Impact of abnormal Glycans expression in Cancer Immunoediting:
Gastrointestinal Cancer as model

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Todas as correções determinadas pelo júri, e só essas, foram efetuadas.
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Abstract

Gastrointestinal cancers are in the top 10 of the most common and aggressive malignancies worldwide, being an important target of research.

Glycosylation is a major post translational mechanism that occurs in essentially all cells, being fundamental for inter- and intracellular signaling, immune defense, inflammation, cell adhesion, molecular recognition and pathogen invasion. In the cancer context, glycans have been shown to be implicated in various processes of tumor cell development and progression. During malignant transformation, tumor cells undergo a significant alteration of the glycosylation profile, displaying high levels of complex β1,6-GlcNAc branched N-linked glycans. The presence of this abnormal N-glycans in cancer cells has been associated with invasive and metastatic phenotype and with poor prognosis in gastric cancer patients.

In homeostasis, a cellular mutation is often detected by the immune system that has the capacity to recognize the transformed cell controlling the tumorigenesis, in the so-called process of cancer immunosurveillance. The process of tumor development and progression appear to be associated with escape of anti-tumor immune response within a process called cancer immunoediting.

The main aim of this study was to characterize the glycosylation profile during gastrointestinal carcinogenesis assessing its relationship with the local immune response. Moreover, the impact of abnormal expression of β1,6-GlcNAc branched N-linked glycans on cancer immunoediting was evaluated.

Our results, in human clinical samples, demonstrated an increased expression of β1,6-GlcNAc branched glycans along the gastrointestinal carcinogenesis, that was accompanied by a decreased frequency of effector Th1-type immune cells. Co-cultures of gastric tumor cells with different branched glycans profile with human peripheral blood mononuclear cells (PBMCs) and monocyte derived dendritic cells (moDCs) revealed the impact of MGAT5 overexpressing cells (with overexpression of β1,6-GlcNAc branched N-glycans), in suppressing, or at least diminuishing the magnitude, of pro-inflammatory responses. Namely, by reducing the expression of Th1-associated transcription factor expression and by inhibiting the secretion of the pro-inflammatory cytokine IFN-γ by PBMCs, as well as IL-6 and IL-8 cytokine production by moDCs.

Our findings support for the fist time that abnormal N-glycans expressed in gastrointestinal cancer cells are implicated in tumor development and progression, through immune modulation and anti-tumor immune escape.

Key words: Cancer, gastrointestinal, β1,6-GlcNAc branched N-linked glycans, glycosylation, immunosurveillance, immunoediting, T lymphocytes, dendritic cells, cytokines, immunosuppression, histochemistry, co-cultures.

Resumo

Cancro gástrico e colorretal estão entre os 10 cancros mais comuns e malignos em todo o mundo, representando um importante foco de estudo.

A Glicosilação é um dos mais importantes mecanismos pós-tradução que ocorre de um modo geral em todas as células, sendo essencial para a inter- e intra-sinalização, resposta imunológica e inflamação, adesão celular, reconhecimento molecular e invasão de agentes patogénicos. No contexto do cancro, os glicanos estão envolvidos em diversos processos de desenvolvimento e progressão tumoral. Durante a transformação maligna, as células tumorais alteram o seu perfil de glicanos, passando a expressar altos níveis de N-glicanos complexos *β1,6-GlcNAc branched*. A presença destes glicanos anormais tem vindo a ser associada a um fenótipo de invasão e metastização e a um prognóstico desfavorável em pacientes com cancro gástrico.

Numa situação de homeostasia, uma célula mutada é detetada pelo sistema imunológico, que a reconhece e elimina o foco tumorigénico, processo chamado immunovigilância. No entanto, um tumor desenvolve-se e progride devido à sua capacidade de se camuflar e escapar ao sistema imunológico.

O objetivo deste estudo foi, por um lado, caracterizar o perfil de glicanos durante a carcinogénese gastrointestinal e relacioná-lo com a resposta imunológica local. Por outro lado, avaliar o impacto da expressão anormal dos glicanos *β1,6-GlcNAc branched* das células gástricas cancerígenas na resposta imunológica.

Os nossos resultados em biópsias humanas, demostraram um aumento da expressão de N-glicanos *β1,6-GlcNAc branched*, ao longo da carcinogénese gastrointestinal, acompanhada por diminuição da frequência de células do tipo Th1. Co-culturas de células mononucleares isoladas de sangue periférico (PBMCs) e células dendríticas derivadas de monócitos (moDCs) com células da linha MKN45, cuja expressão de MGAT5 está aumentada levando à sobre-expressão de N-glicanos *branched*, levaram a uma diminuição da expressão do factor de transcrição Tbet associado a células do tipo Th1 e a uma redução na produção e secreção de citocinas pró-inflamatórias, como IFN-γ, pelas PBMCs e de IL-6 e IL-8 por moDCs.

Este trabalho suporta a hipótese de que os N-glicanos anormais presentes no cancro gastrointestinal estão envolvidos no seu desenvolvimento e malignidade, através da modelação da resposta imunológica, tendo um impacto negativo na resposta anticancerígena pro-inflamatória, nomeadamente na resposta T efetora.

