then stained with CFSE dye and analyzed after 5 (total of 10 days) and 9 days (total of 14 days). Proliferation index was given by ModFit software (n=4; $p< 0.05$ (*)). **B:** Gene expression of growth factors expressed by 2-FF treated CF1_T cell line was compared to the one expressed by non-treated CF1_T cell line. Relative quantification (RQ) indicates the relative change in mRNA expression levels from the 2-FF treated cells relative to the untreated cells (n=6; $p< 0.05$ (*), $p< 0.01$ (**) and $p< 0.005$ (***)).

3.7. Suppression of MAPK signaling pathways by 2-FF treatment

Finally, the involvement of MAPK signaling pathways in 2-FF treated CF1_T cell line was examined. ERK1/2 MAPK (extracellular signal-regulated kinases 1 and 2) and p38 MAPK activation are involved in the proliferation and migration of breast cancer cells [32, 33]. The ratio of p-ERK1/2 / ERK1/2 expression and p-p38 / p38 expression was assessed by flow cytometry upon 7 and 14 days of 2-FF treatment. Concerning p38 signaling pathway, we significantly detected a lower expression of phospho-p38 in the 2-FF treated cells than in non-treated cells after 7 days of treatment (71.20±6.49 to 77.09±6.77, respectively), being this difference more evident after 14 days of treatment (71.68±9.91 to 80.42±9.16) (Fig. 6A). Regarding ERK1/2 signaling pathway, we just verified a significantly reduced expression of phospho-ERK1/2 in the cells treated with 2-FF comparing with the non-treated cells after 14 days of treatment (57.88±7.24 to 69.30±5.94, respectively), but not after 7 days (91.46±4.27 to 91.81±5.86) (Fig. 6B). The ratio of p-ERK1/2 / ERK1/2 and p-p38 / p38 expression was also measured by western blot upon 14 days of 2-FF treatment (Fig. 6C and 6D, respectively). Similarly, 2-FF treatment downregulates the expression of phospho-ERK1/2 and phospho-p38 in comparison with non-treated cells (0.92±0.22 to 1.23±0.27 and 0.82±0.14 to 1.05±0.12, respectively). Overall, these data suggest that fucosylated structures, such as E-selectin ligands, contribute to cell proliferation and migration through ERK1/2 and p38 MAPK pathways in breast cancer cells.

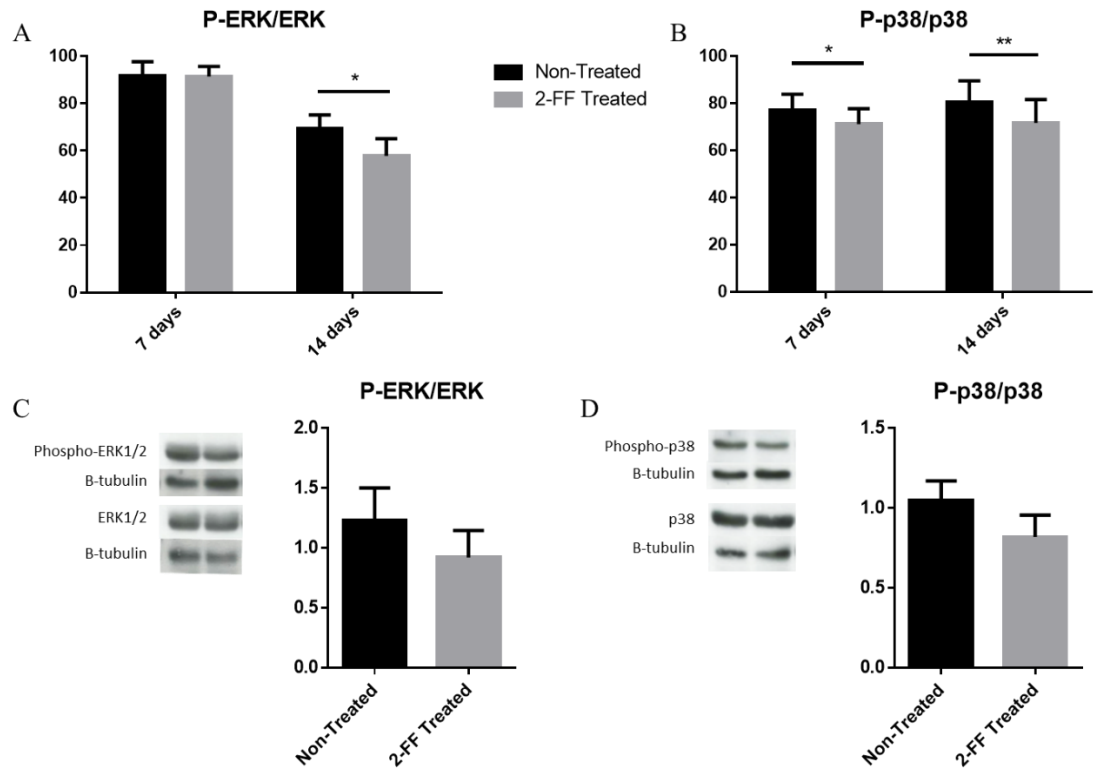

Fig 6: ERK1/2 and p38 MAPK pathways are negatively influenced by 2-FF treatment on CF1_T cell line. A and B: CF1_T cell line treated with 2-FF for 7 and 14 days were analyzed regarding their expression of phospho-ERK1/2 (P-ERK), total ERK1/2 (ERK), phospho-p38 (P-p38) and total p38 (p38) kinases by flow cytometry. Ratio between P-ERK and ERK expression ((P-ERK/ERK)*100) and P-p38 and p38 expression ((P-p38/p38)*100) are represented in the graphs A and B, respectively (n=4; $p< 0.05$ (*) $p< 0.01$ (**)). **C and D:** CF1_T cell line was treated with 2-FF inhibitor for 14 days. Total lysate proteins were stained with anti-P-ERK1/2, anti-ERK1/2, anti-P-p38 or anti-p38 mAb and analyzed by western blot. B-tubulin expression was analyzed as a loading control. Band intensity was measured in ImageJ software and P-ERK/ERK ratio (C) and P-p38/p38 ratio (D), after normalization with loading control, is represented in the graphs (n=4).

4. Discussion

In this work, we aimed to understand the role of E-selectin ligands on breast malignant features. Starting by characterizing the expression of E-selectin ligands in the two main histopathological classes, invasive ductal carcinoma and invasive lobular carcinoma, we determined that poorly-differentiated invasive ductal carcinoma tends to express more E-selectin ligands than well-differentiated one or invasive lobular carcinoma. Taking in account this preliminary results, we focused our work in invasive ductal carcinoma, which is also the most common type of breast cancer, comprising 80% of all invasive breast cancers [34]. Primary cancer cell cultures were established from invasive ductal carcinoma tissues, originating 8 different primary cultures from patients in different clinical stages of disease (Stage I and II) and all from Luminal subtype (Luminal A and

B), which includes approximately 70% of all invasive breast cancers [35]. All 8 primary breast cancer cell cultures express E-selectin ligands, which is composed of a scaffold protein or lipid decorated by the tetrasaccharide sLe^X. Although the levels of E-selectin ligands expression are different among the primary cancer cells, the profile of glycoprotein decorated with sLe^X is identical among them when analyzed by western blot. One of the reasons for this seems to be that all cell cultures express the same group of α1,3/4-fucosyltransferases, specifically FUT4, FUT5, FUT10 and FUT11. In other reports, the main fucosyltransferases associated with sLe^X biosynthesis in breast cancer are FUT3, FUT4, FUT6 and FUT7 [14-16], while in this study other FUTs seems to be involved. Considering that FUT4 and FUT10 are mainly associated with the biosynthesis of unsialylated glycans [36], although the latter is not well described yet [37], FUT5 and FUT11 should be the main enzymes responsible for sLe^X fucosylation in these breast cancer cultures, since they were already described as being involved in its biosynthesis [36, 38].

We selected the breast cancer cell culture with the higher expression of E-selectin ligands as a representative of this group of cell cultures in order to study the effect of E-selectin ligand removal on malignant features. Since primary cell cultures have a limited time in culture, we immortalized CF1 cells, creating the CF1_T breast cancer cell line, which allowed us to continue this study. The chosen process to immortalize CF1 cells was already described as being able to extend the life span without altering the characteristic phenotypic properties of the cells [39, 40]. However, in order to confirm it, we checked that CF1_T cell line continued to express E-selectin ligands and FUT4, FUT5, FUT10 and FUT11, as well as maintaining a similar profile of glycoproteins decorated with sLe^X. In the further assays, we used a fluorinated fucose (2-FF) compound that was able to reduce the adhesion of colon carcinoma cells to E-selectin, being describe as a fucosylation inhibitor [19]. Testing this compound in CF1_T cells, we verified that sLe^X glycan expression was reduced after 5 days of treatment. Specifically, high molecular weight glycoproteins that are normally decorated with sLe^X glycans recognized by HECA-452 mAb were just slightly detected by this antibody after treatment, indicating that 2-FF is able to inhibit the biosynthesis of this sialofucosylated glycan in CF1_T cells. Since sLe^X glycan structure is the main structure needed for engagement with E-selectin, we tested then the ability of 2-FF-treated, or not, CF1_T cell line to adhere to E-selectin in flow condition. As expected, since sLe^X biosynthesis was highly reduced by 2-FF

treatment, CF1_T cells treated with 2-FF were not able to bind to E-selectin in flow conditions.

Expression of E-selectin ligands, such as sLe^X, has been positively associated with cell migration and motility in several cancer types [41, 42], including breast cancer [22]. Here, we demonstrated that E-selectin ligands expression is associated with F-actin presence in CF1_T cell line, indicating a role for these ligands in cell migration. In order to assess this topic, we analyzed 2-FF-treated CF1_T cells, which did not express functional E-selectin ligands, regarding their migration capacity and we verified that, in fact, the removal of functional E-selectin ligands negatively influenced the migratory ability of these cells, proving the role of fucosylated structures, such as sLe^X, in cell migration.

It has been shown that when the expression of sLe^X was increased in cancer cells, they have a higher cell proliferation rate [43]. Herein, we assessed the proliferation index of CF1_T cells when treated with 2-FF compared with non-treated cells. We demonstrated that 2-FF-treated cells, which have a highly reduced sLe^X content, have a significantly reduced proliferation index than non-treated cells that express sLe^X. Since cell proliferation is mostly stimulated by growth factors, which are low molecular peptides that are highly produced in cancer [44], we evaluated the expression level of several growth factors (IL1-β, IL-6, IL-8, TGF-β, Her2, VEGF, VEGFR1 (Flt-1), VEGFR-2 (KDR) and FGF2), which were already described as promoting cancer cell proliferation [26-29, 31, 45-47], on 2-FF treated CF1_T cells compared with non-treated cells. We verified that the expression of IL-8, TGF-β, VEGF, VEGFR1, VEGFR-2 and FGF2 was significantly reduced on 2-FF treated cells, justifying the low proliferation index detected in these cells. The reduction of sLe^X structures was never before linked with the low expression of growth factors, however most of them have been associated with improved invasion ability and metastasis formation in breast cancer [25, 29, 48].

The regulation of cell proliferation is a complex process, being primarily regulated by external growth factors that activate different MAPK pathways. Taking this into account, we assessed the influence of 2-FF treatment in the activation of two main MAPK pathways, ERK1/2 and p38 pathways, which have been described as being involved in breast cancer cell proliferation and migration [32, 33, 49]. We verified that CF1_T cells treated with 2-FF overtime leads to a diminished activation/phosphorylation of ERK1/2 and p38 kinases. Among the affected growth factors, FGF2 have already been shown to be associated with ERK1/2 activation in breast cancer [46], which indicates that the low

expression of growth factors could be the main cause for the reduced activation of MAPK pathways observed in this study.

Taken together, all these data indicate that 2-FF treatment of CF1_T breast cancer cell line, which leads to the removal of functional E-selectin ligands, decreases the expression of growth factor that will negatively influence the activation of ERK1/2 and p38 pathways, leading to a reduced cell proliferation and migration.

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Chapter 5

CD13 and CD44 as E-selectin ligands in breast cancer cells

(Paper III – Manuscript in preparation)

CD13 and CD44 as E-selectin ligands in breast cancer cells

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Abstract:

Metastasis are the major cause of death in breast cancer patients. It involves a series of steps, including tumor cell dissemination through bloodstream and shear-resistant interactions with endothelium followed by tumor cell extravasation into tissues. Engagement of tumor cells by endothelial E-selectins are crucial in metastasis formation, however the nature of the E-selectin counter receptors expressed in breast cancer cell remains unclear and are the focus of the present investigation. Using a recently established breast cancer cell line, CF1_T we developed an affinity chromatography to isolate glycoconjugates that bind E-selectin. The majority of E-selectin ligands in CF1_T cells are N-glycosylated glycoproteins. By mass spectrometry, we identified several proteins belonging to the adhesion functional category. By western blotting and immunoprecipitation, we identified the CD44 and CD13 glycoproteins as two main E-

selectin ligands in CF1_T cells. CD44 glycoprotein was already defined as an E-selectin ligand in breast cancer. However, CD13 glycoprotein is a new identified E-selectin ligand. Although CD13 expression has been associated with invasion and metastasis, its role as counterreceptor for E-selectin had never been described before. This novel role for CD13 makes this glycoprotein an interesting target for novel anti-cancer therapy.

1. Introduction

Breast cancer is still the most common cancer in women and their second leading cause of death. The reason of its mortality relies mostly on its high tendency to form bone metastasis [1, 2]. During tumour metastasis, cancer cells spread from the primary tumor, entering into the blood and lymph vessels and consequently colonizing new organs. The process of extravasation of cancer cells from the bloodstream to originate new metastasis is comparable to the migration of leukocytes during inflammation, which includes a cascade of events initiated by tethering and rolling contacts of circulating cells with the endothelium (step-1), chemokine receptor engagement, triggering G-protein-coupled integrin activation (step-2), with subsequent firm adhesion by integrins to endothelial cells (step-3) and culminating in transendothelial migration (TEM) (step-4) [3]. While all these events are decisive, the earliest obstacle is the contact of flowing cells along the endothelium and the establishment of strong adhesive interactions to overcome the hemodynamic shear forces, which is accomplished by selectins.

E-selectin is a calcium-dependent lectin expressed on endothelium upon inflammation and constitutively expressed in marrow microvessels, mediating specifically the cellular recruitment to bone [4]. Several *in vivo* studies have demonstrated the importance of E-selectin-mediated interactions in cancer metastasis, where metastasis in mice was decreased by the blockage of E-selectin and/or E-selectin ligand activity [5, 6]. The minimal binding structural domain for E-selectin is a terminal glycan structure containing sialic acid and fucose, where the major representative is sialyl-Lewis x (sLex). sLex expression in breast cancer has been correlated with metastasis formation [7]. However, E-selectin binding is modulated not only by the carbohydrate scaffold's nature (the glycan tree) but also by the protein scaffold that carries sLex [8].

Although some E-selectin ligand protein scaffolds have been identified in cancer, such as CD44, CEA, PSGL1, ESL1, MUC16 and CD43 [9-13], the ones expressed by breast cancer cells still need to be clarified and are the focus of the work herein.

Of potential interest in this work is CD44 and CD13 proteins. CD44 has been recognized as a breast cancer stem cell marker [14], and a subpopulation of CD44-expressing tumor cells seems to possess high metastatic potential [15]. Others have linked CD44 as an E-selectin ligand, specifically two isoforms, CD44v4 decorated with sialofucosylated structures recognized by HECA-452 mAb and another CD44v that is not detected by HECA-452 mAb [16, 17].

CD13, also known as the protease aminopeptidase-N, is expressed in several cancer types, including 36% of breast carcinomas [18]. CD13 is described as a multifunctional protein with enzymatic as well as other functions, including inflammatory and immunological responses, signal transduction, antigen processing, neuropeptide and cytokine degradation as well as extracellular matrix degradation [19]. In breast cancer, aminopeptidase activity was significantly increased in tumor tissue than in adjacent unaffected tissue [20]. CD13 has also been associated with a number of malignant characteristics, such as cell proliferation, secretion, invasion and angiogenesis [21-23]. Specifically in breast cancer, CD13 expression was significantly associated with tumor type, e.g. CD13 expression was more often found in ductal invasive breast carcinoma than in other histological types, being also more frequently expressed in lymph-node positive patients suggesting a role in tumor metastasis. In the same study, CD13 expression was also negatively correlated with overall survival time [18].

Here we showed that most E-selectin ligands in CF1_T breast cancer cell line are N-glycosylated glycoproteins. Mass spectrometry analysis, identified several possible core glycoproteins as E-selectin ligands, including CD44, ESL-1, CD13, CD29 and NG2. At this point, we proved that both CD44 and C13 glycoproteins are decorated with sialofucosylated glycans, playing a role as E-selectin ligands in CF1_T breast cancer cells.

2. Material and methods

2.1. Reagents

Recombinant mouse E-Selectin/CD62E Fc chimera (E-Ig) and anti-human CD44s pan specific monoclonal antibody (mAb), clone 2C5, were purchased from R&D Systems

(Minneapolis, MN). Anti-CLA mAb, clone HECA-452 was purchased from Biolegend (San Diego, CA). Anti-human CD29 was from Immunotools (Germany). Anti-mouse Ig - HRP and anti-rat Ig – APC mAbs were from BD Biosciences (San Jose, CA). Anti-CD13 and anti-NG2 mAbs were from Santa Cruz Biotechnology (Dallas, Texas) and and anti-rat IgM – HRP mAb was from Southern Biotech (Birmingham, AL). Anti-human Ig – FITC mAb was from Sigma-Aldrich (St. Louis, MO). Anti-mouse Ig - FITC was from Dako. Peptide-N-Glycosidase F (PNGase F) enzyme was acquired from New England Biolabs (Ipswich, MA). Neuraminidase from *Clostridium perfringens* was purchased from Roche Diagnostics (Basel, Switzerland). Bromelain was purchased from Sigma-Aldrich. 2-fluorofucose (2-FF) is a fucosyltransferases inhibitor kindly given by Professor Peter Senter from Seattle Genetics.

2.2. Cell culture

The human breast cancer cell line, CF1_T, was stablished from an invasive ductal carcinoma tissue, as described in chapter 4 and was grown in Dulbecco's modified Eagle medium (DMEM) (Sigma), supplemented with fetal bovine serum, glutamine, penicillin and streptomycin.

2.3. Confocal laser scanning microscopy

Cells were cultured in cover glasses overnight and fixed with 3.7% paraformaldehyde. After blocking with 1% bovine serum albumin (BSA), cells were stained using E-Ig (1 µg) plus anti-human Ig FITC (1,5 µL) in PBS-Ca. Negative control with only anti-human Ig FITC mAb was performed. Then, cells were permeabilized with 0.1% TritonX-100 and cell nuclei were stained with TO-PRO3 iodide dye (Molecular Probes, Leiden, Netherlands). Images were acquired with a Leica TCS SP2 AOBS confocal microscope (Leica Microsystem, Mannheim, GmbH). Representative confocal cross-section images were selected after Z-stacking.

2.4. Flow cytometry

Expression levels of E-selectin ligands on bromelain treated and non-treated breast cancer cells was analyzed by flow cytometry (Attune Flow Cytometer). Specifically, CF1_T cells were collected by centrifugation and resuspended in DMEM medium (no serum) and treated with 0.1% bromelain for 1 hour at 37°C. Then, stop the reaction by diluting and washing in DMEM medium. Cell suspensions were incubated with E-Ig chimera plus

anti-human Ig FITC in PBS-Ca (Sigma-Aldrich) or, as negative control, in PBS with 2mM EDTA (Merck) for 1 hour at 4°C to detect E-selectin ligands.

Analysis of CD44, CD13, CD29 and NG2 expression was performed using protein-specific antibodies for 30 minutes at 4°C followed by incubation with anti-mouse Ig - FITC antibody for 30 minutes at 4°C. Background levels were determined by incubating cell suspensions with only fluorescent-labeled secondary antibodies.

2.5. Immunoprecipitation

Cells were solubilized in lysis buffer (150 mM NaCl, 2 mM CaCl2, 50 mM Tris (pH 7.4), 1 mM PMSF, 2% Nonidet P-40 and 1 EDTA-free protease inhibitor cocktail tablet (Roche, Indianapolis, IN)) and incubated overnight in agitation at 4°C, being then centrifuged for 10 min at 10,000 g. Supernatants were collected and stored at -80°C until use. Protein concentration was determined using Pierce BCA protein assay kit (Thermo scientific).

Firstly, Protein lysate was precleared with Protein G-agarose. Precleared samples were incubated with anti-CD44 or anti-CD13 mAb, or E-Ig (in presence of calcium) for 2 hours and then incubated with Protein G-agarose beads overnight. Mix with beads was washed three times with lysis buffer and boiled in SDS-PAGE sample loading buffer for 5 minutes. Then E-Ig immunoprecipitate (IP) is treated with PNGase F enzyme as recommended by the manufacturer. Finally, PNGase F treated and non-treated E-Ig IP and CD44 and CD13 IP were subjected to reducing 8% SDS-PAGE, transferred to PVDF membrane, and immunoblotted with mAb HECA-452, CD44 or CD13.

2.6. SDS-PAGE and Western blot

CF1_T protein sample representing 100g of protein, or equivalent immunoprecipitated, was diluted with SDS-PAGE sample loading buffer (NZYTech, Portugal) and electrophoresed through 8% polyacrylamide SDS-PAGE gels. Prestained molecular mass standard (NZYTech, Portugal) were included in adjacent lanes in all experiments. SDS-PAGE separated proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad) and blocked with TBS/0.1% Tween 20/10% dry milk for 1 hour. Immunoblots were stained with anti-CLA (HECA-452 clone), anti-CD44 (2C5 clone) or anti-CD13 mAbs and then rinsed with TBS/0.1% Tween 20. Subsequently, blots were incubated with appropriate HRP-conjugated secondary antibodies. Lumi-light western blotting substrate (Roche) was used as developing reagent.

2.7. Mass spectrometry

Mass spectrometry analysis of E-selectin reactive protein E-Ig chimera immunoprecipitate isolated from CF1_T cell lysate was carried over after SDS-PAGE separation. Polyacrylamide gel was stained with PageBlue protein staining solution (Thermo Scientific) and the stained bands were excised. Then, the gel fragments were washed with 50mM ammonium bicarbonate buffer (buffer 1) and 50% acetonitrile to remove the stain. Fragments were dehydrated in 100% acetonitrile and dried in the speedvac system. Subsequently, the fragments were treated with 10mM DTT and 100mM iodoacetamide and washed in buffer 1, 50% acetonitrile and finally dehydrated with 100% acetonitrile and dried in the speedvac system. Subsequently, the fragments were digested at 37ºC, overnight with MS grade trypsin. Peptide recovery was carried out by washing with 10% formic acid and with 10% formic acid plus 100% acetonitrile, saving the supernatant. Finally, the peptide containing supernantant was dried in a centrifugal evaporator and analyzed by nano-liquid chromatography – electrospray ionization - tandem mass spectrometry (nanoLC-ESI-MS/MS). The experimental set up and instrument operation conditions were those described elsewhere [24], except for sample size (10 µl) and the peptide separation gradient, which was reduced to 60 min. MS-data were analyzed automatically using the SequestHT search engine with the Percolator algorithm for validation of protein identifications (Proteome Discoverer 1.4, Thermo Scientific). LC-MS runs from the sample, but different SDS-PAGE gel fractions were combined in a single database base search using the Proteome Discoverer Deamon application (Thermo Scientific). Search was conducted against the human proteome obtained from the SwissProt database on 22/11/2015, selecting trypsin as the enzyme and allowing for up to 2 missed cleavage sites a precursor ion mass tolerance of 10 ppm and 0.6 Da for product ions. Carbamidomethylcysteine was selected as a fixed modification while oxidation of methione oxidation (+15.99491), was defined as variable modification. Protein data regarding cellular localization, glycosylation types, molecular weight and function were obtained based on gene ontology annotation using STRAP bioinformatics tool version 1.5 [25].

2.8. Cell adhesion to E-selectin using alternative Stamper-Woodruff assay

For analysis of CF1 cells adherence to E-selectin, shear-dependent Stamper-Woodruff assays were performed as previously described [26]. Briefly, recombinant E-Ig was placed on glass slides and let dry. The adhesion area was blocked with 1% BSA and then

let dry. CF1_T cells that were treated or not with 1mM 2-FF for 5 days, or with 100 mM neuraminidase (per $1x10^6$ cells) for 1h, were resuspended in Hank's Balanced Salt Solution (HBSS) containing 2mM $CaCl_2$ (HBSS-Ca) or 5mM EDTA (HBSS-EDTA) (negative control). E-Ig spots were incubated, or not, with 20µg/mL of rat anti-mouse CD62E (BD Biosciences, San Jose, CA), or 20µg/mL rat IgG2a isotype control (BD Biosciences, San Jose, CA), in order to block E-selectin. The CF1_T cells were overlaid onto E-Ig spot in the glass slides and incubated on an orbital shaker at 80 rpm for 30 minutes at 4°C. The slides were placed in HBSS-Ca or HBSS-EDTA to remove the non-adherent cells and then adherent cells were fixed with 3% glutaraldehyde. CF1_T cell adherence to E-selectin was examined under light microscopy at ×100 magnification, and representative photomicrographs were taken for analysis. The number of adherent cells in each photomicrographs was counted using ImageJ software.

3. Results

3.1. E-selectin ligands expressed on the CF1_T cell surface are mainly N-linked glycoproteins

The expression of E-selectin ligands on CF1_T cell surface was observed using E-Ig staining by confocal microscopy and flow cytometry. We observed that E-selectin ligands are highly expressed by these cells, spreading throughout the cell membrane including the focal adhesion points of CF1_T cells (Fig. 1A), showing their role in cell adhesion. Since E-selectin ligands on breast cancer cells can be glycoproteins and/or glycolipids [27], we used bromelain treatment, which remove proteins, to verify which glycoconjugate is mainly playing a role as E-selectin ligands. The data has showed that E-Ig staining was almost completely abrogated by bromelain treatment, thus suggestion that the majority of E-selectin ligands on CF1_T cells are glycoproteins (Fig. 1B). Analysis of E-selectin ligands by western blot showed that the main glycoproteins that bind to E-selectin have a high molecular weight, specifically there are two major bands, one around 150KDa and another between 180 and 200KDa (Fig. 1C). Since glycoproteins are mainly divided in N- and O-linked glycans, we used PNGase F enzyme, which cleaves the link between N-acetylglucosamines and asparagine residues of N-linked glycoproteins releasing the N-linked oligosaccharides [28]. Specifically, E-selectin ligands immunoprecipitate were denaturated and treated with PNGase F enzyme and then run in SDS-PAGE and stained with anti-CLA mAb (HECA-452 clone), which recognize

$sLe^{x/a}$ glycans. The majority of E-selectin ligands were not detected after treatment, which means that E-selectin ligands are mainly N-linked glycoproteins in CF1_T cells.

3.2. The functionality of E-selectin ligands on CF1_T cells under flow conditions is depended on sialofucosylated glycans

In order to mimic the physiological role of E-selectin ligands during transendothelial migration in the bloodstream, specifically the tethering and rolling of circulating cells over endothelium, we tested the ability of CF1_T cells to bind to E-selectin (E-Ig) under flow conditions. CF1_T cells were able to specifically bind to E-selectin in calcium buffer (Fig. 1D). The use of EDTA buffer or blocking antibody for E-selectin (negative controls) resulted in a significantly reduced binding (98.5% and 55% of reduction, respectively), proving the specificity of this interaction between these breast cancer cells and E-selectin. When cells were previously treated with sialidase, which remove sialic acids, or 2-FF, which inhibits the addition of fucose residues during glycan biosynthesis, the cell adhesion to E-selectin was also highly decreased (98% and 99.6%, respectively). In sum, this results confirmed that functional E-selectin ligands on CF1_T cells bind to E-selectin through sialofucosylated structures, such as $sLe^{X/A}$ glycans.

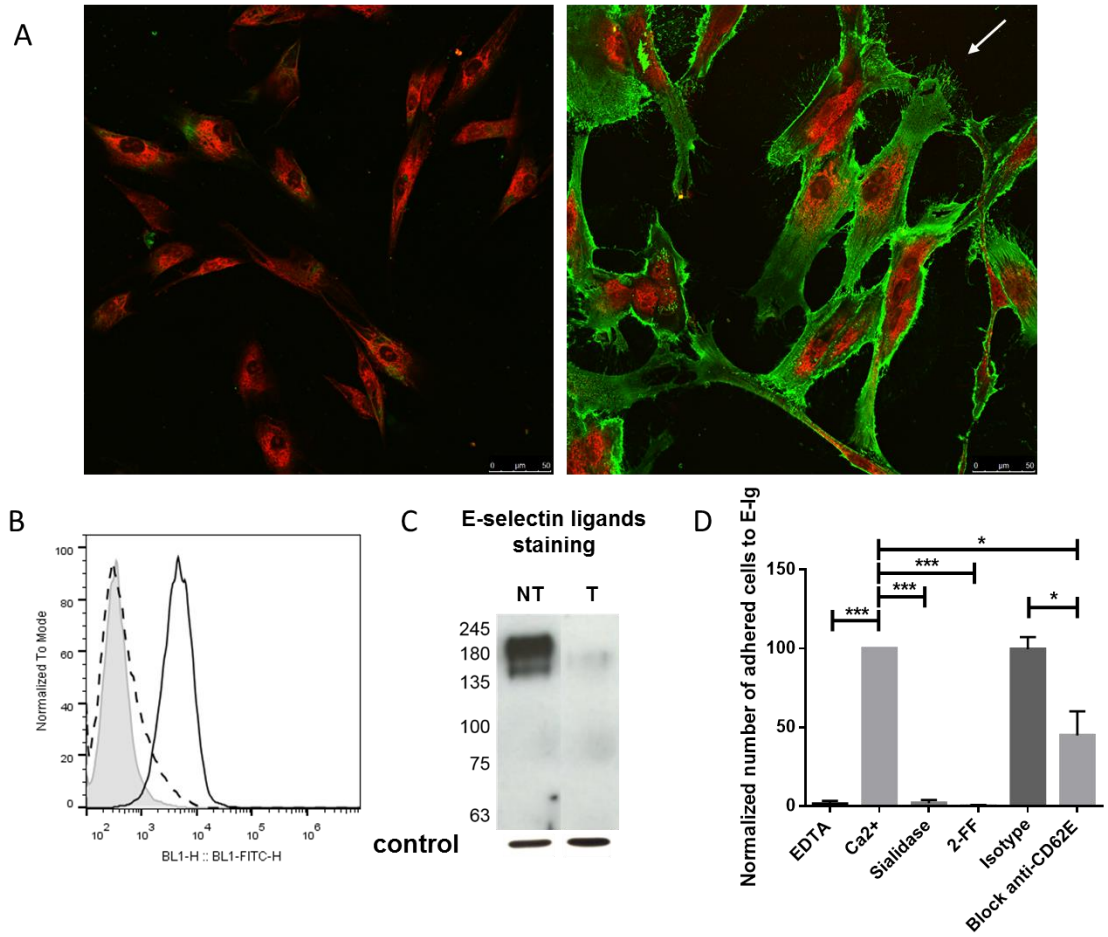

Fig 1: CF1_T breast cancer cells express functional E-selectin ligands. A: E-selectin ligands in cancer cells were stained using E-Ig chimera plus anti-human IgG FITC and nucleus using TO-PRO3 dye (right image) and was analyzed by confocal microscopy. White arrow indicates the tip of the cell responsible for the cell adhesion (focal adhesion point). Control stained with just fluorescent secondary antibody and TO-PRO3 dye was also analyzed (left image). **B:** CF1_T breast cancer cells were staining using E-Ig chimera plus anti-human IgG FITC after treatment with bromelain (dashed line), or not (black line), and analyzed by flow cytometry. Control stained just with fluorescent secondary antibody (grey filled pick) was also analyzed. **C:** E-selectin immunoprecipitate obtained from CF1_T total lysate proteins using E-Ig chimera was treated (right lane), or not (left lane), with PNGase F enzyme and then stained for sialofucosylated structures recognized by HECA-452 mAb by western blot. **D:** The adhesion of CF1_T cells to E-Ig was analyzed in flow conditions by an alternative Stamper-Woodruff assay. Breast cancer cells were added in calcium buffer over an E-Ig spot in a shaker. EDTA buffer, sialidade or 2-FF treatment of cancer cells and blocking antibody against E-selectin were used as negative control.

3.3. Most E-selectin ligands identified proteins in MS are adhesion proteins

To identify the carrier proteins that play a role as E-selectin ligands, E-selectin ligands IP from CF1_T cells were run in SDS-PAGE, the most prominent bands were excised and digested by trypsin and protein fragments were analyzed by mass spectrometry (Fig. 2A). The resulted list of identified proteins was examined regarding their cellular localization (cellular component) and molecular function. In general, most of the identified proteins

are associated with “binding” functions (89,54%), being typically localized in the plasma membrane (78,15%) or in the extracellular region (79,15%) (Fig. 2B).

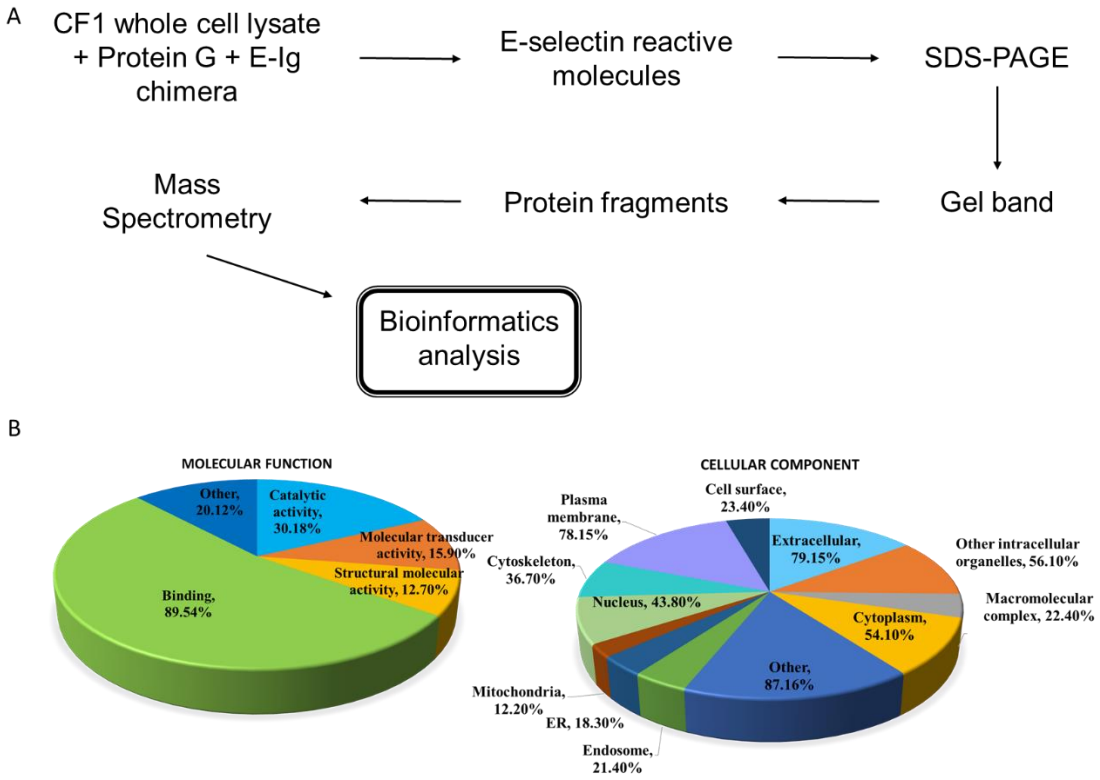

Fig 2: Mass spectrometry analysis uncovered mainly binding molecules expressed on plasma membrane or extracellular region. A: The steps followed for identification of E-selectin reactive proteins. CF1_T cells were lysed and immunoprecipitated using E-Ig chimera. Immunoprecipitates were resolved by SDS-PAGE. Bands were excised and digested with trypsin into protein fragments, which were analyzed by mass spectrometry. **B:** Distribution of identified proteins by their molecular function and cellular localization displayed in pie charts.

3.4. CD44, ESL1, CD13, CD29 and NG2 are the most probable proteins to play a role as E-selectin ligands following MS analysis

The general list of proteins identified by MS contains around 150 proteins (table SI), which had to be narrow down to the most probable proteins to play a role as E-selectin ligands, considering that they should be plasma membrane proteins, N-glycosylated with high molecular weight (between 80 and 250KDa) and associated with cancer progression. Specifically, when considering plasma membrane proteins, the number of proteins was narrowed down to 101 proteins, being then reduced to 32 and next to 18 when we selected only N-glycosylated and high molecular weight proteins, respectively. Finally, 11 from the 18 proteins were identified as being already correlated with tumor progression (table

SII). From this reduced list of proteins, we chose the 5 proteins with higher coverage in MS analysis to continue this work, CD44, ESL1, CD13, CD29 and NG2 (table I).

Table I: List of identified proteins by mass spectrometry

Accession number	Entry name	Protein name
P16070	CD44_HUMAN	**CD44**
Q92896	GSLG1_HUMAN	**Golgi apparatus protein 1 (ESL1)**
P15144	AMPN_HUMAN	**Aminopeptidase N (CD13)**
P05556	ITB1_HUMAN	**Integrin beta-1 (CD29)**
Q6UVK1	CSPG4_HUMAN	**Chondroitin sulfate proteoglycan 4 (NG2)**

3.5. CD44, CD13 and CD29 are expressed on CF1_T cell surface

Since E-selectin ligands have to be expressed on the cell surface to be physiological significant, we checked if these 5 identified proteins were expressed on the cell membrane of CF1_T cells by flow cytometry. Here, we show that CD44, CD13 and CD29 glycoproteins are expressed on the cell surface of these cells, while NG2 proteoglycan is almost absent in the cell membrane (Fig. 3), being mostly expressed intracellularly (data not shown).

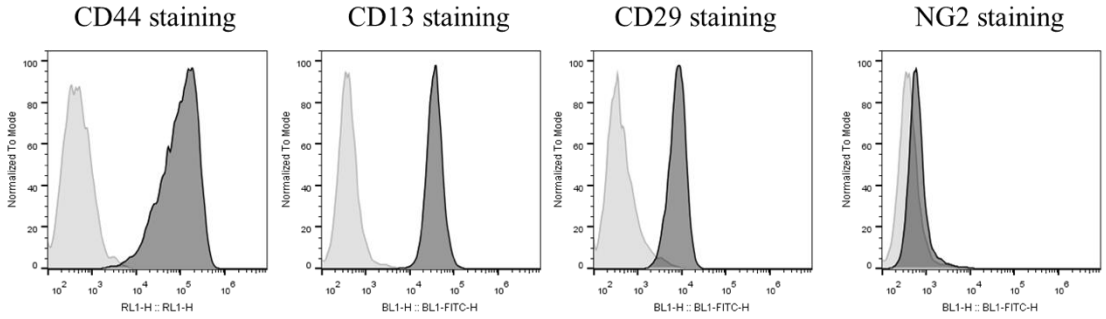

Fig 3: CF1_T breast cancer cells express CD44, CD13 and CD29 on their cell surface. Breast cancer cells were stained with anti-CD44-APC, anti-CD13 plus anti-mouse Ig-FITC, anti-CD29 plus anti-mouse Ig-FITC and anti-NG2 plus anti-mouse Ig-FITC and analyzed by flow cytometry.

3.6. CD44 and CD13 are E-selectin ligands on CF1_T cells

To prove that CD44 and CD13 glycoproteins were E-selectin ligands in CF1_T cells, we immunoprecitated these proteins and stained them with anti-CLA (clone HECA-452) to detect $sLe^{X/A}$ glycans. By western blot, we were able to detect a major isoform of CD44 (≈90KDa) and a minor one (≈180KDa) expressed by CF1_T cells. Comparing with E-

selectin ligands staining in these cells, there are a match between the minor isoform of CD44 and the main band of E-selectin ligands detected by western blot around 180KDa. Checking CD44 IP stained for $sLe^{X/A}$ glycans, we demonstrate that the minor isoform of CD44 is decorated with $sLe^{X/A}$ glycans, being an E-selectin ligand in CF1_T cells (Fig. 4A). Similarly, we tested CD13 protein, which is a ≈150 KDa protein that match the minor band of E-selectin ligands detected on these cells. We demonstrated that CD13 IP was decorated with $sLe^{X/A}$ glycans, giving its role as E-selectin ligand in these breast cancer cells (Fig. 4B). In general, when we analyze these results, it's clear that other proteins with the same molecular weight, predominantly between 180-200KDa, are also involved as E-selectin ligands, being decorated with $sLe^{X/A}$ glycans, being yet to be identified.

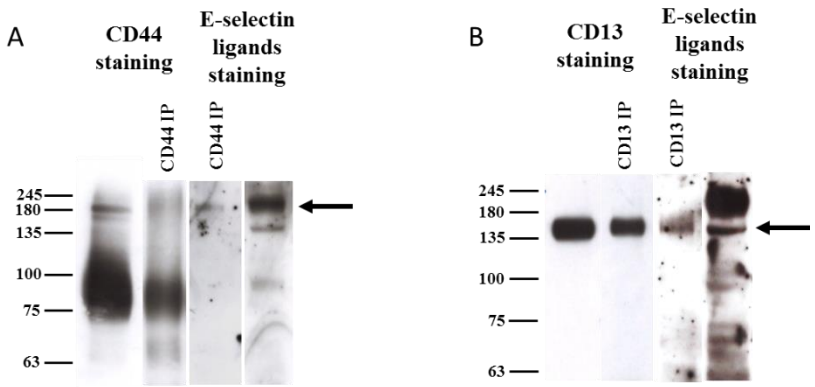

Fig 4: Identification of CD44 and CD13 glycoproteins as E-selectin ligands. A: Lysate from CF1_T cells was subjected to western blotting with anti-CD44 (2C5 clone) mAb (first lane) or HECA-452 mAb (fourth lane). CD44 IP from CF1_T cells were also stained with anti-CD44 (2C5 clone) mAb (second lane) or HECA-452 mAb (third lane). **B:** Similarly, lysate from CF1_T cells was stained with anti-CD13 mAb (first lane) or HECA-452 mAb (fourth lane) and analyzed by Western blot. CD13 IP from CF1_T cells were also stained with anti-CD13 mAb (second lane) or HECA-452 mAb (third lane).

4. Discussion

Changes in glycosylation leading to the expression of aberrant glycans in cancer cells are well documented [29, 30]. High expression of sialyl Lewis-x and -a ($sLe^{x/a}$) glycans in cancer has attracted particular interest because of their function in cancer cell extravasation. In distance metastasis, circulating cancer cells that express $sLe^{x/a}$ engage with the endothelial cell adhesion molecules, mainly E-selectin, leading to the attachment of these cancer cells to the vasculature of a new tissue or organ and further migration into them [31, 32]. Cancer cell adhesion to endothelial cells has been shown to be mediated

by E-selectin in several cancer types, including colon and prostate [9, 10, 12, 33, 34]. However, there are relatively fewer reports characterizing breast cancer cell E-selectin ligands. In the present report we aim to contribute to the better knowledge of breast cancer cell adhesion to E-selectin, identifying two glycoproteins, CD44 and CD13, as E-selectin ligands in a newly established breast cancer cell line.

All previous studies of E-selectin ligands role in breast cancer cells have been performed using well known breast cancer cell lines [16, 27, 35, 36]. Here, we used CF1_T breast cancer cell line, which was recently established from primary tumor tissue from the most common breast cancer cell type, invasive ductal carcinoma [37], in order to avoid possible glycan alterations occurred during long term cell cultures. In addition, this breast cancer cell line came from luminal B subtype, which is one of the breast subtypes to preferentially metastasize to the bone [2]. Taking this together with the fact that E-selectin is constitutively expressed on endothelial cells from bone microvasculature [38], E-selectin ligands seem to play a major role in the formation of bone metastasis in breast cancer.

E-selectin ligands detected using E-selectin chimera molecule in CF1_T cells are expressed throughout their cell surface, extending until the cellular protrusions. This E-selectin ligands expression pattern were already reported before in another breast cancer cell line, MDA-MB-231, which has E-selectin ligands molecules strongly expressed on their cell migrating front, particularly in the “spiking” cellular protrusions [16]. Since not only glycoproteins but also gangliosides (sialylated glycolipids) have been associated with E-selectin engagement in breast cancer [27], we analyzed the expression of E-selectin ligands on CF1_T cells treated, or not, with bromelain peptidase, discovering that glycoproteins were the main glycoconjugates engaging E-selectin in this cell line. It is believed that cell surface glycoproteins are preferred E-selectin ligands since they can extend farther from the cell surface than lipids and therefore can easily make initial contact with E-selectin. Since there are two main glycan types in glycoproteins, O- and N-linked glycans, we characterized the type of E-selectin ligand glycoproteins expressed by CF1_T cells as mostly being N-linked glycans. Currently, the only reported glycoproteins associated with E-selectin engagement in breast cancer are CD44, Mac2bp and CD24. From these three, only CD44 was characterized in terms of the type of glycosylation, which depending on the CD44 variant, the glycans responsible for E-selectin binding can be O-linked or N-linked glycans [17].

Similar to other breast cancer cell lines [16, 27], CF1_T cells adhere to E-selectin under flow conditions, being completely dependent on sialofucosylated glycans. Since fucose residue is involved in multiple interactions with E-selectin lectin domain [39], it's not a surprise that all described E-selectin ligand carbohydrate is fucosylated. Regarding these results, the main carbohydrate structure involved in CF1_T cell adhesion to E-selectin should be sialyl-Lewis x and/or sialyl Lewis a, being both recognized by anti-CLA (HECA-452 clone).

Analyzing an E-Ig immunoprecipitate from CF1_T total protein lysate by mass spectrometry, we obtained a list of proteins that seems to bind to E-selectin. In general, almost 90% of the identified proteins were already described as having a binding property and around 78% as being expressed in the plasma membrane. These results are positive as it assures the used protocol since these are two characteristics that we are looking for in E-selectin ligands. When we narrowed down the total list considering just plasma membrane proteins that can be N-glycosylated, with a high molecular weight (between 80 and 250KDa) and involved in cancer progression, we obtained a list of 11 proteins. Taking in account just the proteins with more than 10% coverage in mass spectrometry analysis, we found the 5 glycoproteins that are more probable to bind to E-selectin in CF1_T cells. ESL-1 have not been described yet in breast cancer cells, but its role as E-selectin ligand have already been highlighted in prostate cancer [40]. In this report, we did not explore ESL-1 role in CF1_T cell adhesion to E-selectin.

So far, we verified that CD44, CD13 and CD29 are expressed on CF1_T cell surface. Since NG2, also known as chondroitin sulfate proteoglycan 4, has already been described as a P-selectin ligand in metastatic breast cancer cells, although this binding was not mediated by $sLe^{x/a}$ structures [41], it is highly probable that NG2 is also able to be recognized by E-selectin. There are reports of other proteins that can bind to two different selectins, such as CD44 that recognize both E- and L-selectin [42, 43]. However, although NG2 is expressed intracellularly (data not shown), it's almost absent on the CF1_T cell surface, not being then the main molecule that mediates E-selectin engagement in these breast cancer cells. CD29 (β1 integrin) and CD49d (α4 integrin) form together the very late antigen 4 (VLA4), which is normally expressed on leukocytes and mediate the firm adhesion of these cells to endothelial cells in the inflamed vasculature through VCAM-I binding [44]. On leukocytes, integrins usually exist in a resting (inactive) non-adhesive state, which are extended to a high affinity (active) adhesive state upon cytokine/chemokine stimulus [45]. In CF1_T cell surface, CD29 are constitutively

expressed in its active form, being able to bind to fibronectin (data not shown) and possibly VCAM-I. Here, we did not explore CD29 role as E-selectin ligand, however it's possible that CD29 is able to bind to both E-selectin and VCAM-I, similarly to MUC1in breast cancer cells that can recognize both E-selectin and ICAM-I [46].

CD44 is a molecule known for its capacity to bind extracellular matrix molecules, such as hyaluronic acid [47] and, when modified with sialofucosylated structures, to mediate E-selectin adhesion [9, 16]. In breast cancer cells, two isoforms of CD44 has already been identified as E-selectin ligands [16, 17]. In CF1_T cells a minor part of CD44 is decorated with sialofucosylated structures recognized by HECA-452 mAb. This CD44 seems to be CD44v4 isoform, which was described before as being able to bind E-selectin through sialofucosylated structures recognized by HECA-452 mAb in breast cancer cells [16], as well as having a similar molecular weight (≈170-180KDa) to the CD44 detected in CF1_T cells. However, analyzing these results, it is clear that CD44 is not the major E-selectin ligand with high molecular weight (≈180-200KDa) in these cells and it will be needed further analysis to identify these proteins.

CD13 expression in cancer has been associated with a shorter overall survival rate and with a number of malignant features, such as cell proliferation, motility, invasion and angiogenesis [18, 23, 48, 49]. Here, we described CD13 as an E-selectin ligand, being decorated with sialofucosylated glycans that are recognized by HECA-452 mAb. CD13 was never before described as being able to bind to E-selectin, however there are studies that associate the expression of CD13 with higher motility, invasion and metastatic potential in cancer [50-52]. For a long time, CD13 association with invasion was mainly linked to its enzymatic function, which was able to degrade collagen from the extracellular matrix (ECM), promoting cell invasion through ECM [51]. In the last 10 years, it has been shown that CD13 binding capacity also play a role in cancer cell motility and invasion [52]. In cancer, CD13 knockdown reduces the formation of lung metastasis, showing the role of CD13 in metastasis development [50]. However, it's still unclear which mechanisms are behind CD13 role in invasion and metastasis. Herein, we uncovered a new mechanism for which CD13 facilitates invasion and metastasis, specifically by binding to E-selectin expressed on endothelial cells. However, more studies are needed to clarify this new role for CD13 glycoprotein in cancer.

Our study thus attributes a role to CD44 and CD13 glycoproteins in cell adhesion by identifying them as E-selectin ligands, which potentially explains one of their functions

in cancer metastasis. In particular, this new role for CD13 as E-selectin ligand turn this glycoprotein a more interesting target for novel anti-cancer therapy approaches.