Palavras-chave: Cancro, gastrointestinal, N-glicanos β1,6-GlcNAc *branched,* glicosilação, vigilância imunológica, imunoevasão, linfócitos T, células dendríticas, citocinas, imunossupressão, histoquímica, co-culturas.

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Abbreviations

18S	18S ribosomal RNA
- APC	Allophycocyanin
APC	Antigen presenting cells
Asn	Asparagine
BSA	Bovine serum albumin
CD	Cluster of differentiation
CEA	Carcinoembryonic antigen
CHSJ	from Portuguese Centro Hospitalar do São João
CHUP	from Portuguese *Centro Hospitalar Universitário do Porto*
CLR	C-type lectins receptors
CRD	Carbohydrate recognition domains
CTL	Cytotoxic T lymphocyte
Cy 5/7	Cyanine 5 or 7
DAB	3,3'-Diaminobensidine tetradrocloride
DC-ASGPR	DC-asialoglycoprotein receptor
DCs	Dendritic cells
DC-SIGN	DC-specific intercellular adhesion molecule-3-grabbing non-integrin
DMSO	Dimethyl sulfoxide
E-cadherin	Epithelial cadherin
ECM	Extracellular matrix
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
ERAD	Endoplasmic reticulum -associated degradation
FBS	Fetal bovine serum
FFPE	Formalin-fixed paraffin-embedded
FITC	Fluorescein isothiocyanate
Fuc	Fucose
Fuc-VIII	Fucosyltransferase VIII
G418	Geneticin
Gal	Galactose
GalNAc	N-acetylgalactosamine
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GBR	Glycan-binding receptors
Glc	Glucose

GlcNAc	N-acetylglucosamine
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GnT-I to V	N-acetylglucosaminyltransferase I to V
H_2O_2	Oxygen peroxide
HLA	Human leukocyte antigen
IBD	Inflammatory bowel disease
iDC	Immature dendritic cell
IL	Interleukin
IPO	from Portuguese Instituto Português de Oncologia
ITIM	Immune-receptor tyrosine inhibitory motifs
LPS	Lipopolysaccharide
Man	Mannose
MFI	Mean fluorescence intensity
MGAT3/5	Mannosidase acetylglucosaminyltransferase 3/5
MGL	Macrophages galactose lectins
MHC	Major histocompatibility complex
MoDC	Monocyte-derived dendritic cell
MR	Mannose receptor
Muc-1	Mucin-1
Neu5Ac	N-acetylneuraminic acid
NK	Natural Killer
NKT	Natural Killer T cell
OST	Oligosaccharyltranferase
PBMC	Peripherical blood mononuclear cell
PBS	Phosphate buffer saline
PE	Phycoerythrin
Pen-Strep	Penicillin-streptomycin
PerCP	Peridinin chlorophyll protein
PolyLacNac	Poly-N-acetyllactosamine
POMT2	Protein O-mannosyltransferase 2
RorγT	RAR-related orphan receptor gamma
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute
RT-PCR	Real time polymerase chain reaction
Ser	Serine
Siglecs	Sialic acid-binding immunoglobulin-like lectins

SLe^a	Sialyl Lewis a
SLe^x	Sialyl Lewis X
sTn	Sialyl Tn
TAA	Tumor-associated antigens
TAM	Tumor-associated macrophage
Tbet	T-box transcription factor TBX21
TCR	T cell receptor
TGF-β	Transforming growth factor β
Th	T helper cell
Thr	Threonine
TIL	Tumor infiltrate lymphocytes
TNF	Tumor necrosis factor
Treg	Regulatory T cell

Introduction

1. Cancer

Cancer is a global problem with around 14.1 million new cancer cases, 8.2 million cancer deaths and 32.6 million people living with cancer (within 5 years of diagnosis) in 2012 worldwide [1], making this disease an important investigation target.

Tumor is the name given to a collection of related diseases characterized by the uncontrolled growth of cells from some tissue, resulting in a loss of differentiation and altered control of cell cycle. Tumor is called cancer when malignancy is achieved, being characterized by loss of cell-cell adhesion, in which cells detach from each other and from the extracellular matrix and spread throughout the body organs. The leading cause of this abnormal cell transformation was traditionally studied as a genetic issue. Mutations are common in some proliferative-regulatory genes, such as proto-oncogenes (growth factors, receptors, signal transducers, transcription factors, cyclins) responsible for the progression of the cell cycle, tumor suppressors and apoptosis-regulatory genes [2].

Carcinoma is called to a cancer that has origin in epithelial tissue. There are several subtypes of carcinoma, including adenocarcinoma, basal cell carcinoma, squamous cell carcinoma, and transitional cell carcinoma [3, 4].

In this study, we will focus in gastrointestinal carcinogenesis, namely in colorectal and gastric carcinoma.

1.1. Colorectal carcinogenesis

Colorectal cancer is the third most common cancer in both sexes and the fourth leading cause of cancer-related death worldwide [1].

In colorectal carcinogenesis, most of cancers arise from a polyp beginning with an aberrant crypt, which then progresses into an early small adenoma, or low grade dysplasia, (<1 cm in size, with tubular or tubulovillous histology). This adenoma advances to high grade dysplasia, a large adenoma (>1 cm in size, and/or with villous histology) before finally becoming a colorectal cancer (Figure 1) [5]. This process is generally driven by the accumulation of mutations and epigenetic alterations and takes 10–15 years to evolve [6, 7]. Although histology of tubular adenomas is homogeneous, the molecular biology of these polyps is heterogeneous, which might explain why some adenomas progress to colorectal cancer (approximately 10% of polyps) and some do not [8, 9].

In addition to the substantial risk associated to diet habits and life style factors [10], that it is well known, it has been established an important relationship between

inflammation and carcinogenesis [11]. In this regard, immune cells, cytokines, and other immune mediators as well as disturbance of the host/microbiome mutualism have been demonstrated to play important roles in almost all steps of colon tumorigenesis from initiation, promotion, progression to metastasis [10-13].

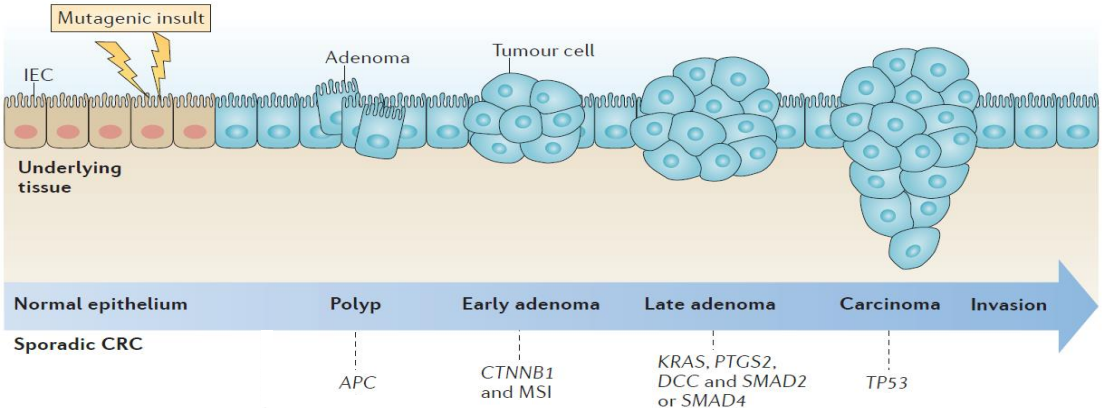

***Figure 1* | Colorectal carcinogenesis and associated mutagenic steps.** In sporadic colon rectal cancer (CRC), the normal epithelium suffers a mutagenic insult leads to appearance to polyp with aberrant crypt, them progresses to early adenoma and, posteriorly, to late adenoma, until carcinoma. Along carcinogenesis, several mutagens determine each step of carcinogenesis. Adapted from [13].

1.2. Gastric carcinogenesis

Gastric cancer is the fifth most common malignancy in the world, after cancers of the lung, breast, colorectum and prostate, and the third leading cause of cancer-related death worldwide [1]. The incidence has been declining over the past few decades due mostly to reduction of infection of *Helicobacter Pylori* cases (through eradication prgrammes) and early diagnosis. However, the survival rate is highly dependent on the tumor stage at diagnosis [14].

Two major histological classifications of gastric cancer was described by Laurén, the intestinal and diffuse subtypes, that display different clinical pathological profiles and distinct epidemiological settings [15].

The intestinal type of gastric cancer represents almost 70% of the cases, being more frequently observed in older male patients [16]. The main carcinogenic event associated with intestinal subtype cancer is *H. pylori* infection which leads to a sequence of histological lesions that culminate in a malignant lesion. A cascade of events take place to several years before diagnostic of cancer: chronic non-atrophic gastritis, multifocal atrophic gastritis, intestinal metaplasia (precancerous conditions), dysplasia (precancerous lesions) and carcinoma [17] (Figure 2). The progression of gastric carcinogenesis pathway has been showing to be related to host characteristics, such as

genetic polymorphisms of pro-inflammatory interleukins [18, 19], and molecules involved in adhesion of the bacteria and genetic variations of bacteria [20-22].

In contrast to intestinal gastric cancer, diffuse subtype is generally diagnosed in young female patients and represent nearly 30% of the gastric cancers. The diffuse gastric cancer develops without precancerous lesions and it is characterized by poorly cohesive cells, organized in cords or micro-glands, displaying a poor prognosis than the intestinal type [23, 24]. Diffuse-type tumors have an important carcinogenic process based in defective intercellular adhesions mainly resulting from Epithelial cadherin (E-cadherin) dysregulation. This is mostly due to abnormal expression of E-cadherin that could result, among other genetic, transcriptional and epigenetic factors [25, 26], by alterations in E-cadherin glycosylation (Figure 2) [27-29].