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Supplementary data:

Table SI: List of all proteins detected by mass spectrometry

Accession number	Entry name	Protein name	Plasm Membrane	N-glycosylation	Mw (KDa)	Coverage in MS
P07355	ANXA2_HUMAN	Annexin A2	Present	Negative	38,604	54,87%
P04083	ANXA1_HUMAN	Annexin A1	Present	Negative	38,714	39,88%
P02786	TFR1_HUMAN	Transferrin receptor protein 1	Present	Positive	84,871	37,04%
P14618	KPYM_HUMAN	Pyruvate kinase PKM	Absent	Negative	57,937	34,65%
P04264	K2C1_HUMAN	Keratin, type II cytoskeletal 1	Absent	Negative	66,039	34,63%
P35613	BASI_HUMAN	Basigin	Present	Positive	42,2	34,39%
P60709	ACTB_HUMAN	Actin, cytoplasmic 1	Absent	Negative	41,737	34,13%
Q9NQC3	RTN4_HUMAN	Reticulon-4	Present	Negative	129,931	33,91%
P08133	ANXA6_HUMAN	Annexin A6	Present	Negative	75,873	33,28%
P07437	TBB5_HUMAN	Tubulin beta chain	Absent	Negative	49,671	32,43%
Q71U36	TBA1A_HUMAN	Tubulin alpha-1A chain	Absent	Negative	50,136	30,60%
P68363	TBA1B_HUMAN	Tubulin alpha-1B chain	Absent	Negative	50,152	30,60%
Q09666	AHNK_HUMAN	Neuroblast differentiation-associated protein AHNAK	Present	Negative	629,101	29,97%
P68133	ACTS_HUMAN	Actin, alpha skeletal muscle	Absent	Negative	42,051	29,62%
P16070	CD44_HUMAN	CD44	Present	Positive	81,538	29,13%
P21333	FLNA_HUMAN	Filamin-A	Present	Negative	280,739	29,04%
P68371	TBB4B_HUMAN	Tubulin beta-4B chain	Absent	Negative	49,831	28,99%
P01857	IGHG1_HUMAN	Ig gamma-1 chain C region	Absent	Positive	36,106	27,88%
O43707	ACTN4_HUMAN	Alpha-actinin-4	Absent	Negative	104,854	27,44%
P13645	K1C10_HUMAN	Keratin, type I cytoskeletal 10	Absent	Negative	58,827	26,71%
P35579	MYH9_HUMAN	Myosin-9	Present	Negative	226,532	25,31%
Q92896	GSLG1_HUMAN	Golgi apparatus protein 1	Present	Positive	134,552	24,85%
P08670	VIME_HUMAN	Vimentin	Present	Negative	53,652	23,20%
P80723	BASP1_HUMAN	Brain acid soluble protein 1	Present	Negative	22,693	22,91%
Q9NZM1	MYOF_HUMAN	Myoferlin	Present	Negative	234,709	22,03%
P08758	ANXA5_HUMAN	Annexin A5	Present	Negative	35,937	21,56%
P35527	K1C9_HUMAN	Keratin, type I cytoskeletal 9	Absent	Negative	62,064	21,19%
P04899	GNAI2_HUMAN	Guanine nucleotide-binding protein G(i) subunit alpha-2	Present	Negative	40,451	20,85%
P50995	ANX11_HUMAN	Annexin A11	Present	Negative	54,39	20,00%
P15144	AMPN_HUMAN	Aminopeptidase N	Present	Positive	109,54	19,86%
O43852	CALU_HUMAN	Calumenin	Present	Positive	37,107	19,73%
Q03135	CAV1_HUMAN	Caveolin-1	Present	Negative	20,472	19,66%
P06576	ATPB_HUMAN	ATP synthase subunit beta, mitochondrial	Absent	Negative	56,56	19,61%
P35908	K22E_HUMAN	Keratin, type II cytoskeletal 2 epidermal	Absent	Negative	65,433	19,56%

P11142	HSP7C_HUMAN	Heat shock cognate 71 kDa protein	Present	Negative	70,898	19,46%
Q14315	FLNC_HUMAN	Filamin-C	Present	Negative	291,022	19,19%
P62820	RAB1A_HUMAN	Ras-related protein Rab-1A	Absent	Negative	22,678	19,08%
O00461	GOLI4_HUMAN	Golgi integral membrane protein 4	Absent	Positive	81,88	18,53%
P12814	ACTN1_HUMAN	Alpha-actinin-1	Present	Negative	103,058	18,39%
P05556	ITB1_HUMAN	Integrin beta-1	Present	Positive	88,415	18,17%
P04406	G3P_HUMAN	Glyceraldehyde-3-phosphate dehydrogenase	Present	Negative	36,053	17,61%
Q14764	MVP_HUMAN	Major vault protein	Present	Negative	99,327	17,58%
Q14847	LASP1_HUMAN	LIM and SH3 domain protein 1	Absent	Negative	29,717	16,27%
P27824	CALX_HUMAN	Calnexin	Present	Negative	67,568	16,22%
E9PJC4	E9PJC4_HUMAN	Transmembrane 9 superfamily member	Absent	Negative	24,263	16,19%
Q10471	GALT2_HUMAN	Polypeptide N-acetylgalactosaminyltransferase 2	Present	Negative	64,733	16,11%
G3XAM7	G3XAM7_HUMAN	Catenin (Cadherin-associated protein), alpha 1, 102kDa, isoform CRA_a	Absent	Negative	92,722	15,81%
P26038	MOES_HUMAN	Moesin	Present	Negative	67,82	15,08%
Q99805	TM9S2_HUMAN	Transmembrane 9 superfamily member 2	Present	Negative	75,776	15,08%
P10314	1A32_HUMAN	HLA class I histocompatibility antigen, A-32 alpha chain	Present	Positive	41,048	15,07%
Q01995	TAGL_HUMAN	Transgelin	Absent	Negative	22,611	14,93%
P12236	ADT3_HUMAN	ADP/ATP translocase 3	Absent	Negative	32,866	13,92%
P35222	CTNB1_HUMAN	Catenin beta-1	Present	Negative	85,497	13,70%
O60716	CTND1_HUMAN	Catenin delta-1	Present	Negative	108,17	13,65%
P68104	EF1A1_HUMAN	Elongation factor 1-alpha 1	Present	Negative	50,141	13,64%
Q9HC07	TM165_HUMAN	Transmembrane protein 165	Absent	Negative	34,906	13,27%
Q6UVK1	CSPG4_HUMAN	Chondroitin sulfate proteoglycan 4	Present	Positive	250,537	13,26%
P02751	FINC_HUMAN	Fibronectin	Present	Positive	262,625	13,03%
P49419	AL7A1_HUMAN	Alpha-aminoadipic semialdehyde dehydrogenase	Absent	Negative	58,487	12,92%
Q07065	CKAP4_HUMAN	Cytoskeleton-associated protein 4	Present	Negative	66,022	12,29%
P23634	AT2B4_HUMAN	Plasma membrane calcium-transporting ATPase 4	Present	Negative	137,92	12,25%
H3BPS8	H3BPS8_HUMAN	Fructose-bisphosphate aldolase A	Absent	Negative	30,427	12,23%
Q05682	CALD1_HUMAN	Caldesmon	Present	Negative	93,231	12,03%

G3XAJ6	G3XAJ6_HUMAN	Raft-linking protein, isoform CRA_c	Absent	Negative	58,763	11,99%
P49755	TMEDA_HUMAN	Transmembrane emp24 domain-containing protein 10	Present	Positive	24,976	11,42%
A0A087WZH7	A0A087WZH7_HUMAN	Myristoylated alanine-rich C-kinase substrate	Absent	Negative	31,596	11,21%
P04843	RPN1_HUMAN	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit 1	Present	Positive	68,569	11,03%
Q53TN4	CYBR1_HUMAN	Cytochrome b reductase 1	Present	Positive	31,641	10,84%
H0Y3Z3	H0Y3Z3_HUMAN	Protein disulfide-isomerase	Absent	Negative	31,544	10,58%
E7EQD9	E7EQD9_HUMAN	Unconventional myosin-Ib	Absent	Negative	34,15	10,56%
Q06830	PRDX1_HUMAN	Peroxiredoxin-1	Absent	Negative	22.110	10,55%
O75955	FLOT1_HUMAN	Flotillin-1	Present	Negative	47,355	10,54%
P10321	1C07_HUMAN	HLA class I histocompatibility antigen, Cw-7 alpha chain	Present	Positive	40,649	10,38%
Q15149	PLEC_HUMAN	Plectin	Present	Negative	531,791	9,99%
Q99536	VAT1_HUMAN	Synaptic vesicle membrane protein VAT-1 homolog	Absent	Negative	41,92	9,92%
P18084	ITB5_HUMAN	Integrin beta-5	Present	Positive	88,054	9,89%
B4E3S0	B4E3S0_HUMAN	Coronin	Absent	Negative	41,604	9,76%
Q07954	LRP1_HUMAN	Prolow-density lipoprotein receptor-related protein 1	Absent	Positive	504,606	9,68%
P00387	NB5R3_HUMAN	NADH-cytochrome b5 reductase 3	Present	Negative	34,235	9,63%
Q7Z7M9	GALT5_HUMAN	Polypeptide N-acetylgalactosaminyltransferase 5	Absent	Positive	106,266	9,57%
Q15942	ZYX_HUMAN	Zyxin	Present	Negative	61,277	9,40%
Q8IVF2	AHNK2_HUMAN	Protein AHNAK2	Present	Negative	616,629	9,37%
P30101	PDIA3_HUMAN	Protein disulfide-isomerase A3	Present	Negative	56,782	9,31%
A0A087X0S5	A0A087X0S5_HUMAN	Collagen alpha-1(VI) chain	Absent	Positive	108,339	9,26%
Q8NBN3	TM87A_HUMAN	Transmembrane protein 87A	Present	Positive	63,43	8,83%
Q00610	CLH1_HUMAN	Clathrin heavy chain 1	Present	Negative	191,615	8,54%
P06396	GELS_HUMAN	Gelsolin	Present	Negative	85,698	8,44%
O75131	CPNE3_HUMAN	Copine-3	Present	Negative	60,131	8,38%
P11021	GRP78_HUMAN	78 kDa glucose-regulated protein	Present	Negative	72,333	8,26%
Q9UKX5	ITA11_HUMAN	Integrin alpha-11	Present	Positive	133,47	8,25%
P48668	K2C6C_HUMAN	Keratin, type II cytoskeletal 6C	Absent	Negative	60,025	7,98%
P02538	K2C6A_HUMAN	Keratin, type II cytoskeletal 6A	Absent	Negative	60,045	7,98%

O00159	MYO1C_HUMAN	Unconventional myosin-Ic	Present	Negative	117,907	7,89%
O14786	NRP1_HUMAN	Neuropilin-1	Present	Positive	103,134	7,73%
P08238	HS90B_HUMAN	Heat shock protein HSP 90-beta	Present	Negative	83,264	7,73%
Q9Y490	TLN1_HUMAN	Talin-1	Present	Negative	269,767	7,67%
F5GWF6	F5GWF6_HUMAN	T-complex protein 1 subunit beta	Absent	Negative	56,806	7,55%
P14625	ENPL_HUMAN	Endoplasmin	Present	Positive	92,469	7,47%
P26572	MGAT1_HUMAN	Alpha-1,3-mannosyl-glycoprotein 2-beta-N-acetylglucosaminyltransferase	Present	Negative	50,878	7,19%
Q92544	TM9S4_HUMAN	Transmembrane 9 superfamily member 4	Present	Negative	74,519	7,17%
P02452	CO1A1_HUMAN	Collagen alpha-1(I) chain	Absent	Positive	138,941	7,10%
Q13813	SPTN1_HUMAN	Spectrin alpha chain, non-erythrocytic 1	Present	Negative	284,539	7,04%
P09525	ANXA4_HUMAN	Annexin A4	Present	Negative	35,883	7,02%
P21589	5NTD_HUMAN	5'-nucleotidase	Present	Positive	63,368	6,97%
P62873	GBB1_HUMAN	Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-1	Present	Negative	37,377	6,76%
P13797	PLST_HUMAN	Plastin-3	Absent	Negative	70,811	6,73%
P00558	PGK1_HUMAN	Phosphoglycerate kinase 1	Present	Negative	44,615	6,71%
Q13740	CD166_HUMAN	CD166 antigen	Present	Positive	65,102	6,58%
P26006	ITA3_HUMAN	Integrin alpha-3	Present	Positive	116,612	6,57%
P02533	K1C14_HUMAN	Keratin, type I cytoskeletal 14	Absent	Negative	51,561	6,57%
P06733	ENOA_HUMAN	Alpha-enolase	Present	Negative	47,169	6,45%
P20073	ANXA7_HUMAN	Annexin A7	Present	Negative	52,739	6,35%
Q14254	FLOT2_HUMAN	Flotillin-2	Present	Negative	47,064	6,31%
Q9NZN4	EHD2_HUMAN	EH domain-containing protein 2	Present	Negative	61,161	6,26%
Q16555	DPYL2_HUMAN	Dihydropyrimidinase-related protein 2	Present	Negative	62,294	6,12%
P08648	ITA5_HUMAN	Integrin alpha-5	Present	Positive	114,536	6,10%
O75976	CBPD_HUMAN	Carboxypeptidase D	Present	Positive	152,931	5,65%
P05362	ICAM1_HUMAN	Intercellular adhesion molecule 1	Present	Positive	57,825	5,64%
O15427	MOT4_HUMAN	Monocarboxylate transporter 4	Present	Negative	49,469	5,59%
O75369	FLNB_HUMAN	Filamin-B	Present	Negative	278,164	5,50%
Q16181	SEPT7_HUMAN	Septin-7	Present	Negative	50,68	5,36%
P04259	K2C6B_HUMAN	Keratin, type II cytoskeletal 6B	Absent	Negative	60,067	5,14%
Q14247	SRC8_HUMAN	Src substrate cortactin	Present	Negative	61,586	5,09%
Q02952	AKA12_HUMAN	A-kinase anchor protein 12	Present	Negative	191,482	5,05%

P07900	HS90A_HUMAN	Heat shock protein HSP 90-alpha	Present	Negative	84,66	5,05%
Q9NR12	PDLI7_HUMAN	PDZ and LIM domain protein 7	Absent	Negative	49,845	5,03%
P05023	AT1A1_HUMAN	Sodium/potassium-transporting ATPase subunit alpha-1	Present	Negative	112,896	4,79%
O75083	WDR1_HUMAN	WD repeat-containing protein 1	Absent	Negative	66,194	4,79%
A0A087WTA8	A0A087WTA8_HUMAN	Collagen alpha-2(I) chain	Absent	Negative	129,151	4,62%
Q14108	SCRB2_HUMAN	Lysosome membrane protein 2	Present	Positive	54,29	4,39%
P23229	ITA6_HUMAN	Integrin alpha-6	Present	Positive	126,606	4,34%
P22413	ENPP1_HUMAN	Ectonucleotide pyrophosphatase/phosphodiesterase family member 1	Present	Positive	104,924	4,32%
Q16706	MA2A1_HUMAN	Alpha-mannosidase 2	Present	Positive	131,141	4,28%
P18206	VINC_HUMAN	Vinculin	Present	Negative	123,799	4,23%
P06756	ITAV_HUMAN	Integrin alpha-V	Present	Positive	116,038	4,20%
P07996	TSP1_HUMAN	Thrombospondin-1	Absent	Positive	129,383	4,19%
O15460	P4HA2_HUMAN	Prolyl 4-hydroxylase subunit alpha-2	Absent	Positive	60,902	4,11%
Q9H4M9	EHD1_HUMAN	EH domain-containing protein 1	Present	Negative	60,627	3,93%
Q9HD45	TM9S3_HUMAN	Transmembrane 9 superfamily member 3	Present	Positive	67,888	3,74%
Q9BSJ8	ESYT1_HUMAN	Extended synaptotagmin-1	Present	Negative	122,856	3,53%
P46821	MAP1B_HUMAN	Microtubule-associated protein 1B	Present	Negative	270,634	3,44%
Q01082	SPTB2_HUMAN	Spectrin beta chain, non-erythrocytic 1	Absent	Negative	274,609	3,38%
Q99829	CPNE1_HUMAN	Copine-1	Present	Negative	59,059	3,38%
P55072	TERA_HUMAN	Transitional endoplasmic reticulum ATPase	Absent	Negative	89,322	3,23%
P35580	MYH10_HUMAN	Myosin-10	Present	Negative	228,999	2,83%
Q01955	CO4A3_HUMAN	Collagen alpha-3(IV) chain	Absent	Positive	161,813	2,69%
P46940	IQGA1_HUMAN	Ras GTPase-activating-like protein IQGAP1	Present	Negative	189,252	2,60%
O00410	IPO5_HUMAN	Importin-5	Present	Negative	123,63	2,28%
Q15643	TRIPB_HUMAN	Thyroid receptor-interacting protein 11	Absent	Negative	227,586	1,59%
P11717	MPRI_HUMAN	Cation-independent mannose-6-phosphate receptor	Present	Positive	274,375	1,04%
P46939	UTRO_HUMAN	Utrophin	Present	Negative	394,466	0,84%

Table SII: List of 11 most probable proteins to play a role as E-selectin ligands identified by mass spectrometry

Accession number	Entry name	Protein name	Plasma Membrane	N-glycosylation	Mw (KDa)	Coverage in MS
P16070	CD44_HUMAN	CD44	Present	Positive	81,538	29,13%
Q92896	GSLG1_HUMAN	Golgi apparatus protein 1	Present	Positive	134,552	24,85%
P15144	AMPN_HUMAN	Aminopeptidase N	Present	Positive	109,54	19,86%
P05556	ITB1_HUMAN	Integrin beta-1	Present	Positive	88,415	18,17%
Q6UVK1	CSPG4_HUMAN	Chondroitin sulfate proteoglycan 4	Present	Positive	250,537	13,26%
P18084	ITB5_HUMAN	Integrin beta-5	Present	Positive	88,054	9,89%
Q9UKX5	ITA11_HUMAN	Integrin alpha-11	Present	Positive	133,47	8,25%
O14786	NRP1_HUMAN	Neuropilin-1	Present	Positive	103,134	7,73%
P26006	ITA3_HUMAN	Integrin alpha-3	Present	Positive	116,612	6,57%
P08648	ITA5_HUMAN	Integrin alpha-5	Present	Positive	114,536	6,10%
P23229	ITA6_HUMAN	Integrin alpha-6	Present	Positive	126,606	4,34%

Chapter 6

Staining of E-selectin ligands on paraffin-embedded sections of tumor tissue

(Paper IV – Submitted to BMC Cancer)

Staining of E-selectin ligands on paraffin-embedded sections of tumor tissue

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Abstract

Background: The E-selectin ligands expressed by cancer cells play a critical role in mediating adhesion of circulating cancer cells to endothelial cells (the initial step of hematogenous metastasis) and in mediating adhesive interactions within tissue microenvironments important for tumor progression. The development of procedures to identify E-selectin ligands within cancer tissue could yield new biomarkers for patient stratification and could aid in identifying novel therapeutic targets. The critical binding determinants of selectin ligands consist of sialylated tetrasaccharides, including the sialyl Lewis X and A (sLe^X and sLe^A), which are displayed on protein or lipid scaffolds. Standardized procedures for immunohistochemistry make use of the antibodies against these carbohydrate determinants. However, antibody binding does not define E-selectin binding activity. **Methods:** In this study, we have developed an immunohistochemical staining technique, using E-selectin-human Ig Fc chimera (E-Ig) to characterize the expression and localization of E-selectin binding sites on paraffin-embedded sections of colon adenocarcinoma. **Results:** E-Ig successfully stained cancer cells and the staining

presented higher specificity and less background than standard antibody-based techniques. **Conclusions:** The E-Ig staining technique allows the qualitative and semi-quantitative analysis of E-selectin binding activity on cancer cells. The development of accurate techniques for detection of selectin ligands may contribute to better diagnostic and better understanding of the molecular basis of tumor progression and metastasis.

Keywords: E-selectin ligands, sialyl-Lewis X, sialyl-Lewis A, cancer

Background

Metastasis is initiated when cancer cells leave the primary tumor and disseminate to other parts of the body, where these cells are able to proliferate and form new tumors. The metastasis of vital organs such as the liver, lungs, and bones is commonly initiated from the dissemination of tumor cells through blood stream. A key and early step of the hematogenous metastasis is the contact of blood-circulating cancer cells with the endothelium. Cancer cells expressing relevant sialofucosylated glycan determinants bind to the endothelial selectins, E- and P-selectin, thereby establishing adhesive interactions with endothelium that resist hemodynamic shear forces. This initial "shear-resistant" endothelial adhesion step is requisite for the transendothelial migration of cancer cells from blood into tissues (1). Since the endothelial selectins are inducible by inflammatory cytokines and expressed constitutively on marrow microvasculature (2, 3), cancer cell binding to selectin is likely to contribute for cancer cell tropism to inflammation sites and to the bone. In addition to their roles in cell adhesion and transendothelial migration, binding to selectins also initiates signal transduction that may promote cancer progression. As an example, in colon cancer, diverse cellular functions such as the activation of SAPK2/p38 (4) and tyrosine phosphorylation of several proteins are induced following engagement of E-selectin ligands (5).

The prototypical selectin binding motif consists of the tetrasaccharide sialyl Lewis X (sLe^X; NeuAc-α(2,3)-Gal-β(1,4)-[Fuc-α(1,3)]GlcNAc-R), or its stereoisomer sialyl Lewis A (sLe^A, NeuAc-α(2,3)Gal-β(1,3)-[Fuc-α(1,4)]GlcNAc-R) (6). Therefore, typically, the identification of selectin ligands is performed using monoclonal antibodies against sLe^X and/or sLe^A, such as HECA-452, in standardized protocols (7-9). The expression of both sLe^X and/or sLe^A has been shown to increase in various cancers in a

progressive fashion, increasing in expression from normal tissue to early stage cancer to metastatic disease (10, 11). *In vitro*, the expression of sLe^X by cancer cells correlates with the cancer cell ability to bind endothelial selectins *in vitro* (12). In tumor tissue, sLe^X and sLe^A expression has been correlated with the metastasis formation by several cancer types, such as hepatocellular, breast and colon carcinoma (7, 8, 13, 14). In colorectal cancers, the expression of sLe^X and sLe^A in the primary lesion is considered a good marker for assessing the metastatic proclivity of colorectal cancer (15). Indeed, expression of these determinants is also correlated with the extent of malignancy, high incidence of recurrence and with decreased survival of patients (16). Importantly, the well-recognized clinically-relevant tumor marker CA19-9 is sLe^A (17).

Nevertheless, the prognostic value of the detection of the carbohydrate sLe^X or sLe^A, as a solely measure to evaluate selectin ligands, is controversial (18, 19) and whether antibodies against sLe^X and/or sLe^A mimic E-selectin binding is questionable (20). Binding to E-selectin itself, contrary to the use of anti- sLe^X antibodies, is more specific for identification of E-selectin binding activity displayed on specific protein scaffolds (6, 21). In addition, there are minor sialofucosylated glycans, which are also carbohydrate determinants of E-selectin ligands, that are not recognized by any current monoclonal antibody (22).

In this study, we developed a novel staining protocol for paraffin-embedded slides of colon cancer tissues, using a mouse E-selectin-human Ig Fc chimera (E-Ig). In contrast to antibodies against the tetrasaccharides sLe^X and sLe^A, the staining with E-Ig offers the advantage of mimicking a physiological process defining a functional benchmark for E-selectin binding.

The E-selectin ligand staining protocol described here stains colon adenocarcinoma cells and produces a consistent membrane staining with little background compared to current staining protocols using antibodies against sLe^X and sLe^A.

Results

The use of a chimeric mouse E-selectin fused to the human Fc region of immunoglobulins (i.e., mouse E-selectin-human Ig Fc chimera (E-Ig)) allowed a successful immunohistological staining of E-selectin ligands in colon adenocarcinoma tissue. The E-Ig staining technique is a three-step procedure that includes an incubation with E-Ig, followed by incubation against E-selectin (anti-CD62E) and then incubation with a

polymer of anti-rat Ig Fc conjugated with horseradish peroxidase (HRP), hereafter named HRP polymer (Figure 1). As shown by the brown staining on cancer tissue, obtained after 3,3'-diaminobenzidine tetrahydrochloride (DAB) color - enzyme detection, the E-Ig staining was scattered and stronger within crypts. The strongest staining signal was on the goblet cells in their apical pole (Figure 2A-D). The lamina propria showed E-Ig staining exclusively on nests of neoplastic cells (Figure 2A and 2C). Staining was not detected in the control assays, run in the absence of anti-CD62E (Figure 2E), or absence of E-Ig (Figure 2F), or when assays were performed in presence of a calcium chelator (EDTA) (Figure 2G), thus confirming the specificity of E-Ig staining. In the normal colon tissue, the strongest staining was in goblet cells (Figure 3) and negligible staining in the lamina propria.

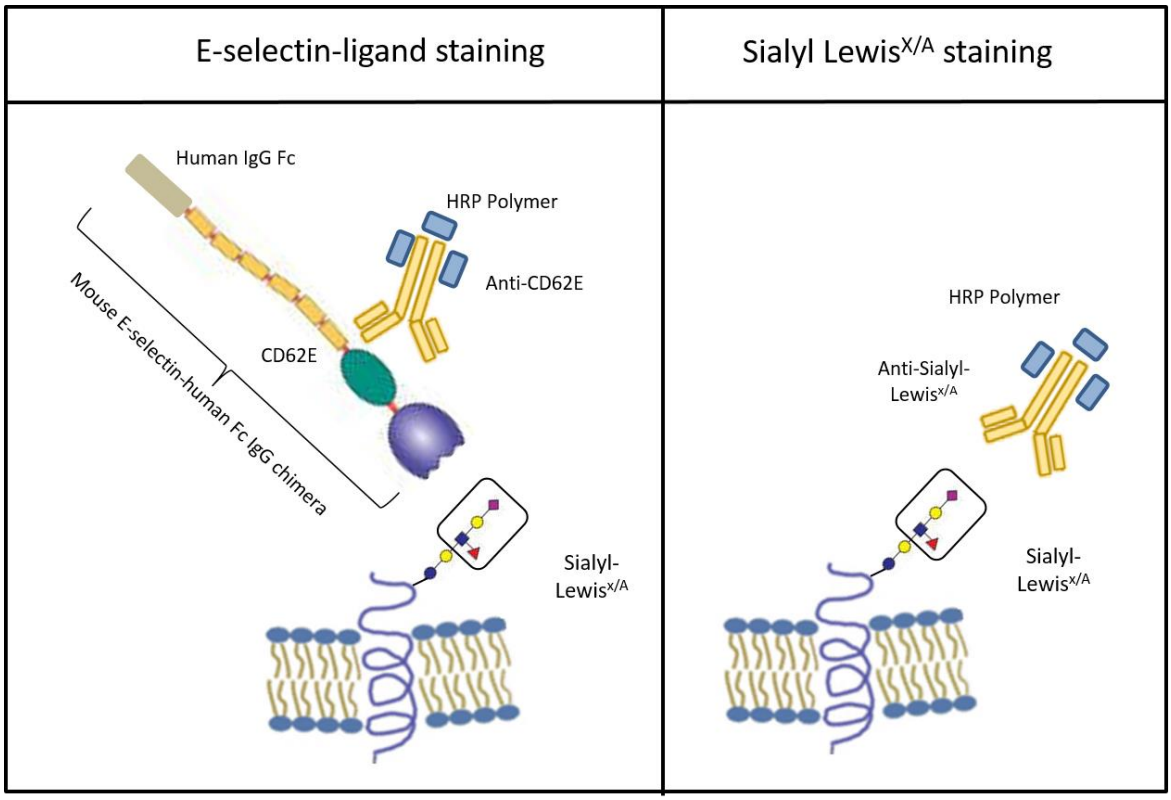

Figure 1. Schematic figure comparing E-Ig and anti-sLe$^{X/A}$ staining technique. E-selectin ligands are recognized by using a three-step staining procedure, where the first staining uses a chimera of mouse E-selectin, i.e. CD62E, with the human IgG Fc (E-Ig). This step is followed by anti-CD62E staining and HRP polymer detection system. The sLe$^{X/A}$ glycan structure is recognized by using anti-sLe$^{X/A}$ antibody followed by HRP polymer detection system.

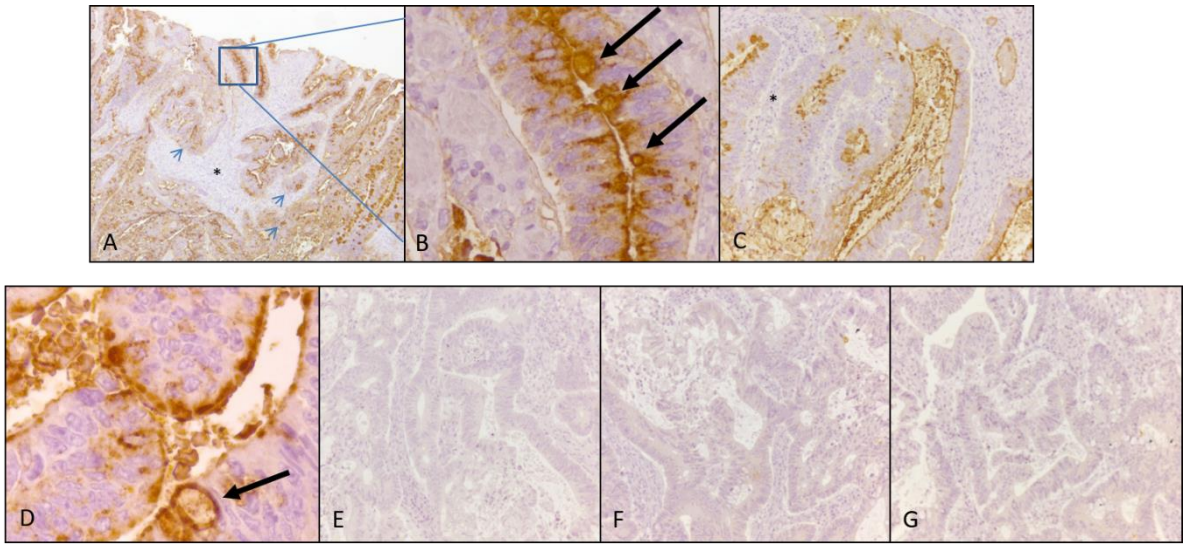

Figure 2. Immunohistochemistry staining of colon adenocarcinoma tissue with E-Ig chimera. Brown color indicates positive reactivity and shows expression of E-selectin ligands in a serial section of the same tissue with 40× magnification **(A)**; 400× magnification **(B)**, 100× magnification **(C)** and 600× magnification **(D)**. B is an increased magnification of the boxed area shown in A, demonstrating the high reactivity with goblet cells (indicated by black arrows in B and D) of the crypts and in particular in their apical pole. In D, it is highlighted the positive reactivity within the lumens of the crypts and in the cellular cytoplasm. In A and C, the asterisks show the lamina propria with no staining, except for nests of neoplastic cells indicated by the blue arrows. Control assays are represented in E, where staining was performed in the absence of anti-CD62E monoclonal antibody; in F where staining was performed in the absence of E-Ig; and in G where assays were performed in presence of a calcium chelant (EDTA).

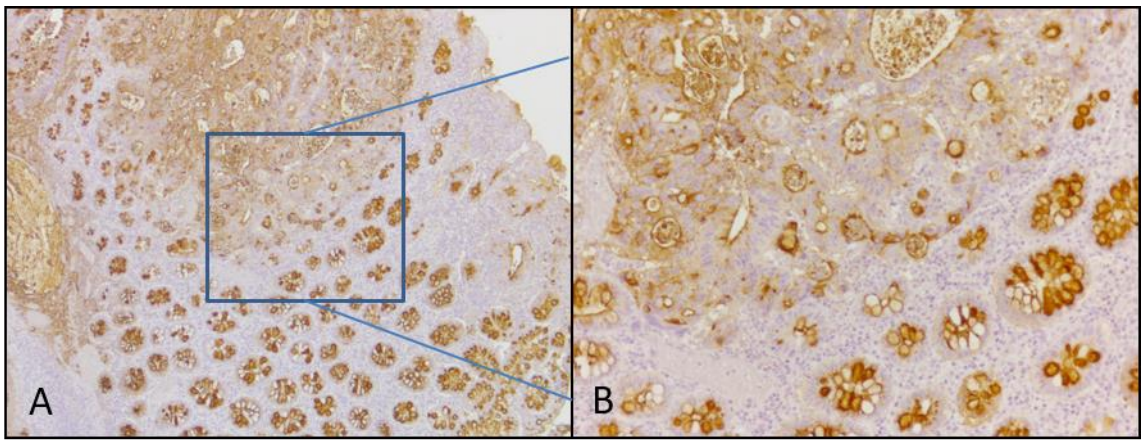

Figure 3. Immunohistochemistry staining of normal colon tissue with E-Ig chimera. Brown color indicates E-Ig reactivity in goblet cells, in a section of normal colon tissue with 40× magnification. **B** is an increased magnification (100×) of the boxed area shown in **A**, demonstrating the high reactivity with goblet cells.

As E-selectin ligands are normally inferred by using antibodies against sLe^X and $sLex^A$ glycans, we compared the E-Ig staining profile with the immunohistochemical expression profile of tissues stained with antibody that recognize both-sLe^X and sLe^A, the HECA-452 clone. HECA-452 staining was stronger in the goblet cells of the crypt similarly to the histochemical profile with E-Ig staining, both in colon adenocarcinoma and normal colon tissue (Figure 4). The lamina propria showed scattered HECA-452 staining, which was not exclusive of neoplastic cells (Figure 4). Comparison of results obtained from E-Ig or HECA-452 staining protocols, suggest that both protocols stain goblet cells and tumor cells. However, E-Ig generates a specific and clearer staining, while HECA-432 staining is scattered, mainly within the lamina propria.

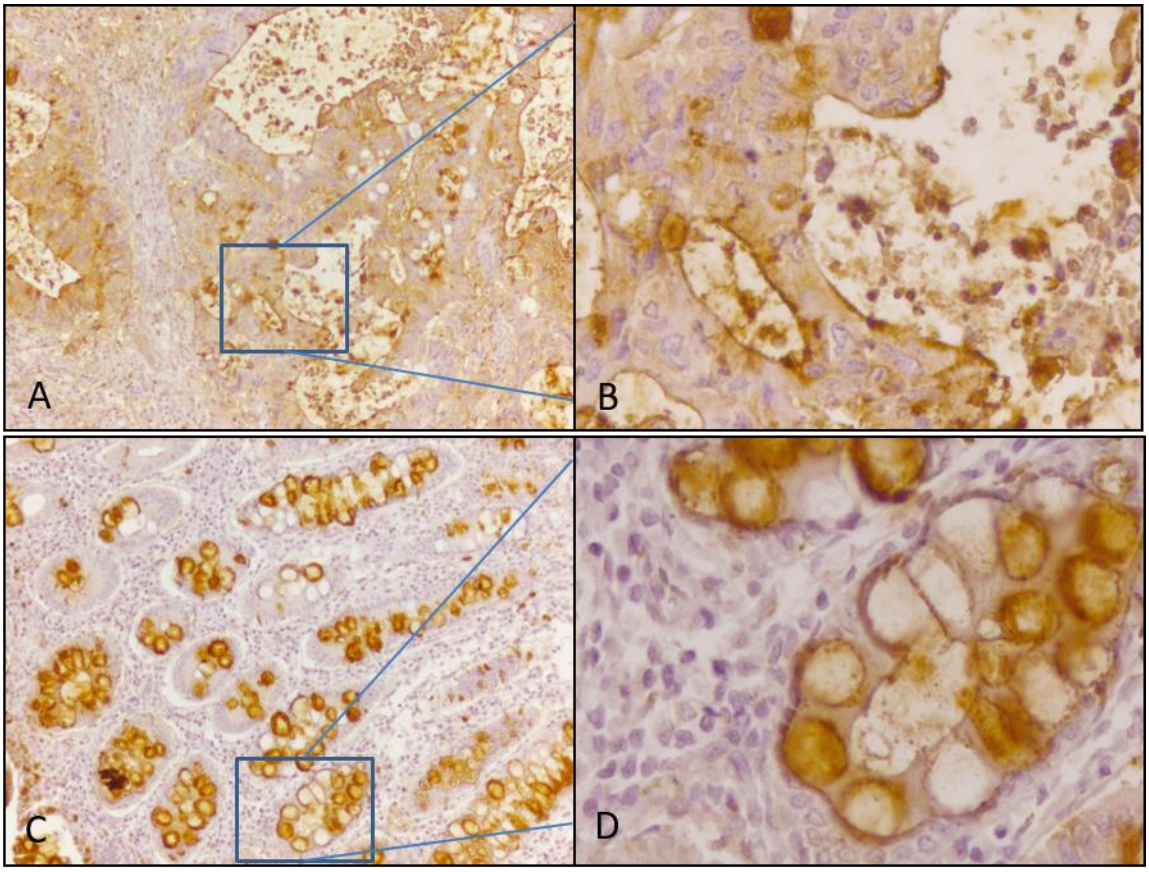

Figure 4. Immunohistochemistry staining of colon adenocarcinoma and normal tissue with HECA-452 antibody. The HECA-452 antibody, that recognizes sLe^X and sLe^A glycans, was used to stain colon adenocarcinoma **(A, B)** or normal colon **(C, D)** tissues. Brown color shows that HECA-452 stains the lumens of the crypts and, in particular, the goblet cells. B and D are increased magnifications (400×) of the boxed area shown in A and B, respectively.

Discussion

Immunohistochemical staining of selectin ligands has been inferred in prior studies for a variety of cancers, based on the expression of sLe^X and sLe^A, detected by appropriate antibodies (7-9). These studies have highlighted that the aberrant expression of sLe^X and sLe^A epitopes by cancer cells is usually associated with higher propensity for cancer progression and metastization. For instance, in gastric cancer, sLe^X expression is an independent risk factor for liver metastasis (7). In mammary carcinoma, sLe^X expression is associated with a higher risk of metastasis (8), and in prostatic carcinoma, sLe^X is also associated with poor prognosis (9). However, despite the fact that expression of sLe^X and sLe^A is closely associated with selectin ligand binding, it is not in itself predictive of E-selectin ligand activity (20).

Here, we have provided evidence of successful staining of paraffin-embedded colon carcinoma tissue with a three-step procedure using an E-selectin chimera (E-Ig). The three-step procedure staining using E-Ig is able to amplify the signal intensity to levels comparable to those obtained using antibodies for immunohistochemistry. Moreover, the use of this E-Ig staining strategy enabled the detection of cancer cells and, compared with anti- sLe^X and sLe^A staining, E-Ig staining was more specific for cancer cells, with anti-sLe^X and -sLe^A staining presented a more diffuse pattern. These relative differences may be due to the fact that while sLe^X and sLe^A serves as a binding determinant, only the clustered display of these tetrasaccharides on specific protein and/or lipid scaffolds determines E-selectin ligand activity (23). The scaffold is necessary to create sufficient sLe^X and sLe^A density to engender E-selectin binding (6, 21, 24). Notably all available anti-sLe^X antibodies, such as HECA-452, cannot block E-selectin binding (25) and blockade of E-selectin, with anti-E-selectin antibodies, is much more effective in blocking cell adhesion to endothelium (26, 27). The E-Ig staining was also able to bind to goblet cells, and in particular to their apical membrane. This is consistent with the fact that goblet cells are the main producers of mucus in colon and mucins are well known E-selectin ligands in colon cancer (28).

The immunohistochemical staining with E-Ig here described was performed using the Lab Vision PTM, which combines deparaffinization, rehydration, and unmasking in one step. As alternative, classic methods of de-paraffinization can be used, and citrate buffer

can also be utilized during 30 min at 98ºC to perform antigen retrieval. Blockade of non-specific binding is critical to obtain specific results. In this protocol, Tween 20 containing buffers, such as TBST has been used and all antibodies/chimera dilutions were performed using Diamond antibody diluent, which contains bovine serum albumin, as blocking agent. As alternative, antibody and E-Ig dilution can be performed in TBST following a pre-blocking step with 5% BSA in TBST for 10 minutes, being then necessary to adjust the dilution of E-Ig and rat anti-mouse CD62E antibody as appropriate. One of the critical aspects of the E-Ig staining protocol described here is incubation in the presence of calcium, since the binding of E-selectin receptor to E-selectin ligands is calcium-dependent. Therefore, it's necessary to add up to 2mM of $CaCl_2$ to both staining and washing buffer solutions. Binding specificity must be always confirmed using EDTA chelation, which has the ability to "sequester" calcium, diminishing its reactivity and inhibiting the binding of E-Ig to E-selectin ligands. In this protocol, we verified that adding 10mM EDTA to both staining and washing buffer solutions, there was an efficient inhibition of the E-Ig staining to colon tissues. During E-Ig staining, the use of a secondary antibody, rat anti-mouse CD62E, is critical since the HiDef amplification and HRP polymer solutions bind preferentially to rat Ig Fc regions and do not recognize the human Ig Fc region contained in E-Ig chimera molecules. Importantly, non-specific binding of the secondary antibody must always be excluded. As an alternative to HiDef amplification and HRP polymer solutions, one can use an anti-rat IgG antibody conjugated with HRP.

Conclusions

The E-Ig staining technique here described allowed for qualitative and semi-quantitative analysis of E-selectin ligands expression and location on colon adenocarcinoma cells and it may be applied to stain other tissues. The development of cancer-specific immunohistochemical staining methods is of paramount relevance, since the tumor tissue samples are often obligatory stored and used in the form of paraffin-embedded blocks, for diagnosis and for patient stratification. As E-selectin ligands play a fundamental role in the metastatic processes of cancer cells of several cancer types, including various adenocarcinomas, this staining technique will also facilitate our understanding of the molecular basis of tumor progression and metastasis.

Methods

Formalin fixed paraffin-embedded colon cancer tissue are sectioned and placed onto slides using standard paraffin microtomy. The Lab Vision PreTreatment Module (PTM) from Thermo Scientific, is used for de-paraffinization and antigen retrieval on tumor sections. After blocking endogenous peroxidases, slides are stained using a three-step procedure with the E-Ig, anti-CD62E and HRP polymer. All the steps in these protocols take place at room temperature. All reagents used are listed in Table 1. All procedures were performed under the approval of the Ethics Committee of Hospital CUF Descobertas.

1. De-paraffinization and heat-induced antigen retrieval

1.1. Place 2-µm thick sections of formalin fixed paraffin-embedded tissue on glass slides (Superfrost Plus, Thermo Scientific) made to ensure firm electrostatic attraction of the tissue sections.

1.2. Dry slides on oven at 37°C overnight.

1.3. Prepare the laboratory instrument, Lab Vision PreTreatment Module (PTM) from Thermo Scientific, to perform de-paraffinization and antigen retrieval of tumor tissue:

1.3.1. Fill each of the PTM tanks with 1.5L of the Trilogy Pretreatment Solution 1x and program PTM to preheat to 60°C and heat to 94°C for 20 minutes.

1.3.2. Start the pre-heating cycle and then mount the slides into the racks and place them into the PTM.

1.3.3. Start the heating cycle, which will heat to 94°C and then cool down to 60°C.

1.3.4. After the heating cycle has finished, take racks out of PTM and wash slides well with distillated water for 1-5 minutes.

1.4. Immerse the slides in 70% ethanol for 10 seconds.

1.5. Immerse the slides in 96% ethanol for 10 seconds.

1.6. Immerse the slides in 100% ethanol for 10 seconds. Repeat once.

2. Blockade of endogenous peroxidases

2.1. Place slides in peroxidase block solution (3% H_2O_2) for 15 minutes.

2.2. Wash the slides with tap water for 1-2 minutes.

2.3. Place the slides in a humidity chamber to avoid the drying of the tissue during the entire staining process. Rinse slides with Tris-Buffered Saline solution with 0.1% Tween 20 (TBST), and leave in TBST for 5 minutes; repeat one more time.

2.4. Wipe off the excess of solutions around the tissue on the slides. Mark a circle around the tissue with a hydrophobic pen.

2.5. Rinse slides with TBST and then leave for 5 minutes in TBST.

3. Staining with chimeric molecules & antibody:

3.1. Mouse E-selectin-human Ig Fc chimera (E-Ig)

3.1.1. Incubate slides with 1:300 dilution of recombinant mouse E-selectin-human Ig Fc chimera (E-Ig) in "Diamond: Antibody Diluent", for 30 minutes. The final E-Ig concentration is 1.67 μg/mL.

3.1.2. Rinse slides with TBST, then place in TBST for 5 minutes. Repeat once.

3.1.3. Incubate slides with 1:250 dilution of rat anti-mouse E-selectin (CD62E) monoclonal antibody in 100 μL of "Diamond: Antibody Diluent", for 30 minutes. The final antibody concentration in TBST is 2 μg/mL.

3.1.4. Rinse slides with TBST, then place in TBST for 5 minutes. Repeat once.

3.1.5. Incubate slides with the "HiDef Detection HRP Polymer System", which detects the primary antibody by amplification of antibody (Amplifier) followed by HRP polymer (Detector), explicitly:

3.1.5.1. Incubate slides with "HiDef Detection Amplifier", for 10 minutes.

3.1.5.2. Rinse slides with TBST, then place in TBST for 5 minutes. Repeat once.

3.1.5.3. Incubate slides with "HiDef Detection HRP Polymer Detector", for 10 minutes.

3.1.6. Rinse slides with TBST, then place in TBST for 5 minutes. Repeat once.

Notes: All solutions in the staining process should have 2mM $CaCl_2$. As negative control, the same staining protocol run without the E-Ig or without the rat anti-mouse CD62E monoclonal antibody or by adding EDTA (final concentration of 10mM) to all the solutions (TBST and "Diamond: Antibody Diluent") during the staining process.

3.2. Staining with anti-sLe^X and anti-sLe^A antibody HECA-452

3.2.1. Incubate slides with 1:50 dilution of rat anti-sLe^X and -sLe^A monoclonal antibody (clone HECA-452) in 100 µL of "Diamond: Antibody Diluent", for 1 hour. The final antibody concentration is 10 µg/mL.

3.2.2. Rinse slides with TBST, then place them in TBST for 5 minutes. Repeat once.

3.2.3. Incubate slides with "HiDef Detection HRP Polymer System":

3.2.3.1. Incubate slides with "HiDef Detection Amplifier", for 10 minutes.

3.2.3.2. Rinse slides with TBST, then place in TBST for 5 minutes. Repeat.

3.2.3.3. Incubate slides with "HiDef Detection HRP Polymer Detector", for 10 minutes.

3.2.4. Rinse slides with TBST, then place in TBST for 5 minutes. Repeat once.

4. Chromogenic detection of HRP and Hematoxylin staining

4.1. Combine 1 part of chromogen concentrate, the DAB, with 20 parts of DAB substrate and mix thoroughly.

4.2. Apply the mix substrate solution to the slide. Incubate slides for 3 minutes. Positive staining produces a dark brown reaction product.

4.3. Immediately after the colour development, wash slides with tap water for 1-2 minutes.

4.4. Incubate slides in hematoxylin, which stains nucleic acids, for 3 minutes.

4.5. Wash slides with warm tap water for 1-2 minutes in order to blueing hematoxylin stain.

5. Dehydration and Mounting

5.1. Immerse the slides in 70% ethanol for 10 seconds.

5.2. Immerse the slides in 96% ethanol for 10 seconds.

5.3. Immerse the slides in 100% ethanol for 10 seconds. Repeat once.

5.4. Immerse the slides in xylene for 10 seconds. Repeat once.

5.5. Add one drop of Quick-D mounting medium to the slide and place the coverslip.

Table 1. List of commercial reagents used in this study.

Name	Company (Country)	Catalog Number	Comments
Lab vision PT Module	Thermo scientific (USA)		
Trilogy Pretreatment Solution	Cell Marque (USA)	920P	Diluted to 1x with ddH_2O
Ethanol	AGA (Portugal)	4.006.16.00.00	Used in 70% and 96% solution and pure.
Peroxidase block solution	Atom Scientific (United Kingdom)	GPC8054-E	
TBS IHC wash buffer with Tween 20	Cell Marque	935B-09	Diluted to 1x with ddH_2O
Diamond antibody diluents	Cell Marque	938B-09	
Hi-Def Detection HRP Polymer System	Cell Marque	954D-30	A polymer containing anti-rat Ig Fc conjugated with HRP
DAB Chromogen/Substrate Bulk Pack (High Contrast)	ScyTek Laboratories (USA)	ACV500	A pack of chromogen concentrate, the 3,3´-Diaminobenzidine tetrahydrochloride (DAB) and DAB substrate
Xylene	Klinipath Netherland (The Netherland)	4055.9005	
Mayer's Hematoxylin	Bio-Optica (Italy)	05-06002/L	

Quick-D Mounting Medium	Klinipath Nederland	7281	
Coverslips	Thermo Scientific	4951PLUS4	
Humidity chamber	Bio-Optica		
E-Ig chimera	R&D Systems (USA)	575-ES-100	Diluted to 1:300 in Diamond: Antibody Diluent
Rat anti-mouse CD62E	BD Biosystems (USA)	550290	Diluted to 1:250 in Diamond: Antibody Diluent
Anti-sLe$^{X/A}$ (HECA-452)	Biolegend (USA)	321302	Diluted to 1:50 in Diamond: Antibody Diluent

List of abbreviations

sialyl Lewis X (sLe^{X})

sialyl Lewis A (sLe^{A})

E-selectin-human Ig Fc chimera (E-Ig)

Competing interests

The author(s) declare that they have no competing interests.

Authors' contributions

MAC carried out the immunohistochemistry protocols and wrote the manuscript. CT and CP helped establishing the immunohistochemistry protocols. PB analyzed and compared both protocols (staining with E-Ig and HECA-452 antibody). MM helped the analysis of the results. SB contributed for science discussion. RS contributed for science discussion and revised the manuscript. PAV conceived he study, and participated in its design and coordination and wrote the manuscript. All authors read and approved the final manuscript.

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Chapter 7

Immunohistochemical staining of E-selectin ligands in Triple Negative Breast Cancer

(Paper V – Manuscript in preparation)

Immunohistochemical staining of E-selectin ligands in Triple Negative Breast Cancer

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Abstract

Triple negative breast cancer (TNBC) has a more aggressive course and increased likelihood of distant recurrence, compared with other breast cancers. E-selectin ligands (E-SL), such as sialyl-Lewis X and A ($sLe^{X/A}$), are expressed by cancer cells and are involved in the first step of cancer cell adhesion to endothelium and further extravasation during the establishment of a new metastasis.

The aim of this study was to determine the relevance of analyzing E-SL expression by immunohistochemistry in TNBC, with regard to clinicopathological features. E-SL were compared with the expression of predictive biomarkers, such CK5/6, EGFR and P-cadherin. Our results show that the expression of basal cytokeratin CK5/6 is negatively correlated with the patient age at diagnosis, while EGFR expression is negatively correlated with tumor size. About the expression of E-SL, it is negatively correlated with the expression of CK5/6 expression, suggesting that E-SL expression is possibly more associated with a less aggressive phenotype in TNBC. Overall, these are the first findings of E-SL expression in TNBC and its correlation with CK5/6. Future studies are warranted to establish the causality of the reported associations.

1. Introduction

Triple-negative breast cancer (TNBC) is associated with an advanced stage, increased risk of visceral disease and worse outcome [1]. It is characterized by not expressing the

three main breast molecular markers: estrogen receptor (ER), progesterone receptor (PR) and epidermal growth factor receptor type 2 (HER2), representing approximately 15% of all types of breast cancer [2]. These tumors are generally larger in size, are of high histological grade (III), with lymph node involvement at diagnosis, and are biologically more aggressive with worse prognosis [3]. The aggressiveness of this subtype of cancer is illustrated by the patient's shorter time to recurrence and median time to death (2.6 versus 5.0 years, and 4.2 versus 6.0 years, respectively) compared to other breast cancer subtypes. More than 70% of women with metastatic TNBC does not survive more than 5 years and all die of the disease, despite having undergone adjuvant chemotherapy and subsequently chemotherapy for advanced disease [4].

TNBC is a heterogeneous disease, including different molecular subtypes. Nearly 80% of all TNBC are classified as the basal-like subtype, based on the expression of molecules usually found in normal basal cells of the breast, such as cytokeratin 5/6 (CK 5/6). Other molecules, such as epidermal growth factor receptor (EGFR), cytokeratin 14 (CK 14), P-cadherin and mast/stem cell growth factor receptor (c-KIT or CD117), have been associated to further stratify TNBC patients, according to their clinical features [5-7].

In fact, there is an unmet clinical need of markers to align TNBC patients to the most appropriate therapies. TNBCs constitute an important clinical challenge, as they do not respond to endocrine treatment and anti-HER2 directed therapy, resulting in an absent of specific treatment guideline for this subtype.