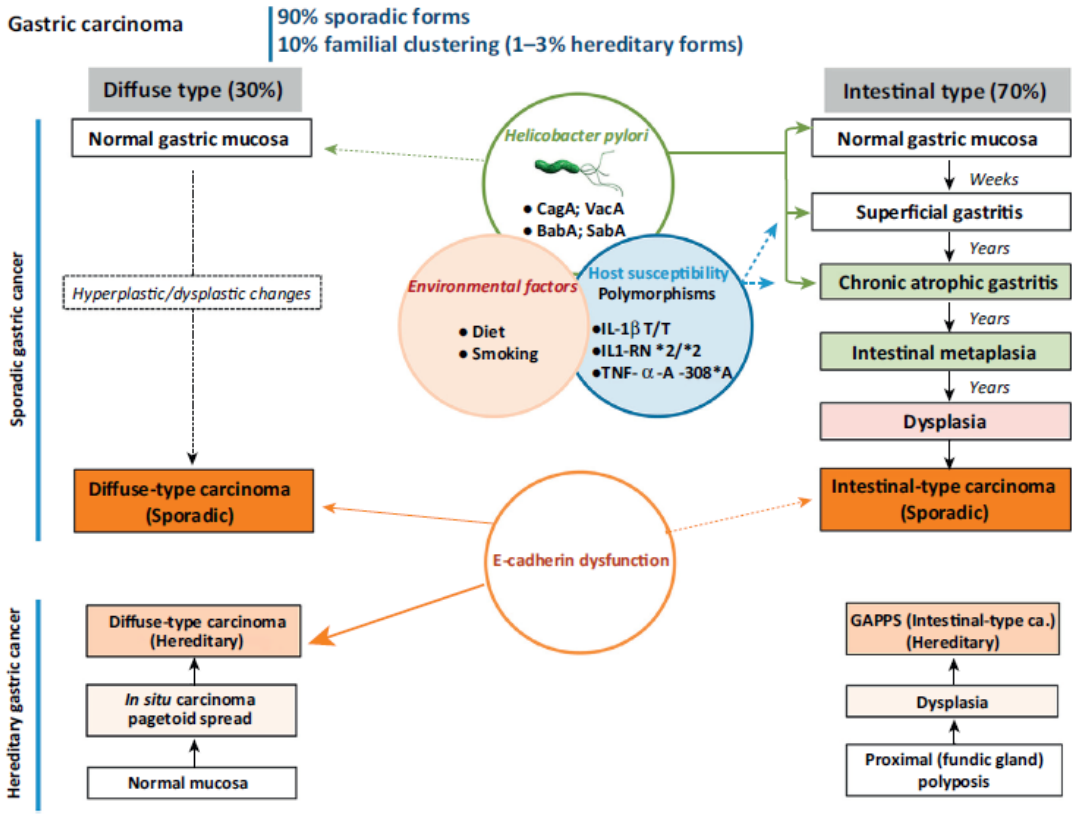

***Figure 2* | Gastric carcinogenesis and commonly factors transformation.** In intestinal type, normal tissue is damaged by Helicobacter pylori or other factors and appear a superficial gastritis that progresses to chronic atrophic gastritis, intestinal metaplasia and dysplasia, leading to intestinal-type carcinoma. E-cadherin dysfunction is also implicated in this process. Adapted from [27].

2. Glycosylation

Glycans, are considered a building block of cells, playing key roles in several physiopathological processes, such as inter- and intracellular signaling, immune defense, inflammation, cell adhesion, molecular recognition and pathogen invasion [30].

Glycosylation is defined as the enzymatic process that produces glyosidic linkages of saccharides to other saccharides, lipids, proteins and other organic molecules producing different families of glycoconjugates: Glycoproteins, Glycolipids and Glycosaminoglycans (Figure 3). [31, 32].

Glycosylation occur in the secretory pathway, that includes the lumen of the endoplasmic reticulum (ER) and the Golgi apparatus [31, 33]. This process involves the action of a portfolio of different glycosyltransferases and glycosidases enzymes, which, together with glycans availability, sugar nucleotides donors, and other biological factors, determine the type of glycoconjugates and glycan structure within a cell and giving rise to the so-called **glycome** - the biological repertoire of all glycan structures of an organism. The mammalian glycome is estimated to be between hundreds and thousands of glycan structures and can be larger than the proteome and the genome [31].

Glycoproteins are proteins that may carry one or more glycans attached to a polypeptide backbone. The two main types of glycans known to be attached to proteins are the O-glycans and N-glycans. O-linked glycans are attached to Serine (Ser) or Threonine (Thr) residues of a protein and are particularly found on secreted or membrane-bound mucins [34]. N-glycosylation is a highly frequent post-translational process occuring in asparagine (Asn) residues of nascent proteins in the consensus peptide sequence Asn-X-Ser/Thr, where X is any amino acid except proline [35].

This study will be focus in N-glycosylation.

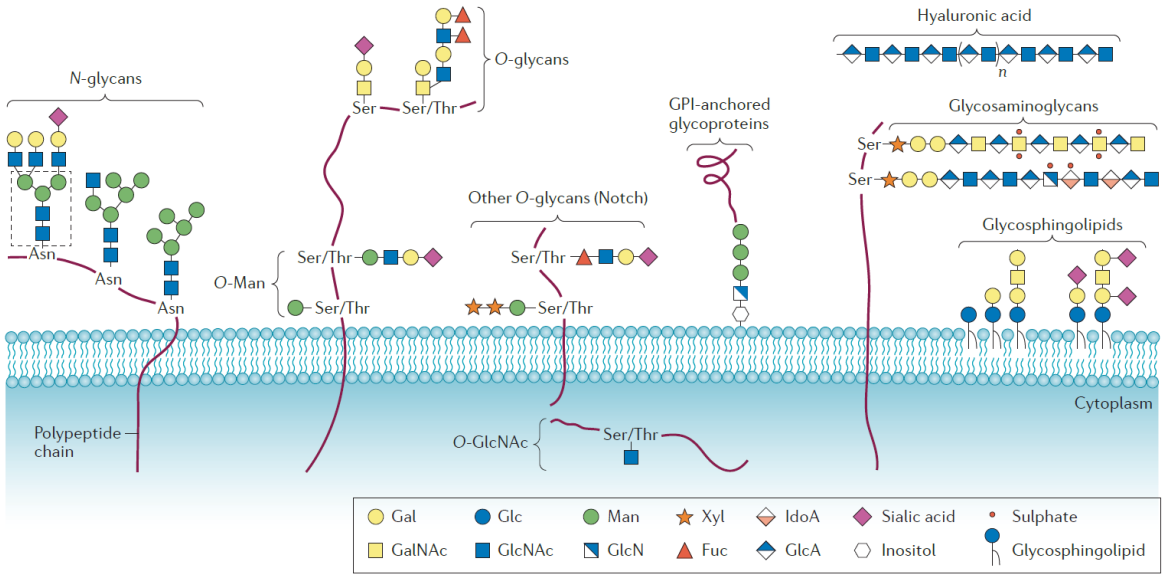

***Figure 3* | Common classes of glycoconjugates in mammalian cells.** Glycans can be attached to various types of macromolecules, such as glycoproteins, in Ser/Thr (O-glycans) or Asn (N-glycans) or GPI-anchored glycoproteins, glycosphingolipids, glycosaminoglycans. Adapted from [32].

2.1. N-Glycosylation

In eukaryotic cells, more than 90% of all mammalian glycoproteins are known to carry N-linked glycans with an average of 2 N-linked glycans per polypeptide chain, which increase the interest in the study of this subtype of glycans [36].

The process initiates in the extra-membrane of the Endoplasmic reticulum (ER) where N-acetylglucosamine (GlcNAc) residue is attached to dolichol phosphate, a lipid carrier in ER membrane. The glycan is building upon this residue where is added one more GlcNAc and 5 mannose residues (Man). In this step, the glycan is translocated to ER luminal side and it is decorated with more 4 Man and 3 Glucose residues (Glc). This specific 14-residue oligosaccharide, consisting of $Glc_3Man_9GlcNAc_2$, is *enbloc* transferred to the side-chain Asn in the consensus sequence Asn-X-Ser/Thr during the protein translation (co-translational) by a oligosaccharyltransferase (OST) [37].

Before living the ER, the glycoprotein is submitted to a quality control system controlled by the calnexin-calreticulin cycle. These two homologous lectins chaperones, calnexin (membrane-bound) and calreticulin (soluble), bind to glycans and control protein folding thought a deglucosylation-reglucosylation system. When the protein is correctly folded, the glycan structure missing the last Glc goes through the Golgi apparatus where the glycans are further elongated and extended. On the other hand, if the protein is misfolded, it is maintained in the calnexin-calreticulin cycle and it will be targeted to ER-associated degradation (ERAD) pathway [38].

Once in the Golgi, the glycans are trimming by glycosidases and further elongated by a sequential activity of different glycosyltransferases that add a specific monosaccharide through a specific glycosidic bond in a step-wise manner. The resulting N-glycans can be classified in three main categories according to the diversity of sugar residues: High Mannose, constituted only with mannose residues; Complex, where one or more “antennae” are yielded by the N-acetylglucosaminyltransferases; and Hybrid, in which mannose residues are attached to the Manα1-6 glycosidic bond and one or two antennae are on the Manα1-3 arm [34].

N-acetylglucosaminyltransferase I (GnT-I) catalyzes the addition of the first GlcNAc residue, and it is a crucial step for the conversion of high-mannose to hybrid or complex-type N-glycans. Afterward, two outer mannose residues are removed by α-Mannosidase II and a second GlcNAc residue is added to mannose α1-6 in the core by the action of N-acetylglucosaminyltransferase II (GnT-II). Further, branching may occur with the addition of GlcNAc residue to the Manα1-3 arm via β1,4 linkage by N-acetylglucosaminyltransferase IV (GnT-IV); or/and the addition of β1,6GlcNAc branch to the Manα1-6 arm by N-acetylglucosaminyltransferase V (GnT-V) to form tri- and tetra-antennary N-glycans. By the action of N-acetylglucosaminyltransferase III (GnT-III), hybrid and complex N-glycans may carry a “bisecting” GlcNAc residue linked on the internal β4Man residue. The presence of a bisecting GlcNAc impedes several otherwise possible elongation and branching reactions [34, 39]. All this process is represented in *Figure 4*.