Extravasation of circulating cancer cells from the bloodstream to new tissues or organs is a critical step during the formation of new metastasis. The first step of extravasation consists in the tethering and rolling of circulating cells over E-selectin expressed by endothelium upon inflammation, which slow down the circulating cells promoting their adhesion to the endothelium [8, 9]. The major ligands of E-selectin on the tumor cell surface have been identified as the sialofucosylated glycan determinants, $sLe^{X/A}$, which are recognized by anti-CLA (HECA-452 clone) mAb [10, 11]. The expression of $sLe^{X/A}$ was higher in metastatic lesions compared with primary tissues being associated with distant metastases in breast cancer [12, 13]. However, the relevance of E-SL expression, such as $sLe^{X/A}$, have not been addressed in TNBC.

Here we aimed to study the significance of E-SL expression in TNBC, comparing it with several basal-like markers and clinical features. We analyzed the expression of $sLe^{X/A}$, total E-SL, CK5/6, EGFR, P-cadherin and androgen receptor (AR), which is expressed in more than 70% of breast cancers and has been implicated in breast carcinogenesis [14],

in 20 cases of TNBC. In this study, we reported for the first time a negative correlation between the expression of $sLe^{X/A}$, and total E-SL, and basal marker CK5/6 in TNBC.

2. Material and Methods

2.1. Patients

This study involved 20 triple negative breast cancer patients who underwent surgery, in Hospital CUF Descobertas (Lisbon). Median age of diagnosis was 59 years (Min= 33 years; Max= 87 years). For each patient, tissue specimens were fixed in formalin and embedded in paraffin. The determination of the molecular subtype was performed by the Hospital's pathology laboratory. A summary of the clinical data is available in Table I.

2.2. Immunohistochemistry

Paraffin-embedded sections of breast cancer were heated in trilogy pretreatment solution 1x (Cell Marque) at 94°C for 20min to remove paraffin and recover antigens in Lab Vision PreTreatment Module (Thermo Scientific). After blocking endogenous peroxidase (peroxidase block solution from Atom Scientific), sections were stained with anti- $sLe^{X/A}$ (1:50; HECA-452 clone; Biolegend), anti-CK5/6 (1:200; Cell Marque), anti-androgen receptor (AR) (1:100; Cell Marque), anti-P-cadherin (1:1000; Abcam) or anti-EGFR (1:100; Abcam) for 1h in "Diamond: Antibody Diluent" (Cell Marque) or E-Ig chimera (1:100) for 30min in TBS-T with 2mM $CaCl_2$ (TBS-T-Ca). Then slides were washed with TBS-T (Cell Marque) or TBS-T-Ca. In the latter staining slides, they were still stained with anti-CD62E mAb (1:50; BD Biosystems) for 30 min in TBS-T-Ca. After this step, slides were rinsed with TBS-T or TBS-T-Ca and were stained using "HiDef Detection HRP Polymer System" (Cell Marque) and washed again in TBS-T or TBS-T-Ca accordantly. Then, the color is develop using DAB solution (ScyTek Laboratories). Subsequently, sections were washed with distilled water and stained with hematoxylin (Bio-Optica), prior to dehydration, clearing and mounting with Quick-D mounting medium. The expression of stained cells was divided into four categories: absence or weak labeling (0); weak staining and less than 1/3 of the cells (1); moderate intensity staining of 2/3 of cells (2) and strong staining of 3/3 (3).

2.3. Statistical analysis

The correlation between data was analyzed using Spearman Correlation and unpaired student's t-test was used for the comparison of features. Tests were considered statistically significant when $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***) and marginally significant when $0.05 < p < 0.1$. Statistical analysis was performed using GraphPad Prism 6 (GraphPad Software, Inc).

3. Results

3.1. Patient age at diagnosis is negatively correlated with tumor size

In this study, 20 cases of TNBC were analyzed by immunohistochemistry (detailed results in Table SI and Fig. SI). From these 20 cases, the median patient's age at diagnosis was 59 years (Min= 33 years; Max= 87 years). Regarding tumor size, 25% (5/20) of the tumors were smaller than 10mm, 50% (10/20) were comprised between 10 and 25mm and 10% (2/20) were bigger than 25mm, while 15% (3/20) of the cases were not determined. The majority of the tumors (75%) was high grade (Grade III), poorly differentiated, while the others (25%) were moderate (Grade II). Looking at the numbers of sentinel lymph nodes invaded by tumor cells, 45% (9/20) of the cases reported more than 10 lymph nodes occupied with tumor cells, while 25% (5/20) of the case had less than 10 affected, the others (30%) were not determined (Table I).

Correlating all these patient and tumor features, we detected a negative correlation between patient age at diagnosis and tumor size ($r=-0.542$; $p=0.024$), indicating that, within this TNBC population, the younger patients had the bigger tumors.

Table I. **Patient or tumor features from 20 cases of TNBC.**

Patient or tumor Features	*No. TNBC (N= 20)*	*%*
Age at Diagnosis (y)		
<35	2	10%
35-50	3	15%
51-65	9	45%
>65	6	30%
Tumor Size (mm)		
<10	5	25%
10-25	10	50%
>25	2	10%
not determined	3	15%
Tumor Grade		
1 (well)	0	0%
2 (moderate)	5	25%
3 (poorly)	15	75%
Number of invaded lymph nodes		
<10	5	25%
>10	9	45%
Not determined	6	30%

3.2. CK5/6 and EGFR expression on TNBC are negative correlated with patient age at diagnosis and tumor size, respectively

Immunohistochemical staining of expression of CK5/6 was observed in the cytoplasm (Fig. 2C). In general, CK5/6 expression was absent or very weak in most of the analyzed cases (55%; 11/20). 20% of the cases (4/20) had a weak expression, another 20% (4/20) had a moderate expression and just 1 case (5%) had a strong expression of this basal-like marker. One of the other analyzed basal-like marker was EGFR, which could be detected in the cell membrane (Fig 2E). Similar with CK5/6, half of the TNBC cases (50%; 10/20)

did not had any expression, or very weak, of EGFR. 4 cases (20%) had a weak staining, while another 4 (20%) had a moderate expression of EGFR. Only 10% of all cases (2/20) highly expressed EGFR in their cell membrane (Table II; Fig 2). Testing if these markers were correlated with patient or tumor features, we detected that CK5/6 expression is negatively correlated with patient age at diagnosis (r=-0.459; p=0.042) and EGFR expression is negatively correlated with tumor size (r=-0.519; p=0.033). This mean that younger patients have a higher expression of CK5/6, as well as bigger tumors, and that, in general, patients with bigger tumors have a lower expression of EGFR. We did not find any correlation between $sLe^{X/A}$, E-SL, P-cadherin, which was expressed in 90% of the cases, or AR expression, which was negative in most of the cases (85%), and patient or tumor features. Although $sLe^{X/A}$ and E-SL are associated with lymph node infiltration in breast cancer [10], we did not find any statistically significant correlation between their expression and the number of lymph nodes invaded by tumor cells in these cases. However, when we separate the cases by the median number of affected lymph nodes, $sLe^{X/A}$ expression seems to be slightly higher in the cases with superior number of lymph nodes invaded by tumor cells than the cases with lower number of affected lymph nodes (Fig. 1).

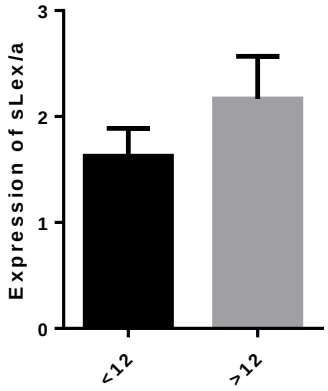

Fig. 1 –TNBC with higher number of lymph nodes invaded with tumor cells tend to have a higher expression of $sLe^{X/A}$ than the TNBC samples with fewer lymph nodes infiltrated with tumor cells. Separating TNBC cases by the median number of lymph nodes invaded with tumor cells (12 lymph nodes), we verified that $sLe^{X/A}$ expression tend to be slightly higher in the cases with more affected lymph nodes (1.62 vs 2.17; p=0.26).

Table II. **Expression of sLe$^{X/A}$, E-SL, CK5/6, P-cadherin, EGFR and AR in 20 cases of TNBC.**

Molecules	***No. TNBC (N= 20)***	**%**
sLe$^{X/A}$ glycans		
0	0	0%
1	8	40%
2	6	30%
3	6	30%
E-SL		
0	0	0%
1	4	20%
2	5	25%
3	11	55%
CK5/6		
0	11	55%
1	4	20%
2	4	20%
3	1	5%
P-cadherin		
Negative	2	10%
Positive	18	90%
EGFR		
0	10	50%
1	4	20%
2	4	20%
3	2	10%
AR		
Negative	17	85%
Positive	3	15%

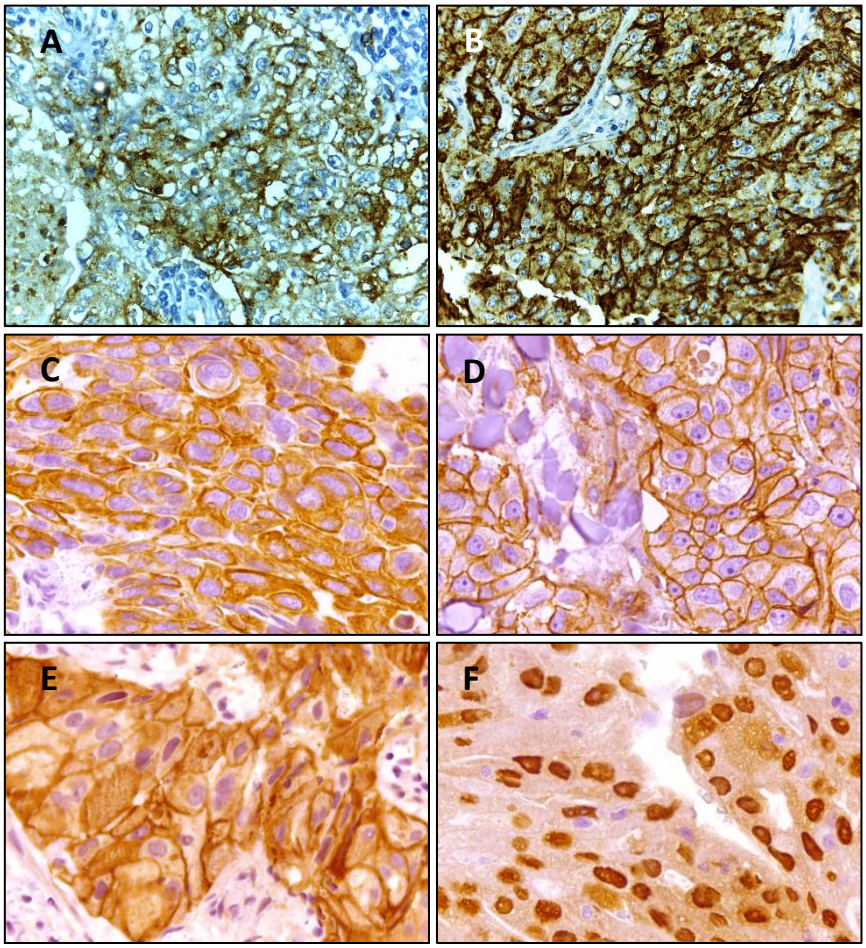

Fig. 2 – Representative microphotographs of immunohistochemical staining against $sLe^{X/A}$ (A), E-SL (B), CK5/6 (C), P-cadherin (D), EGFR (E) and AR (F) in TNBC sections.

3.3. $sLe^{X/A}$ and E-SL expression are positively correlated, being both negatively correlated with CK5/6 expression

E-SL and $sLe^{X/A}$ were expressed in all TNBC cases however with different expression levels. Specifically, $sLe^{X/A}$ expression was weakly expressed in 40% of the cases (8/20), moderated expressed in 30% (6/20) and strongly expressed in another 30% (6/20) of the cases, while total E-SL was weakly expressed in just 20% (4/20), moderated expressed in 25% (5/20) and strongly expressed in 55% of the cases (11/20) (Table II). Here, we verified that total E-SL expression is significantly higher than $sLe^{X/A}$ expression (Fig. 3), indicating that TNBC express not only $sLe^{X/A}$ glycans able to bind E-selectin, but also other glycan structures, such as possibly 3'-Sulfo-Le^{A} or 6-sulfo sLe^{X} [15, 16].

In terms of cell localization, 15% of the cases (3/20) has just cytoplasmic expression of $sLe^{X/A}$, 20% (4/20) express $sLe^{X/A}$ mainly on their cell surface, while 65% (13/20) have

expression of $sLe^{X/A}$ on both cytoplasm and plasma membrane. Regarding the expression of total E-SL, 25% of the cases (5/20) presented just cytoplasmic staining, 15% (3/20) have expression only on their cell membrane, while 60% (12/20) express E-SL on both cytoplasm and plasma membrane. Inflammatory cells within the tissue were also stained for both total E-SL and $sLe^{X/A}$, which is expected since they are known to express E-SL decorated with $sLe^{X/A}$.

As expected, $sLe^{X/A}$ and E-SL expression is positively correlated (r=0.661; p=0.002), showing that the general increase of E-SL expression reflects the intensification of $sLe^{X/A}$ expression.

Comparing the expression of these two markers with the expression of the other analyzed molecules, we verified that $sLe^{X/A}$ expression was negatively correlated with CK5/6 expression (r=-0.479; p=0.033). Similarly, total E-SL expression also tend to be negatively correlated with the expression of CK5/6 (r=-0.418; p=0.066). Comparing the expression of $sLe^{X/A}$ and total E-SL between the negative (expression=0) and positive (expression=1 or 2 or 3) CK5/6 cases, we verified that both markers are significantly more expressed in negative CK5/6 cases compared to the positive ones (Fig. 4).

Comparing the other markers, we did not find any other correlation between their expression.

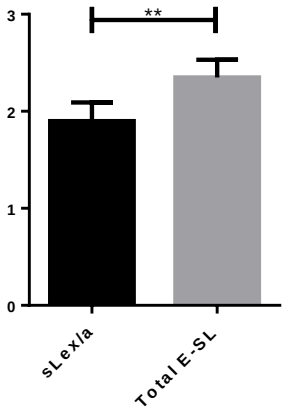

Fig. 3 – Total E-SL expression is higher than just $sLe^{X/A}$ expression in TNBC. Detection of E-SL using E-selectin chimera produce a higher general staining than when just $sLe^{X/A}$ glycan structures are being detected with HECA-452 clone (1.9 vs 2.35; p=0.008).

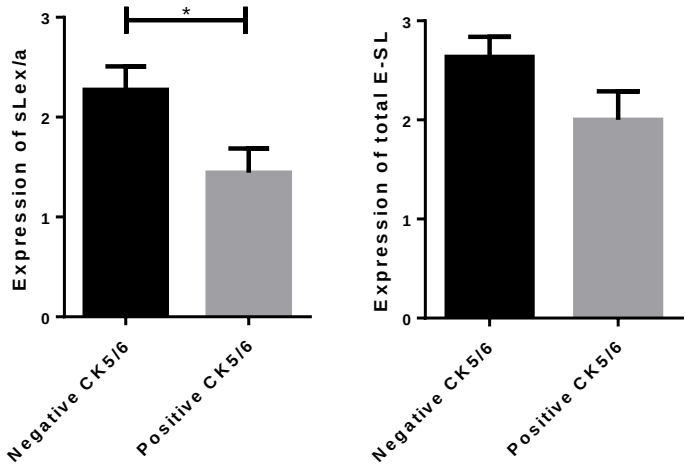

Fig. 4 – CK5/6-positive TNBC have lower expression of sLe$^{X/A}$ and total E-SL than the TNBC samples that do not express basal-like CK5/6 marker. Separating TNBC cases by the expression (values equal to 1, 2 or 3), or not (value of 0), of CK5/6, we verified that the 11 cases that do not express CK5/6 have higher expression of sLe$^{X/A}$ (2.27 vs 1.44; p=0.026) and total E-SL (2.63 vs 2; p=0.081).

4. Discussion

TNBC is strongly associated with a younger patient age at diagnosis, larger tumor sizes and higher grade tumors [4, 5]. The nodal status and tumor size have been shown to be inversely associated with both disease-free interval and overall survival in TNBC [17]. Here we show that younger patients with TNBC have larger tumors than the older ones, indicating that younger patients would have probably a lower overall survival time.

Most of TNBC belongs to the basal-like subtype, which is now characterized by estrogen receptor negative, progesterone receptor negative, HER2 negative and CK5/6 and/or EGFR positive [18]. However, basal-like subtype is not well defined immunohistochemically yet and have being under characterization in the last years. Although the most commonly used basal-like markers are CK5/6 and EGFR molecules, several other markers have been associated with basal-like subtype in later studies, such as c-Kit, P-cadherin, nestin, osteonectin, vimentin, and laminin. So, in fact, the most correct immunophenotype of basal-like tumors would be the positive expression of basal CKs (such as CK5/6 and CK14), EGFR, p53, P-cadherin, caveolins 1 and 2, cyclin E, Ki-67, c-Kit, fascin, Sox2, moesin, vimentin, nestin, and laminin [19]. Here, we selected three of this basal-like markers, CK5/6, EGFR and P-cadherin, in order to show the diversity in this TNBC population, which belongs in fact to basal-like subtype since all samples stained for at least one of the basal-like markers. When analyzing just the

expression of CK5/6 and EGFR, since this is the most common characterization of basal-like subtype, we verified that 75% of the TNBC cases were positive for at least one of this markers, which is in concordance with results obtained from other studies in which detected that 74% of TNBC cases were positive for CK5/6 and/or EGFR [20].

Positivity for CK5/6, which is probably the most frequently used basal cytokeratin, has been largely used to define basal-like cancer. However, studies have demonstrated that only 61-62% of basal-like tumors express CK5/6 [21, 22], not being recommended to be used alone in defining basal-like tumors. Here, only 45% of TNBC cases expressed CK5/6, being this expression negatively correlated with patient age at diagnosis, meaning that younger patients have higher expression of CK5/6. These results are not in agreement with another study that reported that older TNBC patients presented a higher frequency of CK5/6 expression compared to those of younger patients [23].

EGFR expression in this study was observed in 50% of TNBC cases, which is in agreement with other studies that have detected EGFR expression on approximately 57% of basal-like breast cancers [21]. EGFR overexpression in breast cancer has been associated with large tumor size, poor differentiation, and poor clinical outcomes [24]. However, as shown in this study, when we focus in TNBC, the expression of EGFR is negatively correlated with tumor size, indicating that the higher expression of EGFR in TNBC is associated with smaller tumors.

The expression of E-SL, such as sLe^X, has been associated with metastatic lesions in breast cancer and invasive micropapillary carcinoma with lymph node metastasis [10, 12]. However, its expression has never been studied in TNBC. In this study, we analyzed the expression of total E-SL, detected using a E-selectin chimeric molecule, and $sLe^{X/A}$ glycan structures, detected using HECA-452 mAb. We verified that all TNBC cases expressed E-SL and that the results obtained with both methods (detection of total E-SL and of $sLe^{X/A}$ glycans) were significantly correlated. However, since the expression of total E-SL was significantly higher than the expression of $sLe^{X/A}$ glycans, TNBC expresses probably other glycan structures besides $sLe^{X/A}$ that can bind to E-selectin.

On opposite to other breast cancer subtypes, the expression of E-SL was not related with the number of lymph nodes affected by tumor cells in TNBC. However, the expression of both $sLe^{X/A}$ and total E-SL was negatively correlated with CK5/6 expression. In detailed, CK5/6 negative TNBC samples express significantly more E-SL than the CK5/6 positive samples, suggesting an association between E-SL and a non-basal-like phenotype in TNBC. Although there is not an agreement yet regarding the outcome of patients with

CK5/6 expression, there are recent studies that report a worse prognosis with lower overall survival in the patients' group negative for 5 markers (5 negative phenotype), specifically ER, PR, HER2, EGFR and CK5/6, comparing with basal-like subtype (ER-, PR-, HER2-, EGFR+ and/or CK5/6+) [25, 26]. Based on these results, the correlation of E-SL in this studies with negative eCK5/6 can indicate an association with a worse prognosis in TNBC. However, further evaluations are needed to elucidate this matter.

In sum, our results indicate that, within the heterogeneity of TNBC, the expression of CK5/6 is associated with younger patients while EGFR expression is associated with patients with larger tumors. Concerning E-SL expression, $sLe^{X/A}$ and total E-SL expression is negatively correlated with CK5/6 expression, suggesting that E-SL expression is associated with a more aggressive phenotype that would be translated in a lower overall survival in TNBC patients. However, future studies are necessary to clarify the clinical value of these associations.

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Supplementary data

Table SI. Detailed data from patient or tumor features and molecules expression from 20 cases of TNBC.

Tumor Number	*Immunohistochemical staining*						Age	Tumor size (mm)	Tumor grade	Number of invaded lymph nodes
	$sLe^{X/A}$	E-SL	CK5/6	P-cadherin	EGFR	AR (%)				
1	1	1	0	0	1	0%	73	17	3	12
2	1	3	2	1	0	0%	35	25	3	8
3	1	1	2	1	1	0%	59	25	2	15
4	1	3	0	1	0	0%	52	nd	2	3
5	3	3	0	1	0	100%	82	21	3	nd
6	3	3	0	1	2	0%	52	20	3	1
7	1	1	2	1	0	0%	62	23	3	16
8	3	3	0	1	1	0%	67	6	2	15
9	2	3	0	1	1	0%	78	6	3	nd
10	3	3	1	1	0	0%	34	15	3	nd
11	2	3	1	1	3	0%	87	2	3	12
12	1	2	2	1	0	0%	33	70	3	nd
13	2	2	3	1	2	0%	42	25	3	16
14	1	1	1	1	0	0%	65	15	3	1
15	2	2	0	1	0	50%	51	nd	3	nd
16	1	2	1	1	3	100%	56	nd	3	nd
17	3	3	0	1	0	0%	59	25	2	15
18	3	3	0	0	2	0%	45	8	3	19
19	2	3	0	1	0	0%	64	40	2	8
20	2	2	0	1	2	0%	87	2	3	12

Absence or weak labeling (0); weak staining and less than 1/3 of the cells (1); moderate intensity staining of 2/3 of cells (2) and strong staining of 3/3 (3); Not determined (nd)

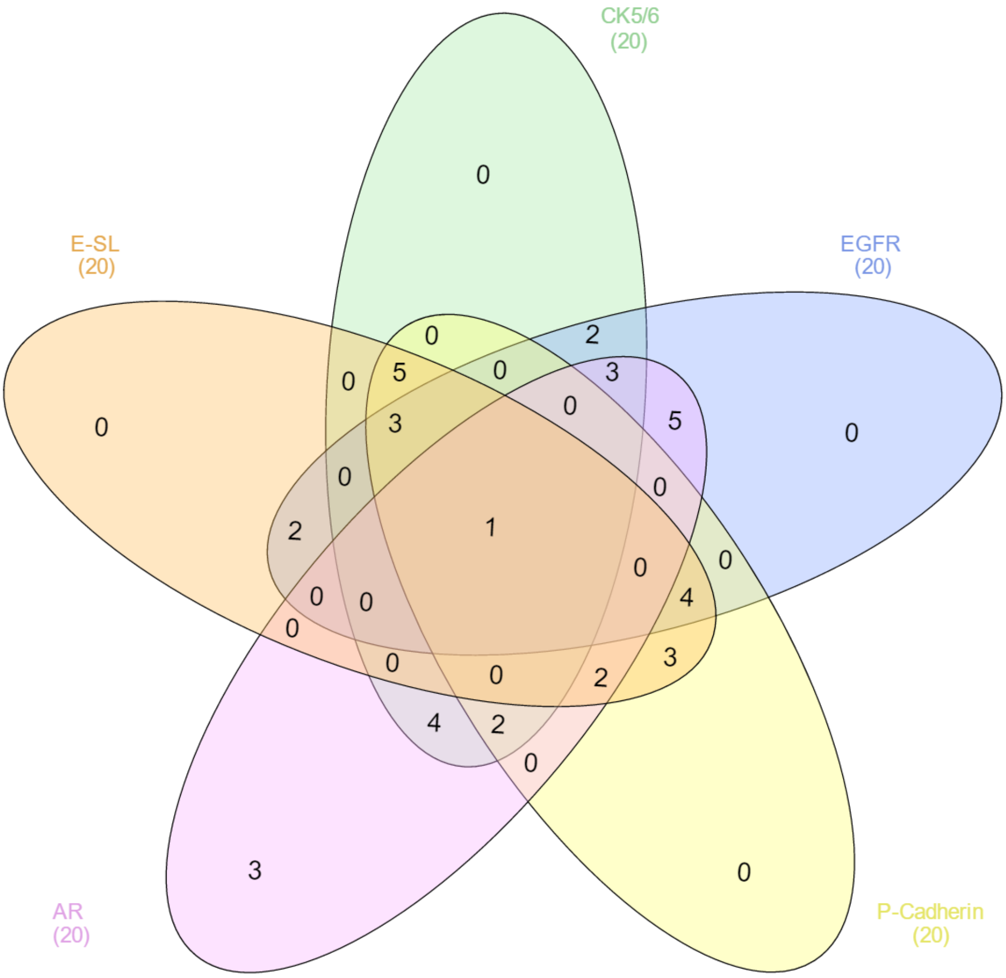

Fig. SI – Expression of E-SL, CK5/6, EGFR, P-cadherin and AR in 20 cases of TNBC represented in a Venn diagram.

Chapter 8

General Discussion

8. General Discussion

Glycosylation is the most frequent posttranslational modification of proteins and lipids. Glycans regulate a diversity of biological processes, including protein folding and intracellular trafficking, cell-cell and cell-matrix interactions, cell motility, cellular differentiation and the immune response [1]. Changes in glycosylation result in alterations in these processes and have long been associated with the development and progression of cancers. In fact, aberrant cell surface glycans, typically found in cancer, modulates cell adhesion, motility and signal transduction [2]. A few distinct glycan structures have been shown to contribute to specific mechanisms leading to gain of functions in cell fitness and survival. This suggests that specific glycosylation patterns, such as increased sialylation, are associated with malignant transformation and tumor progression [3].

Cancer-associated changes in glycosylation include under- or over-expression of glycan structures, as well as the appearance of novel or truncated glycan structures. In addition one of the most commonly reported cancer-associated alterations in protein and lipid glycosylation is an increased content on sialic acid (sialylation), with the increased expression of sialylated glycans such as sialyl Tn (STn) and sialyl-Lewis X (sLe^X) [4]. In this context, the characterization of sialylated glycans in cancer and its association with a specific clinical features and a given cancer type is crucial to understand how those glycans, or the resulting glycoconjugates, are worth as clinical biomarkers. Understanding the underlying mechanism on how glycans affect a number of pathophysiological processes may reveal clues to use them as therapeutic target for that cancer type.

8.1. Elucidation of STn role in dendritic cells (DCs) immune response – A contribution to the development of anti-cancer therapies

When we initiated this thesis it is known that STn was expressed by more than 80% of human carcinomas including gastric, colon, breast, bladder, lung, oesophageal, pancreatic, prostate and endometrial cancer. A high level of STn in these cancers has been mostly associated with tumor size, metastasis and with decreased overall survival of patients [5].

In bladder cancer, we were the first reporting that STn was expressed mostly in high-grade bladder tumors that presented elevated proliferation indexes and high risk of recurrence/progression and invasion [6]. This glycan was not detected in normal bladder

epithelium and its expression in cancer has been associated with a poor prognosis and the invasive capacity of the tumors [6]. This results are in agreement with other studies that have correlated the expression of STn in breast and gastric cancer cells with an increased migration and invasion [7, 8].

Early studies have contributed to elucidate the potential of STn as a biomarker for diagnosis and prognosis in cancer [9-12]. Following studies have suggested a potential for STn as predictive biomarker to assess response to therapy [13]. Interestingly, later works are now focused on using STn as a potential therapeutic target. In this context, it was developed STn-based vaccines, such as Theratope, which consists of STn anchored to the keyhole limpet hemocyanin (KLH) molecule. The immunization with Theratope vaccine, in clinical trials, usually induces the production of STn-specific antibodies. However, in spite of the development of anti-STn antibodies, no significant overall benefit in time to progression or patient survival was observed [14]. In the future, a better understanding of the immune response against STn would contribute in the development of new STn-based immunotherapeutic approaches.

In **Chapter 3**, we report our studies on the influence of STn antigen in the modulation of DCs, which are recognized by their pivotal role in the setting of immune responses. Firstly, in tumor tissues, we reported, in high grade bladder tumors, a significant correlation between the expression of ST6GalNAc1 enzyme, which is responsible for STn biosynthesis [6] and an immature profile of DCs. This immature profile included a higher expression of CD1a immature marker and lower levels of pro-inflammatory cytokines (IL-12 and TNF-α). To further investigate how STn affects DCs maturation, which is a crucial process to effectively develop T cell-specific immune response against cancer cells, we used bladder cancer cell lines overexpressing, or not, ST6GalNAc1, which leads to a high expression of STn glycan. Our data suggests that STn-expressing cancer cells inhibits DCs maturation by reducing the expression of MHC-II and co-stimulatory molecules. This result is also observed using STn-expressing breast cancer cells, which shows that this effect on DCs is specifically mediated by the overexpression of STn in cancer cells. Exploring other DCs functions, we demonstrated that the phagocytic capacity of DCs was increased when presented with apoptotic STn-expressing cancer cells compared with STn negative cancer cells; however, the downstream activation of T cells was negatively affected by STn-expressing, resulting in lower activated T cells, in which predominantly was present $FOXP3^{high}$ IFN-γ^{low} regulatory T cells. These results

are in agreement with previous studies that demonstrated the role of STn-mucins in inducing the immature DC phenotype [15, 16].

DCs have been used as anti-tumor cell-based vaccines, where one of the most common strategies is the upload of patient's DCs *ex vivo* with the tumor cell antigens (lysates, cells fusion or apoptotic bodies), their readministration into the patient where it's expected them to induce anti-tumor T cell responses leading to tumor elimination [17]. As shown in the present study, the use of DC-based immunotherapy would not be efficient in eliminating STn-expressing tumors, since DCs pulsed with immunosuppressive STn+ cancer cells showed a tolerogenic/regulatory profile, resistant to further maturation stimuli and limited capacity to activate T cells. In the context of STn-expressing bladder tumors, DC-based immunotherapy would always have to be combined with other immunotherapeutic approaches that would lead to DC activation, such as *Bacillus Calmette-Guérin* (BCG) immunotherapy, which is a potent inducer of Th1 responses [13]. Our preliminary data also indicates that this would be a good approach since BCG seems to be able to maturate DCs even in STn-expressing cancer cells environment (data not published). Furthermore, there are also other immunotherapies in the clinic that would also enhance the efficiency of DC-based immunotherapy in STn-expressing cancers, such as the use of antibodies against programmed cell death protein 1 (PD1) immune checkpoint, which will inhibit the binding of PDL1 to PD1 that normally leads the inhibition of cytotoxic T-cell response [18].

The development of therapeutic antibodies (Ab) against STn is other promising therapeutic strategy. This would safely direct this therapy just to cancer cells since this glycan is rarely expressed in normal tissue and simultaneously activate the immune cells against them. Recently we did a literature search on the existing anti-STn antibodies and their potential clinical /diagnostic interest, which are reviewed in Loureiro and Carrascal, et al [1]. In sum, the three main produced antibodies that recognize STn are B72.3 clone, which was the first antibody produced against STn, TKH2 clone and HB-STn1 clone. So far, none of these antibodies have been integrated into the clinic, mostly because their specificity is still debatable and the potential therapeutic effect on cancer cells and patient tissues needs to be thoroughly assessed prior to their use as therapeutic agents. However, in the future, the use of these or novel and more specific antibodies against STn would contribute to anti-cancer therapies, by stimulating the immune response as well as by directing it specifically to STn+ cancer cells. Additionally, several engineered antibodies

are currently being used in the clinic, such as antibody-drug conjugates, which consists in an anticancer drug coupled to an antibody that specifically targets a certain tumor marker [19], and bispecific antibodies that are composed of fragments of two different monoclonal antibodies, binding consequently to two different types of antigen [20]. All these strategies are promising in the development of novel anti-STn antibodies.

8.2. Comprehensive analysis of E-selectin ligands expression by breast cancer – Biological behavior and new therapeutic target identification

One of the main concern in cancer progression is the development of metastasis, since it decreases dramatically the probability of patient survival. In breast cancer the leading cause of death compared with other cancer types are metastasis. The sLe^X glycans can participate in the metastatic process by allowing the adhesion of circulating cancer cells to the endothelial selectins, i.e. selectin ligand, thus initiating the dissemination of cancer cells from blood to tissues. In breast cancer, the preferential metastatic site is bone [21, 22]. Interestingly, bone marrow microvasculature is one of the sites in the body where E-selectin is constitutively express [23]. This suggests that breast cancer metastasis to the bone is favored by E-selectin ligand molecules expressed by breast cancer cells and are crucial for the establishment of bone metastasis. Thus, there are an urgent need to better understand the role of E-selectin ligands in breast cancer progression.

The most common histopathological subtype in breast cancer is invasive ductal carcinomas, while in the molecular classification, Luminal A and B subtypes are the most frequent. Thereby, we mainly dedicated our study of sLe^X biological role in the most common subtypes of breast cancer. Nevertheless, since the role of sLe^X glycan in less frequent breast cancer subtypes, such as triple negative breast cancer (TNBC) have never being addressed before, TNBC subtype is also one of the focus of this thesis.

8.2.1. Biological role of E-selectin ligands expression in breast cancer cells – Use of fucosylation inhibitor

All known glycan structures capable to bind E-selectin are fucosylated, including the most common glycans, sLe^X and sialyl-Lewis A (sLe^A) [24]. Thus, the best way of understanding the role of E-selectin ligands during tumor progression is by inhibiting fucosylation, which would prevent the formation of E-selectin ligands. With this aim, we used a fluorinated fucose analog, named 2-fluorofucose (2-FF), which hinder the addiction of fucose residues to glycan structures [25].

In **Chapter 4**, we focus in invasive ductal carcinoma, which is not only the most frequent histopathological subtype, but also the one that seemed to express more E-selectin ligands within the most common subtypes. Thus, we established primary cell cultures from tissues of patients diagnosed with this breast cancer subtype. Since luminal molecular subtype is the most frequent in breast cancer patients, all the primary cell cultures studied were consequently from this subtype, allowing the inclusion of the maximum number of breast cancer patients in this work. Comparing the primary cells, we showed that the expressed E-selectin ligand glycoproteins seem to be identical among the primary cells, suggesting that most of these glycoproteins are specific for this subtype and could be used as new anti-cancer targets.

There are several mechanisms involved in tumor progression, such as tumor proliferation, migration, invasion, angiogenesis and metastasis [26]. In this study, we explored the role of E-selectin ligands expressed by a newly immortalized cell line (CF1_T) from one of the primary cell cultures, which will represent this population in study, in most of the mechanisms characteristic of tumor progression. Specifically, we presented that, when CF_1 breast cancer cell line was treated with 2-FF, the most common glycoproteins that used to bind to E-selectin lose that capacity. This effect was even more evident when these cells were tested in flow conditions adhesion assays, which confirms that the use of fucosylation inhibition with fluorinated analogs of fucose leads to the reduction of metastatic ability. We also showed that the removal of functional E-selectin ligands by this method will also affect negatively other features associated with tumor progression, such as proliferation and migration.

Tumor growth is one of the major features of carcinogenesis, being dependent on cells gaining the potential to replicate limitless, which is reached when the cells are able to avoid apoptosis, to be autonomous in the production of growth factors and insensitive to anti-growth signals [26]. We presented here that cells that do not express functional E-selectin ligands, express less growth factors, which by consequence negatively influence the activation of ERK1/2 and p38 pathways that are responsible for cell proliferation and migration [27, 28]. These results prove that functional E-selectin ligands are essential for the development of metastasis but also for tumor cell proliferation and migration. Since the expression of VEGF, VEGFR-1 and VEGFR-2 molecules, which are highly associated with the development of angiogenesis [29], are negatively affected by the removal of fucosylated structures, such as E-selectin ligands, it is probable that E-selectin ligands can also play a role in the formation of new vessels in tumor environment.

Obviously we can't ignore that the observed outcome by the treatment of 2-FF can also be partially an effect of other fucosylated glycans that are being down-regulated by the treatment with 2-FF.

With this work, it is clear that fucosylation is a promising target for cancer therapeutics. Specifically, the anti-tumorigenesis effect of 2-FF compound in breast cancer indicates that 2-FF would be a potential new drug to be used in this cancer in order to avoid metastasis, specially bone metastasis. However, the effect of 2-FF compound in other cells from the organism, such as immune cells, is too harmful, which would cause severe side effects. This was observed when 2-FF compound was administrated in mice and their immune cells, such as neutrophils, lost the ability to extravasate to inflammatory sites [25]. Therefore, in order to use this compound, or any similar fucosylation inhibitor, in cancer therapy, would have to be using a method to direct the drug specifically to cancer cells, such as targeted drug delivery system based on nanoparticles, or antibody–drug conjugates [30].

In combination with this therapy, inhibitors of RAS/RAF/MEK/ERK pathway, such as Trametinib and Dabrafenib, which are already approved by FDA in melanoma treatment [31], or growth factor inhibitor, as for example Ramucirumab, which is an antibody that blocks the interaction of the human vascular endothelial growth factor- receptor 2 (VEGF-R2) with its ligands [32] or Bevacizumab, which binds VEGF and prevents the interaction of VEGF to its receptors [33], can be used to effectively prevent tumor growth and progression.

8.2.2. Discovery of new E-selectin ligand glycoproteins – A contribution for novel cancer targets

Since E-selectin ligands are so important for tumor progression, there are a need to identify them to develop specific therapeutic agents to each possible target. In **Chapter 5**, we characterize the most common E-selectin ligands expressed by CF1_T cell line as being mainly N-glycosylated proteins. Then, these glycoproteins were analyzed by mass spectrometry, which identified the 5 most probable glycoproteins able to bind E-selectin. So far, we identified CD44 and CD13 glycoproteins as presenting the correct glycan structures ($sLe^{X/A}$) to bind E-selectin, suggesting that they have a role in the first step of extravasation (tethering and rolling of circulating cells by binding with E-selectin expressed by vascular endothelium). Further studies are still needed to uncover other glycoproteins that can play a role as E-selectin ligands in breast cancer.

Over the last 10 years, clinical studies have investigated the role for the high expression of CD44 found on breast cancer cells. However, most studies did not report any statistical significance between CD44 overexpression and growth/metastasis of breast cancer. Nevertheless, several *in vitro* studies have reported that CD44 seems to be involved in several cell functions, including cell proliferation, migration, adhesion, and signaling [34]. Here, we demonstrated that part of CD44 expressed by CF1_T cell line is a sialofucosylated glycoform able to bind E-selectin. However, the overall analysis of these results indicates that CD44 is not the major E-selectin ligand expressed by this cell line. Although CD44 seems like a promising therapeutic target in cancer treatment, it's believed that further investigation is needed to clarify the role of CD44 completely before its use as a successful marker or anti-cancer target.

The other identified glycoprotein is CD13, which has been described in many tissues, organs, and cell types, including breast cancer cells. CD13 is a metalloproteinase involved in the degradation of extracellular matrix, which promotes malignant cell invasion into the bloodstream [35]. For a long time, CD13 association with invasion was mainly linked to its enzymatic function, however lately CD13 binding capacity has been shown to also play a role in cancer cell motility and invasion [36]. In this work, we uncovered a new mechanism for which CD13 facilitates invasion and metastasis, specifically by binding to E-selectin expressed on endothelial cells, showing that CD13 have a dual function during cancer invasion. Therefore, CD13 would be a key target in cancer metastasis. In this context, some synthetic compounds capable of inhibiting the enzymatic activity of CD13 have been studied regarding their effect in cancer metastasis. One of the most studied is bestatin, which is a cancer chemotherapeutic drug in some countries, and has been shown to effectively reduce the formation of metastasis *in vivo* [37]. In the future, a drug, or a mixed drugs, that could effectively abrogate both functions of CD13 during cancer metastasis would be the ideal anti-CD13 cancer therapy to prevent metastasis development.

8.2.3. Characterization of E-selectin ligands expression in Triple Negative Breast Cancer (TNBC)

One of the most commonly used techniques to analyze the expression of markers in cancer samples, helping in the diagnosis and treatment of cancer, is immunohistochemistry. This technique also allows us to relate the expression of several potential markers with clinical cancer features [38].

Since there was a need for a protocol by immunohistochemistry that could detect all molecules able to bind E-selectin protein, in **Chapter 6**, we presented a novel established protocol using E-selectin chimera (E-Ig) molecule. This new protocol allows the screening of all E-selectin ligands expression in paraffin-embedded sections of cancer by immunohistochemistry, which was not possible before, since the protocols using antibodies against the $sLe^{X/A}$ glycan structures missed some minor glycan structures that bind E-selectin [39].

Therefore, in **Chapter 7**, we used both protocols, using E-Ig and HECA-452 antibody (anti-$sLe^{X/A}$), to detect E-selectin ligands expression and relate it to clinical cancer features in TNBC by immunohistochemistry. TNBC represents approximately 15% of all types of breast cancer and is associated with an advanced stage and worse outcome [40]. Basal-like subtype, which includes the majority of TNBC, is not well defined immunohistochemically yet and have being under characterization in the last years. The most commonly used basal-like markers are CK5/6 and EGFR molecules, however, there are several other markers associated with this subtype, including P-cadherin, c-Kit and CK14 [41]. In this study, we showed that the detection of E-selectin ligands expression is higher when using the novel E-Ig staining protocol than when the staining is performed using just the anti-$sLe^{X/A}$ antibody, demonstrating that in fact the antibody in use is not able to detect all molecules that bind E-selectin, being the expression of E-selectin ligands in this case not complete. The expression of both $sLe^{X/A}$ and total E-selectin ligands was negatively correlated with CK5/6 expression in TNBC. Specifically, CK5/6 negative TNBC samples express significantly more E-selectin ligands detected with both protocols than the CK5/6 positive samples. The clinical relevance of CK5/6 expression in breast cancer remains unclear and. However, the five negative phenotype (ER-, PR-, HER2-, EGFR- and CK5/6-) has been associated with a worse prognosis and lower overall survival compared to basal-like phenotype in TNBC [42, 43]. Therefore, the negative correlation of E-selectin ligands with CK5/6 expression indicates that the high expression of E-selectin ligands can be associated with a more aggressive phenotype that would translate in a worse overall survival and prognosis in TNBC patients. However, further analysis is being conducted to explore the overall survival rate of the patients involved in this study and in the future, we hope to clarify the role of E-selectin ligands expression in TNBC patients' outcome.

8.3. Conclusion

Overall, we uncovered the role of two sialylated glycans, STn and $sLe^{X/A}$, in tumor progression. Specifically, STn-expressing cancer cells induce a more immature profile in DCs, which leads to a tolerogenic immune response, helping the tumor to escape of being eliminate by the immune system. Since the immune response of DCs can be prone by the use of antibodies against STn antigens and STn+ glycoproteins, these molecules are potential therapeutic targets for circumventing tumor-induced tolerogenic mechanisms. Furthermore, we showed that the inhibition of fucosylation led to a complete absent of functional E-selectin ligands on breast cancer cells, which decreased cell proliferation and migration, affecting negatively the expression of growth factors and consequently reducing the activation of ERK1/2 and p38 MAPK pathways. These data show that fucosylated structures, mainly functional E-selectin ligands decorated with $sLe^{X/A}$ glycans, have a major impact on malignant features, contributing to tumor progression. Analyzing the protein scaffolds that constitute E-selectin ligands in breast cancer, we identified two glycoproteins, CD44 and CD13, which are decorated with $sLe^{X/A}$ glycans. Both glycoproteins are promising targets to avoid the development of metastasis. As a therapeutic target, CD13 glycoprotein, which had never before been described as an E-selectin ligand, are of particular interest since it plays a dual role in extravasation, by binding the cancer cells to endothelium through CD13/E-selectin interaction and by promoting the degradation of extracellular matrix leading to cell invasion. Finally, the expression of E-selectin ligands evaluated in TNBC is correlated with no or low expression of CK5/6. Since the negative expression of CK5/6 has been associated with poor prognosis in TNBC, it suggests that the high expression of E-selectin ligands can also be related with a worse outcome in TNBC; however, further studies are needed to clarify this association. Overall, these findings will contribute to the development of novel anti-cancer therapies based on sialylated glycans and/or their protein scaffolds.

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Chapter 9

Other Contributions

Herein included:

- Chapter 4 “Sialylation and dendritic cells: bridging innate and adaptive immune responses” From the book: Carbohydrate Chemistry: Volume 37 (2011)
- Overexpression of tumour-associated carbohydrate antigen sialyl-Tn in advanced bladder tumours - Molecular Oncology 2013; 7(3):719-31
- Challenges in Antibody Development against Tn and Sialyl-Tn Antigens - Biomolecules 2015; 5(3):1783-809
- CEA as an E-selectin ligand in non-small cell lung cancer – Manuscript in preparation

Not included here (co-author):

- The phagocytic capacity and immunological potency of human dendritic cells is improved by α2,6-sialic acid deficiency – Immunology 2013;138(3):235-45
- Expression of sialyl-Tn sugar antigen in bladder cancer cells affects response to Bacillus Calmette Guérin (BCG) and to oxidative damage – Under major revision on Oncotarget
- Genetic instability and BCG response in bladder cancer cells expressing sialyltransferase ST3GAL1 – Submitted to Journal of Cellular and Molecular Medicine
- Effect of the triterpene 3β,6β,16β-trihydroxylup-20(29)-ene isolated from the leaves of Combretum leprosum Mart. on in vitro scratch wound assay – Submitted to Wound Repair and Regeneration

Sialylation and dendritic cells: bridging innate and adaptive immune responses

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Dendritic cells (DCs) surface, like all mammalian cells, is covered by proteins and lipids that are glycosylated. The attached glycan chains are complex structures with a high content of sialic acids. These sugars usually occupy a terminal position, thus representing key structural determinants for a number of receptors, such as lectins, and are potential modulators of DC responses. DCs are crucial players as innate and adaptive immune responses linkers, and have been exploited, with limited success, as therapeutic tools to trigger immunity against tumours or pathogens. However, an unmet clinical need is finding means to shape DC immune functions, to improve and diverse its applicability. This chapter will focus on the regulation of sialylation and sialic acid patterns in DC development and maturation and on evidences of DC immune responses modulation by sialic acids. Recently known roles of sialylation in specialized processes, such as endocytosis, recruitment, capacity for T cell priming and pathogen and tumour cell recognition, are herein reviewed. The study of DC *sialome* and its relevance is a work in progress, with promising implications for DC biology and therapeutic applications.

1. Introduction

Dendritic cells (DCs) are essential components of innate immunity due to their pivotal role in triggering and regulating both innate and adaptive immune responses. One of their major features is their capacity to uptake foreign antigens in the periphery and then migrate to draining lymph nodes, to present the captured antigens to T cells, thus triggering the adaptive immune responses [1]. In this way, DCs can protect the host from pathogenic microorganisms and elicit cytotoxic T cell immunity against tumour cell. DCs are also involved in the recognition of self antigens and therefore contribute to the maintenance of peripheral tolerance and prevention of autoimmune diseases. For a general overview of DC main function see Figure 1.

The need for harnessing and, in some aspects, improving DCs' immune potential led to a significant increase of research and generated substantial knowledge about these immune cells. Presently, DCs are being exploited in adoptive immune therapy to treat human malignancies, infectious diseases or dampen hypersensitivity responses [2, 3]. The most common approach involves the use of DCs derived *in vitro* from precursors such as monocytes

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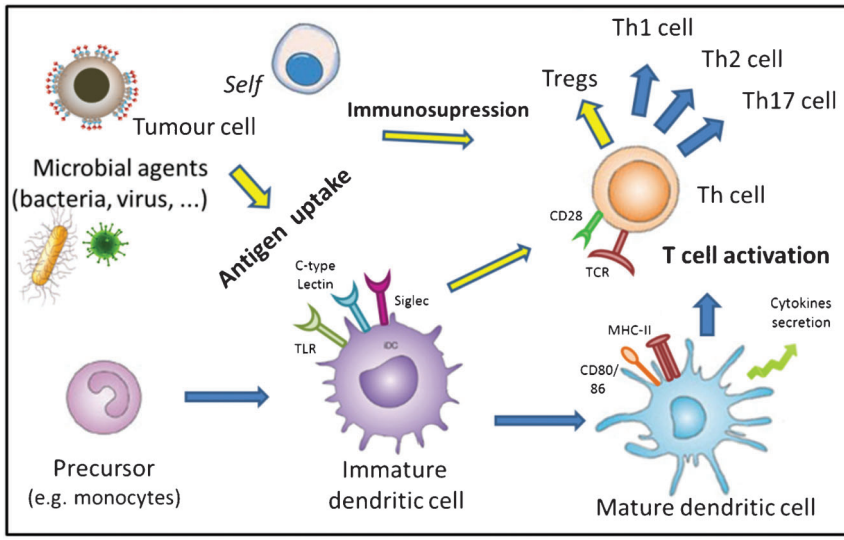

Fig. 1 General overview of dendritic cell main functions.

(mo-DCs), or isolated from patients. These DCs after being loaded with tumour antigens, proved to grant protective and therapeutic actions in cancer patients [4]. Hitherto, the FDA has approved Provenge (Dendreon Corp.'s), a DC-based vaccine for the treatment of advanced prostate cancer [5].

While extremely promising, the benefits of DC-based therapy are still modest and further studies focused on *ex vivo*-generated DCs interaction with tumour cells and pathogens will allow us to find means of eliciting *in vivo* an appropriate immune response.

The surface of DCs, like all mammalian cells, is covered by glycolipids and glycoproteins, many of which are terminated by sialic acids attached to the underlying glycans. Sialic acids are negatively charged nine-carbon sugars, that when added to glycans alters significantly the glycan complexity, thus influencing a number of biologic processes [6]. Sialylation of DCs is regulated both during differentiation and maturation and is variable among DC subtypes and subsets. Recent findings highlight the pleiotropic sialic acids' role in the modulation of DC immune functions. Added to this, lectin receptors (glycan-binding proteins) that recognize sialic acid-bearing glycans on DCs include Siglecs, selectins, and other lectins with roles in intracellular signalling, leukocyte adhesion and migration and antigen recognition [7]. For a general overview of the DC functions that are known to be modulated by sialylation see Figure 2.

Understanding the sialic acids' participation in DC function, its recognition by other cells, including pathogens, as well as the DC lectins' role in intra- and inter-cellular communication is essential to control DC-mediated immunity.

In this chapter, we first describe DC functions, including pathogen and tumour recognition. We then discuss general evidences of the modulation of immune responses by sialic acid, followed by an exhaustive updated

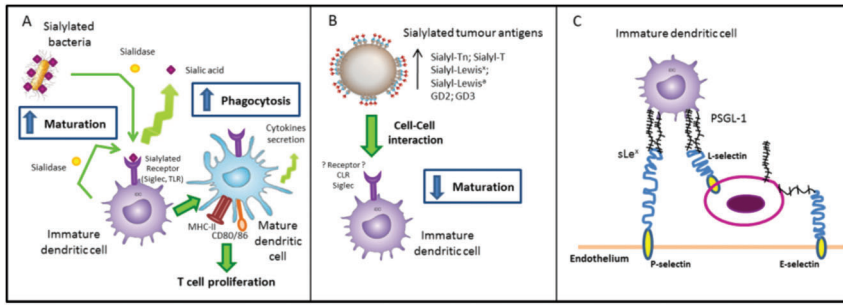

Fig. 2 General overview of the dendritic cell functions that are known to be modulated by sialylation. A - Microbial recognition and immune responses. B - Tumour cell recognition. C - Recruitment and interaction with endothelium.

description of this modulation in the case of DCs. In particular, the regulation of sialylation and sialic acid patterns in DC development and maturation and recently known sialylation roles in specialized processes, such as endocytosis, recruitment, capacity for T cell priming and pathogen and tumour cell recognition. The role of lectin receptors on DC functions and sialic acid recognition will be also addressed.