During subsequent terminal glycosylation, in trans-Golgi, further sugar additions may occur, such as 1) fucose (Fuc) residue addition in first GlcNAc residue adjacent to Asn, 2) elongation of the branch GlcNAc structures, with, for example, poly-N-acetyllactosamine (polyLacNac), and/or “capping” and “decoration” of elongated branches with the addition of N-Acetylneuraminic acid (Neu5Ac), Fucose (Fuc), Galactose (Gal), N-acetylgalactosamine (GalNAc) and Sialic Acid [34].

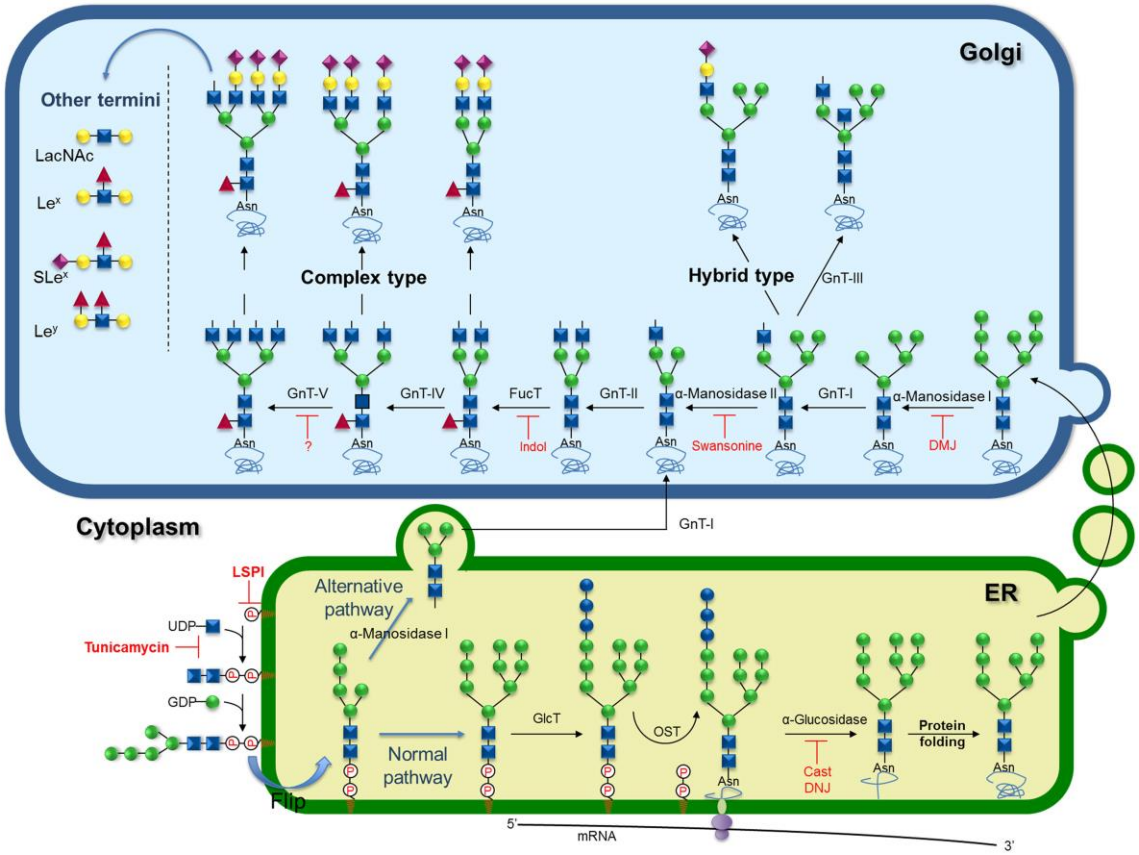

***Figure 4* | N-glycosylation pathway.** N-glycosylation starts in ER where glycans are building in P-dolichol and transferred to Asn in consensus sequence Asn-X-Ser/Thr by OST. The glycoprotein undergoes quality control, regulated by calnexin-calreticulin cycle, and enters into the Golgi apparatus where glycans are trimmed and elongated In Golgi, the glycans structures can be subdivided in high mannose, hybrid and complex type glycans, dependingon the expression of glycosidases and glycosyltransferases. Adapted from [40].

3. Glycosylation alterations in cancer

The glycosylation profile of malignant cells significantly changes when compared to the normal counterparts [32]. Expression of aberrant glycans have been implicated in different steps of tumor progression, such as proliferation, invasion, metastasis and angiogenesis [32, 41]. The altered protein glycosylation can be due to under- or overexpression of glycosyltransferases, their mislocalization in the ER/Golgi compartments, dysregulation of chaperone function and availability of the sugar nucleotide donors and cofactors [32].