2. Dendritic cells

DCs play a pivotal role in immune responses. They are special antigen-presenting cells that uptake, process and present to lymphocytes, antigens found in peripheral blood and tissues; thus regulating cellular and humoral immune responses [1]. Due to their unique features, DCs are essential elements at the interface of the innate and adaptive immunity.

In the first place, antigen (e.g. pathogens) uptake by immature DCs is performed by plethora of highly efficient endocytosis mechanisms: i) by receptor mediated endocytosis, which consists in the uptake of small molecules after their recognition by surface membrane receptors, ii) by phagocytosis or ''cell eating'', the uptake of large particles, such as apoptotic and necrotic cells, viruses and bacteria, while also involving specific cell-surface receptors, and iii) by macropinocytosis, a non-selective uptake process of solute molecules, nutrients and antigens which is constitutive and the major pathway of antigen capture in DCs [8, 9] The uptake of antigens, as well as specific inflammatory stimuli induce DC maturation, enabling then the proficient DC interaction with T cells and the initiation of adaptive immune responses [10]. Interestingly, DCs constitutively capture and present self antigens, in steady state conditions, without undergoing through maturation changes, in which case they mostly participate in self-tolerance induction processes [11, 12].

DC maturation is characterized by phenotypic and physiologic changes. The antigen-uptake machinery is rapidly downregulated [13] and the lysosomal compartments are acidified to optimize antigen processing. The reorganization of major histocompatibility complex class I and class II (MHC I and MHC II) molecules allows the antigen presentation for T cell

recognition, which happens mainly in secondary lymphoid organs to where antigen-loaded DCs migrate during their maturation process. The expression of co-stimulatory molecules, such as CD80 and CD86 [14] and the synthesis of DC specific cytokines [15] are also essential to initiate and boost T cells responses. All the factors and stimulus received by DCs, namely, the type of recognized and endocytosed antigens and the cytokine exposition are fundamental to modulate DC functions. Ultimately, according to their acquired functions, DCs will activate either cytotoxic or helper T cell, driving the latter into different types, Th1, Th2, Th17 or T regulatory (Tregs) thus leading to cell-mediated, humoral or tolerogenic immunity, respectively [16, 17]. For an overview of the DC main function see Figure 1.

This heterogeneity of DC functions, together with the existence of diverse DC subtypes, has afforded investigators different strategies to define and manipulate the immune response using DCs.

2.1. Pathogen glycan recognition by dendritic cells

The interactions between DCs and microorganisms are rather complex but progress in the past few years has shed light on several aspects of these interactions. DCs can directly identify pathogens by recognizing distinct microbial patterns. Those patterns that are not found associated with mammalian cells and are shared by related microbes are called pathogen-associated molecular patterns (PAMPs) and include lipopolysaccharide (LPS) from the gram-negative cell wall, peptidoglycan and lipoteichoic acids from the gram-positive cell wall, the sugar mannose, bacterial and viral unmethylated CpG DNA, bacterial flagellin, the amino acid N-formylmethionine found in bacterial proteins, double-stranded and single-stranded RNA from viruses, and glucans from fungal cell walls.

For pathogen recognition, DCs express several pattern recognition receptors (PRRs) with functions in endocytosis and in signalling. One family of these PRRs are the Toll-like receptors (TLR), which recognize a panoply of PAMPs outside and inside the cell [18]. Specific binding of microbial ligands to TLRs activates different intracellular signalling cascades, results in the transcription of various genes coding molecules with direct microbicidal activity and collectively provide immediate host protection [18–20].

Another important family of PRRs expressed by DCs are the C-type lectin receptors (CLRs), which recognize carbohydrate structures on pathogens. These CLRs' family includes the DC-specific intercellular adhesion molecule-3 grabbing non-integrin (DC-SIGN), macrophage galactose/N-acetyl-galactosamine specific lectin1 (MGL1), mannose receptor (MR), DEC205, dectin-1 and the blood DC antigen 2 (BDCA2). Except for Dectin-1, which is specific for a fungus-glycan, the others CLRs also bind glycan structures that are also expressed by mammalian cells, reflecting their dual function in both host and pathogen recognition [21].

It is now becoming clear that CLRs may not only serve as antigen recognition receptors, but also function in the recognition of glycosylated self antigens for homeostatic control. Contrasting with TLRs, CLRs

recognize and internalize pathogens for presentation, without inducing DC maturation, i. e., the uptake of antigens by CLRs does not necessarily results in the induction of a robust immune response [2]. Thus pathogens may exploit CLR's route to evade immune surveillance [21–24].

This clearly indicates that the receptor's nature, as being TLR or CLR, that is engaged upon microbial recognition can tilt the balance towards immunity or tolerance [21, 23, 24].

DCs also express Siglecs (sialic acid binding immunoglobulin-like lectins), selective for sialic acid-containing glycans and with a fundamental physiological function in self antigen recognition. However, as CLRs, Siglecs are also able to recognize pathogens, namely sialylated glycans pathogens, and have a role in the innate immune responses [25–27]. The importance of these receptors in endocytosis, signalling and therefore in modulation of the immune response, will be reviewed in detail below.

Scavenger receptors are other membrane receptors that also function as PRRs in DCs, by directly binding both Gram-positive and Gram-negative bacteria.

DCs also express multifunctional, cytoplasmic PRRs such as Retinoic acid-inducible gene (RIG)-I-Like receptors (RLRs), sensing viral RNA molecules [28] and NOD-like receptors (NLRs), probing for microbial products, such as peptidoglycan components in the cytoplasm [29].

Recognition of microbes and internalization of microbial particles by DCs may also be mediated by receptors that bind to opsonins in "coated" (opsonised) microbes, thus broadening the range of captured antigens. DCs possess two main mechanisms of opsonic recognition. One involving fragments of complement cascade that recognize several PAMPs and opsonize microbes, allowing their recognition by DCs through complement receptors. The other, a mechanism involving specific antibodies (soluble components of the adaptive immunity) which react with appropriate, specific antigens on the microorganism's surface and are simultaneously recognized by appropriate receptors on the surface of DCs [30, 31].

In summary, there are several mechanisms allowing the interaction between DCs and microorganisms, with different particularities, modulating DC functions in the appropriate direction. Thus, the instructions that DCs receive in the beginning of an immune response may critically influence the development and the outcome of further immune responses.

2.2. Tumour glycan recognition by dendritic cells

Tumour cells accumulate drastic changes, such as DNA mutations or alterations in gene expression that are responsible for their progression and survival. The immune system, and particularly DCs, can specifically identify tumour cells based on the recognition of tumour-specific antigen (TSA) – almost exclusively expressed by tumours - or tumour-associated antigen (TAA), which are expressed in normal tissues as well as being over-expressed on tumours [32]. Antigens derived from dying tumour cells are also recognized by the host immune system [33]. Since the recognition of such antigens leads to DC activation, this is the key feature to foster anti-tumour immune responses.

However, tumour cells have multiple strategies to elude immune responses through their ability to generate a tolerance-inducing microenvironment, activation of negative regulatory pathways and secretion of inhibitory factors [10, 33]. In fact, although DCs can present tumour antigens to T cells, most of the tumours shape DC function and activate a plethora of immunosuppressive mechanisms [10], such as the production of bioactive molecules that negatively affect DC maturation and function [34]. During the past few years, it has been found that defects in the DC system are one of the main factors responsible for tumour escape, which contributes in various ways to defective T cell responses in cancer and failure of immunotherapeutic strategies [33]. These defects are translated by the accumulation and recruitment of immature and tolerogenic DCs at sites of rapidly growing tumours [33]. DCs after recognizing tumour antigens can induce immune tolerance in a number of ways, including T cell deletion, induction of T cell unresponsiveness and the activation of Tregs [35]. Consequently, the presence of infiltrating DCs in tumour tissues and its recognition does not always correlate with a good prognosis [36].

A common phenotypic change in cancer is the expression of deranged glycan structures on membrane or secreted glycoproteins or glycolipids. Glycan antigens, TSAs or TAAs, take a variety of forms, such as highly varied expression of certain glycan structures, the persistence of incomplete or truncated structures, the accumulation of glycan precursors and, less commonly, the appearance of novel structures [37].

In eukaryotes, more than half of the proteins are glycosylated, presenting two main types of glycans - N-glycans and O-glycans - whose expression or structures can be affected during cancer progression.

N-glycans have a functional role in cell adhesion, and their modifications in cancer cells are associated with invasion and metastatization. For example, N-glycosylation changes on E-cadherin have been implicated in the modulation of cadherin-dependent cell-cell adhesion in association with tumour progression [38].

O-Glycosylation of glycoproteins, such as mucin glycoproteins that are highly overexpressed in carcinomas, contributes to a substantial part of cancer biomarkers [39]. Here the most important changes are the generation of truncated versions of normal oligosaccharides and generation of unusual forms of terminal structures, namely sialylated versions of their normal counterparts.

The Thomsen-Friedenreich (TF) antigens are a group of simple structures comprising the T (Galβ1,3GalNAc-), sialyl T (Neu5Acα2,3Galβ1,3GalNAc-), Tn (GalNAc-) and sialyl Tn (Neu5Acα2,6GalNAc-) O-linked to serine or threonine residues of the polypeptide backbone. Their expression is largely increased in many types of cancers (kidney, prostate, cervix, ovary, breast, etc.) and the sialylated forms (sialyl T and sialyl Tn) are associated with a worse prognosis [40, 41]. TF sialylation irreversibly blocks the glycosidic chain elongation that occurs in normal cells, thus affecting the cell-cell and cell-extracellular matrix interactions, and therefore influencing the invasive and progression capacities of the cancer cells, angiogenic potential, as well as their recognition by immune cells (reviewed in 42–44).

To date, the overall contribution of DC receptors in tumour cell recognition and induction of immunosuppressive responses (generally observed in tumour microenvironment) is not fully understood. However, some studies have evidenced the importance of some receptors, such as MGL1 and DC-SIGN, in these aspects [23, 45]. MGL1 is highly expressed on immature or tolerogenic DCs and it has been shown to interact with tumour-associated glycoforms of MUC1 mucin, probably contributing to immunosuppressive effects [45]. DC-SIGN, which is expressed by immature DCs, recognizes colorectal cancer cells through tumour-specific glycosylation, in particular, Lewis X (Le^x) and Lexis Y moieties, present in carcinoembryonic antigen (CEA). Therefore, the interaction between DC-SIGN and CEA may play a role in tolerance induction to colorectal tumour cells [23].

3. Sialic acids - crucial modifiers of glycan structure

All mammalian cells are covered by the glycocalyx, a complex assembly of glycolipids and glycoproteins. The glycosylation status of these glycoconjugates is determined by many factors, including the specific set of glycosyltransferases expressed by a given cell, as well as the enzymatic activity of glycosidases that amend glycan chains on glycoconjugates during their synthesis and at cell surface, thus working in a finely regulated balance.

Sialic acids are a large family of negatively charged monosaccharides that includes N-acetylneuraminic acid (Neu5Ac) and N-glycolylneuraminic acid (Neu5Gc). Neu5Ac is ubiquitous, while Neu5Gc is not found in healthy human tissues but is found in some tumours [46]. This chapter will refer only to the Neu5Ac type and, for the sake of simplification, the term ''sialic acids'' will be the only used.

Sialic acids are typically found on the outermost ends of cell surface glycoconjugates. In humans, sialylation of glycoconjugates is mediated by twenty different sialyltransferase enzymes, which catalyze the transfer of sialic acid residues from CMP-Neu5Ac, an activated sugar donor, to terminal non-reducing positions of oligosaccharide chains of glycoproteins and glycolipids.

Each sialyltransferase preferentially recognizes a specific saccharide acceptor substrate. However, *in vivo*, sialylation is much more complex, as the sialyltransferases compete with other sialyltransferases or other glycosyltransferases for the same acceptor substrates. It's, thus, the induced glycosyltransferases' overall expression, including sialyltransferases, at a specific time point, that determines the sialylation profile of glycoproteins.

Sialyltransferases are usually integral membrane proteins of the Golgi apparatus and generate sialic acid content and diversity during the biosynthesis of glycoconjugates [47]. Depending on their nature, sialyltransferases may establish different types of linkages via α2,3, α2,6 or α2,8 and are currently divided into four families. The ST3Gal family comprises six members and catalyzes the transfer of Neu5Ac residues in α2,3-linkage to galactose (Gal), while the ST6Gal family has only two known members and

catalyzes α2,6-linkages to Gal. There are six known members in each of the ST6GalNac and ST8Sia families, which are able to establish α2,6-linkage to the N-acetylgalactosamine (GalNAc) or α2,8-linkages to another sialic acid, respectively (reviewed in (48)).

The sialylated content on the cell surface is not only the product of sialyltransferases, which add sialic acids, but also of sialidases, which remove sialic acids from glycans. To date, four human sialidases (also called neuraminidases) have been described, termed Neu1, Neu2, Neu3, and Neu4 [49]. These four sialidases reside in different cellular compartments: Neu1 is lysosomal, Neu2 is cytosolic, Neu3 is in the plasma membrane and Neu4 is an intracellular-associated protein. All have different substrate preferences: Neu1, Neu2 and Neu4 cleave sialic acid from glycoproteins; Neu2 and Neu4 also act on sialic acid-bearing glycolipids termed gangliosides; and Neu3 prefers solely gangliosides [49].

On the cell surface, sialic acids can be part of specific N-glycans and O-glycans on glycoproteins or part of gangliosides and contribute to the recognition of the glycans by endogenous lectins. For example, sialyl Le^x (sLe^x) antigen is a well-known signature motif for recognition by the selectin family of the cell adhesion molecules [50]. On the other hand, the addition of terminal sialic acid residues can mask the underlying glycans and thus block binding by lectins that recognize those non-sialylated glycans. As an example, α2,6-sialic acids on N-glycans blocks galectin binding to β-galactosides [51].

This chapter will focus on the roles of α2,3 and α2,6 sialic acids due to the growing body of evidence showing their role in the modulation of immune responses.

3.1. Role of sialylation in the modulation of immune responses

Due to their terminal position, sialic acids are exposed to the cellular environment functioning in intrinsic and extrinsic communication between leukocytes and in immune defence. Since they participate directly or indirectly in a myriad of cellular events, it is difficult to describe their role in modulating the immune responses. Yet, in terms of immunity, their functions may be divided as follows.

On the one hand, due to their hydrophilic and electronegative charge, sialic acids act as a biological mask, shielding the host from pathogen recognition or autoimmune responses. As an example, sialic acid moieties prevent the deposition of complement on cell surface. In addition, the augment of α2,6- and α2,3-linked sialic acid in serum proteins and sialyltransferase expression and secretion, during acute phase responses is thought to reinforce a cell surface shield against pathogens (among other effects) [52]. In this way, sialic acids contribute to host cells being considered as self by the immune system.

On the other hand, sialic acids function in the opposite way, by being recognized by a number of cell surface receptors involved in the immune response, such as the Siglecs and selectins (reviewed in (27, 50)). Siglecs are members of the immunoglobulin (Ig) superfamily characterized by their

specificity for sialic acids containing glycans [27]. There are currently 16 known Siglecs in humans and 9 in mice, and they are predominantly expressed on myeloid and lymphoid cells. These receptors seem to be unique in their capability to interact not only *in trans* with sialylated ligands found in other cells, but also *in cis* with ligands found at same cell surface [53]. Siglecs' physiological functions are still poorly characterized but, nevertheless, recent data pinpoints their roles in the positive and negative regulation of immune and inflammatory responses [27, 54, 55]. For example, CD22 is an inhibitory receptor of B cells that regulates multiple B cell functions and survival [56].

Selectins are CLRs that belong to the family of cell adhesion molecules, crucial for leukocyte migration to sites of inflammation and secondary lymphoid organs. They mediate the capture of free flowing leukocytes to the vessel wall and leukocyte rolling. These CLRs bind to carbohydrate determinants on selectin ligands, which are usually decorated by sLe^x antigen [50].

Mouse gene knockout (KO) studies have also provided an irrefutable evidence of the immune-relevant function of the α2,3- and α2,6-sialylation [57–59]. ST6Gal-1 KO mice are reported as having an impaired humoral immune response, evidenced by reduced levels of circulating and surface IgM, impaired B cell proliferation in response to various activation signals and impaired antibody production in response to antigens [58].

Interestingly, a soluble form of ST6Gal-1, expressed by the liver, was found to be essential for limiting myelopoiesis in mice and thus avoid excessive neutrophilic and eosinophilic inflammatory responses [60–62]. In contrast, mice deficient for ST3Gal-1, which generates sialylated O-glycans, has shown the relevance of this sialyltransferase in controlling CD8+ T cell homeostasis and survival [57].

There are several other examples of important roles played by sialic acids in immune responses, derived from their role in host-pathogen interactions and in the progression and spread of human malignancies.

4. Modulation of dendritic cell functions by sialylation

Impelled by the search of markers and targets to modulate DC functions, researchers, in the past decade, have started to investigate the pattern of glycosylation of human DCs. Important and distinct modifications of the sialylation content in DCs undergoing differentiation from precursors, and upon maturation, were found with important biological consequences [63–65].

To investigate the glycosylation changes and the involvement of glycosylation in DCs functions, several strategies have been applied, from the gene expression analysis, mass spectrometric analysis of N- and O-glycans and flow cytometry using plant lectins and glycan-specific antibodies. Although many issues are still unresolved, the study of DC sialome and its relevance is a work in progress, with promising implications for DC biology and therapeutic applications.

Below it will be described the DC parameters that are presently known to be modulated by sialylation (for a general overview see Figure 2).

4.1. Sialylation and dendritic cell subtypes

As referred above, DCs are essential elements at the interface of the innate and adaptive immunity. Yet, different DC subtypes and subsets contribute differently for this interface and the understanding of their pathophysiology will permit their rational manipulation for therapy (reviewed in (66, 67). The two major subtypes are the myeloid DCs and the plasmacytoid DCs. Although not identical, they share many phenotypic and immunostimulatory features with cytokine-generated DCs *in vitro*, the ones that have actually been used in many studies. In fact, the development of cytokine-driven methods for expanding and differentiating DCs *in vitro* allowed the assessment of sufficient quantity for large-scale study and applications, otherwise impossible to achieve due to tissue specificity but, mostly, to their scarceness.

Regarding sialylation, the major focus of attention has been placed on mo-DCs, a subset of myeloid DCs. Immature mo-DC show a high content of α2,6- and α2,3-sialylated structures, when comparing with its precursors, the monocytes [65, 68]. This has been showed by different authors, using the *Sambucus nigra* lectin, which recognizes sialic acid α2,6-linked to lactosamine (Neu5Acα2,6Galβ1,4GlcNAc-), and *Maackia amurensis* lectin, recognizing sialic acid α2,3-linked to lactosamine (Neu5Acα2,3Galβ1,4GlcNAc-). Conversely, the overall reactivity with *Arachis hypogaea* lectin, specific for nonsialylated Galβ1,3GalNAc- (core 1 structure of the O-linked chains), to mo-DCs is very low, increasing dramatically upon sialidase treatment, supporting the idea that these cells express the Core 1 structure of the O-linked chains (T antigen) masked by sialic acid.

The analyses of mRNA, either by microarray analysis and quantitative real-time PCR, have also enlightened significant alterations in the expression of sialyltransferase and sialidase genes, not only during differentiation, but also upon maturation [63, 65, 68, 69]. A particular relevance has been pointed out by different researchers, to the ST3Gal-1 and ST6Gal-1 sialyltransferases, since they increase significantly during mo-DC differentiation. This increase has been confirmed at protein level by determining the overall sialyltransferase activity towards precise ST3Gal-1 and ST6Gal-1 substrates. In fact, a straight correlation between ST3Gal-1 and ST6Gal-1 gene expression, enzymatic activities and α2,3- and α2,6-sialylation phenotype, has been found in mo-DCs [65]. These sialyltransferases are, thus, considered to be the major contributors for the biosynthesis of α2,3- and α2,6-sialic acid-containing structures specific for mo-DCs and probably related with mo-DC functionalities. Nevertheless, a potential contribution of other sialyltransferases, also significantly expressed in mo-DCs, should not be excluded. For instance, ST3Gal-4 and ST3Gal-6, which are involved in sLe^x antigen's biosynthesis [42] may interfere with cell-cell interactions. Besides sialyltransferases, the expression of sialidases is also modulated, with Neu1 and Neu3 being significantly up-regulated upon mo-DC differentiation [69].

During mo-DC maturation, the analysis of the glycosylation changes highlighted the increase in α2,3-sialylation (ST3Gal-1) and a decrease in α2,6-sialylation (ST6Gal-1) [64, 65, 68]. However some variations were

reported regarding on the modulation of other sialyltransferases probably reflecting the fact that this is a stimulus-dependent phenomenon.

The functional consequences of sialic acid content modulation, during maturation, are still far from being elucidated. It has been suggested that immature and tolerogenic DCs, by showing high levels of α2,6-linked sialic acid, are likely to be recognized by inhibitory Siglecs on the surface of effector T cells [68]. In another study, a correlation between the up-regulation of sialic acid expression by mature DC and the increased binding of Siglec-1, -2, and -7 has been shown [64]. This anticipates a role for sialic acids on the Siglec-mediated interactions between mature mo-DCs and several other leukocytes expressing Siglec receptors (such as lymphocytes, macrophages, natural killer cells and DCs themselves), although further studies are still required.

4.2. Functional aspects of dendritic cell's receptors that recognize sialic acids

As mentioned above, DCs have several receptors specialized in the recognition of sialylated glycoconjugates. The most important family of such receptors is the Siglecs, which are well represented in DCs. There are two main categories of Siglecs on the basis of their sequences similarity: the group that includes sialoadhesin (CD169, Siglec-1), CD22 (Siglec-2), myelin associated glycoprotein (MAG; Siglec-4) and Siglec-15, and the group designated as CD33 (Siglec-3)-related Siglecs, which comprise a rapidly evolving subfamily (CD33rSiglecs, Siglec-3, 5–14, and -16 in humans).

DCs express several CD33rSiglecs, namely Siglec-3, -5,-7, -9 and -10 [25], mainly functioning as negative immunoregulators of their functions. In fact, the cytoplasmic domains of these Siglecs contain immunoreceptor tyrosine-based inhibitory motifs (ITIMs) [26, 27] which are involved in cell immunomodulation [70]. ITIMs associate to receptors with immunoreceptor tyrosine-based activation motifs, dampening their signalization, with consequent inhibition of cellular activation, proliferation, induction of apoptosis and modulation of cytokine secretion or endocytosis [55]. Specifically, when engaged, Siglec ITIMs recruit cytoplasm phosphatases, which inhibit signalling pathways [54].

Siglecs are usually masked by *cis* interactions with sialic acids expressed on the same cell membrane. Unmasking can occur following exposure to sialidase or due to cellular activation [26, 71] allowing *trans* interactions of Siglecs with sialylated glycans from other cells or eventually microorganisms. As a result, the phosphorylation status of the Siglec ITIMs is affected, influencing the activation state of the cell [27].

Given the pivotal role played by DC in driving T cell responses, the negative immunomodulation mediated by Siglecs may have a clear significance in dampening immune responses and thus preventing autoimmunity. Other than potential cellular activation modulators, CD33rSiglecs may function as endocytic receptors [25, 72], since many pathogens possess or acquire sialic acids [73–76]. It is possible that CD33rSiglecs, expressed by DCs, may be exploited by sialylated pathogens as a mean to modulate DCs activation and consequently the adaptive immune response.

Besides the CD33rSiglecs, Siglec-15 is known as being expressed by human DCs, functioning as an activating molecule which may, at least in part, be an adaptation of the host to counteract the manipulation of inhibitory Siglecs by sialylated pathogens [54].

4.3. Dendritic cell sialylation and endocytosis

The sialic acid's role on endocytosis has long been studied on the perspective of the pathogen. Evidence exists pointing that several pathogenic bacteria have evolved to use sialic acid beneficially by coating themselves with sialic acid, thus granting protection from host's immune response [77].

Nevertheless, it is now also evident that the role of the sialic acid is far more complex than the pathogen perspective and the importance of the sialylated structures expressed by the "eater cells", such as DCs, is now being elucidated. As referred above, DCs are able to endocytose pathogens by extremely variable and efficient ways, which include receptor mediated endocytosis, phagocytosis and macropinocytosis [8, 9]. In this section, a special attention will be given to what is currently known about the functional relevance of DC sialylation in these mechanisms.

4.3.1. Sialidase effect: Macropinocytosis vs. Phagocytosis. Terminal sialyl residues may be removed from glycoconjugates by sialidases (neuraminidases), which comprises a family of glycoside hydrolase enzymes that, physiologically, can be from endogenous or exogenous sources. Some soluble bacterial and viral sialidases have been cloned and characterized and exhibit substrate-specificity towards sialic acid linkage or substituent groups present on the glycan. These soluble sialidases have been an excellent tool to investigate the sialome and, since they have been employed to elucidate the role of sialic acids on the endocytic capacity of human DCs, they were included in this section. Other tools used for the understanding of sialic acid's role in DC functions involve specific sialyltransferase-knockout mice usage, allowing a more linkage-specific, structural-dependent analysis of observed facts.

DCs, as previously mentioned, possess different endocytic mechanisms, including phagocytosis and macropinocytosis, two actin-dependent mechanisms that result in the internalization of particles or the formation of fluid-filled macropinosomes, respectively. The effect of sialidase treatment in these mechanisms has been investigated by comparing the capacity of human mo-DCs treated or not with sialidase, to uptake antigens, such as ovalbumin, mostly internalized through macropinocytosis, and *Escherichia coli* (K-12 derived strain) as a bacterial model of phagocytosis.

In desialylated mo-DCs, the macropinocytosis is significantly reduced [65] but, in contrast, phagocytosis is improved (Cabral et al, submitted). Therefore, it was suggested that sialic acid removal differentially influences specific endocytic mechanisms.

The sialidase treatment and/or the absence of specifically-linked sialic acids were also implicated in the enhancement of mo-DC and bone

marrow-derived DC maturation [78]. The sialidase-mediated macropinocytosis downregulation can be indirectly justified by the induction of maturation, where the endocytic capacity is significantly decreased [79]. However, this feature does not elucidate the improved phagocytic capacity of sialidases treated mo-DCs. In fact, although several authors have reported that mature DCs may continue uptaking antigens by receptor mediated endocytosis and phagocytosis [80, 81], an improvement of phagocytosis capacity of mo-DCs concomitant with their maturation was only recently attributable to sialylation shortage (Cabral et al, submitted).

Rho GTPases, namely Rac1 and Cdc42, are known to function as molecular switches and to control the actin-dependent events of macropinocytosis and phagocytosis [13, 79, 82–84]. Similarly to the maturation effect [79], sialidase treatment leads to attenuation of Rac1 and Cdc42 activation, in parallel with cytoskeleton disorganization in mo-DCs. Since both endocytic mechanisms depend on these features, probably the *E. coli* phagocytosis improvement is positively influenced by a distinct effect of sialidase, which subverts the typical maturation outcome. This idea was additionally supported by the observation that sialidase treatment also significantly improves the *E. coli* uptake, by mature mo-DCs. Interestingly, *E. coli* phagocytosis improvement, induced by sialidase, is dependent on the presence of sialic acids on bacteria (Cabral et al, submitted). Based on these recent data, it is now hypothesized that sialidase, while affecting the actin organization within the cells, benefits phagocytosis, probably due to the engagement of receptors such as Siglecs which became more accessible for bacteria recognition after mo-DCs desialylation.

There are several microorganism-recognizing DC receptors containing sialic acids moieties and that can be modulated by sialidase. For instance, desialylation of specific TLRs has an essential role in LPS-induced TLRs activation and induced cytokine production [69, 85]. It is worth remembering that human DCs also express significant levels of Neu1 and Neu3 endogenous sialidases and are continuously exposed to exogenous sialidases. It should also be referred that, for instance, in mice, Neu1 activates the phagocytosis by DCs through desialylation of surface receptors [86]. Siglecs are other receptors that can also be desialylated and have their functions altered by sialidase [26, 27]. It is probable that a complex combination of receptors, including others than Siglecs and TLRs, are simultaneously involved after mo-DC desialylation resulting in upregulation of *E. coli* phagocytosis and probably in triggering distinct intracellular signals. Thus, and in agreement with these facts and ideas, is the observation that sialidase treatment notably enhances the expression of both pro-inflammatory and anti-inflammatory cytokines (Cabral et al, submitted).

In summary, recent findings brought to light a new role for sialidases from endogenous and pathogenic origins, as affecting antigen uptake. This is an issue to be considered in the future mo-DC-based antibacterial therapies development.

4.4. Sialylation and T cell priming

As referred earlier in this chapter, the final goal of DCs is the presentation of antigens to T cells, thus activating them and triggering the adaptive immune responses.

Evidences from different groups have suggested that the sialylation content of DCs may dampen their capacity to prime T cells, probably by interfering in the MHC-mediated antigen presentation and co-stimulation [68, 87]. Concordantly, human DCs when submitted to sialidase treatment show a more mature phenotype with increased ability to prime T cells and induce their proliferation [78]. This can be attributable to the observed increased expression of MHC molecules and co-stimulatory molecules by DCs, thereby resulting in increased antigen presentation to T cells or to the fact that desialylation modulates the protein-protein interaction, thus accounting for improved cellular signalling, or a synergistic effect of both.

Interestingly, sialic acid shortage in DCs leads to an upregulation of IL-1α, IL-6, TNF-α and IL-12 expression, which drive the differentiation of T helper 1 cells and the secretion of IFN-γ from T cells [78]. Similar observations were made by another group of investigators, who highlighted Neu3 – a ganglioside specific sialidase – as responsible for these changes during mo-DC differentiation [69]. These cytokines are known to contribute to cell-mediated immune responses and increased immunogenicity [17].

Overall, and considering these and all the above referred facts, sialylation has been proposed as an issue to be considered when refining the immunological parameters of vaccination with DCs.

4.5. Sialylation and dendritic cell recruitment

The recruitment of leukocytes from blood to tissues is a well-coordinated process that involves adhesion and chemotaxis, through the participation of several molecules including, selectins, integrins and chemokines and respective receptors. Selectins are C-type lectins expressed by the endothelium (E-, and P-), platelets (P-) or leukocytes (L-selectin). E-selectin expression is rapidly inducible by inflammatory stimuli, while P-selectin is both inducible and stored in the Weibel-Palade bodies of endothelial cells and alpha granules of platelets, from which it can rapidly be mobilized. E- and P-selectins bind to their respective counterreceptors (ligands) expressed by leukocytes and mediate the initial tethering and rolling along the blood vessel endothelium, leading to the recruitment to tissues at sites of inflammation.

L-selectin is constitutively expressed on the surface of almost all types of leukocytes. L-selectin ligands are constitutively expressed on High Endothelial Venules of peripheral lymph nodes and are mainly involved in trafficking of leukocytes from blood to lymph nodes (homing). L-selectin can also bind to inducible ligands on endothelium at sites of inflammation or to ligands on other leukocytes [50, 88].

The initial tethering and rolling of leukocytes mediated by selectins is reversible and is followed by firm arrest involving interaction between chemokines and respective receptors and adhesive interactions between

integrins expressed by leukocytes and intercellular adhesion molecule-1 (ICAM-1) expressed by the endothelium [88].

Cell surface sialic acids play a critical role in leukocyte recruitment since they participate in the interaction with the endothelial adhesion molecules. One of the most acknowledged functions of sialic acids related to leukocyte recruitment is their relevance in selectin ligands [89]. However, sialylated glycans are also involved in chemokine receptor neutrophils firm arrest [90] and in α4β1 integrin function [91]. Selectins (E-, P- and L-selectin) share the ability to recognize a sialic acid and fucose (Fuc) containing tetrasaccharide, the sLe^x (Neu5Acα2,3Galβ1,4[Fuc α1,3]GlcNAc-). sLe^x is a terminal component of glycans attached to glycoproteins and glycolipids on most circulating immune cells and some endothelial cells. The interaction between selectins and sLe^x decorated glycans mediates the initial tethering and rolling of leukocytes along the endothelial cell surface, which is followed by a cascade of molecular events culminating in the leukocyte extravasation and migration into inflamed tissue [88]. The expression and role of sLe^x as selectin ligand has been extensively studied in neutrophils and lymphocytes [50, 92]. Likewise, the pool of immature DCs circulating in the blood is recruited into tissues via interaction with E- and P- selectins expressed by the endothelium [93, 94]. The ability of mo-DCs to tether and roll over immobilized P- , E-, and L-selectins, under shear flow indicates that this particular type of cells also express functional selectin ligands. Mo-DCs adhere more extensively to P-selectin than to E-selectin and roll at similar velocities on both endothelial selectins [95]. These results supported and expanded previous studies with blood DCs or $CD34^+$-derived DCs [94, 96]. The functional role of sLe^x surface expression by mo-DCs in selectin-dependent binding under dynamic flow conditions, has been investigated by blocking experiments with anti-sLe^x blocking antibody or the sLe^x itself. The binding inhibition attained by this strategy proved that mo-DC-selectin interaction is sLe^x mediated [95].

The non-endothelial, L-selectin mediated interaction are important for secondary capture events, where circulating cells tether to other cells already prebound to endothelium [97]. While expressed by many leukocytes, L-selectin is not expressed by mo-DCs [98]. L-selectin can bind mo-DCs, although less extensively and with higher rolling velocity than P- or E-selectins [95].

To act as an effective selectin ligand, sLe^x determinant must be carried by specific glycoconjugates [99]. To date, P-selectin glycoprotein ligand-1 (PSGL-1) is the sole glycoprotein known to contain sLe^x in mo-DCs [100]. PSGL-1 is a mucin-like glycoprotein expressed on the microvilli of most leukocytes [101, 102]. Recently, it was found that that PSGL-1 is the exclusive P-selectin ligand in mo-DCs, with less affinity to L-selectins, being dispensable for E-selectin binding [95].

To effectively accomplish their task, DCs should migrate to secondary lymphoid tissues after antigen capture. This step is guided by the chemokines and chemokine receptors. Recently, it was reported that polysialic acid present on mature DCs enhances chemokine directed migration toward lymphoid tissues. The enhanced effect depends on the

polysialylation of the acceptor molecule neuropilin 2 and the polysialyltransferase ST8Sia-4 [103]. On the other hand, chemokine receptor-dependent DC migration was not affected in ST3Gal-4 deficient mice, contrarily to other leukocytes [89].

The use of DCs in cell-based therapy is still hampered by mo-DCs defective mobility given that only 1–2% of the cells arrive to lymphatic tissue after administration of DC vaccines [104]. In this context, every effort towards improvement of mo-DCs recruitment into tissues and into secondary lymphoid organs is valuable and, in our opinion must take into account a modulation of mo-DC sialic content.

4.6. Sialic acids in tumour cell recognition by dendritic cells

There are distinct changes in sialylation associated with malignant transformation, both N- and O-glycan structures (e.g. sLe^x and sialyl Lewis A (sLe^a) antigens, sialyl Tn and T antigens) and in glycolipids (e.g. GD2 and GD3 gangliosides). Tumour-associated sialylated structures are usually related with progression and poor prognosis of most carcinomas. This association can be explained by the recognition of malignant cells by lectin receptors, causing interactions of the circulating tumour cells with endothelium and immune cells, and thereby facilitating metastasis and immune evasion [105]. For example, recent studies have shown that high levels of α2,3-linked sialic acid residues were associated with the metastatic potential of gastric cancer [106]. Furthermore, the over-expression of some sialyltransferases, such as ST3Gal-1 and ST6Gal-1, is associated with progression, poor prognosis and tumour development [107–109]. Specifically, it has been shown that ST3GalI-1 over-expression in breast cancer is not just a collateral effect of carcinogenesis but one actually purposeful advantage for tumour development. Given the microevolutionary nature of cancer, subclones with up-regulated sialyltransferases, presenting lower differentiation and higher proliferation rates would stand immune selective pressure and would, thus, be selected. Therefore, sialyltransferase inhibition might prove to be useful for cancer therapy [110, 111].

Sialic acid can mask important cell receptors, e.g. ST6Gal-1-mediated sialylation of β1-integrins on colon tumour cells blocks cell adhesion to galectin-3 (Gal-3), protecting cancer cells against Gal-3-mediated cell apoptosis. This implicates a role for ST6Gal-1 in regulating tumour cell survival and explains why α2,6-linked sialic acid upregulation in a number of tumours, correlates with poor prognosis [112]. Furthermore, sialylation of β1-integrins was found to be enhanced by higher ST6Gal-1 gene expression during exposure to ionizing radiation, resulting in a stronger cell survival due to apoptosis-impairment of exposed tumour cells [113].

Sialylation changes in tumour cells can also influence the response of immune cells, affecting their recognition by immune receptors. For instance, the MUC1-based glycopeptides carrying the tumour-associated Tn antigen (Tn-MUC1) bind selectively to immature DCs through MGL1 receptor, being afterwards internalized, processed and presented on an MHC-I and -II context [114]. However, it has been demonstrated that the sialylation of Tn antigens reduces or completely abrogates the interactions between MUC1

and MGL1 receptors [53], suggesting that sialylated molecules are differently recognized than their respective unsialylated homologues.

As previously mentioned, DCs express the inhibitory receptors, Siglec-3, -5, -7 and -9, which recognize sialylated structures and can be involved either in endocytosis and intracellular signalling pathways [53]. The recognition of tumour-associated sialylated molecules by such inhibitory receptors can explain why DCs cultured with a glycoprotein MUC1 carrying sialyl T antigen (ST-MUC1) show a low level of maturation, increased expression of inhibitory cytokines, decreased expression of pro-inflammatory cytokines, and a reduced antigen-presenting ability, which contribute to the immunosuppression observed in cancer patients [115].

Concordantly, it has been reported that the presence of MUC2 mucins, expressing sialyl Tn antigens, during mo-DC differentiation leads to their apoptosis and a decrease in co-stimulatory molecules expression. In these conditions, Siglec-3 was constantly expressed and bound to MUC2 probably through sialyl Tn, being partly responsible for the apoptosis of DCs [116]. Similarly, it has been demonstrated that MUC2 mucins and bovine submaxillary mucin, expressing sialyl Tn antigen, bind to Siglec-9 in immature mo-DCs, affecting their cytokine repertoire and leading to an immunomodulation of mo-DC functions. It was shown that Siglec-9 can also bind to sLe^a present in mucins obtained from sera of cancer patients [117]. These results indicate that Siglecs participate in the recognition of sialylated molecules present in tumour cells by DCs.

Using sialyl Tn-expressing, and -non-expressing bladder cancer cell lines, it has been recently found that the overexpression of sialyl Tn antigens lead to a less mature mo-DC phenotype. This was translated by a low expression of mo-DCs maturation markers, MHC II, CD80, and CD86. Nevertheless, mo-DCs adhere better to sialyl Tn expressing cancer cells and the differences found in maturation are exclusively related with cell-to-cell contact. Furthermore, the expression of sialyl Tn antigens negatively influences the expression of critical cytokines, supporting the creation of an immunosuppressive microenvironment (Carrascal et al, manuscript in preparation).

Overall, the identification of DC receptors critically involved in the recognition of tumour-associated sialylated molecules is complex. According to the present data, it is notorious that all lectin receptors expressed by DCs, namely Siglecs, CLRs and galectins, have their quota in dampening DC functions and creating an immunosuppressive environment, which may favour the tumour progression.

Conclusions

It has becoming clear that sialic acids play critical roles in many of the DC functions. These findings assume an extreme relevance, especially given the importance of DCs as coordinators of the immune system and their potential application as cellular vaccines to trigger an immune response against pathogens or tumour antigens. At this stage, our understanding of the underlying mechanisms linking a certain sialic acid structure and function is still limited and the interpretation of the data assumes a

considerable difficulty when evoking the diversity of receptors and the multiple signalling pathways used by DCs. The information regarding the sialic acid content of several receptors known to mediate functions such as endocytosis and T cell priming is limited, but can in fact unravel important tools to manipulate DCs. While sialylation shortage has been presented as a simple manner to modify DC immune functions with potential therapeutic applications, an elucidation of these mechanisms at molecular level is still demanding answers to guarantee its applicability from bench to bedside. The question of whether the modulation of DC sialic acid content can improve DC-based vaccines, by dictating the type of response that is triggered by DCs, remains open for now.

Abbreviations:

CD33rSiglecs	CD33 (Siglec-3)-related Siglecs
CLR	C-type lectin receptor
DC	Dendritic cell
DC-SIGN	DC-specific intracellular adhesion molecule-3 grabbing non-integrin
Fuc	Fucose
Gal	Galactose
GalNAc	N-acetylgalactosamine
GlcNAc	N-acetylglucosamine
ITIM	immunoreceptor tyrosine-based inhibitory motif
MGL1	Macrophage galactose/N-acetyl-galactosamine specific lectin1
MHC	Major histocompatibility complex
Mo-DC	Monocyte-derived dendritic cell
MR	Mannose receptor
Neu5Ac	N-acetylneuraminic acid
Neu5Gc	N-glycolylneuraminic acid
NLR	NOD-like receptors
PAMP	Pathogen-associated molecular pattern
PRR	Pattern recognition receptors
Siglec	Sialic acid binding Immunoglobulin-like lectin
TAA	Tumour-associated antigen
TSA	Tumour-specific antigen
Th	T helper cell
TLR	Toll-like receptor
Treg	T regulatory cell
sLe^x	Sialyl Lewis X antigen
sLe^a	Sialyl Lewis A antigen

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Review

Challenges in Antibody Development against Tn and Sialyl-Tn Antigens

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Abstract: The carbohydrate antigens Tn and sialyl-Tn (STn) are expressed in most carcinomas and usually absent in healthy tissues. These antigens have been correlated with cancer progression and poor prognosis, and associated with immunosuppressive microenvironment. Presently they are used in clinical trials as therapeutic vaccination, but with limited success due to their low immunogenicity. Alternatively, anti-Tn and/or STn antibodies may be used to harness the immune system against tumor cells. Whilst the development of antibodies against these antigens had a boost two decades ago for diagnostic use, so far no such antibody entered into clinical trials. Possible limitations are the low specificity and efficiency of existing antibodies and that novel antibodies are still necessary. The vast array of methodologies

available today will allow rapid antibody development and novel formats. Following the advent of hybridoma technology, the immortalization of human B cells became a methodology to obtain human monoclonal antibodies with better specificity. Advances in molecular biology including phage display technology for high throughput screening, transgenic mice and more recently molecularly engineered antibodies enhanced the field of antibody production. The development of novel antibodies against Tn and STn taking advantage of innovative technologies and engineering techniques may result in innovative therapeutic antibodies for cancer treatment.

Keywords: Tn antigen; Sialyl Tn antigen; immune response; therapeutic antibodies; antibody production

1. Introduction

The deranged expression of glycans is a hallmark of cancer. Glycans such as the Thomsen-Friedenreich related antigens, Tn and sialyl-Tn (STn), show a very tumor specific pattern and, in most cancers, they are associated with disease progression and patient's response to treatment. These antigens have therefore triggered enthusiasm among the scientific community as diagnostic and prognostic biomarkers. A relevant number of antibodies were developed and characterized to be used in immunohistochemistry staining protocols. Yet the added value of the identification of STn or Tn as biomarkers has proven insufficient, compared to standard protocols, to justify its regular use in clinical practice. A second and more pronounced enthusiasm arose with the development of immunotherapies targeting these antigens to boost immune responses, *i.e.*, vaccination. Nevertheless, the enthusiasm was rapidly refrained due to the unsuccessful results from clinical trials. More recent findings have helped to understand that these antigens have potential to dampen immune responses which oblige us to revise the efficacy of immunization procedures and to search for more effective strategies for treatment of tumors expressing these antigens.

Interestingly, the antibodies developed against these antigens have been mainly regarded as tools for diagnosis and prognosis. The exploitation of these antibodies as immunotherapeutic tools to elicit anti-tumor immune responses has been completely disregarded. Here we revise the efforts built towards the development of high affinity antibodies against STn and Tn antigens and the methodologies involved. A portfolio of cutting edge methodology is here described that can be used to speed the production of more effective antibodies or novel engineered antibody-based molecules as innovative immunotherapies against STn or Tn.

2. Glycosylation in Cancer

Glycosylation is the most frequent and well-known posttranslational modification of proteins. Glycans are present in all living organisms and regulate a diversity of biological processes, including protein folding and intracellular trafficking, cell-cell and cell-matrix interactions, cellular differentiations and the immune response [1,2]. The relevance of glycosylation is highlighted by the fact that approximately 1%–2% of human genes are required for glycan biosynthesis, which is catalyzed by the enzymatic

reaction of several enzymes that transfer mono or oligosaccharides, *i.e.*, glycosyltransferases, with their strict specificity for both donor and acceptor substrates [3].

In humans, proteins can be glycosylated with two main types of glycans: *N*-glycans and *O*-glycans. *N*-glycans are covalently linked via an amide bond to asparagine in the polypeptide sequence, and it usually involves the Asn-X-Ser/Thr (X ≠ Pro) sequence. *O*-glycans are often covalently linked to the hydroxyl group of a serine or threonine into the polypeptide via *N*-acetylgalactosamine (GalNAc) [4]. As glycan biosynthesis is not a template-based process such as DNA, RNA, or protein synthesis, glycan expression depends on the balance achieved by the expression and activity levels of the different enzymes involved in the glycosylation process, as well as by their localization/organization, for instance in the Endoplasmic Reticulum and Golgi apparatus, and on the availability of precursor monosaccharide molecules [5].

In the mid-1990s there was a peak of interest in glycosylation because a significant correlation between certain types of altered glycosylation and cancer prognosis was observed. In fact, aberrant glycosylation frequently occurs in cancer and plays a pivotal role in cancer progression, angiogenesis and metastasis, cell-cell contact, motility and epithelial-mesenchymal transition (EMT) in cancer cells. These alterations of glycosylation comprises under- or over-expression of glycan structures, as well as the appearance of novel or truncated structures. However, despite the diversity of glycans at the cell surface, only a few distinct structures are associated with malignant transformation and tumor progression. This suggests that specific glycosylation patterns contribute to precise mechanisms such as gain of function, cell fitness and survival [6].

One of the most common aberrant glycosylation in cancer is the neo-expression of Thomsen-Friedenreich-related antigens. This family of incomplete *O*-glycans (Figure 1) includes: The Thomsen-nouveau (Tn) antigen (GalNAc-α1-*O*-Ser/Thr), which consists of one residue of GalNAc alpha-*O*-linked to a serine or a threonine residue in the polypeptide chain; the Thomsen-Friedenreich (T) antigen (Galβ1-3GalNAc-α1-*O*-Ser/Thr) or unmodified core-1 structure, where a residue of galactose (Gal) is β-linked to GalNAc; the STn antigen, in which the GalNAc residue on Tn antigen is bound to a sialic acid (Neu5Ac) on carbon 6; and the sialyl-T (ST), in which the Gal residue on T antigen is bound to a Neu5Ac on carbon 3 (Figure 1). The sialylation of both Tn and T antigens blocks the further elongation of core-1 structures, with the exception of ST (*i.e.*, monosialyl-T) that can still be converted into disialyl-T structure.

Historically, the T antigen was first identified in 1930 by Thomsen, in collaboration with Friedenreich, in a blood sample contaminated by bacteria. Specifically, the action of bacterial neuraminidase released the sialic acid that covered the glycan chain, exposing the T antigen on red blood cells, which were then recognized by anti-T antibodies present in the sera, causing the subsequent hemagglutination [7]. The expression of Tn and STn antigens were later discovered in 1957 by Moreau on subpopulations of blood cells characterizing a rare hematological disorder, the Tn syndrome [8].

The Thomsen-Friedenreich family antigens have been initially called as oncofetal antigens as they are generally precursors in normal complex *O*-glycan chains [9]. Mainly expressed on epithelial cells, the overexpression of these antigens has been reported in several different human tumors, usually due to defects in the secretory pathway organelles (Endoplasmic Reticulum and Golgi) and to altered glycosyltransferase expression [6].

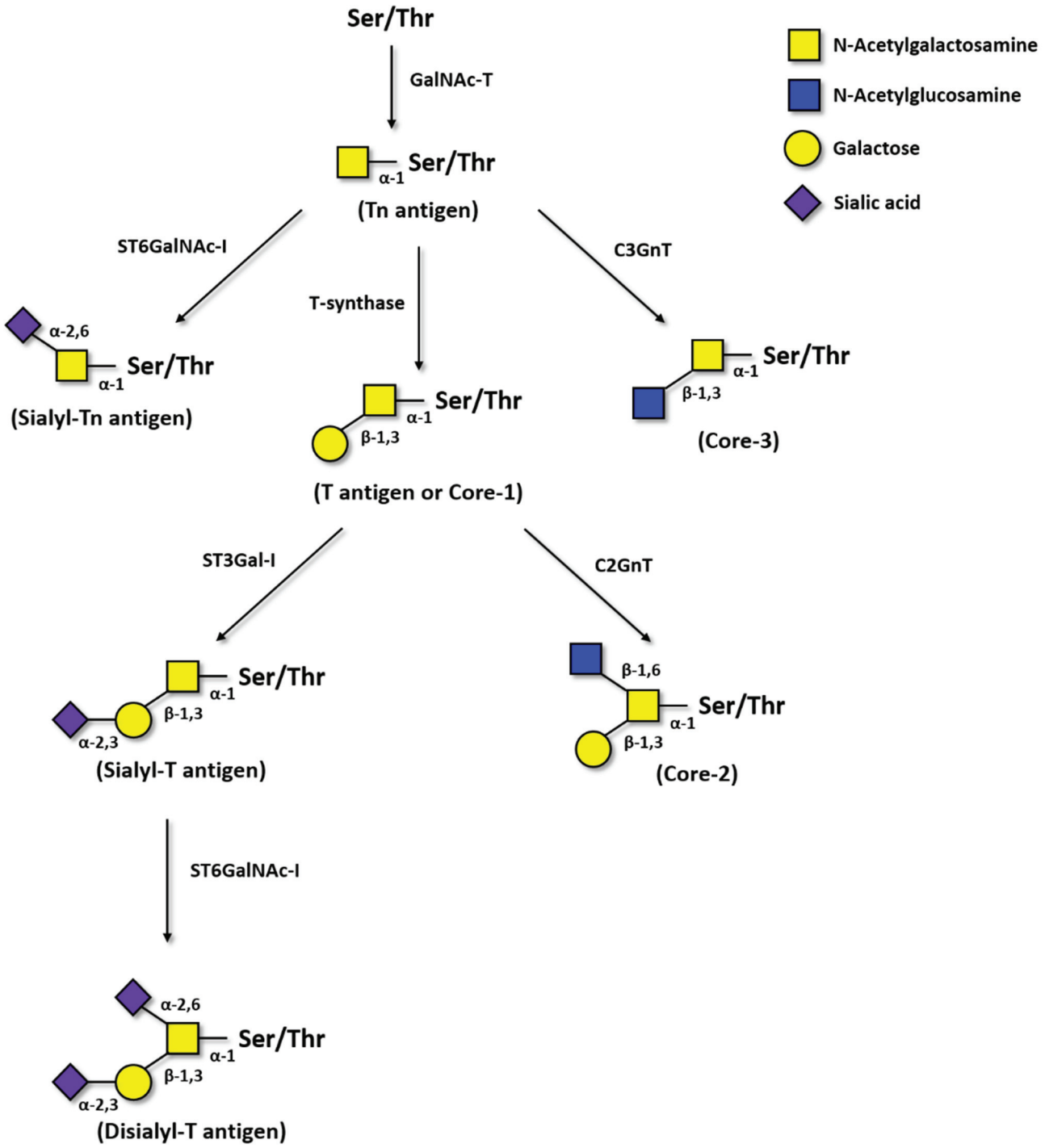

Figure 1. Pathways of Thomsen-Friedenreich antigens biosynthesis. First, GalNAc is transferred to a serine or threonine residue in a polypeptide by peptidyl-*N*-acetylgalactosaminyltransferases (GalNAc-T). GalNAcα1-Ser/Thr, *i.e.*, the Tn antigen, is then converted by T-synthase to Galβ1-3GalNAcα1-Ser/Thr, *i.e.*, the T antigen or core-1 structure. The Tn antigen can be also sialylated by ST6GalNAc-I forming Neu5Acα2-6GalNAcα1-Ser/Thr, *i.e.*, the sialyl-Tn. GalNAcα1-Ser/Thr can also be converted to core-3 structures by the β3GnT-6. 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.