The most-widely occurring changes in glycosylation include an increase in overall sialylation, an increase in branched N-glycans and an increase in core fucosylation. Aberrant glycosylation in cancer frequently involves an increase in sialyl Lewis X (SLe^x), SLe^a and truncated glycans, such as sialyl Tn (STn), in O-linked glycans, and an increase in the α-2,8-linked polysialic acid. Futhermore, overexpression of branching N-glycans,

caused by an increased activity of GnT-V, and an increase of 'core' fucosylation (α1,6-Fuc to the innermost GlcNAc of N-Glycans) by fucosyltransferase VIII (Fuc -VIII) is also considered as important event in tumor development and progression (Figure 5) [32].

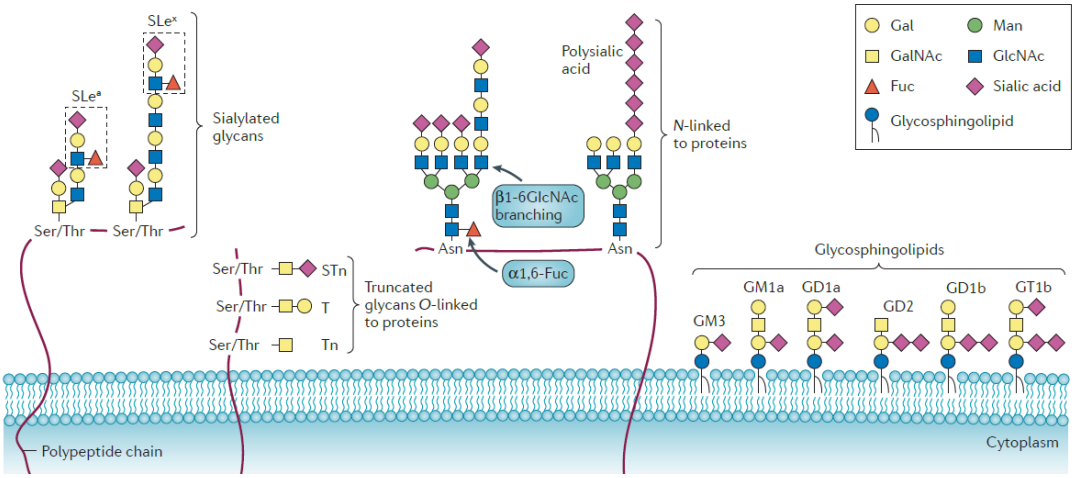

***Figure 5* | Aberrant cancer-associated glycans.** Generally, in cancer context, occur an increase in sialic acid structures, like SLe^a and SLe^x, or truncated glycans O-linked to proteins, like T, Tn and STn antigen. On the other hand, β1,6-GlcNAc branched N-glycans and α1,6-Fuc N-glycans or N-glycans with polysialic acid extension, are frequently found in cancer cells. Adapted from [32].

Aberrant glycosylation is in fact considered an hallmark of cancer being the focus of this thesis [42].

3.1. Altered N-linked glycans in cancer

The abnormal expression of complex β1,6-GlcNAc branched N-Glycans results from the GnT-V activity, which is encoded by mannosidase acetylglucosaminyltransferase 5 (MGAT5) gene. During malignant transformation, the activation of the RAS-RAF-MAPK pathway leads to MGAT5 upregulation and consequently overexpression of β1,6-GlcNAc branching in Asn-linked glycans, mediated by GnT-V [43].

β1,6-branched N-glycans are implicated in several mechanisms occuring in the process of tumor development and progression. Overexpression of MGAT5 in an immortalized lung epithelial cell line results in loss of contact inhibition, increased cell motility and tumor formation in athymic mice [44], as well as in enhanced invasion and metastasis of different types of cancer cells such as in mouse mammary carcinoma cells [45], glioma and colon cancer cells [46, 47]. Accordingly, *MGAT5*-deficient mice displays significant suppression of mammary tumor growth and metastasis [48].

In fact, N-linked glycans have been described to modulate cell-cell adhesion interfering with key functions of E-Cadherin molecule, a transmembrane glycoprotein and

a major epithelial cell-cell adhesion molecule in cancer [26, 29]. GnT-V overexpression in gastric cancer cells induces E-cadherin cellular mislocalization to the cytoplasm concomitantly with its loss of function, and leading to non-functional adherent junctions [28, 49]. On the other hand, the homologous protein O-mannosyltransferase 2 (POMT2) overexpression, that catalyzes the addition of Man residues to Ser or Thr residue (O-mannosylation), contribute to proper adhesive functions of E-cadherin in homestasis, counteracting the function of branched GnT-V-mediated N-glycans [50].

Moreover, the interaction between cell and extracellular matrix (ECM) is also controlled by N-glycosylation. Integrins have a key role in mediating cell-ECM interactions being important receptors for signals in the ECM and connecting many biological functions, such as cell proliferation, protection against apoptosis and malignant transformation [51]. The presence of GnT-V-mediated branched N-glycans on α5β1 and α3β1 integrins enhances the migration and invasion of human fibrosarcoma and melanoma cells, respectively [52, 53].