T antigen is overexpressed in breast, colon, pancreas and lung cancer; but it can also be detected in up to 40% of normal tissues [4] and in inflamed tissues. Similarly, the ST expression is largely increased in some cancers when compared to normal tissues, such as breast and bladder cancer [10–12], but also expressed in normal epithelium, many serum proteins and leukocytes [13,14]. In contrast, the Tn and the STn antigens show a better cancer associated pattern, with absent or very limited expression in adult healthy tissues [15–17], and are therefore more interesting as therapeutic targets.

2.1. Tn Antigen

Tn antigen is a pan-carcinoma antigen, expressed on a majority of carcinomas, such as breast, pancreas, colon, lung and bladder, being less common in hematological malignancies. In normal tissues, the Tn is only expressed in embryonic brain [18] and is shielded in adult tissue. Tn is actually a normal biosynthetic precursor of *O*-glycans however not a normal terminal product, being occasionally observed in adult normal cells in the secretory apparatus, but not found on proteins at cell surface or secreted [4,15].

Tn is normally extended by the β1,3-galactosyltransferase C1GalT1, also termed T-synthase or core-1 synthase, or by the core-3 β1,3-*N*-acetylglucosaminetransferase (C3GnT) to form T antigen (core-1) or core-3 structure, respectively (Figure 1). In cancer, the loss of T-synthase or C3GnT enzymes may lead to overexpression of Tn antigen [19,20]. Altered expression of GalNAc-Ts glycosyltransferases, and re-localization from the Golgi to the endoplasmic reticulum, also results in Tn overexpression. In addition, defects in Cosmc, a chaperone essential for the activity of C1GalT1, due to epigenetic silencing or mutations can also lead to increase expression of Tn antigen in cancer [21,22].

Overexpression of Tn antigens promotes cancer cell proliferation and invasiveness. In fact, in breast cancer, the accumulation of Tn-bearing proteins in lamellipodia at the cell surface promotes cell adhesion, cell motility and invasiveness [23]. Strikingly, the Tn antigen is also detected at early stages of tumor development and may serve as a biomarker, since its expression is associated with invasive and highly proliferative tumors, and metastasis [24].

2.2. Sialyl-Tn Antigen

Sialyl-Tn is also a pan-carcinoma antigen that is expressed early in tumorigenesis. In contrast to Tn, STn is not a normal biosynthetic precursor, meaning that its expression is necessarily pathologic [4]. As a side note, in some normal tissues, such as colon, the sialic acid residue of STn may be *O*-acetylated, thus masking the STn and therefore its recognition by anti-STn antibodies [25]. No other sugars are known to be added to the STn antigen. STn is often co-expressed with Tn and therefore mechanisms that result in Tn overexpression likely apply to STn. Specific mechanisms for STn overexpression, include ST6GalNAc-I upregulation, or re-localization from the Golgi to the endoplasmic reticulum and also loss of *O*-acetyl groups from STn, have been demonstrated in some cancers [26–29].

Specifically, STn expression modulate a malignant phenotype inducing a more aggressive cell behavior in gastric and breast carcinoma cells, such as decreased cell-cell aggregation and increased extracellular matrix adhesion, migration and invasion [30–33].

2.3. Pathological Role of Tn and STn in Cancer

Both Tn and STn antigens have been correlated with cancer progression and poor survival. High expression of Tn and STn in human ovarian carcinoma was associated with disease stage, histological grade and low overall survival time [34]. In addition, Tn has been shown as good biomarker of invasive cervix carcinoma [35]. Moreover, STn expression was associated with invasiveness of liver cancer [36] and with lower survival rate in advanced gastric carcinomas [37]. In breast cancer, STn expression was associated with lymph node metastasis and high histological grade [38], as well as with resistance to adjuvant chemotherapy [39]. STn antigen was also related to high grade tumors, elevated proliferation rates and high risk of recurrence and progression in bladder tumors [29].

The combination of both features: An acceptable tumor specificity and almost absence of expression of Tn and STn in normal cells; together with their association to tumor prognosis and higher malignancy, including metastization, turned Tn and STn antigens as excellent targets to be used in therapeutics.

3. Immune Response and Immunotherapies against Carbohydrates

The immune response is key not only to elicit protection against pathogens, but also to maintain immune surveillance against the development of malignant cells. From this perspective, the development of cancer can be seen as a failure of immune surveillance [40]. Most tumor associated carbohydrates do not elicit strong humoral responses and, in fact, evidences have shown that their aberrant expression is one of the mechanisms developed by cancer cells to prevent effective immune responses against cancer [5].

The mechanisms to provide anti-tumor protection can be broadly categorized into the cellular and the humoral immune responses. While cellular immune response involve mainly cytotoxic lymphocytes such as cytotoxic T cells that may potentially eliminate tumor cells; the humoral response involves B cells and the production of antibodies against specific tumor antigens, that ultimately lead to antibody dependent cytotoxicity, as mentioned in more detail below. Both arms of the immune response involve the activation of a variety of other immune cells, which ultimately converge for both responses. For the elicitation of cellular immune responses, antigen presenting cells such as dendritic cells (DCs), macrophages and B cells, have to uptake antigens and then present through the major histocompatibility complex (MHC) a small portion of peptide antigens (epitopes) to activate both specific $CD8^+$ cytotoxic T cells and $CD4^+$ helper T (Th) cells. The receptors of both cytotoxic and Th cells, *i.e.*, T cell receptors (TCR), are restricted to recognize peptides, presented in the context of MHC class I or class II, respectively, by the antigen presenting cells. The cellular immune response is therefore mainly formatted to fight peptide antigens and this may impose a limitation to develop strong immune responses against carbohydrates. While it has been reported that cytotoxic T cells may recognize mono- and disaccharides attached to peptides with Ser or Thr [41–45], further investigations are necessary to better understand the relevance of such recognition in the context of antitumor immune responses.

The humoral immune response against carbohydrates plays a key role in antitumor responses. In contrast to TCR, the B cell receptors (BCR) can recognize directly carbohydrate antigens with no need for presentation through MHC. Cross-linking of the antigen on BCR activates B cells, which initiate the secretion of IgM antibodies. IgM is the first immunoglobulin isotype to be secreted by plasma B cells (effector B cells), characterized by a pentameric structure with relatively low affinity to antigens and short time period, as compared with the other isotypes. This process is called the T cell-independent B cell activation (Figure 2—upper panel), and is able to provide immunity however with limited duration.

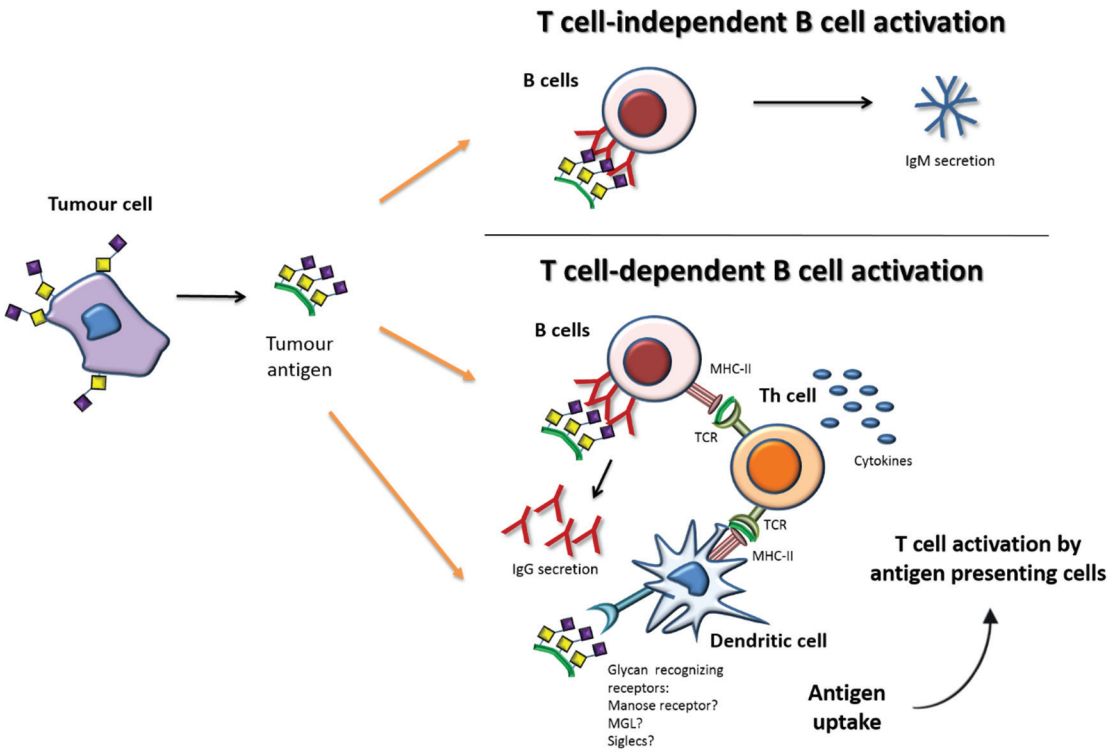

Figure 2. Simplified depiction of tumor antigen recognition and the induction of humoral immune responses. Upper panel—Glycan antigens can be directly recognized by B cells and the cross-linking of the B cell receptors lead to IgM secretion through T cell-independent B cell activation. Lower panel—Tumor antigens can be endocytosed by antigen presenting cells, such as dendritic cells, or B cells and then presented to helper T cells (Th) cells thus leading to T cell activation and subsequent T cell-dependent B cell activation. Adapted from [46].

For the purposes of long lasting anti-tumor humoral immunity, the production of other antibodies isotypes such as IgG is desirable. Physiologically, this usually implies the involvement of Th cells, in a process called T cell-dependent B cell activation (Figure 2—lower panel). Th cells specific for particular peptides are initially activated by antigen presenting cells. Then carbohydrate specific B cells receive stimulatory signals (cytokines) from Th cells, which allow the switching of antibody subtypes from IgM to high-affinity IgGs and the differentiation of plasma cells into memory B cells [47]. These high-affinity IgG antibodies can bind to the target cancer cells, marking them for destruction by either the complement (complement dependent cytotoxicity (CDC)) or by Natural killer (NK) cells (antibody-dependent cell-mediated cytotoxicity (ADCC)).

The knowledge of these physiological mechanisms raised, in recent years, efforts to elicit increased immunogenicity of carbohydrate antigens, aiming to generate potent immunotherapies, such as vaccination. The approaches to improve carbohydrate immunogenicity include the covalently coupling of carbohydrates to immunologically active protein carriers, such as keyhole limpet hemocyanin (KLH) or adjuvants or to physiological/pathological carriers, such as mucin 1 protein (MUC1), to guarantee a range of relevant epitopes [48]. Interestingly, several studies have demonstrated that the conjugation of glycosylated MUC1 peptides with a peptide T helper cell epitope, a Toll-like receptor 2 agonist, tetanus toxoid, or a combination of these active protein carriers induces a potent antigenic and cellular immune response that can kill the tumor cells and reduce the tumor burden [4,49–53].

Immune Response and Tn/STn Antigens

There are a relevant number of reports describing immune responses against the Tn or STn antigens. For instance, autoantibodies against STn and Tn mucin 1 glycopeptides were identified in breast and colorectal cancer patients [54,55]. These autoantibodies were found at higher levels in early stage breast cancer patients and associated with reduced metastases rate [54], suggesting a role in inducing antitumor immune response. Significantly, these antibodies react with the carbohydrate only when carried on the MUC1 protein, emphasizing the role of the peptide backbone in anti-carbohydrate response. A later study with a higher patient cohort showed that autoantibodies to MUC1 peptides or glycopeptides could not be used for cancer screening [56].

Pre-clinical and clinical studies have also shown that immunization with vaccines containing STn or Tn usually induces antigen specific antibodies [57–59]. The elicitation of such immune responses after vaccination could explain the observed delay in tumor growth in mice and the increased time to progression in patients [58,59]. Interestingly, patients receiving the vaccine Theratope, which consisted of STn anchored to the KLH, developed anti-STn antibodies, whose abundance was directly correlated with disease free survival [60]. However, the observed immune responses against STn and Tn antigens, seem to be insufficiently robust to induce protective immune responses. In fact, in spite of the fact that STn and/or Tn-based cancer vaccines have already entered clinical trials (NCT00030823) including the case of Theratope that reached Phase III (NCT00046371) [58], no vaccine has been approved for clinical use yet. Most tested vaccines failed to induce robust T cell-mediated immunity and did not bring any survival benefit for patients. A better understanding of the immune response against these antigens is advised to elicit increased immunogenicity to improve potency of immunotherapeutic approaches.

In recent years, a better understanding of the immunomodulation role of the Tn and STn antigens has elucidated their contribution to immune tolerance, thus explaining in part the failure with vaccination protocols. For example, Tn antigen expressed in mucin 6 (MUC6) protein abrogates Th1 cell responses and promotes interleukin 17 (IL-17) response, which might favor immune escape of tumor cells [61]. Moreover, Tn is recognized by the tolerogenic lectin—macrophage galactose *C*-type lectin (MGL), expressed by DCs and macrophages, which endows these cells to suppress T cell immunity, thus playing a role in tumor progression [62].

The overexpression of STn is associated with low infiltration of nest $CD8^+$ lymphocytes in endometrial cancer [63]. Furthermore, STn present in secreted colon cancer mucins can interact with CD22 and down-modulate B cell signal transduction [64]. Moreover, siglec-15 recognizes the STn antigen expressed by cancer cells and transduces a signal for enhanced transforming growth factor beta (TGF-β) secretion in tumor-associated macrophages, which can contribute to tumor progression by the TGF-β-mediated modulation of intratumoral microenvironment [65]. More recently, our group demonstrated that STn-expressing cancer cells impair maturation of DCs, endowing a tolerogenic function and therefore limiting their capacity to trigger protective anti-tumor T cell responses [66]. We have also observed that blockade of STn antigens expressed by cancer cells was able to lower the induction of tolerance *in vitro* and DCs become more mature [66]. These findings suggest that targeted therapies based on antibodies may provide efficient means to enhance immune responses against STn tumor cells.

4. Antibodies

For the last 20 years, monoclonal antibody-based treatment has been one of the most successful therapeutic strategies in different fields, including cancer. Due to their unique features, such as high specificity and engagement with the immune system, antibodies' impact has been recognized in many therapeutic areas [67,68]. The unceasing development and optimization of methods involved in antibodies engineering, production and purification, as well as the increasing knowledge of the interplay between antibodies, cancer cells and immune system have contributed to the development of innovative next generation antibodies which are more effective, safer and with broader applications [69–71]. Nowadays, the market for therapeutic antibodies has been growing significantly within the healthcare industry so that 40 years after the generation of the first monoclonal antibody (mAb) [72], around 47 recombinant monoclonal antibody products have been approved in the United States or Europe for the treatment of a wide variety of diseases, ranging from cancer to infectious and cardiovascular diseases to autoimmune diseases [73,74].

4.1. Structure and Role of Antibodies

Antibodies are glycoprotein molecules with a remarkable ability to recognize and bind to antigens with high affinity and specificity, thus further promoting their inactivation or elimination [68]. Typically, antibodies (or immunoglobulins (Igs)) are composed of two antigen-binding fragments (Fab) linked via a flexible region (hinge region) to a constant (Fc) region (Figure 3). Antigen specificity is conferred by the antigen binding site of the antibody, which is formed by the hypervariable complementarity-determining regions (CDRs) on the variable regions of each heavy and light chain, present in the Fab portions. On the other hand, the Fc region is responsible for immune effector functions of the antibodies, promoting the binding to various effector molecules and cells of the immune system [75,76].

Antibodies' mechanism of action involves ADCC, CDC, or blockage of the action of specific molecules. Additionally, antibodies can also function as signaling molecules [77]. Specifically, ADCC is an effector mechanism in which antibodies direct NK cells to kill antigen-expressing cells. This mechanism relies on the engagement of Fc receptors expressed by NK cells leading to their activation and exocytosis of the cytolytic granule complex perforin/granzyme, resulting in the destruction of target cells by apoptosis [78]. CDC, also known as the classical pathway of the complement system, is a cytolytic cascade mediated by a series of complement proteins (C1–C9) that are abundantly present in the serum. It starts with the binding of the complement molecule, C1q, to the Fc domain of the antibody (IgG or IgM) bound on the surface of the target cell [77]. This triggers the subsequent complement cascade that leads to the lysis of target cells. Antibodies can also present a blocking activity, which prevents growth factors, cytokines and other soluble mediators from reaching their target receptors [75]. Finally, antibodies can induce the crosslinking of receptors that are connected to mediators of cell apoptosis, such as caspases, leading to cell death [79]. Antibodies that retain these functions represent an important therapeutic strategy in many different fields.

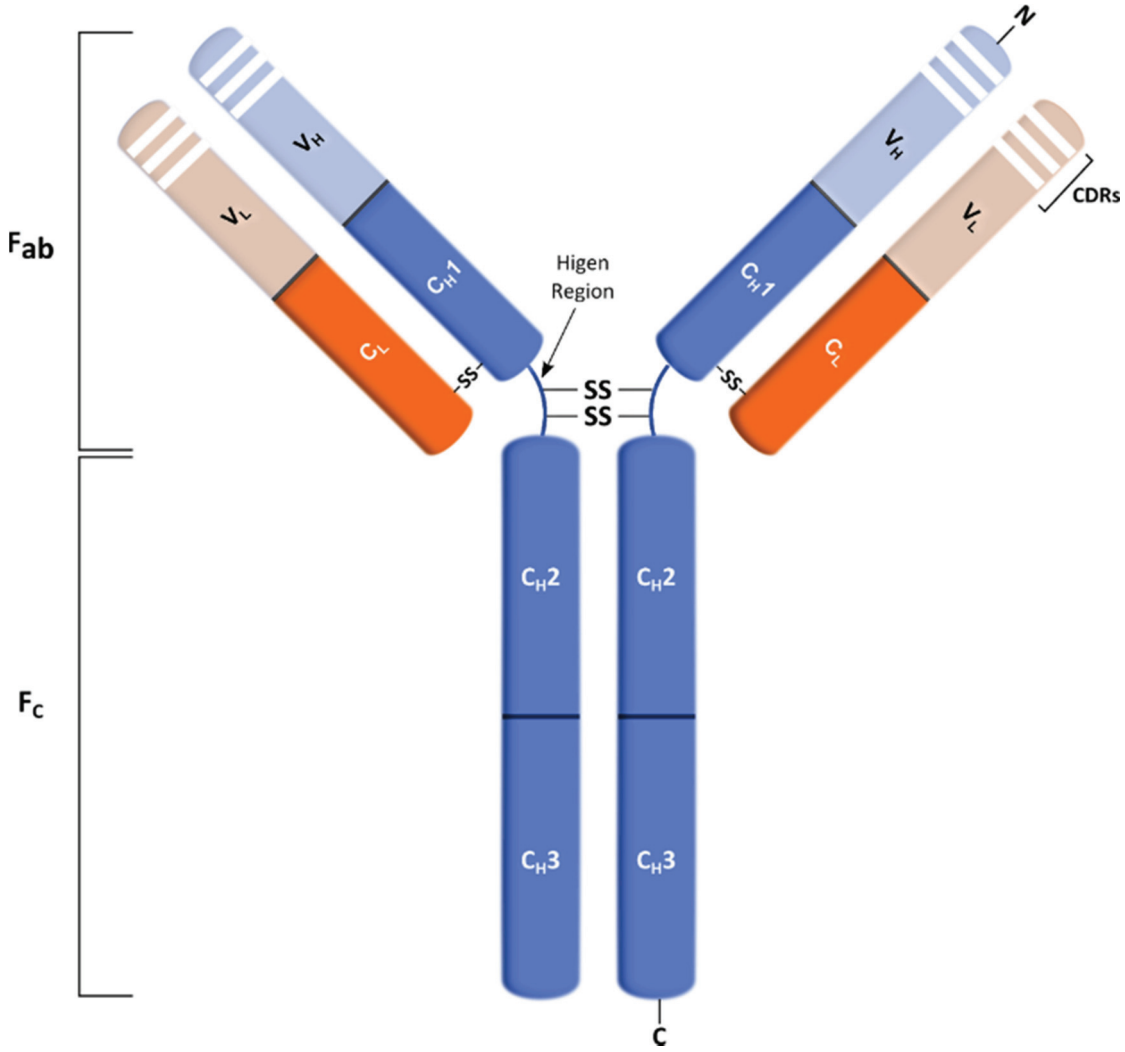

Figure 3. Schematic representation of an immunoglobulin G (IgG) mAb structure. The IgG molecule is composed by constant (C) and variable (V) domains for each light (L) or heavy (H) chain. The heavy chain comprises three constant domains (C_H) and one variable (V_H), and the light chain contains one constant (C_L) and one variable (V_L) domain. This molecule can be divided into F_c and F_{ab} regions in which the latter includes the variable regions, called complementarity-determining regions (CDRs).

4.2. Therapeutic Antibodies in Cancer

Therapeutic antibodies are now one of the most successful and important strategies for treating cancer patients. In their early years, therapeutic antibodies were derived from murine or based on a mouse antibody amino acid sequence, which led to potential issues concerning the generation of human anti-mouse antibodies resulting in immunogenicity or allergic-like reactions. The use of murine antibodies also has the problem of low Fc effector function since murine Fc region are not correctly recognized by human Fc receptors. This results in low efficiency in recruiting the human immune system, including ADCC and CDC, which prevents the effective use of murine antibodies in therapy. In alternative, the next generation of therapeutic antibodies involved genetically engineered fusion of mouse variable with human constant domains, which resulted in a more favorable safety profile and half-life, while retaining the specificity of murine antibodies [80].

Therapeutic antibodies can function through mediating alterations in antigen or receptor functions, modulating the immune system or delivering a specific drug that is conjugated to an antibody that targets a specific antigen. Nowadays, there are molecular techniques that can alter antibody effector function, size and immunogenicity contributing to the development of new and more effective antibody-based therapies [81].

Therapeutic antibodies can kill tumor cells by several mechanisms, by direct action of the antibody, which includes: The blocking of receptors or agonist activity; induction of apoptosis or delivery of a drug or cytotoxic agent; or immune-mediated cell killing mechanisms, which involves CDC, ADCC, and induction of phagocytosis (Figure 4). Furthermore, T cells activation can be improved by antibody-mediated targeting and further cross-presentation of antigen to DCs, T cells targeting to the tumor is improved by antibody-mediated blockade of T cell inhibitory check points (e.g., CTLA4 and PD1) are also among the strategies for tumor cell elimination. All of these approaches have been successfully applied in the clinic [82–85].

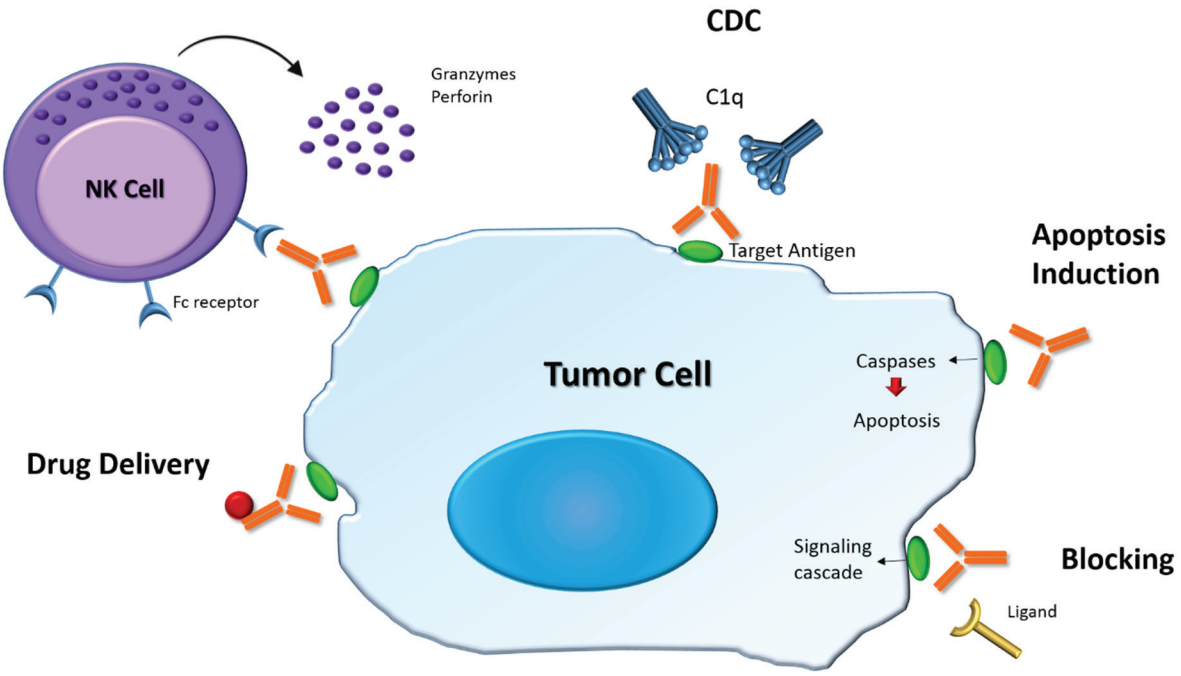

Figure 4. Main functions of therapeutic mAb. mAb can induce antibody dependent cell-mediated cytotoxicity (ADCC) by inducing the release of cytotoxic granules in effector cells (e.g., NK cells). It can also induce complement dependent cytotoxicity (CDC), which starts with the binding of C1q to the antibody triggering the complement cascade. mAb can induce apoptosis as well by activating caspases. In addition, mAb can block receptor/ligand interactions preventing signaling cascade activation, as well as specifically deliver drugs into tumor cells.

Particularly, the target antigen of therapeutic antibodies should be expressed on the surface of cancer cells and be abundant and accessible to antibodies. Depending on the desired mechanisms of action, therapeutic antibodies could be quickly internalized, in case of delivery of toxins into cancer cells and downregulation of cell surface receptors. On the other hand, in ADCC and CDC they should remain on the cell's surface since the Fc region should be available to immune cells and complement proteins [81].

5. Therapeutic Antibodies against Tn and STn Antigens

Due to the association of the Tn and STn antigens with several types of cancers as well as to tumor progression and metastasis, efforts have been made to develop antibodies against these glycosylated structures. In addition, the fact that cancer patients are able to produce autoantibodies, prompted its use as biomarkers for early detection of cancer [86] or response to therapy. Interestingly, antibodies found in cancer patients have not proven to be efficient in activating the immune system and eliminating the malignant cells. Therefore, it is essential to produce more effective antibodies that will specifically kill cancer cells.

So far, the majority of the existent antibodies were obtained by hybridoma technology. Since the development of the first anti-STn specific mAb in 1981, named B72.3 [87], more antibodies against STn antigen have been produced. During this quest, different immunogens and techniques were used and these lead to the development of antibodies exhibiting different affinities and specificities towards the STn antigen [46]. The B72.3 mAb was generated following immunization of mice with membrane-enriched fraction of human breast carcinoma cells and found to react with TAG-72, a glycoprotein with mucin-like characteristics, and with ovine submaxillary mucin (OSM), which is rich in STn epitopes [87,88]. The second generation antibody CC49 was generated following immunization of mice with B72.3 affinity purified TAG-72 molecules [89]. Further studies have been developed using this antibody and its humanized format, which is currently undergoing a clinical trial for its use in radioimmunoguided surgery. However the fine specificity of these mAbs is unclear and in addition to bind to STn, CC49 also recognize other glycans. TKH2 [90] and HB-STn1 are two other anti-STn mAbs that were generated by immunization of mice with OSM [88]. Ogata and colleagues reported that B72.3 strongly binds glycoproteins bearing STn-trimers, but poorly interacts with monomeric-STn glycoproteins [91]. This raised the question of STn configurations for antibody recognition. Later, the antibody MLS102 generated by immunization of mice with LS 180 colonic cancer cells was reported to be specific for clustered STn [92]. More recently, novel anti-STn antibodies LLU9B4 and 3P9 have been produced. The first was generated by immunization of mice with STn glycoproteins extracted from human colon adenocarcinoma cell line LS174T [93] and 3P9 was obtained using human colorectal adenocarcinoma SW1116 cells to immunize mice [94]. This last mentioned antibody is particularly interesting since it is an IgM mAb that showed significant inhibitory effect on proliferation and migration of STn expressing cells and tumor growth by inducing apoptosis. These results suggested that 3P9 mAb has potential applications, not only in diagnosis and imaging, as reported for most of the previous mentioned antibodies, but also for antibody-based tumor therapy [94].

Several mAbs with different specificities towards the Tn antigen have also been generated using different immunogens. Some of these antibodies are CU-1 [95], MLS 128 [96], BRIC 66 (IgM) and BRIC 111 (IgG1) [97], PMH1 [98], and more recently the mAbs KM3413 [99], 2154F12A4 [100] and GOD3-2C4. Monoclonal antibodies, such as MLS128, KM3413 and GOD3-2C4 have been shown to have a therapeutic potential due to their ability to inhibit the growth of certain cancer cell lines [101], and particularly limit the growth of a xenografts of a human lung carcinoma, in the case of the mAb GOD3-2C4 [102].

While most of these antibodies are promising, their specificity is still debatable and the potential therapeutic effect on cancer cells and patient tissues needs to be thoroughly assessed prior to their use as

therapeutic agents. A widely used technique to characterize an antibody's specificity is Enzyme-linked immunosorbent assay (ELISA) and many variations of this methodology against glycoconjugates that are immobilized in plastic or alternatively coating plates with the antibodies. However, in most cases the glycan antigens may not be available and the interpretation is difficult. Therefore, there is still the need to develop assays that fully characterize this specificity of anti-carbohydrate antibodies. Recent efforts in this regard have been accomplished with the development of glycan microarray technologies, which allow the study of interactions between antibodies with carbohydrates in a high-throughput manner [103]. Additionally, existing antibodies are carbohydrate reactive antibodies, meaning that they react with any glycoprotein carrying the glycan. Only a few were able to recognize glycan/peptide epitopes [104] though with low affinity. Antibodies to promising STn or Tn glycopeptide epitopes expressed by cell surface glycoproteins would improve specificity and avoid binding to irrelevant extracellular glycoproteins, such as mucins, which may deceive effective binding of antibodies to cell surface.

5.1. Antibody Production

The urge for therapeutic application of antibodies allied to advances and innovation in molecular biology throughout the years allowed for the development of several methods for mAb production. These technologies endorsed the generation of antibodies against virtually any target molecule on human cells allowing their particular employment in cancer therapeutics. Initially, mAbs production was exclusively accomplished using the hybridoma technology [72] and B cell immortalization using Epstein-Barr virus (EBV) [105]. A few years later, the development of alternative methods such as immunization of transgenic mice expressing human Ig loci [106] and phage display [107] enabled further progress with the production of fully human antibodies.

5.1.1. Hybridoma Technology

Hybridoma technology has long been a remarkable and indispensable tool for generating high-quality mAbs. It allows the isolation and production of mAb with high affinity and specificity against soluble or membrane-bound antigens using an animal's immune system [108,109]. This technology was developed in 1975 by Kohler and Milstein [72], who received a Nobel Prize for this achievement, in which the production of antibodies by spleen B cells obtained from immunized mice was immortalized by fusion with cancerous myeloma cells (Figure 5) [110,111]. The initial step in mAbs production by hybridoma technology is stimulation of the immune system of a suitable host, normally a mouse, with the desired antigen. Usually, the antigen is mixed with an adjuvant to enhance the immune response and consequently increase antibody production. After immunization, antibody-secreting B cells are isolated from the mouse spleen and fused, by using polyethylene glycol (PEG) with an immortalized immunoglobulin non-secreting myeloma cell line that lacks the hypoxanthine-guanine-phosphoribosyltransferase (HGPRT) gene. These cells are then cultured in hypoxanthine-aminopterin-thymidine (HAT) selection medium in which only the hybridoma cells are able to survive and proliferate since they have inherited immortality of myeloma cells and selective resistance of B cells to survive in medium with aminopterin. Usually, the screening of the desired antibody-producing cells is performed using techniques such as ELISA, Western Blot and flow cytometry to analyze the reactivity of their culture supernatant. Positive hybridoma cells are further grown and cloned so high amounts of mAbs can thus be produced *in vitro* [74,112–114].

Several technical improvements have been accomplished to solve the troublesome of this technique, namely, the immortalization of mice B cells and development of different partner cells for fusion; use of feeder layers to increase hybridoma survival and stability in culture and use of different methods for cell fusion, namely electrofusion [110,115].

The first antibody to be approved for the market, muromonab-CD3, was an unmodified murine antibody developed using the hybridoma technology [116]. However, murine mAbs cause immune reactivity when applied to humans and also have low Fc effector function due to the inefficient recognition of their Fc region by human Fc receptors, thus limiting their applications in therapeutics [74]. Consequently, the use and production of human mAbs was boosted in a way that alternative methods have been studied and developed, such as immunization of transgenic mice expressing human Ig loci, phage display libraries, and humanization of mouse antibodies via genetic engineering [106,117,118].

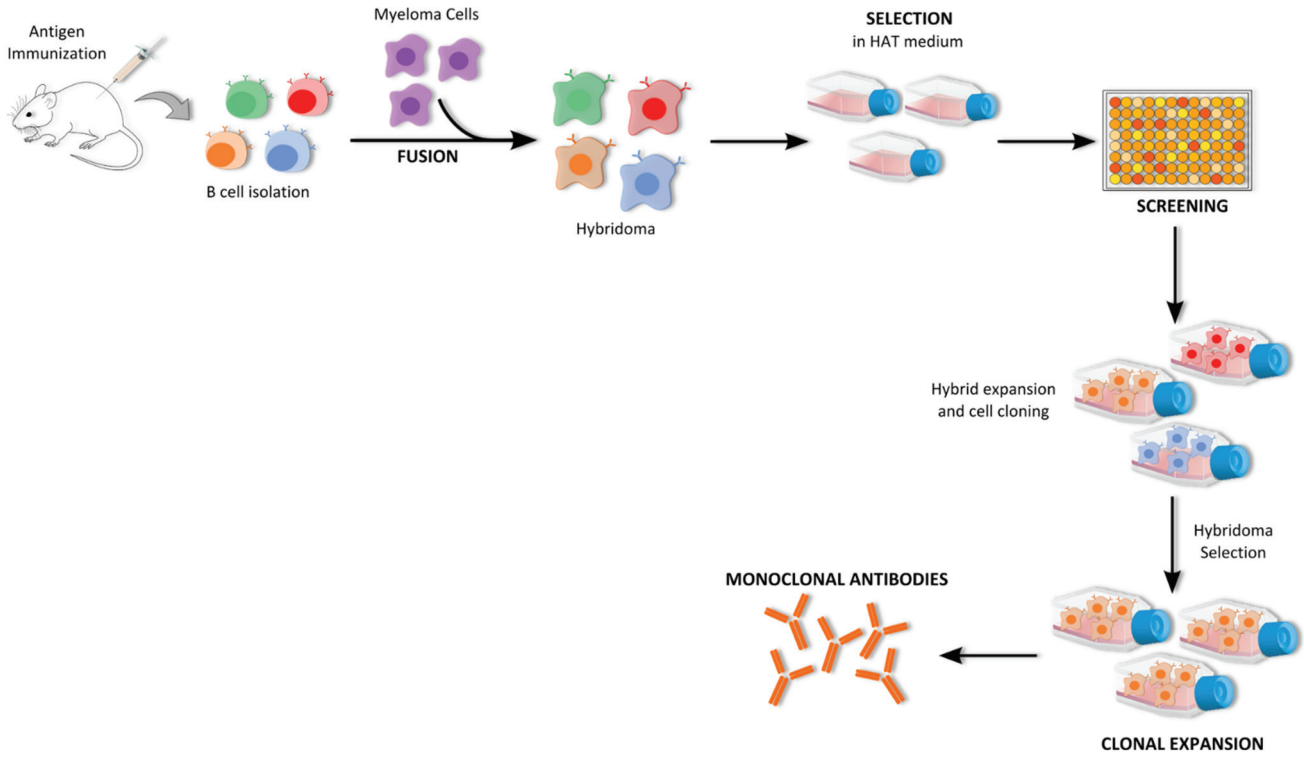

Figure 5. Schematic representation of the hybridoma technology. This technology involves the initial step of mice immunization with desired antigen, followed by the recovery of B cells from mice spleen and consequent fusion with myeloma cells. After that, hybridoma cells are selected in HAT medium and 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.

5.1.2. B Cell Immortalization

Soon after the introduction of the hybridoma technology, an alternative approach to obtain stable human antibody producing B cell lines was created in which antigen-specific plasma B cells are immortalized using the Epstein-Barr virus (EBV) [119]. This virus belongs to the herpes family and has the ability to efficiently immortalize *in vitro* nearly all human B cells. Due to the low frequency of

antigen-specific B cells among peripheral blood lymphocytes of donors, the cells expressing the desired antibody on their surface need to be selected. After selection, target specific B cells are infected with EBV. The B cells are transformed into immortalized diploid cells that usually do not release the virus particles and preserve the characteristics of the initially infected B cells, meaning that they are able to continuously produce and secrete the desirable antibodies [120]. The antibodies produced by this method represent the antibody repertoire of the lymphocytes' donor and, when using a patient's B cells, it offers the possibility of obtaining antibodies with unique specificity against neoantigens. However, the EBV-transformed cells cannot grow indeterminately since they do not have the phenotype of malignant cells. In addition, these cells are difficult to clone, and usually produce only low yields of immunoglobulins [119,120]. In this way, improvements to this method have been carried out, namely fusion of the EBV-immortalized antibody secreting cells with appropriate heterohybridoma fusion partners, which promoted increased stability and higher amount of secreted antibodies [121]. This technique has been successfully applied to generate antibodies against several antigens, such as proteins and carbohydrates [122,123], but never against STn and Tn.

5.1.3. Phage Display Technology

The main struggle in traditional methods is the development of fully human mAbs to apply in therapeutics and consequently, several efforts have been endeavored to overcome this problem. The debut of recombinant DNA technology together with progress of *in vitro* technologies provided several new and powerful ways that lead to the development of the phage display technology to create therapeutic antibodies. This ground breaking technology was introduced in 1985 by Smith [107] centered on the selection of a particular phenotype (e.g., a ligand specific to a target antigen) from repertoires of molecules displayed on phages. In 1990, McCafferty and colleagues demonstrated that the display of antibody fragments on phages was conceivable through the use of vectors to introduce antibody DNA into phage genomes, thus combining genotype and phenotype in one phage particle [117,124]. The concept behind this display technology is that a large library of potentially interesting antibodies is created, from which antibody fragments with desirable specificity and affinity can be selected against a specific antigen [125]. The use of phage libraries carrying human Ig genes-antibody display libraries was one of the most successful applications of this approach [125].

For the construction of an antibody library, the repertoire of V-genes encoding antibodies from B cells is amplified via PCR using a set of specific primers covering all the V gene families. Subsequently the antibody fragments are generated by random combination of VL and VH chain genes [126,127]. Based on the origin of antibody genes, the libraries can be categorized into natural or synthetic. Furthermore, natural source repertoire libraries can be either naive or immune. Naive libraries are derived from non-immunized donors of B cells, and immune libraries are derived from different kinds of immunized animals, human patients with a certain disease or previously exposed [75]. Additionally, several formats of antibody fragments, most commonly Fab and scFv can be cloned and displayed on phage libraries [128]. Once created the repertoire of antibody fragments, this heterogeneous mixture of phage clones is then screened by an affinity selection process called panning, during which the phage populations are presented to specific targets in order to select specific target-binding phages (Figure 6). The antigen of interest is immobilized on a solid support and the antibody phage library is applied to

promote the binding of specific phages against the selected antigen. Extensive washes are performed to remove unbound phages which do not display specificity against the target antigen. Positive binding phages are then eluted and amplified through bacterial infection and grown to produce high phage titer and use in further rounds of selection. Typically, several rounds of panning are performed (2 to 5 rounds) in order to progressively enrich the phage pool with specific binding phages. Antibodies specific for the desired antigen are usually identified by ELISA by screening a set of random clones that were chosen to be analyzed [125,129].

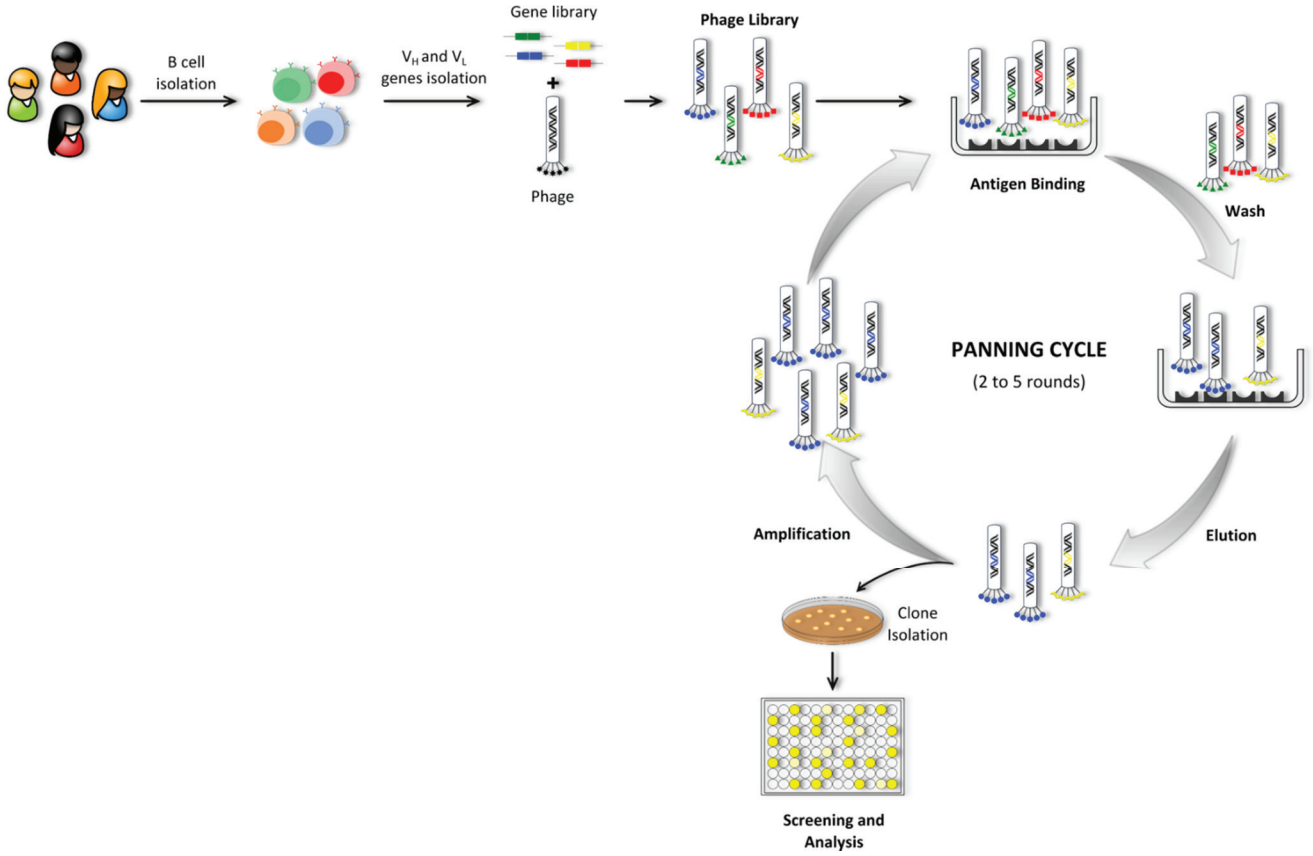

Figure 6. Schematic representation of the phage display technology. This technique includes three main steps that begin with antibody phage library construction and antibody fragments' display onto the phage surface, followed by several rounds of selection against the target antigen (termed panning cycle) and lastly, clone isolation and subsequent screening for fragments with desired specificity.

Once antibody fragments of interest are isolated, due to the link established in phage display between genotype and phenotype, the sequence encoding these molecules can be promptly determined and used to produce the desired antibodies, as well as to refine the isolated antibodies' properties [125,130]. Currently, there are three antibodies on the market created using the phage display technology Humira [131], Benlysta [132], and AbThrax [133]. Particularly, research using this technology has been making progress on the production of antibodies against challenging antigens, namely tumor associated carbohydrates. In particular, this strategy can be an alternative to conventional hybridoma technology, especially when aiming to obtain antibodies against simple and small conformational antigens, with low immunogenicity [134]. Kubota and colleagues have successfully used the antibody phage display

technology to identify an anti-Tn scFv antibody [135]. This was reformatted into a human IgG with promise for therapy, since it has been proven to bind specifically to Tn-positive tumor cells and to induce significant cytotoxic activity in ADCC experiments. Moreover, in comparison with wild-type IgG molecules, the scFv-Fc format has potential benefits for therapeutic development and use. These types of antibodies have small molecular weight and therefore are thought to have better tissue penetration. In addition, since they are composed of a single polypeptide chain, heteromeric disulfide association is not required and consequently, expression of functional scFv-Fcs has been reported using yeast [135].

5.1.4. Transgenic Mice

Among the several techniques developed to produce human mAbs, recently a technology focused its efforts on the generation of low immunogenic mAbs using transgenic mice expressing repertoires of human antibody gene sequences. The production of human mAbs using the hybridoma technology and genetically engineered mice expressing human antibody repertoires was first reported in 1994 [136]. The engineered mice were generated through the disruption of the endogenous mouse Ig-heavy chain and Igκ-light chain loci to avoid the expression of mouse heavy or light chains. Simultaneously, in order to replace the mouse chains, transgenes encoding human Ig-heavy chain and Igκ-light chain were introduced. Upon immunization, these knockout/knockin mice were able to produce human antibodies and using conventional hybridoma technology, the spleens were further used in the production of human mAbs by hybridoma cell lines [136].

Over the past years, progress has been accomplished and additional V gene segments can be expressed by transgenic mice expanding the repertoire of the antibodies obtained. Currently, several different versions of similarly engineered mice have been developed and exploited by different biotechnological and pharmaceutical companies. In fact, over 50 human mAbs developed using this technology are in clinical trials and by 2010 six indeed reached the market [119].

5.1.5. Molecularly Engineered Antibodies

The expansion of therapeutic antibodies is a dynamic field that evolves alongside with technological developments. Advances in antibody engineering allowed to overcome the main problems associated with the immune response developed against foreign antibodies introduced into humans [137]. Antibody chimerization, humanization and the development of human antibodies were the major strategies adopted to reduce immunogenicity [68]. Additionally, efforts to improve affinity, pharmacokinetics and avidity of therapeutic antibodies as well as enhance their natural effector functions have been employed. Several approaches can be used to manipulate the variable regions in order to increase the binding affinity for antigens, or tune the binding specificity, namely chain-shuffling, randomization of CDRs and induced mutations in the variable regions [138,139]. On the other hand, modifications in glycosylation or amino acid sequences in the constant region can modulate the Fc effector functions of antibodies, since this region is involved in ADCC and CDC [137,139]. Extension of the IgG half-life in serum is accomplished by engineering the Fc region particularly in modulation of its interaction with the neonatal Fc receptor (FcRn) which is associated with immunoglobulin protection from intracellular degradation [140]. Currently, antibody-drug conjugates, multimerization of antibody fragments and production of a variety of formats not found in nature, such as Fab and scFv fragments, diabodies and minibodies are rapidly

emerging as key strategies in the antibody engineering field [141,142]. As anticipated, antibody engineering has been fostering continuous research and developments in the refinement and manufacturing of innovative antibodies that display new and enhanced properties to stimulate the antitumor immune response and be applied in therapeutics.

6. Conclusions

Antibodies against STn and Tn antigens have initially raised a great interest due to their potential use as diagnostic tools. This interest was further extended to the exploitation of the antibodies' predictive value in vaccinated patients. Unexpectedly, there has been no (or very limited) efforts in extending the therapeutic use of antibodies to boost antitumor immune responses or conjugated to toxic payloads. The fact that no therapeutic antibodies have entered into clinical trials could be explained by an apparent lack of interest given the glaring promise of vaccination or by an interlude while new antibodies are being developed by academic or biopharmaceutical companies. Undeniably, there is the need to develop novel antibodies with improved specificities targeting STn or Tn. Alternatively, antibodies against glycopeptides could be a suitable approach to increase their specificity. Obtaining antibodies with such specificity for the glycan conformation is technically demanding, therefore novel cutting-edge methodologies herein described emerge as very attractive resources to accelerate the development of therapeutic anti-Tn and -STn antibodies that could be particularly useful in enhancing immune responses or as drug delivery systems.

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Conflicts of Interest

The authors declare no conflict of interest.

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Overexpression of tumour-associated carbohydrate antigen sialyl-Tn in advanced bladder tumours

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ABSTRACT

Little is known on the expression of the tumour-associated carbohydrate antigen sialyl-Tn (STn), in bladder cancer. We report here that 75% of the high-grade bladder tumours, presenting elevated proliferation rates and high risk of recurrence/progression expressed STn. However, it was mainly found in non-proliferative areas of the tumour, namely in cells invading the basal and muscle layers. STn was also found in tumour-adjacent mucosa, which suggests its dependence on a field effect of the tumour. Furthermore, it was not expressed by the normal urothelium, demonstrating the cancer-specific nature of this antigen. STn expression correlated with that of sialyltransferase ST6GalNAc.I, its major biosynthetic enzyme. The stable expression of ST6GalNAc.I in the bladder cancer cell line MCR induced STn expression and a concomitant increase of cell motility and invasive capability. Altogether, these results indicate for the first time a link between STn

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Tumour-associated glycans
Proliferative bladder cancer

expression and malignancy in bladder cancer. Hence, therapies targeting STn may constitute new treatment approaches for these tumours.

1. Introduction

Bladder cancer, the fifth most common cancer in Western society, is a growing concern, owing to increased incidence during the past years (Ploeg et al., 2009; van Rhijn et al., 2009). Most of the newly diagnosed bladder cancer cases are superficial, or low-grade non-muscle invasive papillary tumours, being conservatively treated by complete transurethral resection of the tumour (Babjuk et al., 2012). However, approximately half of the patients show a high-percentage of recurrences and an elevated risk of progression to muscle invasive disease, which correlates with poor prognosis (Hussain et al., 2009). The risk of recurrence and/or progression is mostly determined by clinicopathological features (Babjuk et al., 2012). According to the European Organization for Research and Treatment of Cancer (EORTC), this group includes high grade (HG) papillary tumours and carcinoma *in situ* (CIS) and those with multifocal or recurrent lesions (Babjuk et al., 2012). The evaluation of the nuclear protein Ki-67 (Ki-67 proliferation index), an established marker of cell proliferation, is often used to enhance the prognostic accuracy of risk classification given by clinicopathological features (Margulis et al., 2009; Santos et al., 2003), since it is considered a surrogate biomarker of bladder cancer aggressiveness, disease recurrence and progression (Margulis et al., 2009; Santos et al., 2003).

Tumour resection followed by a schedule of intravesical instillations with live attenuated strains of *Mycobacterium bovis* (Bacillus Calmette–Guérin, BCG) is the standard adjuvant therapeutic option for high-risk of recurrence/progression bladder tumours (Askeland et al., 2012; Babjuk et al., 2012). Although BCG has improved the management of high-risk patients, 30–40% of cases either show intolerance or relapse after treatment (Yates and Roupret, 2011). Consequently, these patients require life-long follow-up and repeated courses of treatment making bladder cancer the costliest to treat among solid tumours (Askeland et al., 2012; Dovedi and Davies, 2009; Sievert et al., 2009). Upon therapeutic failure and/or muscle invasion, cystectomy is advocated for oncological control (Askeland et al., 2012; Dovedi and Davies, 2009; Sievert et al., 2009). Furthermore, at the moment there is a lack of specific biomarkers to target aggressive cell phenotypes and direct molecular-based therapy, which may be used to avoid preventive cystectomy (Dovedi and Davies, 2009).

Vaccines using tumour-associated glycans, in association with immunological boosters, are emerging as potential therapeutic strategies against cancer (Hakomori, 2001; Lakshminarayanan et al., 2012; Ryan et al., 2010; Sorensen et al., 2006). In the forefront of these antigens is sialyl-Tn (STn; Neu5Acα2-6GalNAcα-*O*-Ser/Thr) (Gilewski et al., 2007; Julien et al., 2009; Miles et al., 2011). STn has been mostly observed in tumour-associated mucins due to their high number of potential *O*-glycosylation sites (Clement et al., 2004; Conze et al., 2010; Julien et al., 2006; Marcos et al., 2011; Pinto et al., 2012). However, integrins (Clement et al., 2004) and CD44 (Julien et al., 2006), among other proteins, may also carry this posttranslational modification. Overexpression of STn antigen has been detected in breast (Leivonen et al., 2001), oesophagus (Ikeda et al., 1993), colon (Itzkowitz et al., 1989), pancreas (Kim et al., 2002), stomach (David et al., 1996; Marcos et al., 2011), endometrium (Inoue et al., 1991), and ovary (Numa et al., 1995) carcinomas, whereas low or no expression was observed in the respective normal tissues. STn overexpression was also reported in several cancer precursor lesions, such as esophageal dysplastic squamous epithelia (Itoh et al., 1996), gastric intestinal metaplasia (Baldus et al., 1998; Ferreira et al., 2006) and colonic moderate dysplasia (Cao et al., 1997).

STn is known to influence cell recognition by the immune system (Angata et al., 2007), affect processes as cell cycle, apoptosis, and actin cytoskeleton dynamics, decrease cell–cell aggregation and increase extra-cellular adhesion, migration, invasion (David et al., 1996; Julien et al., 2006, 2005; Pinho et al., 2007) and metastization (Ozaki et al., 2012). In line with these observations, STn positive (STn^+) cells have been frequently observed at the invasion front of tumours and in peritoneal and pleural effusions in ovarian cancer patients; yet they are less common in metastatic lesions than in primary tumours (Davidson et al., 2000). In gastric carcinomas, STn was correlated with the depth of invasion and metastization (Ikeda et al., 1993), and thus poor prognosis (Terashima et al., 1998). Conversely, STn was not correlated with the depth of invasion in studies concerning colorectal (Itzkowitz et al., 1989; Ogata et al., 1998) and breast cancers (Schmitt et al., 1995). However, some contradicting results have been presented regarding its association with metastasis and decreased survival in these cancers (Julien et al., 2012). Hence, a recent review suggests that the biological role of STn in tumour development may be dependent on each cancer type or sub-type (Julien et al., 2012).

Despite these observations, there is little information regarding STn in the context of bladder cancer. Given its clinical relevance and the fact that there are available therapies based on this antigen, we addressed the presence of STn in bladder tumours and the mechanisms underlying its expression.

2. Materials and methods

2.1. Patient and sampling

Formalin-fixed, paraffin embedded (FFPE) tissues were prospectively collected from 69 patients, mean age of 69 years (age range 45–89), who underwent transurethral resection (TUR) of the bladder tumour in the Portuguese Institute for Oncology of Porto (IPO-Porto, Portugal), between July 2011

and May 2012. Based on urothelial carcinoma grading and staging criteria of the World Health Organization (WHO), three different groups were considered (Table 1), low-grade (LG, $n = 24$) and high-grade HG non muscle-invasive (NMIBC, $n = 26$) and muscle-invasive (MIBC, $n = 19$) bladder cancers. Of HG NMIBC, 21 were papillary tumours and 5 were carcinoma *in situ* (CIS). None of these patients had received prior adjuvant therapy. Six normal urothelium tissues of necropsied male individuals without bladder cancer history, within the same mean of age range, were also included.

Additionally, FFPE tissues from 16 radical cystectomy cases including the main lesion in each specimen, responsible for therapeutic decision, the adjacent mucosa, which may or may not include a concomitant tumour, and the ureter representing a distant mucosa, were also studied. Mucosa without visible histopathological alterations was defined as "histologically normal" mucosa.

All procedures were performed under the approval of the Ethics Committee of IPO-Porto, after patient's informed consent. All clinicopathological information was obtained from patients' clinical records.

2.2. Tissue expression of STn and Ki-67

FFPE tissue sections were screened for STn and Ki-67 by immunohistochemistry using the avidin/biotin peroxidase method. Briefly, 3 μm sections were deparaffinised with xylene, rehydrated with graded ethanol series, microwaved for 15 min in boiling citrate buffer (10 mM Citric Acid, 0.05% Tween 20, pH 6.0), and exposed to 3% hydrogen peroxide in methanol for 20 min. The expression of STn was then evaluated using anti-STn mouse monoclonal antibody, clone TKH2 (Kjeldsen et al., 1988), that identifies both single and clustered STn residues (Ogata et al., 1998), whereas Ki-67 was evaluated using monoclonal mouse anti-human Ki-67 antibody, clone MIB-1 (Dako). After blockage with BSA (5% in PBS), the antigens were identified with Vectastain Elite ABC peroxidase kit (Vector Lab) followed by incubation with 3,3-diaminobenzidine tetrahydrochloride (DAB, Dako). Finally, the slides were counterstained with haematoxylin for 1 min. Positive and negative control sections of intestinal metaplasia were tested in parallel. The negative control sections were performed by adding BSA (5% in PBS) devoid of primary antibody. STn^+ tissues were also treated with a neuraminidase from *Clostridium perfringens* (Sigma–Aldrich) as previously described by Marcos et al. (2011) in order to remove the sialic acid. The desialyated samples were thereafter screened for STn. The *O*-acetylation of Neu5Ac residues in STn was evaluated after treatment with 100 mM NaOH at room temperature for 30 min as described by Ogata et al. (Ogata et al., 1998) prior to immunohistochemistry with antibody TKH2.

A semi-quantitative approach was established to score the immunohistochemical labelling based on the intensity of staining and the percentage of cells that stained positively. The STn and Ki-67 expression were assessed double-blindly by two independent observers and validated by an experienced pathologist. Whenever there was a disagreement, the slides were reviewed, and consensus was reached. Tumours were classified as proliferative whenever Ki-67 expression was higher than 18%, as described by Santos et al. (Santos et al., 2003).

Table 1 – STn expression in the healthy urothelium and in non-muscle invasive (NMIBC) and muscle invasive (MIBC) bladder cancers of different clinicopathological natures.

	Total	STn expression
Normal urothelium	6	
–		6 (100%)
+		–
++		–
+++		–
Total STn^+		0 (0%)
NMIBC	50	
Low-grade papillary tumours	24	
–		19 (79%)
+		5 (21%)
++		–
+++		–
Total STn^+		5 (21%)
High-grade (CIS + papillary tumours)	26	
Carcinoma in situ (CIS)	5	
–		4 (80%)
+		1 (20%)
++		–
+++		–
Total STn^+		1 (20%)
High-grade papillary tumours	21	
–		5 (24%)
+		9 (43%)
++		4 (19%)
+++		3 (14%)
Total STn^+		16 (76%)
MIBC	19	
–		5 (26%)
+		11 (58%)
++		2 (11%)
+++		1 (5%)
Total STn^+		14 (74%)

–: No reactivity; +: ≤15%; ++: 15–30%; +++: 30–45% of the tumour.

2.3. Cell lines culture

The human bladder cancer cell line MCR and the transduced variants of MCR (MCRnc and $MCRSTn^+$), were grown as described by Videira et al. (2009b).

2.4. Generation of STn^+ bladder cancer cells

MCR cells were transduced with a retroviral vector generated with the ViraPower™ Lentiviral Expression System (Invitrogen), according to manufacturer's instructions. The whole coding region of human ST6GalNAc.I was PCR amplified and cloned in the pLenti6/V5 Directional TOPO cloning vector which drives the expression of inserted genes through the CMV promoter. A negative control retroviral vector was prepared with an empty plasmid. After transduction with negative control- or ST6GalNAc.I-expressing vectors, MCR cells were selected with 4 μg ml^{-1} blasticidin. An additional immunomagnetic enrichment of the STn^+ cells was performed by using mouse anti-STn (HB-STn1 clone from Dako), followed by the secondary antibody anti-mouse IgG associated to paramagnetic microbeads (Miltenyi Biotec). The stable transduction of the enzyme was confirmed by evaluation of ST6GalNAc.I expression and activity. STn expression was

determined by analysis of the mean fluorescence intensity (MFI) ± SE through flow cytometry analysis using monoclonal antibody TKH2.

2.5. Evaluation of STn expression in cell lines

For phenotypic characterization, cells were stained with 1:50 diluted anti-STn TKH2 monoclonal antibody for 16 h at 4 °C, and 1:100 diluted goat fluorescein isothiocyanate (FITC)-labelled anti-mouse IgG (Dako) for 15 min at 4 °C in the dark and then acquired in a FacsCalibur Flow cytometer (Becton Dickinson). Data were analysed using the WinMDI v2.9 software (The Scripps Research Institute, San Diego, CA, USA).

2.6. Analysis of ST6GalNAc.I expression

RNA extraction from FFPE sections was performed after deparaffinization of the tissue using Absolutely RNA FFPE kit (Agilent technologies) while for cell lines it was used the GenElute Mammalian Total RNA Purification kit and DNAase treatment (Sigma), according to the manufacturer's instructions. The purity of RNA extracts was determined based on the A_{260}/A_{280} ratio. Only ratios between 1.9 and 2.1 were considered further.

Approximately 250–500 ng of total RNA (1 μg for cell lines) was converted by reverse transcription into cDNA, using the random-primers-based High Capacity cDNA Archive Kit (Applied Biosystems). The expression levels of ST6GalNAc.I were determined by TaqMan assay (Applied Biosystems), the reference sequences detected by each primer/probe set and the Assay ID provided by the manufacturer were the following: ST6GalNAc1 (NM018414.2/Hs00300842_m1). Real time PCR was performed in a 7500 Fast Real-Time PCR System using the TaqMan Universal PCR Master Mix Fast from Applied Biosystems, as described previously by Videira et al. (2007, 2009a). During the cDNA exponential amplification the product formation was proportional to the fluorescence emission resulting from the TaqMan probe degradation (van der Velden et al., 2003). The ST6GalNAc.I mRNA levels were normalized for the expression of β-actin, which was taken as a suitable endogenous control for bladder cancer cells (Videira et al., 2007). The relative mRNA levels were calculated by adapting the $2^{-\Delta\Delta Ct}$ formula (Livak and Schmittgen, 2001).

2.7. Evaluation of ST6GalNAc.I activity

MCR cell pellets were homogenized in H_2O and the protein concentration was determined using the RC-DC protein quantification kit (BioRad) according to the manufacturer's instructions. Sialyltransferase activity was assayed in whole cell homogenates as previously described by Dall'Olio et al. (1997) with some modifications. Briefly, the reaction mixture contained 80 mM sodium cacodylate buffer pH 6.5, 0.5% Triton X-100, 6 μg μl^{-1} of asialo bovine submaxillary mucin (ABSM, prepared by acid desialylation of BSM) as acceptor substrate, 30 μM (1280 Bq) of CMP-[^{14}C]Sia (Amersham) and 2 μg μl^{-1} of homogenate proteins. Endogenous controls were prepared in the absence of acceptor substrate. The enzyme reactions were incubated at 37 °C for 2 h and the acid insoluble radioactivity was measured as previously described by Dall'Olio et al. (1997). The incorporation on endogenous substrates was subtracted.

2.8. Cell proliferation measurement

To study their proliferative capacity, cells were labelled with CellTrace™ CFSE Cell Proliferation Kit (Invitrogen). The MCR cells were resuspended into medium at final concentration of 1×10^6 cells ml^{-1} and incubated with 10 μM CFSE, following the manufacturer's instructions. Subsequently, the CFSE-labelled cells were seeded into 24-well microplates, incubated in a 5% CO_2 incubator at 37 °C and harvested at 24, 48, 72 and 96 h post-culture. Flow cytometry using a FACSCalibur (Becton–Dickinson) was performed and the data collected were analysed with ModFit LT 3.2 software (Verity Software House, Topsham, ME), allowing to assess the cell proliferation index (PI). The PI represents the average number of cells that were originated from a single cell of the parental generation. The parental generation was set based on the analysis of data obtained from the cells corresponding to the 24 h of culture.

2.9. Analysis of cell motility using a wound-healing assay

Cell motility was tested in a wound-healing migration assay. MCR cells were seed into 12-well microplates and grown to confluency. A scratch was made in the monolayer with a sterile 200 μl pipette tip. After wounding, the suspended cells and debris were washed away and fresh medium was added. At 0 and 24 h after wounding, scratched regions were photographed with an inverted microscope equipped with a digital camera.

2.10. Invasion assay

Invasion assays were performed using BD Biocoat Matrigel™ invasion chambers, comprised by an 8-μm diameter pore size filter coated with a thin layer of matrigel, and placed in a two-compartment system in a 24-well plate. Prior to each experiment, filters were re-hydrated in serum-free DMEM medium for 2 h at 37 °C. After detachment of subconfluent cells with trypsin/EDTA, cells were suspended in culture medium supplemented with 5% inactivated FBS, counted and seeded on the upper side of the matrigel-coated filter at a density of 5×10^4 cells/well. After 24 h at 37 °C, filters were fixed in 4% paraformaldehyde and non-invading cells, present on the upper side, were completely removed, to facilitate analysis. Cells that had invaded the underside of the filters were mounted in Vectashield+4′,6-diamidino-2-phenylindole (DAPI, Vector Laboratories, CA, USA), and visualized through a Zeiss Axiovert 200M fluorescence microscope (Carl Zeiss, Germany). Invasive cells were scored in at least 12 microscopic fields (20× objective) when DAPI-counterstained nuclei passed through the filter pores. Results are presented as means ± SD for each sample. Invasion levels are expressed as a ratio of the results obtained with the mock-transfected control cell line.

2.11. Statistical analysis

Statistical analysis was performed using the Student's *T*-test for unpaired samples. Differences were considered to be significant when $p < 0.05$. A chi-square test was used to analyse correlations between clinicopathological features and STn and Ki-67 expressions.

3. Results

3.1. Expression of STn in bladder tumours

STn expression in bladder tumours was evaluated by immunohistochemistry using mouse monoclonal antibody clone TKH2. As shown in Table 1, STn is not expressed in the healthy urothelium; conversely 46% of the bladder tumours presented cells with STn membrane and cytoplasmic staining (32/69) (Figure 1), demonstrating the tumour-specific nature of this antigen. The removal of sialic acids from the tissue sections with a α-neuraminidase impaired the recognition by TKH2 and confirmed STn expression.

STn expression was lower in low-grade (LG) NMIBC (21% STn^+ tumours; Figure 1A–B) compared to high-grade lesions (HG; 67%), which include papillary tumours (76% STn^+ tumours; Figure 1C–E), CIS (20% of STn^+ tumours; Figure 1F), and MIBC (74% STn^+ tumours; Figure 1G–H). Noteworthy, STn was absent from the majority of CIS (4/5; 80%) and showed an expression comparable to LG tumours. Altogether, these results highlight an association between the STn antigen and high grade NMIBC ($p < 0.002$; Figure 2) as well as with muscle invasive tumours ($p < 0.03$; Figure 2).

The *O*-acetylation of sialic acid residues prevents TKH2 from recognizing STn antigens in certain tissues (Ogata et al., 1998). To exclude this possibility in bladder cancer, the slides were chemically de-*O*-acetylated prior to immunohistochemistry. This procedure did not alter STn expression patterns demonstrating that STn antigens were not encrypted by *O*-acetylation.

3.1.1. Pattern and extension of STn expression in bladder tumours

The STn antigen presented a focal expression that for the majority of the STn positive cases (26/36) did not exceeded 15% of the tumour section (Table 1). Furthermore, in 25% of the STn positive cases (9/36) the antigen was detected in less than 5% of the tissue (data omitted from Table 1). Higher expression patterns were restricted to HG papillary NMIBC, where 27% of the cases (7/26) presented STn levels between 15% and 45% of the tumour section (Table 1) and locally diffuse staining (Figure 1C, D, G). STn was mainly observed in basal layer cells (75% of STn^+ cases; Figure 1A, C–E), but it could be also detected throughout the papillae (Figure 1C–E) and cells of the luminal surface (Figure 1F) in cases presenting locally diffuse staining. STn was further observed in cells invading the basal (50% of STn^+ of HG NMIBC; Figure 1C–E, G) and muscle layers (57% of STn^+ MIBC; Figure 1G, H), suggesting a role in invasion.

3.1.2. STn antigen expression in advanced tumours and in the surrounding areas

The STn antigen was also evaluated in a series of radical cystectomy specimens which included the tumour used for therapeutic decision (termed "main tumour" in Figure 3) and the tumour-adjacent mucosa. The ureters were included as distant mucosa (Figure 3). In agreement with the observations from Table 1, STn was detected in 69% (11/16) of all main tumours as well as in their adjacent mucosa (Figure 3), independently of their histological classification. Noteworthy, STn was absent from 90% of the distant mucosas of STn positive cases; the only exceptions being a ureter with pre-neoplastic and another with a neoplastic lesions (Figure 3). These results point out that the STn^+ tumour-adjacent mucosa may display molecular changes similar to those of the main lesions. Thus, this antigen may be useful as a marker of field carcinogenesis in the bladder.

3.2. Expression of ST6GalNAc.I in bladder tumours

The presence of STn has been strongly associated with the overexpression of ST6GalNAc.I in several human malignancies. To assess this event in bladder tumours, mRNA levels of *ST6GalNAc.I* gene were analysed and normalized in relation to β-actin, which proved to be a stable expressed gene in previous studies concerning bladder tumours (Videira et al., 2007). As shown by Figure 4, low gene expression levels were detected in tumours that did not express STn. In addition, the levels of *ST6GalNAc.I* increased with the expression of STn, and were significantly higher in the tumours with STn expression superior to 15%. Figure 4 also shows that this behaviour was similar in LG and HG tumours. However, as a result of higher STn expression, the average ST6GalNAc.I mRNA levels were more elevated in HG (53%) tumours than LG (9%). These observations suggest that overexpression of *ST6GalNAc.I* gene is one of the main events leading to STn expression in bladder tumours.

3.3. STn expression and tumour proliferation

As shown above, the expression pattern of STn correlates with HG tumours, known to present elevated proliferation rates (Margulis et al., 2009; Santos et al., 2003). To assess a possible association between STn and proliferation, 24 cases from the initial series of 69 bladder tumours, comprehending 12 LG and 12 HG tumours (7 NMIBC, none of them CIS, and 5 MIBC), were screened for STn and Ki-67 expression. Tumours presenting Ki-67 expression superior to 18% were classified as proliferative. As highlighted by the graphical matrix in Figure 5A, 8% (1/12) LG and 75% (9/12) HG cases showed elevated Ki-67, confirming the higher proliferation of HG tumours ($p < 0.0012$). Similarly, Figure 5A also shows an association between proliferative phenotypes and STn expression ($p < 0.001$). However, in all STn positive cases, the examination of sequential sections revealed that STn antigen expression was mainly seen in areas that did not express Ki-67 (Figure 5A), although some overlap was present in 25% of the cases (3/12; Figure 5B). This indicates that the STn antigen is mostly expressed in non-proliferative areas of the tumour. Nevertheless, the majority of the non-proliferative tumours also did not express STn (12/14), demonstrating an interdependence between both phenomena.

3.4. In vitro assessment of the biological significance of STn expression

3.4.1. Development of a high-grade bladder cancer cell line overexpressing STn

To further corroborate the role of ST6GalNAc.I in the expression of STn antigen by bladder cancer cells, we induced the overexpression of ST6GalNAc.I in a bladder cancer cell line. The MCR bladder cell line, that showed negligible expression

of ST6GalNAc.I and no STn (data not shown), was transduced with a lentivirus expressing the coding region of the human *ST6GalNAc.I* gene. The obtained cell line variant, herein named MCRSTn$^+$, showed markedly increased expression of *ST6GalNAc.I* mRNA levels (Figure 6A). It also showed significantly higher sialyltransferase activity towards the ABSM, a substrate for the ST6GalNac.I enzyme, when compared with the negative control cell line (MCRnc) transduced with void lentivirus (Figure 6A). The overexpression of STn antigen by MCRSTn$^+$ cell line variant was confirmed by flow cytometric analysis (Figure 6B).

3.4.2. *STn influence on cell proliferation, migration and invasion*

STn expression was correlated with tumours with higher proliferative indexes (Figure 5). To assess the influence of STn in

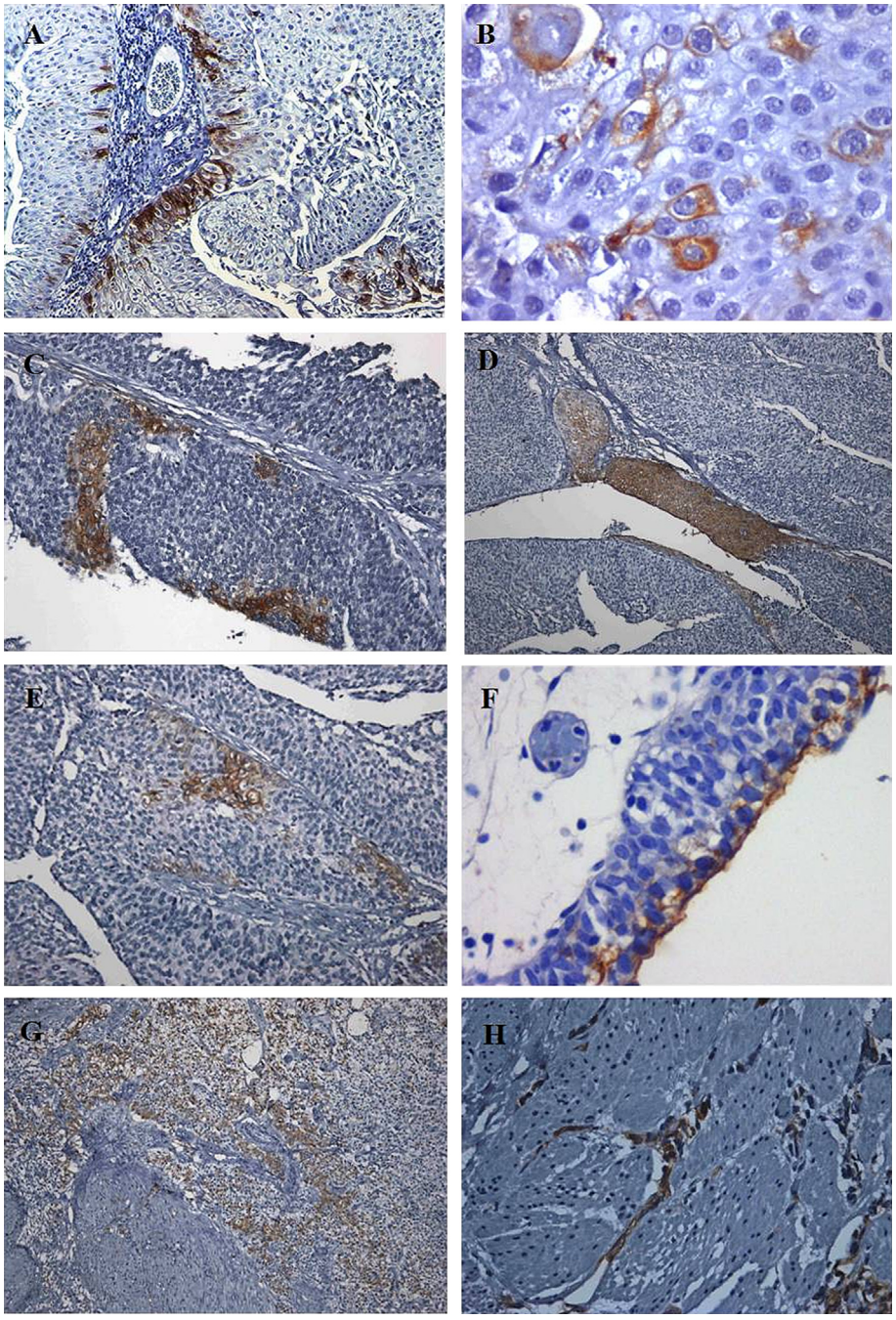

Figure 1 – Expression of STn in FFPE bladder tumours. A) Low-grade papillary tumour showing a predominance of STn$^+$ cells in the basal layer; B) Magnification which shows tumour cells with membrane and cytoplasmic STn$^+$ staining; C) High-grade papillary tumour evidencing the focal nature of STn expression. Positive cells were found both in the basal layer and throughout the papillae; D) High-grade papillary tumour showing locally extensive STn positivity; E) High-grade papillary tumour evidencing STn$^+$ in the basal layer; F) CIS showing STn$^+$ in the cells facing the lumen of the bladder; G) MIBC showing locally extensive STn expression including at the muscle invasive front; H) MIBC highlighting STn$^+$ cells invading the muscle layer.

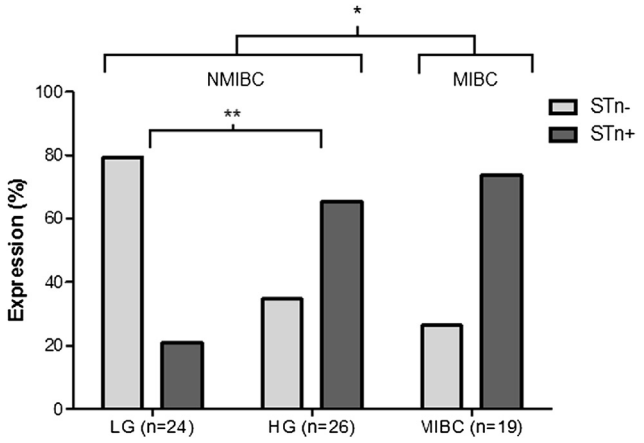

Figure 2 – Association between STn expression and HG NMIBC and MIBC. The percentage of STn$^+$ tumours was higher in HG when compared to LG and also in MIBC when compared to NMIBC (LG + HG). "*" $p = 0.03$; "" $p = 0.002$ (Chi-square test).**

proliferation, MCR cells (MCRnc and MCRSTn$^+$) were cultured for 48, 72 and 96 h and then evaluated in relation to their proliferation index. The comparison between the two cell line variants showed that the proliferation index of MCRSTn$^+$ cells was generally higher than the index of MCRnc cells, although only statistically different at 72 h of culture ($p < 0.05$; Figure 7). However, this effect was no longer significant at 96 h of culture (Figure 7).

STn positive cells were observed invading the basal and muscle layers (Figures 1 and 2) and in the adjacent mucosa of advanced stage bladder tumours (Figure 5), suggesting a correlation of STn with invasion and migration. Thus, the influence of STn expression in MCR cell invasion was assessed using the Matrigel invasion assay. Our results evidence that

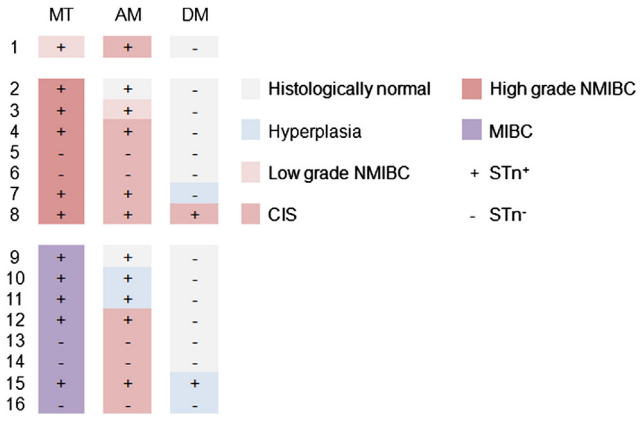

Figure 3 – Expression pattern of STn in radical cystectomy specimens. Radical cystectomy specimens have been organized based on histological grade. They include the tumour responsible by the therapeutic decision termed "main tumour" (MT), an adjacent (AM) and distant mucosa (DM). The graphical matrix highlights that, whenever STn is expressed by the main tumour (13/16; 63%), it is always present in the adjacent mucosa (13/13, 100%). One preneoplastic and one neoplastic distant mucosa also expressed the antigen.

MCR cells transduced with ST6GalNAc.I (MCRSTn$^+$) are approximately four folds more invasive than bladder cells transduced with the negative control (MCRnc; Figure 8A). The effect of STn expression on cell migration was estimated by a wound-healing assay. Therefore, uniform scratches were made in confluent monolayers of MCRnc and MCRSTn$^+$ cell lines and the capability of the cells to migrate and fill the scratches was monitored. As observed in Figure 8A, by 24 h after wounding, the MCRSTn$^+$ cells had almost completely covered the empty space. Conversely, the negative control, MCRnc cells, displayed a large "gap", thus demonstrating their lower capability to closure the wound. Our results evidence that MCR cells expressing STn present increased invasion and wound repair capacities.

4. Discussion

The STn antigen is highly expressed by several human carcinomas and preneoplastic lesions (Julien et al., 2012) and is explored as a tumour marker in serological assays (CA72-4) (Reis et al., 2010).

Despite the clinical relevance of STn in human malignancies, scarce information is available about its role in bladder tumours. Over twenty years ago, Langkilde et al. (1992) addressed this antigen on series of transitional cell carcinomas (currently classified as high-grade urothelial cell carcinomas according to current WHO guidelines (Babjuk et al., 2012)). Normal mucosal biopsy specimens from patients with non-malignant bladder urologic diseases were included as controls. According to the authors, STn was not expressed by the control group, showed a very restricted pattern of expression in bladder tumours and no association with recurrence and progression. Subsequent *in vitro* studies found that

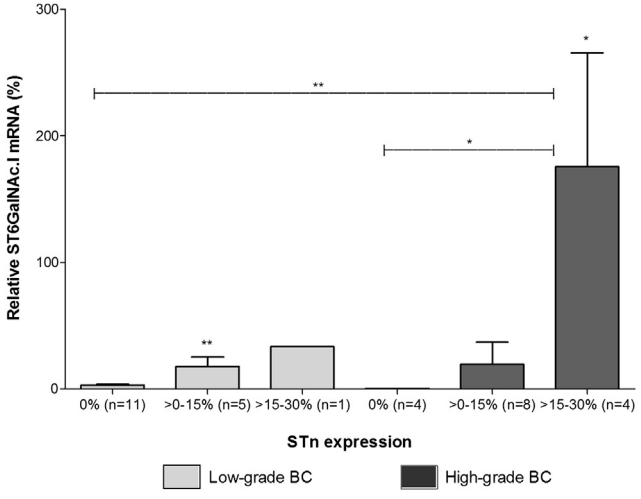

Figure 4 – Association between ST6GalNAc.I and STn expression in LG and HG bladder tumours. The graph shows that ST6GalNAc.I expression is increased in STn$^+$ tumours and increases further for more elevated STn expressions (>15–30% of the tumour section). This suggests that the overexpression of ST6GalNAc.I is one of the main mechanisms underlying the presence of STn in bladder cancers. Furthermore, it shows this event occurs in both LG and HG tumours. "*" $p < 0.05$; "" $p < 0.01$ (Student's *T*-test).**

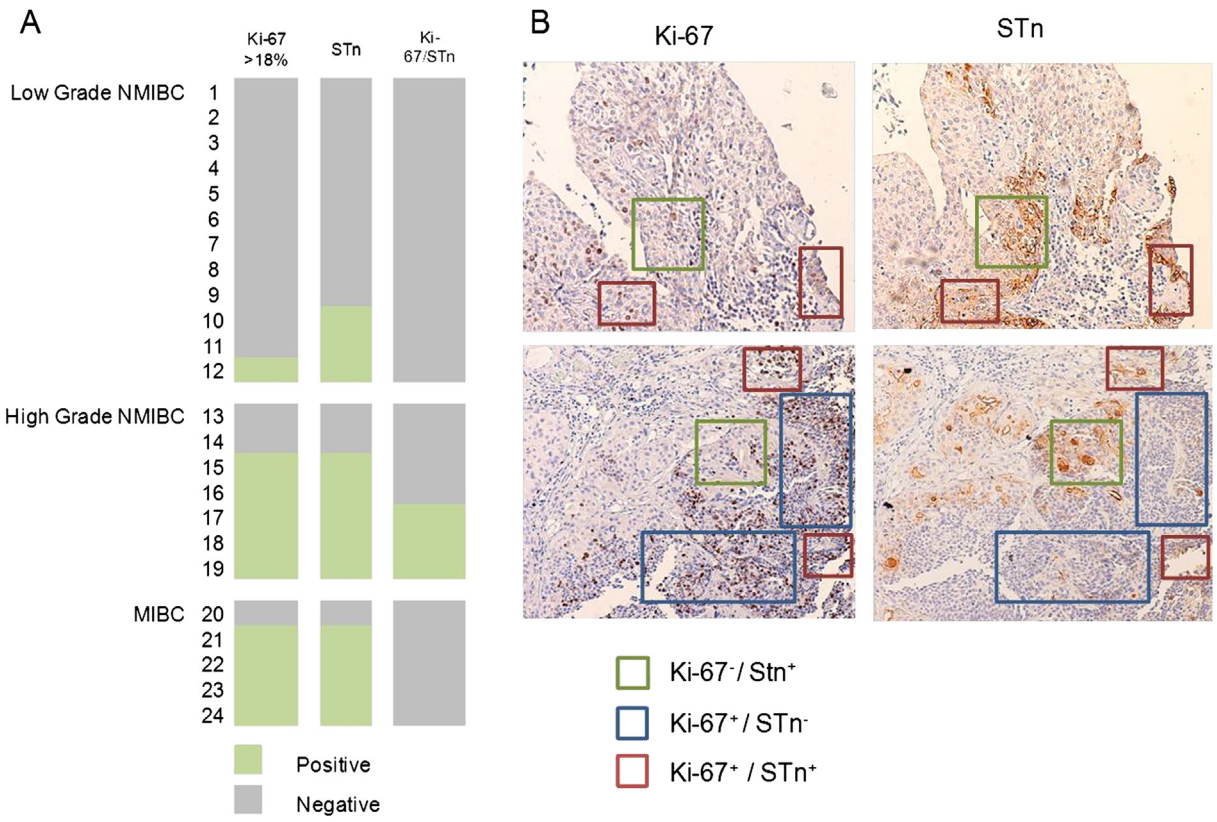

Figure 5 – Expression of STn and Ki-67 in bladder tumours. A) graphical matrix highlighting the association between proliferative tumours (Ki-67 > 18%) and STn expression in bladder tumours. HG NMIBC and MIBC were considered to be proliferative tumours and a significant association was found between STn expression and tumours presenting proliferation phenotypes ($p < 0.001$; Chi-square test). The notation "Ki-67/STn" in the column more to the right refers to tumours presenting areas that appear to exhibit cells expressing both Ki-67 and STn. B) Immunohistochemistry for Ki-67 and STn highlighting Ki-67$^-$/STn$^+$; Ki-67$^+$/STn$^-$; and Ki-67$^+$/STn$^+$ areas.

mucins MUC1, MUC2 and MAUB (mucin antigen of the urinary bladder) isolated from bladder cancer cell lines carried STn (Bergeron et al., 1996, 1997). However, no evidence of such an expression was found in tumours. Herein, we readdressed this matter and found that the STn antigen was associated with advanced stage bladder tumours. More important, STn was absent in the healthy urothelium, which demonstrates its tumour-associated nature. Since this study was performed on a recent prospective series it is not possible, at this point, to determine correlations with disease outcome. Nevertheless, STn was mainly expressed by HG papillary NMIBC, known for their elevated risk of recurrence and progression to muscle invasive disease and MIBC that encompass an elevated risk of metastization and present decreased overall survival (Babjuk et al., 2012). STn expression was further associated with elevated Ki-67, a proliferation-related molecule and a surrogate biomarker of increased risk to recurrence and progression in bladder tumours (Margulis et al., 2009; Santos et al., 2003). In addition, the majority of non-proliferative tumours did not express STn, which demonstrates that the expression of the antigen is indeed a characteristic of proliferative tumours. Still, STn was mainly detected in non-proliferative areas of the tumours. However, the STn antigen was frequently observed in areas of invasion of the basal and muscle layers, suggesting it may be associated with the process of cell migration and invasion. This reinforces the notion that STn is part of a malignant bladder cancer phenotype, as previously observed for other carcinomas (Clement et al., 2004; Julien et al., 2006; Ohno et al., 2006; Ozaki et al., 2012; Pinho et al., 2007). We also found the STn antigen in tumour-adjacent mucosa, which may be explained by the migration of STn$^+$ cells to the tumour surroundings. On the other hand, this may be a consequence of field carcinogenesis previously observed in bladder cancers (Jones et al., 2005; Palmeira et al., 2011). Nevertheless, the STn antigen holds potential as a biomarker of bladder disseminated disease.

STn is a product of an incomplete *O*-glycosylation process due to the premature *O*-6 sialylation of the glycoside GalNAcα1-*O*-Ser/Thr (Tn antigen) by ST6GalNAc.I (Marcos et al., 2004). In several epithelial tumours STn results from an increased ST6GalNAc.I expression and/or activity (Marcos et al., 2011; Sewell et al., 2006; Vazquez-Martin et al., 2004). Previous studies have reported ST6GalNAc.I expression by the urothelium at the mRNA level (Yamamoto et al., 2003); however we and others (Langkilde et al., 1992) have not detected STn expression in the histologically healthy tissues. These observations suggest either the absence of the antigen or the insufficient sensitivity of the method. ST6GalNAc.I localization in the Golgi apparatus and the competitive action of other glycosyltransferases for the Tn antigen may also favour the extension of

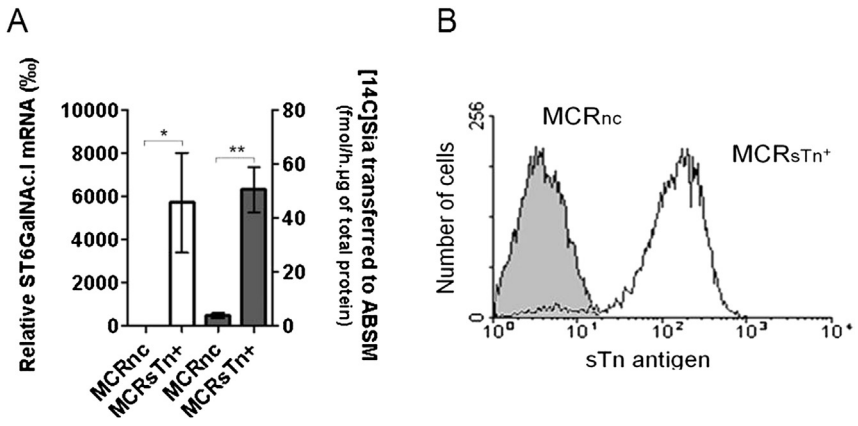

Figure 6 – ST6GalNAc.I mRNA expression and sialyltransferase activity in bladder cancer MCR cell lines. A) ST6GalNAc.I expression and activity in MCR cell lines. The relative mRNA levels of ST6GalNAc.I (open bars) and sialyltransferase activity towards ABSM (grey bars) were analysed as described in the Material and methods section. Both, ST6GalNAc.I mRNA and sialyltransferase activity towards ABSM are negligible in negative control cells and markedly increased upon ST6GalNAc.I transduction. B) Flow cytometry analyses of transduced MCR cells. Both negative control (MCRnc in grey histogram) and ST6GalNAc.I-transduced (MCRSTn$^+$ in open histogram) cell lines were stained with the secondary antibody anti-Ig's-FITC following incubation with the primary antibody anti-STn antigen. 90% of the ST6GalNAc.I-transduced cells expressed the STn antigen (MFI = 216). The data are shown as a mean ± standard deviation of 3 independent studies. "*" $p < 0.05$, "" $p < 0.01$ (Student's *T*-test).**

the O-glycan chain in non-pathological conditions. On the other hand we showed that the levels of STn in bladder tumours were correlated with the expression of ST6GalNAc.I, supporting this as a major molecular mechanism underlying STn biosynthesis in these tumours. Few cases presented STn expression associated with a basal level of ST6GalNAC.I, meaning that other factors may contribute to promote the biosynthesis of STn. A disorganization of secretory organelles (Sewell et al., 2006), somatic mutations in the gene *Cosmc*, encoding a molecular chaperone essential for O-chain elongation (Ju et al., 2008), the down-regulation/decreased activity of several other glycosyltransferases and/or the availability of sugar donors for biosynthesis, may also lead to STn overexpression. The integrated study of metabolic pathways, glycosyltransferases expression/activity, intra-cellular ultrastructures and microenvironmental changes may further enlighten the molecular events leading to abnormal O-glycosylation of bladder cancer proteins.

In addition we have screened HT1376, 5637, T24 and MCR bladder cancer cell lines and found neglectable levels of the STn antigen (data not shown). The same was previously observed in gastric (Ozaki et al., 2012; Pinho et al., 2007) and breast (Clement et al., 2004; Julien et al., 2005, 2006) cancers cell models, demonstrating that tumour cells may lose the ability to express this antigen *in vitro*. Microenvironmental factors may play a determinant role in the induction of STn biosynthesis, yet these events remain unknown. Following the association of STn with invasive cases, we elected the invasive bladder cancer cell line MCR to evaluate the biological role of STn in these tumours. We started by stably transducing the MCR cells with ST6GalNAc.I, which resulted in the overexpression of STn. The expression of STn did not promote a significant enhancement of MCR cell proliferation, which is agreement with observations made for breast (Clement et al., 2004; Julien et al., 2005, 2006) and gastric cancer models (Pinho et al., 2007). These findings associated with the absence of the antigen from most bladder tumours non-proliferative areas strongly suggests that STn expression does not play a direct role in tumour proliferation.

On the other hand, STn expression significantly enhanced the migration and invasive capacity of MCR cells, demonstrating that this antigen plays an important role in bladder cancer cell invasion, as suggested by the observation of bladder tumours. Enhanced migration capabilities of STn$^+$ cells on components of the extracellular matrix, such as fibronectin and collagen, have been described for other cancer cell lines (Julien et al., 2005, 2006; Pinho et al., 2007), and result, among several factors, from impaired integrin binding (Clement et al., 2004). In addition, STn expression has been shown to increase the invasion potential of tumour cells (Clement et al., 2004; Julien et al., 2006; Ohno et al., 2006; Ozaki et al., 2012; Pinho et al., 2007), supporting a similar role in bladder tumours. Further experiments are however required to clarify the molecular mechanisms underlying promotion of cancer cell invasion and migration. These findings reinforce however that alterations in the glycosylation patterns of cell-surface proteins may strongly interfere with events like cell–cell adhesion, cell–matrix interaction, tumour growth, motility and invasion (Dall'Olio et al., 2012).

In resume, our work comprehensively describes the expression of the STn antigen in bladder cancer. Namely, it demonstrates the tumour-specific nature of this type of glycosylation and its association with advanced, highly proliferative tumours, invasion and organ disseminated disease. Thus, the evaluation of STn antigen may add valuable information about the aggressiveness of proliferative tumours, complementing the information given by Ki-67. Studies are ongoing in broader retrospective series to determine the association of STn with disease outcome and corroborate these findings. We are also devoted to the identification of the glycoproteins yielding STn, which is expected to bring insights

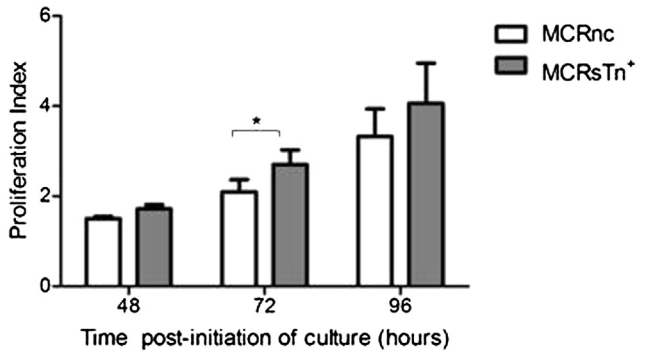

Figure 7 – Comparison between the proliferation capacity of MCRnc and MCRSTn$^+$ cells. The transduced MCR cells were labelled with CFSE and cultured for various periods of time (48, 72 and 96 h). The cells were harvested and analysed by flow cytometry with Modfit software, allowing the calculation of the proliferation index, which represents the average number of cells that was originated by a single cell of the parent generation. At the various periods of culture, MCRSTn$^+$ cells show a higher proliferation index than the negative control, but this difference was only statistically significant at 72 h of culture. The data are presented as a mean ± standard deviation of 3 independent studies. "*" $p < 0.05$ (Student's *T*-test).

about the role of this type of glycosylation in bladder carcinogenesis and provide novel therapeutic vectors. The antigen STn may also be monitored noninvasively in urine or serum using as is the case for other human carcinomas using the CA72-4 test (Reis et al., 2010). This could allow decreasing the number of cystectomies in post-surgery follow-ups of patients with high-grade tumours, a particularly critical matter for the elderly that constitute the majority of the cases.

Furthermore, the STn antigen is associated to high-grade NMIBC which currently constitutes one of the main therapeutics concerns due to their elevated risk of recurrence/progression (Babjuk et al., 2012). Adjuvant immunotherapy with BCG has allowed to delay recurrence and decrease the risk of progression into muscle invasive disease (Babjuk et al., 2012); still more than half of the patients either recur within two-years after TUR of the tumour or show intolerance to the treatment (Askeland et al., 2012; Yates and Roupret, 2011). Due to the lack of efficient therapies, upon therapeutic failure and/or muscle invasion, the patient is faced with cystectomy (Babjuk et al., 2012).

Carbohydrate antigens associated with advanced-stage tumours and malignant phenotypes such as STn, are expressed at the cell surface and, therefore, available for antibody or lectin-mediated recognition (Neutsch et al., 2012). Thus, these antigens may present an opportunity for the introduction of novel therapeutics, such as selective drug-delivery approaches (Neutsch et al., 2012) or carbohydrate-based immunotherapy (Heimburg-Molinaro et al., 2011). An anti-cancer vaccine named Theratope, comprehending a synthetic STn coupled to the immunogenic carrier keyhole limpet haemocyanin has already been developed (Julien et al., 2009; Miles et al., 2011; Sandmaier et al., 1999). Tests in animal models and humans for breast, ovarian, and colorectal cancers have showed that the antigen is safe and produces a strong immune response against these tumours (Julien et al., 2009, 2012; Miles et al., 2011). Even though Theratope failed to improve overall survival of metastatic breast cancer patients in a phase III clinical study, the design of the study disregarded the heterogeneous STn expression between patients (Miles et al., 2011), compromising the outcome (Julien et al., 2012; Zeichner, 2012). Thus, Theratope or other STn-based vaccine designs may constitute valuable therapeutic options for STn positive advanced bladder tumours. However, given the low association of STn with more proliferative areas of the tumour, one is led to speculate that advanced stage bladder cancer patients may better benefit from the combination of anti-STn immunotherapy and antiproliferative drugs. Furthermore, these approaches may allow targeting disseminated disease in the adjacent and distant mucosa from the main tumour.

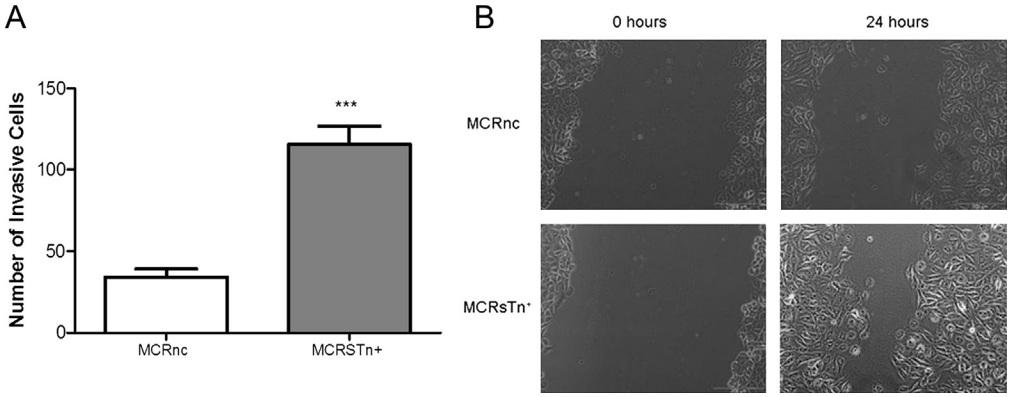

Figure 8 – STn expression promotes MCR cells wound healing closure and invasion. A) Wound healing closure assay. Uniform scratches were made using a 200 μL pipette tip in confluent monolayers of MCRSTn$^+$ and MCRnc cells. Cells were allowed to heal and the extent of closure was monitored by microscopic analysis. After 24 h culture, the MCRSTn$^+$ cells had almost completely covered the wound, in clear contrast to negative control, MCRnc, where unoccupied space was still observed. B) Invasion assay. MCRSTn$^+$ and MCRnc cells were incubated for 24 h, in the upper compartment of Matrigel invasion chambers, in complete DMEM medium and in the absence of other chemoattractants. Invasive cells were determined as described in Materials and methods. The data are presented as a mean ± standard deviation of 4 independent studies. "*" $p < 0.001$ (Student's *T*-test).

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CEA as an E-selectin ligand in non-small cell lung cancer

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Abstract

Lung cancer (LC) is one of the major causes of mortality related to cancer, due to hematogenous metastasis. Adhesion of cancer cells to endothelium is considered one of the crucial steps involved in hematogenous metastasis. In blood cells, this adhesion is initiated by endothelial selectin ligands (E-SL), that are glycoproteins or glycolipids mostly decorated with sialyl-Lewis x (sLe^x) and sialyl-Lewis a (sLe^a) glycan antigens. While LC has been described as expressing these sLex/sLea antigens, its functional role in allowing LC adhesion to endothelium is still poorly understood. Here we analyzed paired tumor and normal tissues samples from non-small cell lung cancer (NSCLC) patients. Immunoblotting assays with anti-sLex/sLea and E-selectin chimera demonstrated that LC tumors tissues significantly overexpress E-SL. The expression analysis of genes involved in sLex/sLea biosynthesis, revealed an overexpression of fucosyltransferases FUT3, FUT6 and FUT7 in LC tumor tissues. The carcinoembryonic antigen (CEA) was identified as one of the major protein scaffold of E-SL in LC. Overall, this work contributes to a better understanding of the glycosidic changes and molecules that can influence tumor progression of LC, allowing identifying in the future new diagnosis/prognosis biomarkers and potential therapeutic targets for NSCLC.

Background

Lung cancer is the leading cause of cancer-related deaths worldwide, with an overall 5-years survival rate estimated between 6-14% among males and 7-18% among

females [1]. 85% of all lung cancers are non-small cell lung cancer (NSCLC) that is divided in three major subtypes: adenocarcinoma (AD), squamous cell carcinoma (SCC) and large cell carcinoma.

Distant metastasis resulting from vascular dissemination of cancer cells is the primary cause of mortality in NSCLC. LC is the most common cause of brain metastases with up to 33% of NSCLC patients developing symptomatic brain metastases during the disease. Besides the brain, LC cells frequently metastasize to the other lung, adrenal glands, bone and liver [2, 3]. Metastatic patterns varied depending on histological subtype, gender and age at the time of diagnosis of the disease; however, it was reported that SCC is less predisposed to metastasize than AD and the last one prefers spreading to the bone. Survival in metastatic LC is worst in patients with liver or bone metastases [2].

Thus, the high incidence of mortality along with the need for an early diagnosis and a proper staging of the disease, determines the urgency to find mechanisms associated with NSCLC progression.

Alterations in glycosylation are one of the main characteristics associated with malignant transformations and tumor progression [4, 5]. Clinical studies shown an overexpression of sLe^x and sLe^a glycans on the surface of tumor cells including colon, gastric, pancreatic and lung cancer [6]. Nowadays, sLe^a is used as a tumor biomarker in pancreatic and colon cancer [7]. sLe^x and sLe^a glycans result from mono-fucosylated substitution of the α-2,3-linked sialic acid containing type 2 or type 1 chains, respectively [8, 9]. These two structures are found at terminal ends mainly on the β-1,6 branching of *N*-glycans or *O*-linked glycans attached to glycoproteins. Besides cell surface glycoconjugates, these antigens can also be detected in serum.

The interaction between circulating cancer cells and the vascular endothelium is one of the most critical steps for hematogenous metastasis. LC is known to overexpress endothelial selectins ligands (E-SL), prototypically the glycans sLe^x or sLe^a. These structures depending on their availability at cell surface have the potential of binding to E-selectin expressed on activated endothelial cells. Higher expression of E-SL in cancer cells is correlated with an enhanced metastatic activity and it is associated with a poor prognosis [10, 11]. However, its functional role in allowing NSCLC adhesion to endothelium is still poorly understood.

In this work, we attempted to investigate the relevance of E-selectin ligands expression in NSCLC and the main enzymes involved in their biosynthesis by comparing their expression in tumor and normal NSCLC tissues. We further evaluated the ability of

these ligands to adhere to E-selectin and identified the main protein scaffolds of E-selectin ligands in NSCLC.

Material and Methods

Patient and tissue specimens

This study involved 18 NSCLC patients who underwent lobectomy/segmentectomy, between July 2011 and January 2012 at *Unidade de Técnicas Invasivas Pneumológicas* in Hospital Pulido Valente (Lisbon). Fourteen patients were further diagnosed with AD and four with SCC. Median age was 65 years (Min= 48 years; Max= 83 years). For each patient, fragments of pulmonary tumor tissue and normal tissue (from a different pulmonary lobule) with a size above 0.5cm^3 were collected and immediately stored in liquid nitrogen until further processing. The determination of the histological type was performed by the Pathology laboratory from Hospital Pulido Valente. Tumor staging was classified according to TNM classification system based on the International Union Against Cancer (UICC) 7th edition [12]. The study was approved by the ethic committee at *Centro Hospitalar Lisboa Norte*. Written informed consent was received from all patients. A summary of the clinical data is available in Table 1.

Table 1: Clinical features of the patients.

Patient	Sex	Age	Diagnostic[1]	Stage[2]	Smoker[3]
1	F	83	AC	IA	0
2	M	66	AC	IB	1
3	M	67	AC	IA	1
4	M	60	AC	IIIA	1
5	M	80	SCC	IA	1
6	F	59	SCC	IA	1
7	M	65	AC	IB	1
8	M	64	SCC	IB	1
9	F	67	AC	IIIA	0
10	M	68	AC	IA	1
11	M	63	AC	IIIA	1
12	M	65	AC	IIA	1
13	F	64	AC	IIIA	0
14	F	71	AC	IA	0
15	M	52	AC	IIIA	0
16	M	49	SCC	IIIA	1
17	M	48	AC	IIB	0
18	M	49	AC	IA	0

[1] Diagnostic: adenocarcinoma (AC) and squamous cell carcinoma (SCC).

[2] Stage according to TNM classification [12].

[3] 0 represents a person that does not smoke and 1 represents a person that smoke.

Immunoblotting and immunoprecipitation

Whole tumor lysates were obtained by homogenizing patient tissues in lysis buffer, containing 150mM NaCl, 2mM $CaCl_2$, 2% Nonidet P-40 (NP-40) and protease inhibitors (Roche). After centrifugation at 17000 ×g, the supernatants, i.e. whole tissue lysates were saved for further use. Immunoprecipitated CEA (CD66E) was obtained by pre-clearing tumor lysates with protein G-agarose, followed by incubation with 1µg/µL of anti-human CD66E. Immunoprecipitate was collected with protein G-agarose beads, boiled and the released proteins were analyzed by western blotting. In some cases, the immunoprecipitate was treated with Peptide-N-Glycosidase F (PNGase-F, New England BioLabs) according to manufacturer's instructions.

For dot blotting, 10µg of whole tissues lysates were applied in nitrocellulose membrane (0.45µm, GE Healthcare Life Sciences). After absorption of the lysates, the membranes were blocked with 10% milk and PBS with 0.1% Tween-20. For Western blot analysis, 20µg of tissue lysates or immunoprecipitates were electrophoresed under reducing conditions on an 8% polyacrylamide gel and transferred to a polyvinylidene difluoride (PVDF) membrane (0.2µm, Bio-Rad).

For sLex/sLea detection, nitrocellulose membranes were stained with HECA-452 antibody (Biolegend), followed by staining with anti-rat IgM conjugated with horseradish peroxidase (HRP) (Southern Biotech). E-SL staining was performed using a 3-step protocol, in the presence of 2mM $CaCl_2$, which includes staining with mouse E-Selectin-human Fc Ig chimera (E-Ig), followed by anti-mouse CD62E antibody (BD Pharmigen) and then HRP-conjugated anti-rat IgG. CEA staining was performed with anti-human CD66E antibody (ImmunoTools), followed by HRP-conjugated anti-mouse Ig (BD Pharmigen). Membranes were incubated with Lumi-Light Western Blotting Substrate (Roche) according to manufacturer's instructions and detected with autoradiography film. Image's analysis was performed using ImageJ 1.48v software.

Gene expression measurements

Tissues samples were homogenized and total RNA was isolated following the instructions of the NZY Total RNA Isolation kit (Nzytech). Conversion to cDNA was performed

using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Real Time-Polymerase Chain Reaction (RT-PCR) was performed using *Taqman* probes methodology. For each primer/probe set, the Assay ID (Applied Biosystems) was the following: *FUT3* (Hs00356857_m1), *FUT4* (Hs01106466_s1), *FUT6* (Hs00173404_m1), *FUT7* (Hs00237083_m1), *ST3GAL3* (Hs00196718_m1), *ST3GAL4* (Hs00272170_m1) and *ST3GAL6* (Hs00196086_m1). The mRNA expression was normalized using the geometric mean of the expression of the endogenous controls, *β-actin* and *GAPDH* genes. The relative mRNA level expression was calculated using the expression $2^{-\Delta Ct} \times 1000$ [13, 14], which infers the number of mRNA molecules of the interest gene for each 1000 molecules of endogenous controls. RT-PCR was performed in 7500 Fast Real-Time PCR System (Applied Biosystems) and the results were analyzed using Sequence Detection Software, version 1.3.

Cells lines

Chinese hamster ovary (CHO) cells transfected with full length human E-selectin (CHO-E) and Mock transfectants (CHO-mock) cells, were grown in Minimum Essential Medium (MEM, Sigma-Aldrich), supplemented with 10% of heat inactivated fetal bovine serum, 2mM of L-glutamine, 100µg/mL of penicillin/streptomycin, 1mM of sodium pyruvate and 0.1mM of non-essential amino acids solution, all from Gibco.

CHO-E cells overexpress E-selectin and are derived from the stably transfection of CHO cells with cDNA encoding full-length E-selectin and CHO-mock cells are derived from CHO cells transfected only with an empty vector [15].

Adhesion assays

Adhesion assays were performed based on modified Stamper-Woodruff binding assay [16, 17]. Briefly, total lysates or CEA immunoprecipitated protein were spotted on glass slides and let dry. The adhesion area was blocked with 1% BSA and then let dry. CHO-mock and CHO-E cells were resuspended in Hank's Balanced Salt Solution (HBSS) containing 2mM $CaCl_2$ (HBSS-Ca) or 5mM EDTA (HBSS-EDTA) (negative control). The cells were overlaid onto protein spot in the glass slides and incubated on an orbital shaker at 80 rpm for 30 minutes at 4°C. The slides were placed in HBSS-Ca or HBSS-EDTA to remove the non-adherent cells and then adherent cells were fixed with 3% glutaraldehyde fixation. CHO-E and CHO-mock cell adherence to protein samples were examined under light microscopy at ×100 magnification, and representative

photomicrographs were taken for analysis. The number of adherent cells in each photomicrographs was counted using ImageJ software.

Immunohistochemistry

Paraffin-embedded sections of lung cancer were heated in trilogy pretreatment solution 1x (Cell Marque) at 94°C for 20min to remove paraffin and recover antigens. After blocking endogenous peroxidase (peroxidase block solution from Atom Scientific), sections were stained with anti-CEA (1:100) or anti-sLex/a (HECA-452 clone) (1:50) for 1h in TBS-T or E-Ig chimera (1:100) for 30min in TBS-T with 2mM CaCl2 (TBS-T-Ca). Then slides were washed with TBS-T or TBS-T-Ca. In the latter staining slides, they were still stained with anti-CD62E mAb (1:50) for 30 min in TBS-T-Ca. After this step, slides were rinsed with TBS-T or TBS-T-Ca and were stained using "HiDef Detection HRP Polymer System" (Cell Marque) and washed again in TBS-T or TBS-T-Ca accordantly. Then, the color is develop using DAB solution (ScyTek Laboratories). Subsequently, sections were washed with distilled water and stained with hematoxylin, prior to dehydration, clearing and mounting with Quick-D mounting medium.

Statistical analysis

All the tests were non-parametric. Data from normal tissues were paired with data from tumor tissues and statistical differences were analyzed using Wilcoxon matched-pairs signed rank test. The correlation between data was analyzed using Spearman Correlation. It was used the Mann-Whitney U-test to investigate relations between gene mRNA expression or sLex/sLea expression with patient categories. Adhesion assay data were analyzed using Kruskal-Wallis test and Dunn's multiple comparison test.

Tests were considered statistically significant when $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***) and marginally significant when $0.05 < p < 0.1$. Statistical analysis was performed using GraphPad Prism 6 (GraphPad Software, Inc).

Results

E-selectin ligands (E-SL) are overexpressed in NSCLC tumor tissues

To ascertain the relevance of E-SL expression in NSCLC, we compared their expression in tumor tissue and matched normal lung tissue. All the analyzed samples stained with

HECA-452 (i.e., sLe^x/ sLe^a expression) and E-Ig chimera (Fig.1). However, both HECA-452 and E-Ig reactivity were significantly higher in tumor samples compared with normal samples (Fig. 1). The expression of both sLe^x/ sLe^a and E-SL showed a statistically significant positive correlation (r=0.7848, p-value<0.0001), suggesting that sLex/sLea antigens are the main E-SL in lung cancer. Altogether, these results suggest that tumor tissues have an increased expression of E-SL compared with normal tissues.

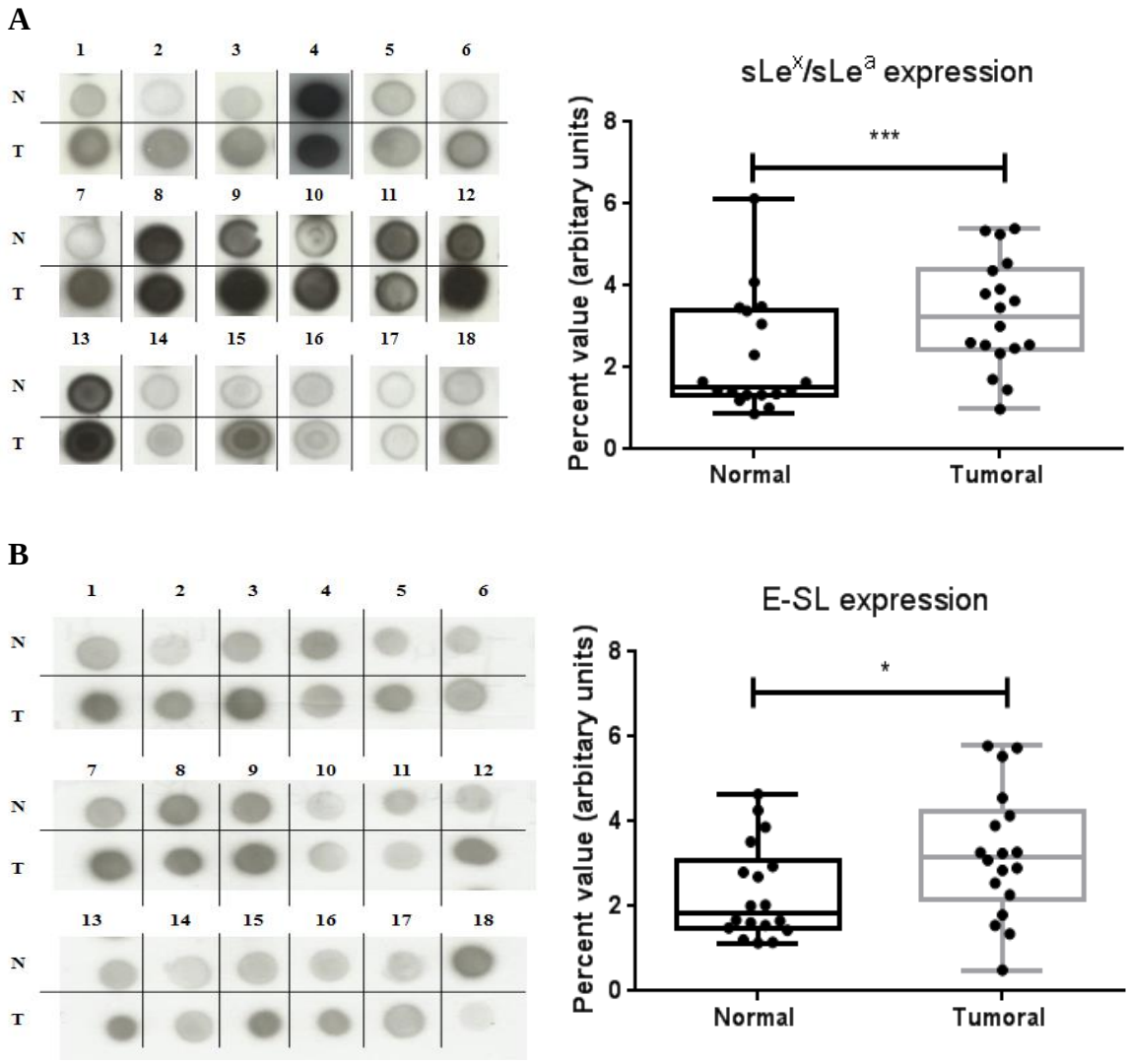


Fig.1: HECA-452 and E-Ig dot blot analysis. 20µg of total homogenates of normal (N) or tumor (T) tissues were spotted on nitrocellulose membrane and stained with antibody HECA-452 (A) and chimera E-Ig (B). The intensity of spots determined by ImageJ 1.48v software and expressed as arbitrary units is represented in the graphics on the right. *Box-and-whisker* plots represent median values, 25 and 75 % percentiles (*boxes*), ranges, and all values for each group (*black dots*). *p<0.05; ***p<0.001

FUT3, FUT6 and FUT7 are upregulated in NSCLC tumor tissues

We next asked whether the expression of enzymes critical to the biosynthesis of sLe^x/ sLe^a (Fig. 2) was upregulated in NSCLC. To asses this, we compared in normal and tumor

tissues, the gene expression of the 1,3/4-fucosyltransferases (FUT3, FUT4, FUT6 and FUT7) and 2,3-sialyltransferases (ST3GAL3, ST3GAL4 and ST3GAL6), which add fucose and sialic acid, respectively, to lactosaminyl glycan precursors of sLe^x/sLe^a [18]. We found that FUT3, F*UT6* and *FUT7* expression was significantly increased by 2.3 times, 5.3 times and 2 times, respectively, in NSCLC tissues compared with normal tissues ($0.05<p<0.1$, $p<0.001$, $p<0.05$, respectively) (Fig.2). On the contrary, the *FUT4*, *ST3GAL4* and *ST3GAL6* expression was significantly decreased by 1.3 times, 2 times and 1.6 times, respectively, in tumor NSCLC tissues compared with normal tissues ($p<0.001$, $p<0.05$, $p<0.05$, respectively) (Fig. 2). The ST3GAL3 expression was not statistically different between tumor tissue and normal NSCLC tissue.

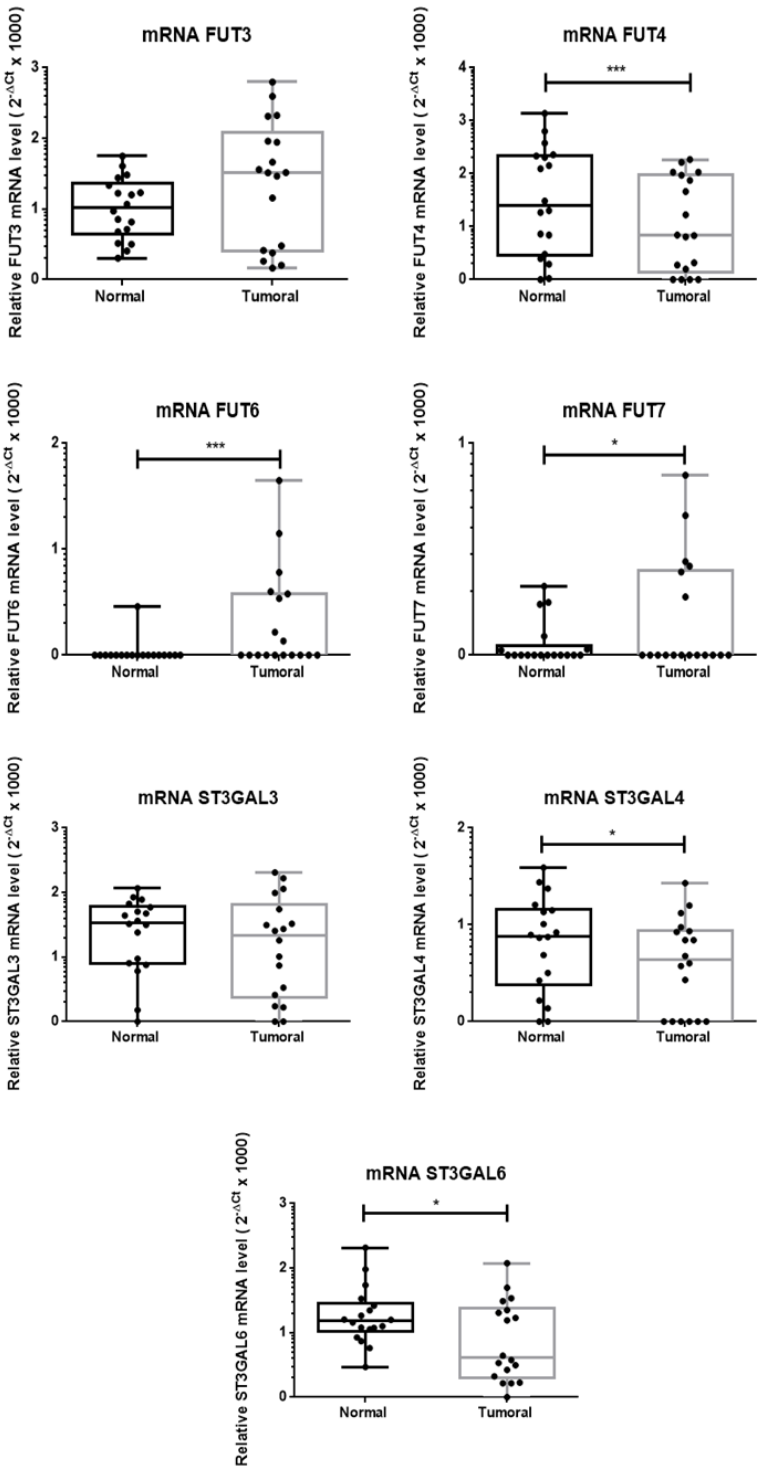


Fig.2: **Comparison of gene expression between normal and tumor NSCLC tissues.** The relative *mRNA* levels of *FUT3*, *FUT4*, *FUT6*, *FUT7*, *ST3GAL3*, *ST3GAL4* and *ST3GAL6* were analyzed by Real-Time PCR as described in Material and Methods section, in normal and tumor tissues from NSCLC patients. Values infer the number of mRNA molecules of a certain gene per 1000 molecules of the average of the endogenous controls (β-actin and GAPDH). Values are present in logarithmic scale. *Box-and-whisker* plots represent median values, 25 and 75 % percentiles (*boxes*), ranges, and all values for each group (*black dots*). Marginally significant when $0.05<p<0.1$; *$p<0.05$; **$p<0.01$; ***$p<0.001$

CEA is expressed in tumor NSCLC samples that highly express sLex/a glycans

CEA is a glycoprotein involved in cell adhesion and has been used as a clinical marker for colorectal cancer [19]. In lung cancer, high levels of CEA have been detected, while its expression is limited in adult normal tissues [20]. Analyzing CEA expression by western blot, we verified that all normal tissues did not express this glycoprotein. Analyzing the tumor tissues that have a higher expression of E-SL comparatively with the respective normal tissue, we verified that 6 of these 15 NSCLC samples expressed CEA glycoprotein (≈180kDa), specifically samples 1, 2, 7, 10, 15 and 18 (Fig. 3A). Comparing the expression of sLex/a glycans between the CEA negative and CEA positive NSCLC samples, we observed that CEA positive samples have a significantly higher expression of sLex/a glycans than the CEA negative samples (Fig. 3B). These results suggest that CEA can be a protein scaffold for sLex/a in NSCLC.

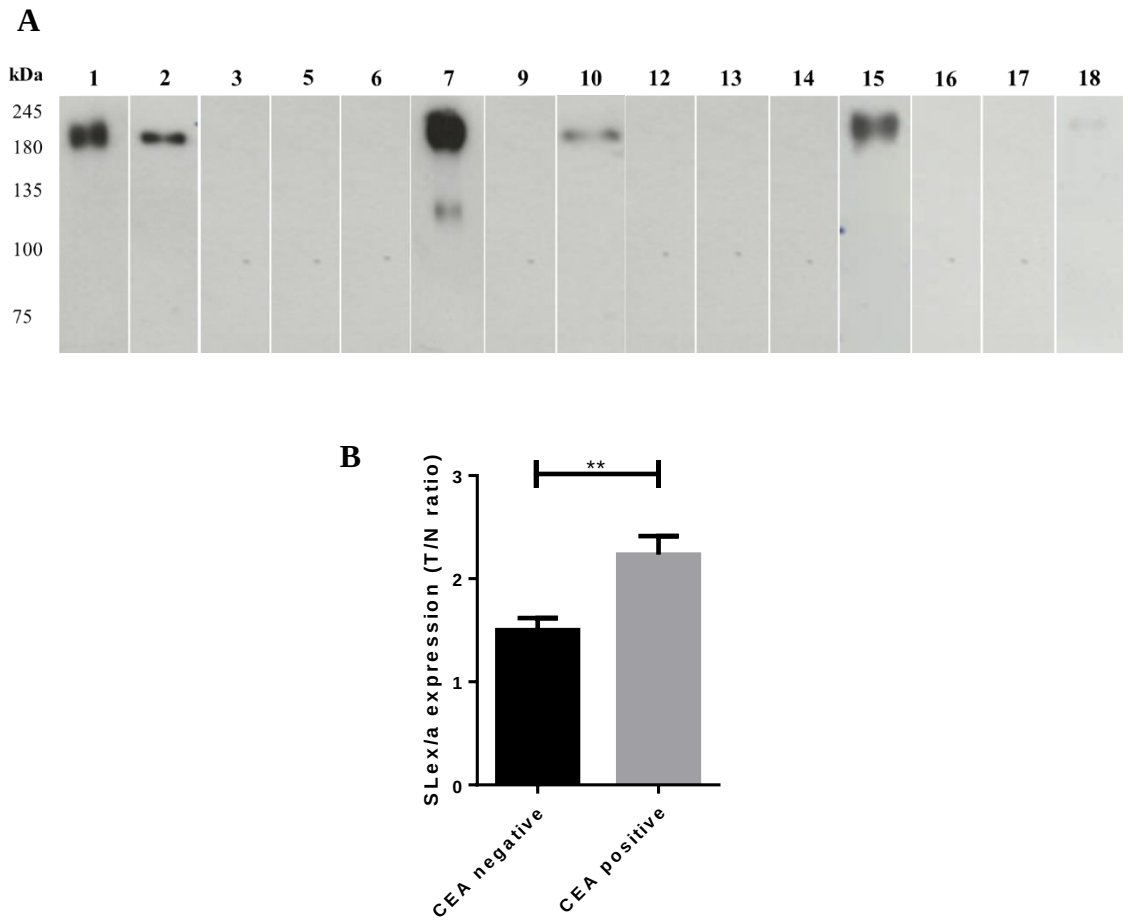


Figure 3: CEA expression in tumor lysates from NSCLC patients. A: Western blotting analysis of CEA glycoprotein present in tumor lysates from fifteen NSCLC patients. The numbers above each lane represent the number of the analyzed patient. **B:** The CEA positive samples have the higher ratio of sLex/a expression in tumor tissues, comparatively with normal tissues, than the CEA negative samples.

CEA shows a similar expression profile as E-selectin ligands in NSCLC tissues

Focusing in these 6 NSCLC samples that express both CEA and E-selectin ligands, as well as sLex/a glycans, we stained paraffin-embedded tumor sections from these samples with anti-CEA mAb and E-Ig chimera. Normal tissue existing in these sections do not stain with anti-CEA or E-Ig, with the exception of inflammatory cells in E-Ig staining. Comparing both stains in tumor regions, we were able to detect a similar cellular staining profile. In general, both CEA and E-selectin ligands are expressed on the membrane with some intracellular staining (Fig. 4). Specificity, sections from patient 2 and 18 mainly express CEA protein and E-selectin ligands in cell membrane while sections from patient 1, 7, 10 and 15 express both target molecules in the cytoplasm with a vast reinforcement in their cell membrane. In general, both CEA and E-selectin ligands expression seem to be co-localized in these tissues, which suggest a role for CEA as an E-selectin ligand.

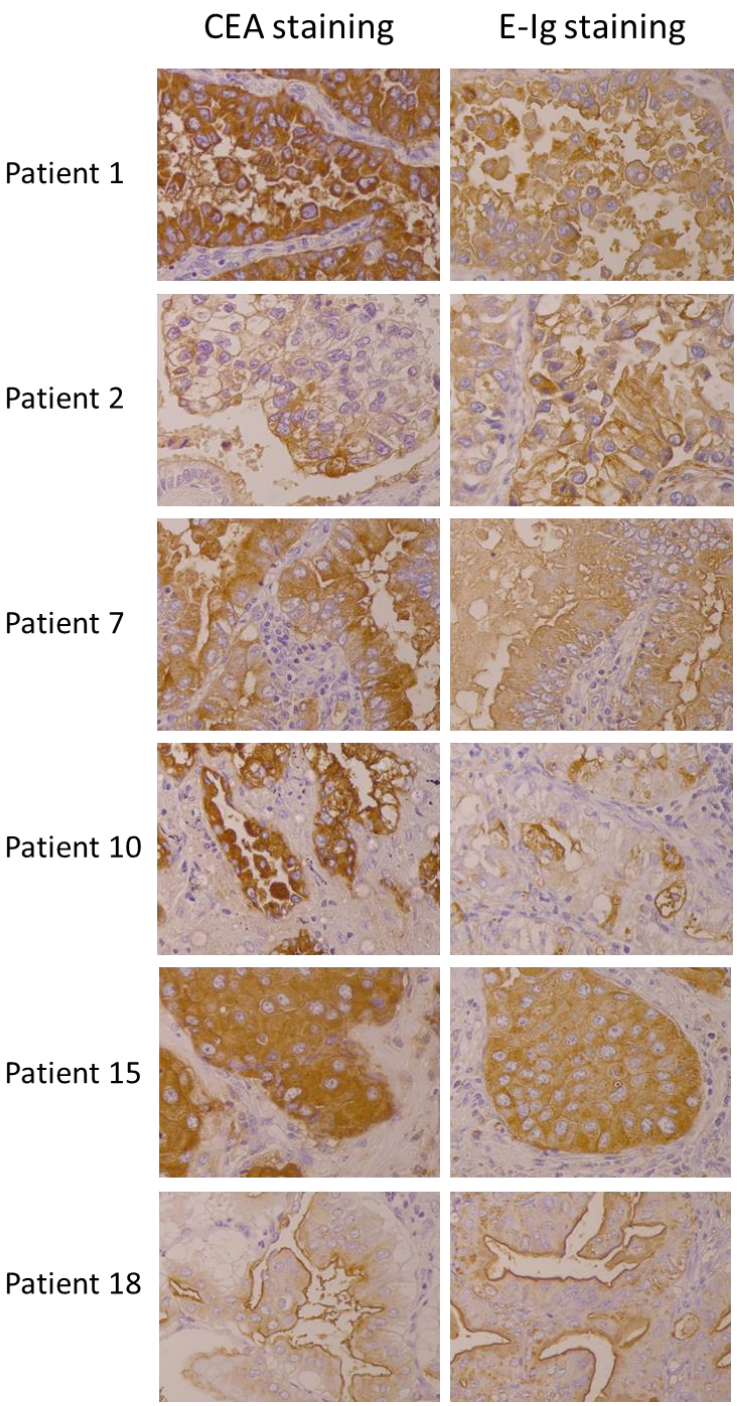
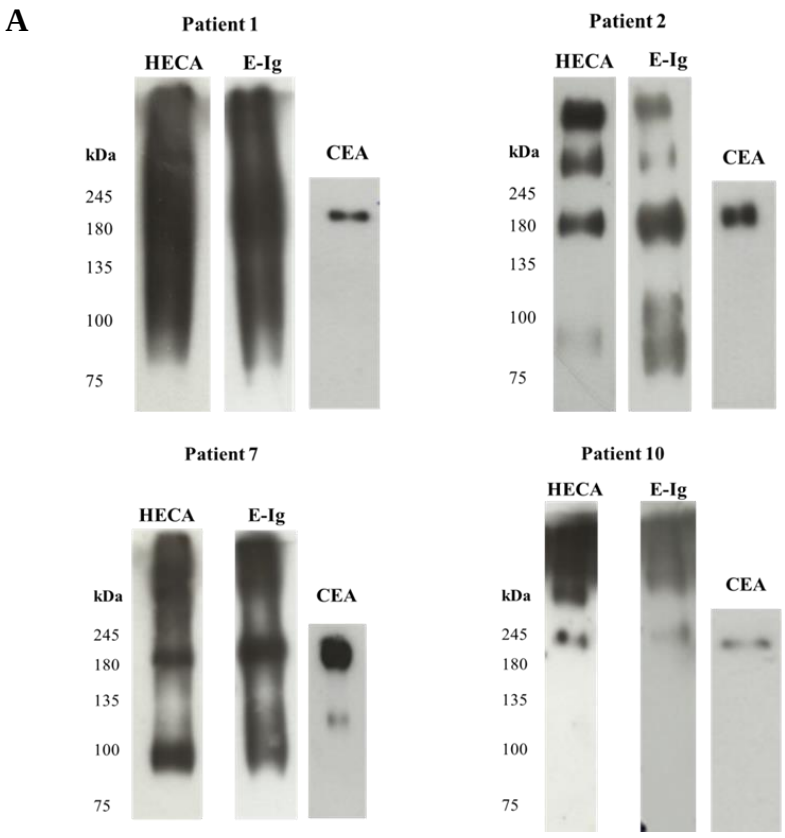


Fig. 4: CEA and E-selectin ligands have a similar staining profile in NSCLC tissues by immunohistochemistry. Paraffin-embedded tumor sections from six NSCLC tissues, specifically 1, 2, 7, 10, 15, 18 patients, were stained with anti-CEA (left microphotographs) and E-Ig chimera (microphotographs on the right) by immunohistochemistry (brown color). Nucleus were stained with hematoxylin (blue color).

CEA is an E-selectin ligand in NSCLC tissues

To show the role of CEA as an E-selectin ligand, protein lysates from the 4 tissues were ran by western blot, and the proteins stained by anti-sLe$^{x/a}$, E-Ig chimera and anti-CEA

were compared in terms of their molecular weight. We found that the 4 protein tumor samples have a protein(s) decorated with anti-sLe$^{x/a}$ and recognized by E-Ig chimera at the same molecular weight as CEA (≈180KDa) (Fig. 5A). This is one more evidence of the role of CEA as E-selectin ligand in NSCLC. Besides CEA, there are clearly other high molecular weight proteins decorated with sLe$^{x/a}$ and able to bind to E-selectin in these NSCLC samples. These proteins, as well as the ≈90KDa protein(s) in samples 2 and 7, have yet to be identified. Specifically, to prove that CEA is an E-selectin ligand in NSCLC, we immunoprecipitated CEA from the protein sample of patient 7, which is the patient with higher CEA expression detected by western blot. Immunoprecipitated CEA was analyzed by western blot to verify if this protein was a carrier of sLe$^{x/a}$ and would bind to E-selectin. As is shown in Fig. 5B, immunoprecipitated CEA is recognized by anti- sLe$^{x/a}$ mAb and E-Ig chimera, meaning that CEA is an E-selectin ligand in NSCLC.



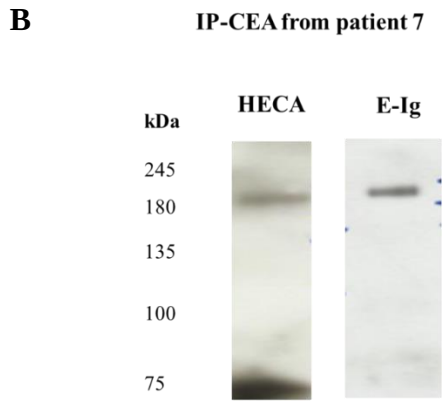


Fig. 5: CEA is an E-selectin ligand in NSCLC. A: Comparison of western blotting analysis with HECA-452 staining, E-Ig staining and CEA staining in tumor lysates from four NSCLC patients. **B:** Western blotting analysis of immunoprecipitated CEA (IP-CEA) from tumor lysate of patient 7. Left lane corresponds to HECA-452 staining (HECA) and the lane on the right corresponds to chimera E-Ig staining.

CEA is a functional E-selectin ligand in flow conditions

Since the main role of E-selectin engagement during transendothelial migration is to slow down the circulating cells in the bloodstream in order to promote the adhesion of these cells to the endothelium, it's crucial to analyze the functionality of E-selectin binding in flow conditions to mimic this physiological role. Therefore, we analyzed if the tumor proteins from patient 7 is able to bind to cells expressing, or not, E-selectin on their surface (CHO-E and CHO-mock cells, respectively) using an alternative Stamper Woodruff assay. We observed that the tumor proteins from patient 7 were able to specifically bind to E-selectin on CHO-E cells when in calcium buffer. When using EDTA buffer or blocking antibody for E-selectin (negative controls), this binding was significantly diminished (Fig. 6A), proving the specificity of this cell adhesion mediated by E-selectin. To demonstrate that CEA protein scaffold is one of the functional E-selectin ligands in NSCLC, CEA immunoprecipitation from tumor proteins of patient 7 was performed and the ability of CEA to bind to CHO-E and CHO-mock cells under flow conditions was assessed. As shown in Fig. 6B, immunoprecipitated CEA was able to bind to CHO-E cells in the presence of calcium, being this adhesion significantly abrogated when EDTA buffer was used. In addition, CHO-mock cells did not adhere to immunoprecipitated CEA, proving the specificity of E-selectin engagement to CEA. In sum, CEA expressed by tumor tissue from NSCLC patient is a functional E-selectin ligand.

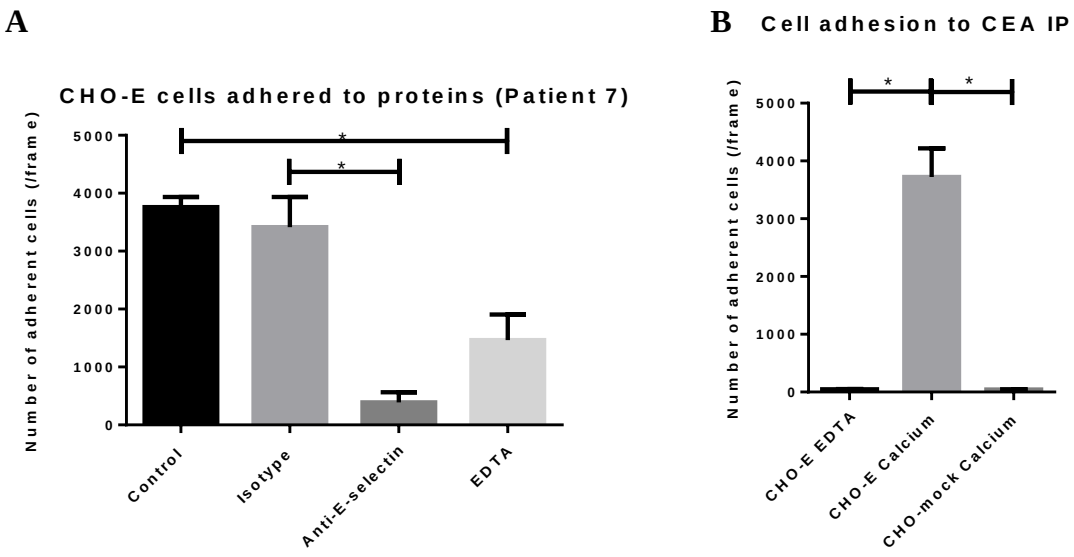


Fig. 6: CEA glycoprotein is a functional E-selectin ligand in NSCLC. A: Adhesion of CHO-E cells to protein lysate of tumor tissue from patient 7. CHO-E cells were ressuspended in calcium buffer (Control), EDTA buffer (EDTA) or cells were incubated with different mAbs before the adhesion experiment: anti-E-selectin (20mg/mL) and isotype antibody (20mg/mL). **B:** Adhesion of CHO-E and CHO-MOCK cells to immunoprecipitated CEA glycoprotein of tumor tissue from patient 7. CHO-E and CHO-MOCK cells were ressuspended in calcium buffer, and CHO-E cells were also ressuspended in EDTA buffer as a negative control.

Discussion

Sialyl Lewis antigens are one of the structures aberrantly expressed in several types of cancers, including LC [21, 22]. These antigens play an important role as E-SL since they are expressed on the surface of glycoproteins in cancer cells, contributing to adhesion of cancer cells to endothelium and subsequent metastasis [23, 24]. Nevertheless, E-SL in NSCLC is still poorly understood.

Herein, we have shown that E-selectin ligands, as well as sLex/a glycans, are overexpressed in NSCLC tissues comparatively with normal tissues, which is in agreement with previous results in serum from NSCLC patients [25, 26] and also NSCLC biopsies [21]. It is known that E-selectin ligands expression, mainly sialyl Lewis antigens, on cancer cells is associated with a poor prognosis due to enhanced metastatic phenotype as well as with increased ability to adhere to E-selectin [6, 27]. Thereby, our results suggest that LC patients that strongly express E-SL and sLex/sLea antigens in their tumor cell surface would have cancer cells with higher ability to bind to E-selectins, facilitating tumor proliferation and promoting hematogenous metastasis of LC. Additionally,

overexpression of both antigens can alter the immune homeostasis of the mucous membrane, favoring cancer progression [28].

The results obtained in this work show that FUT3, FUT6 and FUT7 are upregulated in NSCLC tumor tissues compared with normal tissues. These results are in agreement with the fact that FUTs are overexpressed in several types of tumors, including LC [8, 29, 30]. Since these enzymes catalyze the final step in the biosynthesis of sialyl Lewis antigens, these results suggest that the amount of sLex and sLea epitopes in patients with upregulation of these FUTs should be greater compared with patients without upregulation of these enzymes; however, this association were not detected here.

So far, several glycoproteins scaffolds of E-SL have been identified in several types of cancer, being the most prominent the CD44 glycoform, HCELL, as E- and L- selectin ligand on colon cancer and CEA on colon and prostate cancer [31, 32]. Until now, it is not described any glycoprotein as an E-SL in NSCLC. Exploring CEA as a potential E-SL in NSCLC, we analyzed its expression in 15 NSCLC samples, specifically the ones with higher expression of E-SL and sLex/a antigens in tumor tissues than in the normal tissues. So we detected that 40% of the tumor tissue samples contained CEA glycoproteins. The CEA positive tumor tissues were also the ones that showed a higher expression of sLex/a comparatively with normal tissues, suggesting that CEA could be a protein scaffold for sLex/a glycans. Comparing the cellular expression of CEA and E-SL in NSCLC tissues, we observed a similar staining profile, which indicates that CEA could be a E-SL in NSCLC. In order to confirm it, we immunoprecipitated CEA and proved that it was decorated with sLex/a glycans, being able to bind to E-selectin chimera, as well as to E-selectin expressing CHO cells in flow conditions, which demonstrate its functionality as E-selectin ligand.

Since CEA is a glycoprotein with a limited expression in normal tissues but detected in high levels in tumors with epithelial origin including lung adenocarcinoma, gastric carcinoma and colorectal cancer, it is a promising biomarker and therapeutic target for NSCLC. CEA is already used as a tumor biomarker in colorectal tumors [33]. In NSCLC, CEA expression has been mainly studied in serum with its levels been reported to be correlated with poor therapeutic response and survival, advanced stage of the disease and also early relapse [20, 34]. Overall, our data suggests a new function for this molecule in NSCLC tissues: CEA is an E-SL in NSCLC that may facilitate adhesion to endothelium and consequently cancer metastasis. Further studies with larger cohorts of patients are required to confirm his role in the development of metastasis and to investigate CEA as

potential therapeutic target to combat metastasis in LC and also to confirm their usefulness as a diagnostic marker in NSCLC patients.

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Chapter 10

Published paper

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Sialyl Tn-expressing bladder cancer cells induce a tolerogenic phenotype in innate and adaptive immune cells

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ABSTRACT

Despite the wide acceptance that glycans are centrally implicated in immunity, exactly how they contribute to the tilt immune response remains poorly defined. In this study, we sought to evaluate the impact of the malignant phenotype-associated glycan, sialyl-Tn (STn) in the function of the key orchestrators of the immune response, the dendritic cells (DCs). In high grade bladder cancer tissue, the STn antigen is significantly overexpressed and correlated with the increased expression of ST6GALNAC1 sialyltransferase. Bladder cancer tissue presenting elevated expression of ST6GALNAC1 showed a correlation with increased expression of CD1a, a marker for bladder immature DCs and showed concomitant low levels of Th1-inducing cytokines IL-12 and TNF-α. *In vitro*, human DCs co-incubated with STn^+ bladder cancer cells, had an immature phenotype ($MHC\text{-}II^{low}$, $CD80^{low}$ and $CD86^{low}$) and were unresponsive to further maturation stimuli. When contacting with STn^+ cancer cells, DCs expressed significantly less IL-12 and TNF-α. Consistent with a tolerogenic DC profile, T cells that were primed by DCs pulsed with antigens derived from STn^+ cancer cells were not activated and showed a $FoxP3^{high}$ $IFN\text{-}\gamma^{low}$ phenotype. Blockade of STn antigens and of STn^+ glycoprotein, CD44 and MUC1, in STn^+ cancer cells was able to lower the induction of tolerance and DCs become more mature.

Overall, our data suggest that STn-expressing cancer cells impair DC maturation and endow DCs with a tolerogenic function, limiting their capacity to trigger protective anti-tumour T cell responses. STn antigens and, in particular, STn^+ glycoproteins are potential targets for circumventing tumour-induced tolerogenic mechanisms.

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1. Introduction

The sialyl-Tn (STn) antigen is one of the most common tumour-associated carbohydrate antigen, expressed by more than 80% of human carcinomas but rarely observed in normal tissues (Cao et al., 1996). STn is a posttranslational modification of cell surface glycoproteins commonly resulting from the overexpression of the ST6GALNAC1 sialyltransferase. This enzyme transfers a sialic acid to the *O*-6 position of *N*-acetylgalactosamine residue linked to a serine or a threonine (GalNAcα-*O*-Ser/Thr) on a given protein, blocking the typical elongation of *O*-glycosidic chains in glycoproteins. STn expression in cancer is associated with adverse outcome and decreased overall survival of the patients (Itzkowitz et al., 1990; Werther et al., 1996). It has been reported that STn expression by cancer cells is associated with many malignant features such as invasiveness (Julien et al., 2012; Pinho et al., 2007), and with epithelial to mesenchymal transition (Lin et al., 2009), a loss of cell differentiation that is an important milestone towards cancer metastasis.

STn is highly expressed in high-grade bladder tumours, which present elevated proliferation rates and high risk of recurrence/progression. In bladder cancer, STn enhances motility and invasive capacity of the cancer cells, thus being associated with malignancy (Ferreira et al., 2013).

The expression of STn is clinically relevant, not only as a marker for diagnosis and prognosis in cancer (the CA72-4 serological test), but also as target for therapeutic strategies. One example is a vaccine consisting of STn-clustered epitopes, Theratope, that has been meaningfully used in clinical trials to immunize breast cancer patients (Adis International, 2003; Holmberg and Sandmaier, 2004; Julien et al., 2012). Other STn-based vaccines have also been developed for application in clinical trial, which includes multi-epitope vaccines containing STn and other tumour-associated carbohydrates and STn-expressing glycopeptides (Heimburg-Molinaro et al., 2011; Madsen et al., 2013; Niederhafner et al., 2008; Slovin et al., 2007). Nevertheless, STn-based vaccines have had limited success (Miles et al., 2011) probably because the pathophysiological role of STn-epitopes in cancer cells remains unclear. One of the factors that has been highlighted by many authors is the low immunogenicity of STn-based vaccines (Julien et al., 2012; Lakshminarayanan et al., 2012; Monti et al., 2004). Thus, the elucidation of the immune mechanisms affected by the expression of STn by cancer cells will contribute to improve anti-STn immunotherapy approaches.

Glycans are involved in multiple biological functions, controlling many features of the immune response. In fact the diversity of glycans and of glycan-recognizing receptors, known as lectins, is highly regulated by the immune cells. One of the examples is the modulation of dendritic cell (DC) functions by changes of their glycan phenotype during differentiation and maturation (Crespo et al., 2009; Videira et al., 2008).

DCs play a unique and decisive role in tumour immunity, being capable of activating antigen-specific T cells against cancer cells (Banchereau and Steinman, 1998). To efficiently prime T cells, DCs undergo maturation, which includes the downregulation of the antigen-uptake machinery, the upregulation of antigen presenting molecules, class I and class II MHC; costimulatory molecules, such as CD80 and CD86 and the synthesis of immune-enhancing cytokines (Langenkamp et al., 2000). However, the degree of DC maturation is dependent on the type of stimulus and tumour cells usually prevent maturation, through many immunosuppressive strategies employed *in situ*. Tumours render DC tolerogenic and bias the immune response in favour of their own progression (Almand et al., 2001; Vicari et al., 2002). The presence of immature DCs at tumour sites is consistent with DC involvement in the tumour progression, most likely by inducing immune unresponsiveness, i.e. tolerance against cancer (Almand et al., 2001). We have previously reported, in bladder cancer tissue, that the lower expression of markers of DC maturation, such as MHC-II, was correlated with risk for recurrence (Videira et al., 2009a). Tumour-residing DCs showing limited maturation or anergy, hold back strategies to induce immune responses and create one important obstacle to the efficacy of immune-based-therapies. Concordantly, in bladder cancer tissue, lower levels of mature DCs are associated with low responses to the *Bacillus Calmette-Guerin* (BCG) immunotherapy, the gold standard treatment for the prophylaxis and management of non-muscle invasive bladder cancer (Beatty et al., 2004; Videira et al., 2009a).

It has been described that STn epitope may confer, to different cancer cell, protection from immune defence thus contributing to malignant phenotype and cancer progression (Monti et al., 2004; Ozaki et al., 2012). Mucins, and in particular STn^{+} MUC1 mucins released by cancer cells inhibited DC maturation and modulate DCs towards IL-10^{high} IL-12^{low} regulatory antigen presenting cells with a limited capacity to trigger protective T helper type 1 (Th1) responses (Monti et al., 2004). Interestingly, soluble aberrantly glycosylated MUC1 has also been described to elicit maturation, yet unable to promote Th1 responses (Carlos et al., 2005). While the effect of glycoproteins secreted by tumours is becoming more elucidated, the role of the overall STn expression at tumour cell surface in immunomodulation, remains unknown. Therefore, in this study, we further investigated the influence of STn expression by bladder cancer cells on the immune potency and functionality of human DCs.

2. Material and methods

2.1. Reagents

Fluorescently-conjugated or unlabelled anti-CD14 (M5E2), anti-CD80 (2D10), anti-CD86 (IT2.2) and anti-CD45 (HI30) monoclonal antibodies (mAbs) were purchased from BD Biosciences (San Jose, CA). Anti-MHC-II (L243) and anti-CD1a mAb (HI149) were from Miltenyi Biotec (Bergisch Gladbach, Germany). As anti-STn, we used the HB-STn1 mAb from Dako (Dako Cytomation, Denmark) or clone TKH2 (Kjeldsen et al., 1988). Clone HMFG-2 was used as anti-MUC1 (Griffiths et al., 1986). Interleukin (IL)-4 and Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF) were purchased from R&D Systems (Minneapolis, MN). Sialidase from *Clostridium perfringens* was from Roche Diagnostics (Basel, Switzerland) and carboxi-fluorescein diacetate succinimidyl ester (CFSE) from Molecular Probes (Leiden, The

Netherlands). All other reagents were from Sigma (St. Louis, MO, USA) unless otherwise stated.

2.2. Patient and tissue specimens

This study involved 49 patients, from Hospital São José in Lisbon, who underwent transurethral resection of the bladder tumours. Matched pairs of histologically verified bladder tumours and normal appearing mucosa remote from the tumour were collected and analysed individually. Based on urothelial carcinoma grading and staging criteria of the World Health Organization 132 (WHO), three different groups were considered (Table 1), low-grade (LG, $n = 22$) and high-grade HG non muscle-invasive (NMIBC, $n = 21$) and muscle-invasive (MIBC, $n = 6$) bladder cancers. None of these patients had received prior adjuvant therapy. Patients with *carcinoma in situ* (CIS) were not included, as well as patients with presence of upper tract malignancy, other malignancies, and chronic infections, women expectant or lactating and patients with congenital or acquired immunodeficiency. Prior patient consent and approval from the institute research ethics committee were obtained.

2.3. Histological analysis

The immunohistochemical analysis was performed in automated equipment (Ventana BenchMark® ULTRA). All reagents were from Ventana, USA, unless otherwise stated. Briefly, the slides were heated at 72 °C, deparaffinized with EZ Prep and antigenic recovery at 97 °C. Endogenous peroxidase was blocked with 3% of hydrogen peroxide and the slides were incubated with TKH2 mAb (1:10). This was followed by amplification with HRP UltraView Universal Multimer, revelation with UltraView Universal DAB Chromogen and DAB H202 and the intensification was achieved with UltraView Universal DAB Copper. The nuclear contrast was performed with hematoxylin and bluing. After the immunohistochemical technique, the slides were washed, dehydrated, treated with increasing concentrations of alcohol (75%, 90% and 99%) for 1 min each, cleared in xylene and mounted with synthetic mounting medium (Quick-D-M-Klinipath). A semi-quantitative approach was established to score STn expression based on the percentage of tumour that stained positively in comparison to the tumour bulk. The STn expression was assessed double-blindly by two independent observers and validated by an experienced pathologist. Whenever there was a disagreement, the slides were reviewed, until a consensus was reached.

Table 1 – Information of patients included in this study.

	Number of cancer patients	Age, years median (range)
Total number of cases	49	
Muscle invasive (MIBC)	6	65.1 (56–79)
Non muscle invasive (NMIBC)		
High grade	21	68.7 (47–84)
Low grade	22	69.9 (55–90)

2.4. Cell isolation and culture

Monocytes were isolated by positive selection using anti-CD14 coated magnetic beads (Miltenyi Biotech, Germany) from peripheral blood mononuclear cells (PBMCs) of healthy volunteers, provided and ethically approved by the Portuguese Blood Institute. Monocytes were differentiated into immature mo-DCs (mo-DCs) as described (Videira et al., 2008). Whenever needed, maturation of mo-DCs (mmo-DCs) was induced, at day 5, with 1 μg/ml of lipopolysaccharide (LPS), for 24 h.

2.5. Flow cytometry

Cell purity, differentiation and maturation of mo-DCs was assessed by staining with fluorescein isothiocyanate (FITC)-labelled mAbs against CD14, BDCA-1, CD80 and CD86 or Allophycocyanin (APC)-labelled anti-HLA-DR. Unlabelled mouse anti-STn, -CD44 or -MUC1, followed by anti-mouse Ig-FITC were used to characterize MCR cells. Flow cytometry acquisition was performed, using a FacsCalibur Flow Cytometer. File data was analysed using the CELL QUEST (BD Biosciences) and FLOWING (Turku, Finland) software to discriminate specific populations, and to determine the mean fluorescence intensity (MFI) of the cells.

2.6. Cell lines

The human bladder cancer cell line variants, MCRcont and MCRSTn, were generated as described (Ferreira et al., 2013) and were grown in Dulbecco's modified Eagle medium (DMEM) (Sigma), supplemented with foetal bovine serum, glutamine, penicillin and streptomycin.

2.7. Confocal laser scanning microscopy

Cells were cultured in cover glasses, fixed with 3.7% paraformaldehyde and permeabilized with 0.1% TritonX-100. After blocking with 1% bovine serum albumin (BSA), cells were stained using anti-STn, clone TKH2, or anti-ST6GALNAC1, clone 2C3 (Marcos et al., 2011), followed by fluorescent polyclonal anti-Ig antibody. The cell nuclei were stained with 1 μM TO-PRO-3 dye (Molecular Probes, Leiden, Netherlands). Images were acquired with a Leica TCS SP2 AOBS confocal microscope (Leica Microsystem, Mannheim, GmbH). Representative confocal cross-section images were selected after Z-stacking.

2.8. Establishment of DC: bladder cancer cell lines cocultures

Cancer cell lines were incubated at 0.2×10^6 cells/ml in a 6-well plate, at 37 °C. After 24 h, mo-DCs were added in the proportion of 1:5 (cancer cell: mo-DC) in adhesion buffer (20 mmol/l of trizma hydrochloride, 150 mmol/l of sodium chloride, 1 mmol/l calcium chloride, 1 mmol/l magnesium chloride and 0.5% BSA, pH 8.0) at 37 °C. After 2 h incubation, the non-adherent mo-DCs were washed and the coculture of MCR with adherent mo-DCs was stained with mAbs against MHC-II, which is expressed by DCs, but not by MCR cell lines, thus allowing to assess the percentage of adhering mo-DCs in the coculture. The cell viability was measured by annexin-V

staining. Some experiments were performed in adhesion buffer without calcium or magnesium, to assess the impact of divalent ions. In other experiments, mo-DCs were incubated with 24 h supernatants obtained from MCR cell cultures to assess the requirement for cell: cell interactions. When testing function blocking mAb, to avoid unspecific Fc receptor-mediated mAb binding to mo-DCs, the Fc receptors from mo-DCs were blocked with 10% human serum. In addition, the blocking antibodies were added to the MCR cells and not to mo-DCs. We used 20 μg/ml of anti-CD44 mAb (clone IM7), 1:100 of anti-STn mAb (clone HB-STn1), 1:20 of anti-MUC1 (clone HMFG-2) or isotype control for 30 min, washed and then cocultured the mAb coated MCR cells with mo-DCs, as described above.

2.9. Gene expression analysis by real-time PCR

RNA extraction from formalin-fixed, paraffin embedded sections was performed after deparaffinization of tissues, using Absolutely RNA FFPE kit (Agilent technologies) while RNA from the cocultures was isolated using the GenElute Mammalian Total RNA Purification kit (Sigma), according to the manufacturer's instructions. After DNAase treatment, 1 μg of total RNA was reverse transcribed with random primers and the real-time PCR was performed with Master Mix, TaqMan probes and primers from Applied Biosystems. The assay ID provided by the manufacturer were: Hs00300842_m1 (*ST6GALNAC1*); Hs00168405_m1 (*IL-12α*); Hs00174128_m1 (*TNF-α*); Hs00174086_m1 (*IL-10*); Hs00372324_m1 (*IL-23*), Hs00203958_m1 (*FoxP3*) and Hs00174143_m1 (*IFN-γ*). The relative mRNA levels were normalized against the arithmetic mean of the *β-actin* and GAPDH expression and calculated by adapted formula $2^{-\Delta Ct} \times 1000$ which infers the number of mRNA molecules of the gene of interest per 1000 molecules of the endogenous controls (Videira et al., 2009b). ΔCt stands for the difference between the cycle threshold of the target gene and that of the endogenous control genes. The efficiency for each primer/probe was above 95% (as determined by the manufacturer). When analysing the gene expression of mo-DCs in the coculture, the contamination with MCR cells was disregarded since the analysis of cytokine gene expression in both MCR cell line variants showed a completely absence of *IL-12α*, *TNF-α*, *IL-10*, *IL-23*, *FoxP3* and *IFN-γ* gene expression.

2.10. Phagocytosis assay

Cell lines were labelled with CFSE according to the manufacturer's instructions and then induced to apoptosis with 10 μM of camptothecin. After 48 h, cells were incubated with mo-DCs in the proportion of 1:2, in a 48-well plate, for 6 h at 37 °C or 4 °C. After incubation, cells were stained with anti-MHC-II mAb and the percentage of MHC-II$^+$/CFSE$^+$ cells (mo-DCs that phagocytosed MCR cells) was calculated by flow cytometry and confirmed by confocal microscopy. The values obtained at 4 °C were subtracted from the 37 °C values.

2.11. T cell activation

Human T cells were obtained during monocyte isolation procedure (CD14$^-$ PBMC fraction) and maintained in complete RPMI medium until complete mo-DC differentiation. Autologous T cells were then incubated with mo-DCs following phagocytosis of MCR cells, as described above, in the proportion of 8:1, in a 96-well round bottom plate, throughout 11 days. As controls, similar experiments were performed with unstimulated mo-DC or with the MCR cell lines (negative controls) or with phytohaemagglutinin (positive control). T cell activation was assessed by the percentage of CD69$^+$ cells within the CD3$^+$ population (T cells) and their MFI values were evaluated by flow cytometry.

2.12. Protein extraction, immunoprecipitation and western blotting

Proteins were isolated from cell lines using RIPA buffer (Sigma–Aldrich) and bladder tumour proteins were extracted from formalin-fixed paraffin embedded tissues using the Qproteome FFPE tissue kit (Qiagen). The amount of protein was estimated with RC protein assay kit (BioRad). CD44 protein was immunoprecipitated from total protein extracts (IP) with anti-CD44 monoclonal antibody (2C5 clone; R&D Systems) using Pierce Direct IP Kit (Thermo Scientific). Protein samples were separated in reducing SDS-PAGE gels, using 20 μg per lane, transferred to 0.45 μm nitrocellulose membrane (GE Healthcare Life Sciences) and blocked with 1% Carbo-Free Blocking Solution (Vector Laboratories). TKH2 and goat anti-mouse IgG1 heavy chain horseradish peroxidase conjugate (Abcam) were used as primary and secondary antibodies, respectively. The Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare Life Sciences) was used as developing reagent. Protein extracts treated with sialidase were used as controls.

2.13. Statistical analysis

Statistical analyses were conducted using GraphPad Prism software, version 5.0 (GraphPad Software, La Jolla, CA). Paired student's *t*-test was used when data was normally distributed, and the results expressed as the mean values ±SDs. Alternatively, Wilcoxon signed-rank test was used in the case of non-normal distribution, and the results were plotted individually or as box and whiskers plot. The correlations were analysed using Spearman and Pearson methods. Tests were considered statistically significant when $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.005$ (***) and marginal significance was considered for $p < 0.1$.

3. Results

3.1. The STn antigen expression and DCs are increased in bladder cancer

We have previously reported that STn is highly overexpressed in bladder cancer tissues, showing a markedly high incidence and intensity only in malignant tissue and was barely detectable in normal mucosa (Ferreira et al., 2013) (Figure 1A). Considering the tumour bulk the maximum STn expression was 30%. The STn expression is highly correlated with the gene expression of the sialyltransferase ST6GALNAC1

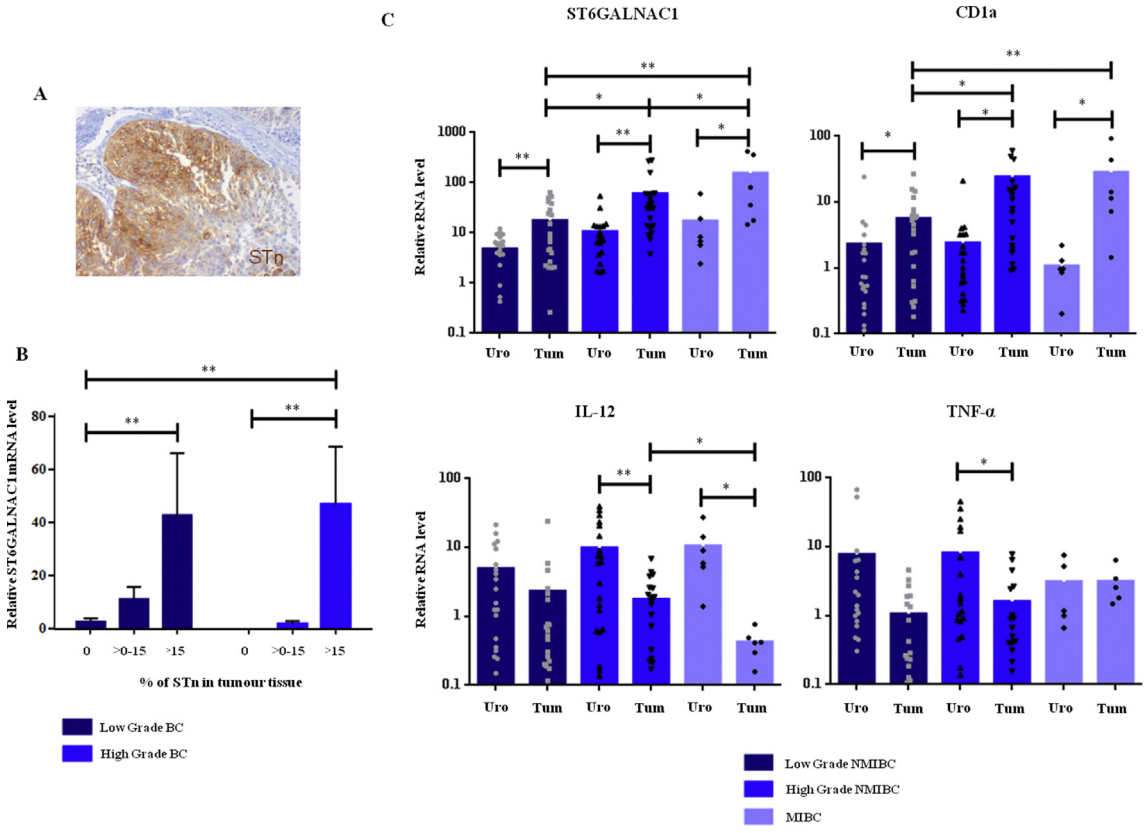

Figure 1 – Bladder cancer tissues show differential expression of STn antigen, CD1a, IL-12 and TNF-α cytokines. A: Analysis of STn expression in high grade tumour bladder cancer tissue. Representative image of a paraffin embedded section, processed for immunohistochemical staining with anti-STn mAb. STn expression was detected in tumour tissue. B: Association between ST6GALNAC1 and STn expression in bladder tumours. STn expression was determined in low and high grade bladder tumours, by immunohistochemistry, using TKH2 mAbs. Specimens were then selected and distributed into three groups, according to their expression of STn related with the tumour bulk: 0%, 0–15% and more than 15% of tissue expressing STn. The relative mRNA levels of *ST6GALNAC1* gene, in the paraffin-embedded sections, was analysed individually, by real time PCR and paired compared with data regarding STn expression. Values infer the number of mRNA molecules of a *ST6GALNAC1* gene, per 1000 molecules of the average of the endogenous controls. C: Gene expression analysis in bladder tissue. The relative mRNA levels of *CD1a*, *IL-12* and *TNF-α* cytokines and *ST6GALNAC1* genes were analysed by real time RT-PCR in specimens from low and high grade non-muscle invasive bladder cancer (NMIBC) ($n = 22$ and $n = 21$, respectively) and muscle invasive bladder cancer (MIBC) ($n = 6$) tissues and also from matched normal urothelium ($n = 49$). Values infer the number of mRNA molecules of each gene per 1000 molecules of the average of the endogenous controls. *CD1a* and *ST6GALNAC1* expression was significantly increased in all tumour tissue, as compared with matched urothelium; while *IL-12* and *TNF-α* cytokines were decreased ($p < 0.05$ (*) and $p < 0.01$ (**)). In general, the differences were more evident in high grade NMIBC.

(Figure 1B) either in low and high grade tumours. To assess whether STn expression was correlated with altered immune function, we quantified the expression of ST6GALNAC1 and CD1a, a marker for immature DC subtype predominant in bladder carcinoma (Figure 1S) (Troy et al., 1999). We also investigated the expression of interleukin (IL)-12, and tumour necrosis factor (TNF)-α, cytokines naturally expressed by DCs and involved in inducing Th1-immune responses. We have analysed tumour tissue and adjacent normal urothelium, and our data showed that both CD1a and ST6GALNAC1 are upregulated in tumour tissue, as compared with corresponding normal urothelium. Interestingly, the upregulation was more pronounced and significantly correlated in muscle invasive and high grade non muscle invasive tumours rather than low grade tumours (Figure 1C). By contrast IL-12 and TNF-α expression, which is associated with DC maturation and Th1-inducing, showed an inverse correspondence with significantly lower expression in high grade tumour tissue than low grade tumour tissue. A correlation was observable between *CD1a* and *ST6GALNAC1* ($r = 0.41$ and $p = 0.003$) expression, pointing out for an association between STn expression by cancer cells and the presence of immature DCs in tumour tissue.

3.2. *Mo-DCs tend to adhere to STn$^+$ bladder cancer cell lines and show significant less mature phenotype*

To further investigate the functional implications of STn-expressing cancer interaction on DC functionality, we established *in vitro* coculture models with STn$^+$ bladder cancer cell lines and human mo-DCs. The transduction of *ST6GALNAC1* sialyltransferase cDNA in MCR bladder cancer cell lines, that natively do not express STn, induced a dramatic expression of STn (Figure 2A), corroborating the role of ST6GALNAC1

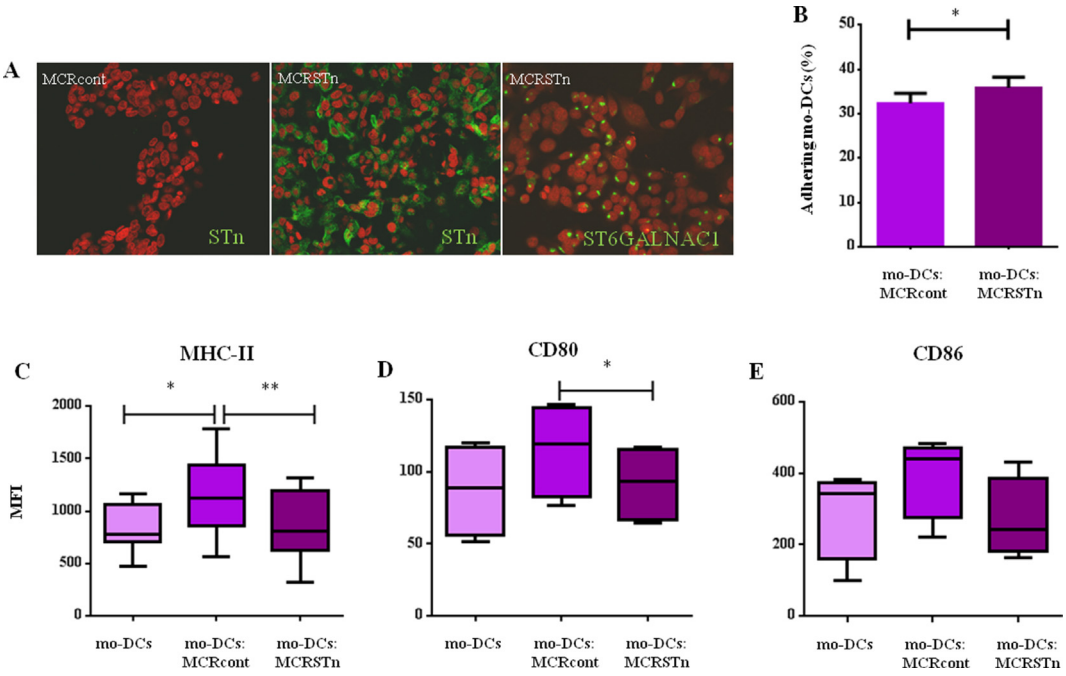

Figure 2 – Mo-DCs adhere preferentially to STn$^+$ MCR cell line and show a less mature phenotype. A: Overexpression of ST6GALNAC1 in MCR bladder cancer cells. MCR cells were transduced or not with a retroviral vector expressing the whole coding region of human *ST6GALNAC1*. Both negative control (MCRcont) and ST6GALNAC1-transduced (MCRSTn) cell lines were stained with anti-STn or anti-ST6GALNAC1 mAbs and then analysed by confocal microscopy. The MCRSTn cell line, but not MCRcont cells expressed the STn antigen and ST6GALNAC1. B: Characteristics of mo-DCs adherent to MCR cell lines. Mo-DCs were cocultured with MCR cell lines and after 2 h incubation, non-adherent mo-DCs were washed and the percentage of mo-DCs adherent to MCR cell lines was estimated by flow cytometry, as the total of MHC-II$^+$ cells in the coculture, following staining with APC-labelled anti-MHC-II mAb. The mean number of mo-DCs adhering to MCR cells was significantly higher in the co-incubation with MCRSTn than with MCRcont ($p = 0.045$ (*), $n = 14$). C-E: Analysis of mo-DC maturation and co-stimulatory profile. Adherent mo-DCs were stained with anti-MHC-II (C), anti-CD80 (D) or CD86 (E) mAbs and then analysed by flow cytometry. The expression level of the antigens was inferred from the mean fluorescence intensity (MFI) of the cells. Values are displayed as box-and-whisker plot with mean and quartiles ± maximum/minimum of 5 independent assays. The expression of MHC-II and CD80 was significantly different in mo-DCs co-incubated with MCRSTn, as compared with MCRcont ($p = 0.0043$ (**) and $p = 0.0438$ (*), respectively). MFI values in mo-DCs alone were comparable to mo-DCs co-incubated with MCRSTn, while mo-DCs co-incubated with MCRcont showed a significant more mature phenotype.

in STn biosynthesis by bladder cancer cells (Ferreira et al., 2013). The obtained STn$^+$ (MCRSTn) and mock transduced STn$^-$ (MCRcont) cell lines were used throughout this study. Co-incubation of human mo-DCs together with MCR cells showed that a significant number of mo-DCs adhered to the cancer cells. The mean percentage of adhering mo-DCs was significantly higher in the coculture with MCRSTn (1.27-fold more) than with MCRcont (Figure 2B). We also investigate whether the observed cellular interactions were dependent on divalent ions, a characteristic of many intercellular interactions. In the absence of divalent ions, mo-DC adhesion to bladder cancer cells was reduced to an average of 10% adherent cells and it was not statistically different between the cocultures with each of two cell line variants (data not shown), suggesting that the differences in adhesion of mo-DCs to MCR cells were dependent on divalent cations.

To characterize the maturation phenotype of the mo-DCs adhering to the bladder cancer cells we assessed the expression of the MHC-II antigen presenting molecule, and co-stimulatory molecules, CD80 and CD86. While the mo-DCs co-incubated with MCRcont cells showed increased expression of MHC-II, CD80 and CD86, mo-DCs adhering to MCRSTn presented an expression level similar to the non-stimulated mo-DCs, suggesting that no maturation was induced (Figure 2C, D and E). To verify whether the immature phenotype of mo-DCs incubated with MCRSTn was dependent on cell contact, parallel experiences were performed where mo-DCs were incubated in the presence of conditioned media, obtained from MCRcont or MCRSTn cell cultures. However, in these conditions the phenotype of mo-DCs incubated with the conditioned medium from either MCR variants was similar (data not shown). The viability of the mo-DCs in culture was approximately 97%, indicating that the observed differences in adhesion and maturation were not due to loss of mo-DC viability. Effects on the MCR cells was also observed, but only when the cocultures were prolonged until 24 h. Specifically, we observed that the proliferative capacity of MCRSTn cells increased 26% ($p = 0.007$), as compared with the proliferative capacity of this cell line in the absence of mo-DCs (Figure 2S).

In order to confirm that the effects on maturation and adhesion to mo-DCs were specifically caused by the enhanced expression of STn, the adhesion assays were performed with a different cell line, namely the breast cancer cell line, MDA-MB-231STn, overexpressing the STn antigen (Julien et al.,

2001). Similarly to MCRSTn, mo-DCs adhered significantly more to MDA-MB-231STn cells when compared to control cells, and these mo-DCs expressed significantly less MHC-II on their surface, showing a less mature phenotype (Figure 3S). Therefore, the same negative effects is obtained after co-incubation of mo-DCs with STn$^+$ cancer cells, irrespective of the cancer type, corroborating the immunomodulatory role specifically for STn.

We then investigated the effect of STn$^+$ cancer cells in semi-matured mo-DCs. We stimulated mo-DCs with LPS, a canonical inducer of mo-DC maturation, which resulted in a remarkable increase of MHC-II expression by DCs (Figure 4SA). In our conditions, LPS-matured DCs could still respond to secondary maturation stimuli and were therefore referred semi-mature. We observed that semi-matured mo-DCs adhere significantly less to both MCRcont and MCRSTn, when compared with immature mo-DCs (55% and 61% less, respectively, data not shown). After co-incubation of semi-matured mo-DCs with MCRcont, a significant increased expression of MHC-II was observed ($p = 0.043$), while no significant effect was obtained by a parallel incubation with MCRSTn cells (Figure 4SA).

To assess whether the contact with MCR cancer cells influenced further mo-DC responses to maturation stimulus, we stimulated mo-DCs with LPS, after coculture with MCR cells. In the presence of the cancer cells, mo-DCs showed defective LPS-induced maturation, while in their absence mo-DCs underwent maturation (Figure 4SB). The resistance to maturation was more evident when mo-DCs were previously incubated with MCRSTn, as compared with MCRcont cancer cells. Thus, the data suggest that the contact with STn$^+$ bladder cancer cells not only hinders maturation, but also prevents further mo-DC maturation.

3.3. *Contact with STn$^+$ cancer cells compromises mo-DC cytokine expression and phagocytosis*

Next, we investigated the function of mo-DCs following contact with STn$^+$ cancer cells. We analysed the expression of several cytokine genes and found that the expression of IL-12, TNF-α, IL-23 and IL-10 was significantly decreased in mo-DCs co-incubated with MCRSTn, as compared with controls (Figure 3A). Analysis of the level of phosphorylated extracellular signal-regulated kinase (ERK), which is involved in cytokine expression, was reduced by 21% in mo-DCs following contact with MCRSTn cells (data not shown).

Since mo-DCs physiologically phagocyte apoptotic cancer cells, we then compared the capacity of mo-DCs to phagocyte both MCR cancer cell line variants. As shown in Figure 4A and B, the percentage of mo-DCs which phagocytosed MCRSTn is significantly higher (1.25-fold more) than those that phagocytosed MCRcont. Confocal microscopy analysis confirmed the internalization of MCRSTn cells by mo-DCs (Figure 4B).

3.4. *T cells activated with mo-DC loaded with STn$^+$ cancer cells show defective activation profiles*

We next analysed the capacity of mo-DCs to activate autologous T cells by measuring the expression of the T cell activation marker CD69. We observed that the mo-DCs that phagocytosed MCRSTn tended to activate significantly less number of T cells, and more weakly, than mo-DCs phagocytosing MCRcont (Figure 5A and B). T cell activation was also lower in T cells primed by mo-DCs that adhered to STn$^+$ cancer cells (Figure 5S), as compared to control MCR cells. T cells alone and T cells incubated with each apoptotic MCR cell lines (with no mo-DCs) were not significantly activated and lost viability (less than 5% viable cells after 11 days). By contrast, after 11 days, more than 35% of the T cells cocultured with mo-DCs remained viable, suggesting that mo-DCs stimulus is necessary to maintain T cell viability. Interestingly, in the T cell: mo-DC (MCRSTn) coculture, the expression of interferon (IFN)-γ and the transcription factor forkhead box P3 (FoxP3) was significantly affected. Namely, IFN-γ was decreased by 54%, while FoxP3 was increased 39% compared with control (Figure 5C and D). While the differences were marginally significant ($p < 0.1$), they indicated a clear tendency for less Th1-skewed T cell response. These data are in agreement with the above mentioned defective maturation profile of mo-DCs, following contact with STn$^+$ cancer cells.

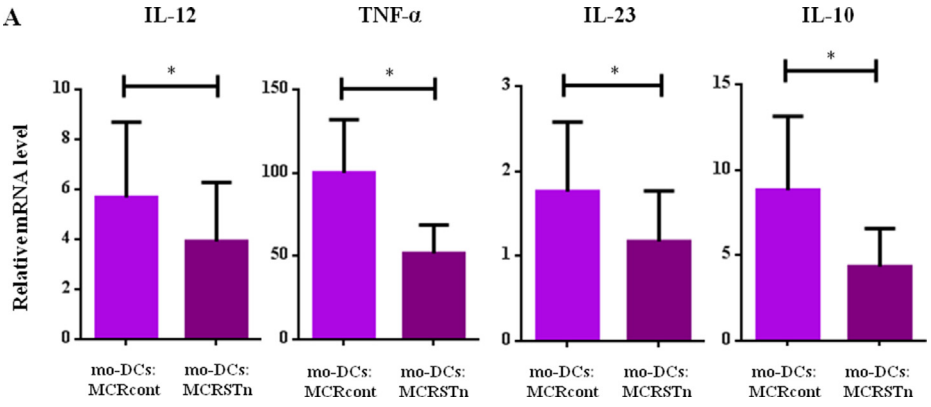

Figure 3 – Co-incubation with STn$^+$ MCR cell line downregulates cytokine expression levels. A: The expression of *TNF-α*, *IL-23*, *IL-12* and *IL-10* cytokine genes was evaluated by quantitative real-time PCR. The relative mRNA levels for each cytokine are expressed as the permillage (‰) of the expression of the endogenous positive controls. Values represent the means of at least 5 independent assays. The expression of *TNF-α*, *IL-12*, *IL-23* and *IL-10* were significantly decreased ($p = 0.015$ (*), $p = 0.031$ (*), $p = 0.034$ (*) and $p = 0.015$ (*) respectively) in mo-DCs co-incubated with MCRSTn as compared with those incubated with MCRcont.

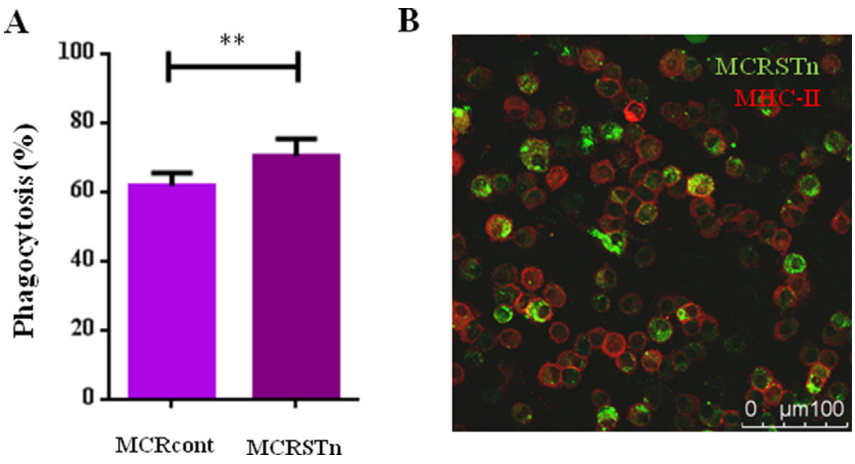

Figure 4 – STn$^+$ MCR cell lines are better phagocytosed by mo-DCs. Both MCRcont and MCRSTn cell lines were labelled with CFSE and then induced to apoptosis. Cells were then incubated with mo-DCs to allow phagocytosis, in the proportion of 1:2 for 6 h at 37 °C or 4 °C and then stained with anti-MHC-II mAb. A: Flow cytometric analysis of percentage of mo-DCs that phagocytosed MCR cells lines. The percentage of mo-DCs that phagocytosed MCR cells was calculated based on the positivity for both MHC-II and CFSE staining. The values obtained at 4 °C were subtracted from the 37 °C values. MCRSTn cells were significantly more phagocytosed than MCRcont ($p = 0.001$ (**), $n = 4$). B: Microscopic analysis of mo-DCs that phagocytosed MCR cells lines. Representative confocal microscopy image showing mo-DCs that phagocytosed MCRSTn cells [MHC-II$^+$ (red)/CFSE$^+$ (green)]. The image is representative of a confocal cross-section image, selected from Z-stack images.

3.5. *STn$^+$ glycoproteins blockade restore DC maturation*

MUC1 and CD44 are glycoproteins equally expressed by both MCR cells (Figure 6A and B) and described as the most likely candidates for being modified by STn by human cancer cells (Julien et al., 2006). The Western blot analysis of MCRSTn cell lysates, using anti-STn mAb, identified one prominent band of approximately 75 KDa, and two weak bands of 150 KDa and 260 KDa each. In all cases, the blot staining was completely abolished after sialidase treatment, proving the STn staining specificity. Immunoprecipitation assays led us to identify the most prominent band (75 KDa) as CD44. Bladder tumours also showed STn in CD44 proteins, as shown by the detection of a STn$^+$ ~75 KDa band in the Western blot analysis (Figure 6C). In order to confirm the role of the STn$^+$ protein scaffold, we blocked STn$^+$ glycoproteins in MCRSTn cells and conducted coculture assays with mo-DCs similar to those described above. In the cocultures, whenever the CD44 was blocked in the MCRSTn cells, they gained the capacity to significantly increase, in mo-DCs, the expression of MHC-II and cytokines, in particular of IL-12 and TNF-α (Figure 6D). Blocking of MUC1, as well as STn antigen in MCRSTn cells also upregulated MHC-II and cytokine expression, indicating a tendency, although not statistically significant (Figure 6D).

4. Discussion

We have recently reported that the majority of high-grade bladder tumours, presenting elevated proliferation indexes and high risk of recurrence/progression and invasion, expressed STn (Ferreira et al., 2013). This glycan is not expressed by normal epithelium and it has been associated with a poor prognosis, and the invasive capacity of the tumours (Ferreira et al., 2013). Similar observations were made for breast and gastric cancers and other solid tumours, supporting the ubiquitous association of STn with malignancy.

The expression of the STn antigen is also accepted to be implicated in the immunogenicity of cancer cells and this has been explored in STn-based vaccines to induce protective immune responses against STn$^+$ cancers. Humoral immune responses against STn have been reported in vaccinated patients and mouse models (Holmberg and Sandmaier, 2004; Julien et al., 2009), demonstrating that the immune system reacts against STn antigens. However, the potency of the immune response is not clinical relevant to provide patients with robust anti-tumour protection.

While, glycans are known to be implicated in several immune responses, the involved biological processes are diverse and complex and thus still poorly understood. Lectin-glycan ligand interactions are implicated not only in mechanisms that establish immune protection, but also in those that establish immunological tolerance. Given the clinical relevance of STn antigens, a deeper investigation on the interaction of STn expressing cancer cells with the immune system is warranted to improve and develop novel STn-based therapeutics.

Here we have studied the influence of STn antigen in the modulation of DCs, which are recognized by their pivotal role in the definition of immune responses. In the tumour tissue, we observed a significant correlation between STn expression and an immature profile of DCs. Indeed, the expression of ST6GALNAC1, which is correlated with the expression of the STn epitopes, in bladder cancer, is correlated with CD1a, a marker of immature DCs. Interestingly, high grade bladder tumours presented not only higher levels of ST6GALNAC1 and CD1a, but also lower levels of the pro-inflammatory, Th1-inducing cytokines, IL-12 and TNF-α, thus supporting the association between STn and DC immature profile.

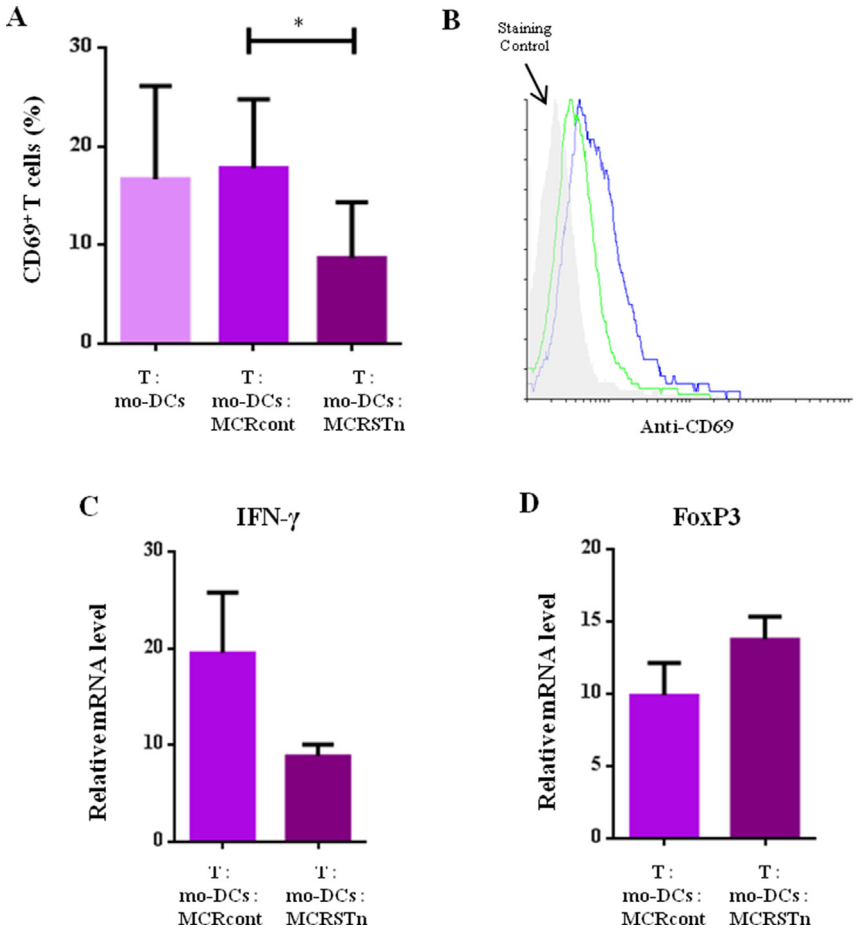

Figure 5 – T cell activation is reduced when primed with mo-DCs that phagocytosed STn$^+$ cancer cells. Mo-DCs were allowed to phagocytose MCR cells and then incubated with autologous T cells (1:8 proportion). A–B: The coculture was analysed by flow cytometry for the expression of the T cell early activation marker CD69. A: Graphical representation of the percentage of CD69$^+$ T cells. Data was determined by the percentage of CD69$^+$, within the CD3 population ($n = 3$). Mo-DCs that phagocytosed MCRSTn cells induce significantly less activation in T cells than mo-DCs that phagocytosed MCRcont cells ($p = 0.026$ (*)). B: A representative CD69 histogram for T cells following priming with mo-DCs that phagocytosed MCRcont (solid line) or MCRSTn (dashed line) cell line. Expression of CD69 by resting T cells is shown as staining control (grey filled peak). C–D: The coculture was analysed by quantitative real-time PCR regarding the expression of *IFN-γ* and *FoxP3* genes. The relative mRNA levels for each gene are expressed as the permillage (‰) of the expression of the endogenous positive controls. C: The expression of IFN-γ was reduced by 54% ($p = 0.074$) in T cells co-incubated with mo-DCs that phagocytosed MCRSTn as compared to controls ($n = 4$). D: T cells co-incubated with mo-DCs that phagocytosed MCRSTn expressed 39% ($p = 0.081$) more FoxP3 than control mo-DCs ($n = 4$).

Maturation is a crucial process that enables DCs to effectively prime T cells to mount responses against malignant cells (Steinman et al., 2003). To confirm a possible inverse association between STn$^+$ cancer cells and DC maturation and to investigate if STn antigen played a role in DC maturation, we used *in vitro* models of STn$^+$ cancer cells cocultured with human DCs. We have found that DCs tend to adhere more to STn$^+$ cancer cells and that this contact inhibits DC maturation and co-stimulation, when compared with DCs cocultured with STn$^-$ control cells. The higher cell adhesion to STn$^+$ cancer cells, which could be due to either stronger or longer cell contacts, was responsible for the defective maturation of mo-DCs. After coculture with STn$^+$ cancer cells, the lower expression of MHC-II and of co-stimulatory molecules could not be rescued by LPS stimulation, suggesting that mo-DC become resistant to further maturation stimuli.

An hallmark of immature DCs is a high phagocytic capacity, which is lowered upon maturation. In agreement with the fact that STn-expressing cancer cells prevents the maturation of mo-DCs, mo-DCs showed better phagocytic capacity for STn$^+$ cancer cells than for control cells. Nevertheless, the effects seen might not only be caused by STn but also some general feature of the MCR cells, since MCRcont cells also induced small maturation arrest.

Mo-DCs incubated with STn$^+$ cancer cells showed defective expression of TNF-α and IL-12, which is consistent not only with DC immature phenotype but also with data observed in bladder tumour tissues. These observations were also in agreement with other reports showing that tumour environment lowers the expression of inflammatory cytokines by DCs (Ishida et al., 2008). We have not detected significantly altered expression of anti-inflammatory

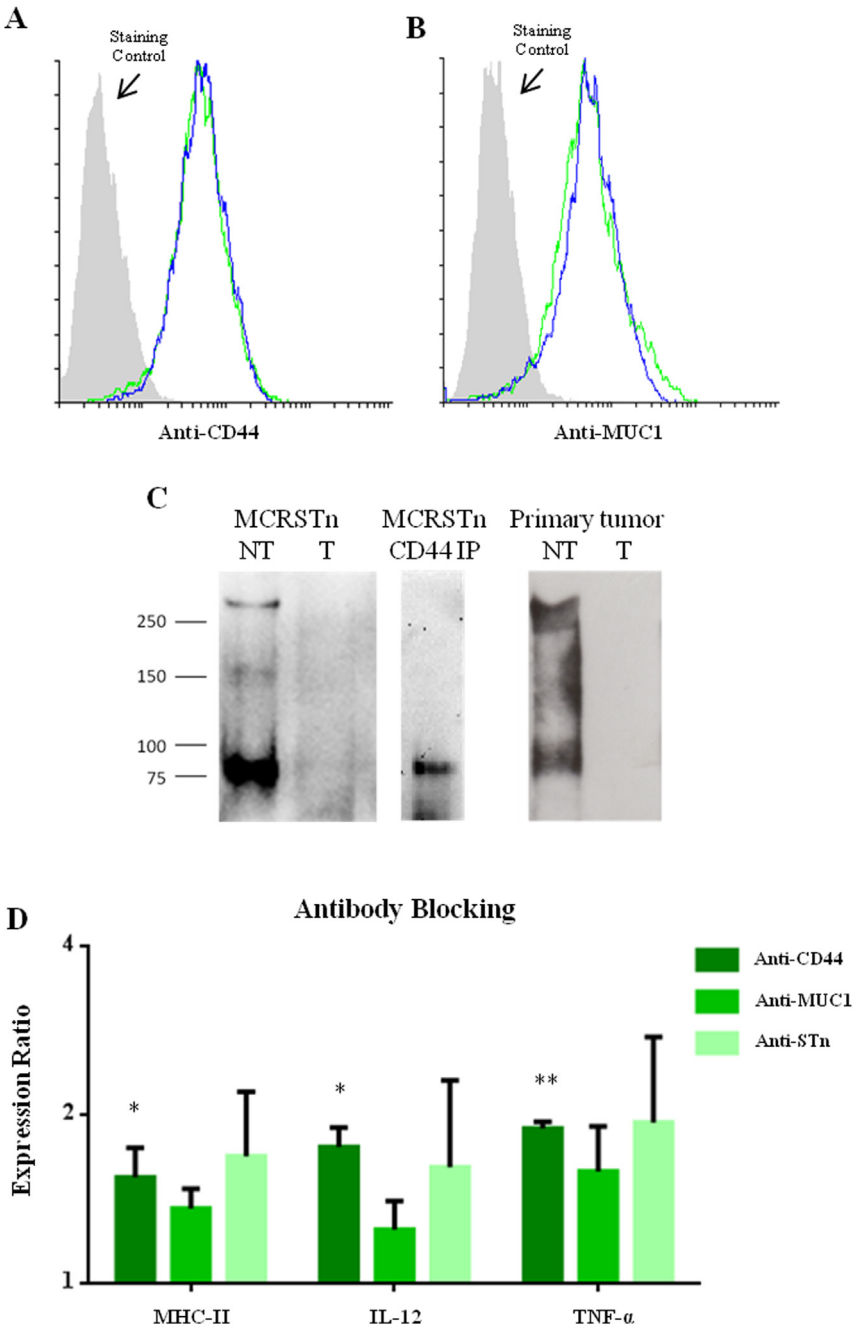

Figure 6 – STn$^+$ glycoproteins blockade restore mo-DCs maturation. A–B: MCR cell lines were analysed by flow cytometry regarding the expression of possible scaffolds of STn, CD44 and MUC1 glycoproteins. MCRcont (dashed line) and MCRSTn (solid line) cell lines were stained with anti-CD44 (A) or anti-MUC1 (B) mAb (not filled peaks) or only with secondary mAb (grey filled peak) as staining control. Both MCR cell lines express similarly CD44 and MUC1 glycoproteins. C: Analysis of STn$^+$ proteins in cancer cells. Total protein lysates from MCRSTn cell line (left image) and primary bladder tumour samples (right image) were treated (T) or not (NT) with sialidase. CD44 immunoprecipitation (IP) from MCRSTn total proteins was performed using Pierce Direct IP Kit (middle image). Cell lysates and CD44 IP were separated and the proteins were transferred to nitrocellulose membrane and stained with anti-STn mAb (clone TKH2). MCRSTn cells showed three proteins (≈75 KDa, 150 KDa and 260 KDa) decorated with STn and the most prominent STn+ protein showed a molecular weight of ≈75 KDa. As control, when the lysate was treated with sialidase, staining with anti-STn was completely abrogated. The CD44 IP analysis showed that CD44 protein is decorated with STn in MCRSTn cells. D: The expression of *MHC-II*, *IL-12* and *TNF-α* is restored when blocking STn and STn$^+$ glycoproteins. Gene expression was evaluated by quantitative real-time PCR in mo-DCs incubated with MCRSTn cell line in presence of anti-CD44, -MUC1 or -STn blocking mAbs. Mo-DC Fc receptors were previously blocked to avoid non-specific Fc receptor-mediated antibody binding. The expression values were calculated as described in the Material and Method section and correspond to the ratio between the expression of mo-DCs incubated with MCRSTn cell line in presence of blocking mAbs and the expression of mo-DCs incubated with MCRSTn in presence of isotypic control. CD44 blockade was able to increase the expression of MHC-II, IL-12 and TNF-α ($p = 0.0294$ (*), $p = 0.0357$ (*), $p = 0.0035$ (**), $n = 3$). Similarly, the expression of MHC-II, IL-12 and TNF-α was also increased by MUC1 and STn blockage.

cytokines, with the exception of IL-10. IL-10 downregulation may be a balanced consequence of the downregulation of pro-inflammatory cytokines, playing a dual proliferative and inhibitory effect (Ogden et al., 2005).

The negative effect of STn^+ cancer cells on the maturation and Th1-inducing profile of mo-DCs were observable in less than 2 h of coculture. On the other hand, STn^+ cancer cells could themselves be influenced by mo-DCs. This was evidenced by the significant increase proliferation of MCRSTn cells in the presence of mo-DCs that was not observable in MCRcont cells. Yet these differences were only observable after 24 h coculture, and the mechanism behind the altered proliferation may be dissociated from the ones implied on the altered mo-DC phenotype. However, in the future, the effect of mo-DCs in tumour cell proliferation should be further clarified and it might be associated with the fact that STn^+ bladder tumours present elevated proliferation indexes *in situ*.

The fact that using other STn^+ cell line, i.e., the MDA-MB-231STn breast cancer cell line, also rendered mo-DCs with an immature phenotype, with an extent similarly to the phenotype induced by MCRSTn bladder cancer line, strongly suggested that the specific STn overexpression in cancer cell was responsible for the induction of tolerogenic DCs reported here. Nevertheless, we cannot disregard the fact that ST6GALNAC1 overexpression in cell lines also alters other glycan structures beyond the STn (Table IS).

To further confirm the role of STn expression by cancer cells in DC maturation, we used different strategies to abrogate the MCRSTn cancer cell interaction with DCs. Blocking of STn antigen in MCR cells, by means of the HB-STn antibody, described to block STn interactions (Pinho et al., 2007), resulted in tendency of MCRSTn cells to induce DC maturation. This suggested that blocking STn antigen may reverse the propensity of STn^+ cancer cells to induce tolerogenic DCs. The fact that we were not able to obtain statistical significant differences may have to do with the role of the protein scaffolds that also participate in the recognition or interaction of glycans (Padler-Karavani et al., 2008). Therefore, anti-glycan mAbs may not have efficient function-blocking properties, as anti-scaffolds have.

We have observed that the major protein scaffold in the MCRSTn cells and bladder tumours is CD44. Moreover CD44 is also decorated with STn in MDA-MB-231STn breast cancer cells (Julien et al., 2006), which in this work induced the same DC immunomodulation as MCRSTn cells. These findings suggested us a possible involvement of STn^+ CD44 in the acquisition of the immature profile by DCs. Concordantly, blockade of MCR CD44 by means of functional blocking antibodies was able to restore the capacity of the mo-DCs to become matured.

CD44 is a rather ubiquitously expressed adhesion molecule, involved in several processes including migration and cell signalling and recognition. The role of CD44 in governing the progression of tumours, including bladder carcinoma (Golshani et al., 2008), is well documented and its expression by leukocytes also plays a role in modulating immune responses (Hegde et al., 2008; Jacobs and Sackstein, 2011; Weiss et al., 1997). However the role of its recognition by immune cells, such as DCs, is unclear. In our model, it is possible that DC receptor for CD44 may recognize differently the protein due to substitution of normal glycosylation by STn in cancer cells. CD44 is a receptor for hyaluronic acid and other extracellular proteins, such as osteopontin and collagens. Very recently it has been reported that the mannose receptor interacts with CD44 (Salmi et al., 2013). Although, mannose receptor primarily binds mannose and fucose, it is possible that in our model, the STn expression affected the CD44 recognition by mannose receptor, leading to the described immunological implications. Nevertheless, other receptors expressed by DCs may also be involved, such as the Sialic acid-binding immunoglobulin-type lectins (Siglec)-9 that have also been reported to recognize STn antigen (Ohta et al., 2010). In addition, the participation of other STn scaffolds such as MUC1 (Monti et al., 2004) should not be excluded and as matter of fact, we also observed that blockade of MUC1 results in a slight induction of DC maturation. Further studies are therefore necessary to better characterize the mechanisms of STn antigen recognition by DCs.

As mentioned above, previous studies were controversial in demonstrating that STn mucins were implicated in inducing the immature DC phenotype (Carlos et al., 2005; Monti et al., 2004). While this controversy may be due to the complexity of the involved mechanisms, these studies were in agreement, when showing that STn expression has a negative influence on the capacity of DCs to activate T cells, resulting in DCs that do not support T cell commitment to Th1 phenotype, which is important for tumour rejection (Carlos et al., 2005; Monti et al., 2004).

DCs are currently used as anti-tumour cell-based vaccines, where one of the most common strategies is the upload of patient's DCs *ex vivo* with the tumour cell antigens (lysates, cells fusion or apoptotic bodies). Tumour antigens are phagocytosed by DCs and these cells are then used to induce anti-tumour T cells responses and further tumour rejection (Palucka and Banchereau, 2013). It has been reported that DCs pulsed with apoptotic cancer cells are able to activate cytotoxic T cells against poorly immunogenic cancer cells (Goldszmid et al., 2003) and in bladder cancer patients increases survival and cure rate (Nair et al., 1997). In the present study, DCs pulsed with immunosuppressive STn^+ cancer cells showed a tolerogenic/regulatory profile, resistant to further maturation stimuli and limited capacity to activate T cells. In these conditions, mo-DCs express lower levels of Th1-inducing cytokines and preferentially polarize T cells towards a $FOXP3^{high}$ IFN-γ^{low} phenotype, typical of regulatory T cells. On the other hand, it has been recently reported by us that STn expression in bladder cancer is associated with better responses to complementary immunotherapy with Bacillus Calmette-Guérin, a potent inducer of Th1 responses (Lima et al., 2013). Therefore, the combination of Th1-inducing therapies with DC-based therapy against STn-bearing cancer cells could be a promising strategy to induce anti-tumour protective responses. Nevertheless, a better understanding of the mechanisms behind the tolerogenic/regulatory profile of DCs induced by STn^+ cancer cells is still necessary.

5. Conclusions

This study shows that STn, a cancer-associated carbohydrate antigen expressed in high-grade bladder cancers, has the

ability to down-regulate the anti-cancer immune-response through different mechanisms. First, it hinders the expression of MHC-II and co-stimulatory molecules by DCs, resulting in impaired ability to present cancer-associated antigens to T cells and making DCs unresponsive to successive activation stimuli. Second, it hinders the expression of inflammatory, Th1-inducing cytokines in DCs, which may result in an attenuation of the Th1 microenvironment. Third, DCs pulsed with STn-expressing cancer cells show a reduced ability to activate and polarize T cells towards the Th1 phenotype, resulting in impaired ability to mount an effective anti-tumour response. Finally, blockade of STn antigens and STn protein may reverse tolerance induced by cancer cells. Altogether, these results highlight the expression of STn by cancer cells as a crucial event in the establishment of the tolerogenic microenvironment which allows cancers to escape from the attack of innate and adaptive immunity.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.molonc.2014.02.008.

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