In contrast, GnT-III-mediated bisecting GlcNAc N-glycans counteract GnT-V activity, having a significant role in a cancer context. GnT-III, encoded by MGAT3 gene, catalyzes the addition of GlcNAc in a β1,4-linkage and suppresses additional processing and elongation. Melanoma B16 cell line, with high metastatic potential, and transfected with MGAT3 displayed a significant suppression of lung metastasis in mice due to a reduction of β1,6 branched N-glycans [54]. Accordingly, the presence of bisecting N-glycans in E-cadherin increases stability of adherent junctions, inhibiting tumor progression and metastasis [28, 49]. Moreover, the overexpression of GnT-III resulted in an inhibition of α5β1 integrin-mediated migration due to reduce of the affinity of the binding of integrin to fibronectin [55]. Similarly, in MKN45 gastric cancer cells, the over expression of GnT-III suppresses α3β1 integrin-mediated cell migration [56].

Overall, GnT-V-mediated branched N-glycans, aberrantly expressed in cancer context, are implicated in tumor progression and metastasis due to loss of cell-cell adhesion and dysregulation of cell-ECM interaction. On the contrary, GnT-III-mediated bisected N-glycans, as well as O-mannosyl glycans, enhance cell-cell adhesion and promote a downregulation of cell-ECM adhesion, suppressing tumor development and metastasis.

4. Immune system, glycosylation and cancer

4.1. Overview of Immune Response

The physiologic function of the immune system is defense against pathogens and foreign substances. Furthermore, mechanisms that normally protect individuals from infection and eliminate foreign substances can also cause tissue injury and disease. Under some situations, even self-molecules can elicit immune responses (so-called autoimmune responses). Immunology is the study of immune responses in its broader sense and of the cellular and molecular events that occur after an organism encounters microbes and other foreign macromolecules [57].

The immune system can be divide in two different lines of defense: innate and adaptive immune responses. The **innate immune response** is the first line of defense against microbes or injured cells products. It consists in cellular and biochemistry defense mechanisms, which are essentially the same to repeated exposures. The main components of innate immunity are (1) physical and chemicals barriers, such as epithelia and antimicrobial chemicals produced at epithelial surfaces, (2) phagocytic cells (neutrophils, macrophages), dendritic cells, natural killer (NK) cells and other innate lymphoid cells, as well as (3) blood proteins, including members of complement system and other mediators of inflammation. Additionally, the dense and complex coat of glycans that cover all epithelia, termed glycocalyx, can also serve as an important physical and biological barrier with key function in immune response. The **adaptive immunity** consists in later mechanisms and develops as a response to specific infections. The adaptive immune response is based in the ability to respond more vigorously to repeated exposures to the same antigen (foreign substance that induce specific immune response), called as memory response. The lymphocytes are the main components of adaptive immunity, as well as their secreted products, like antibodies [57, 58].

Cytokines are a large group of secreted proteins with diverse structures and functions, which regulate and coordinate many activities of innate and adaptive immunity. All cells of immune system secrete at least some cytokines and express specific signaling receptors for several cytokines. The cytokines can be divide in two subgroups: pro-inflammatory cytokines, that led to proliferation and differentiation of pro-inflammatory immune cells, and anti-inflammatory cytokines, that control an exaggerated immune response and inhibit pro-inflammatory response. There is no clear separation between this two groups of cytokines as some cytokines have dual functions depending on the tissue and physiopathological context. Chemokines are a smaller group of cytokines that regulate cell migration and movement [57]. In *Appendix 1* is displayed a summary of cytokines, as well as its cell source and function, that was mentioned along this study.

The **lymphocytes** have a key role in the adaptive immunity and can be T lymphocytes or B lymphocytes. Morphologically, they are very similar, but their heterogeneity and functions are highly diverse. B lymphocytes, B cells or bone marrow-derived lymphocytes, are responsible for producing antibodies, which mediate humoral immunity. T lymphocytes, T cells or thymus-derived lymphocytes, mediate cellular immunity. T cells can be subdivided in different classes with distinct functions: *CD4+ helper lymphocytes* (Th cells), subdivided in Th1, Th2 and Th17; *CD8+ cytotoxic T lymphocytes* (CTL), that have cytotoxic granules that are harmful to different pathogens; *regulatory T lymphocytes* (Treg cells), which their function is to inhibit immune responses; and two numerically smaller subsets, *Natural Killer T cells* (NKT) cells and *γδ T cells* [57-59].

Antigen-presenting cells (APCs), predominantly dendritic cells (DCs) and macrophages, represent the bridge between innate and adaptive immunity. DCs recognise and uptake the antigen, becoming activated and travels to a nearby lymph node, where present antigens to naïve T cells, via major histocompatibility complex (MHC) providing additional stimuli, that are required to trigger T cells responses. Moreover, APCs secrete cytokines that play critical roles in T cells differentiation into effector T cells [57, 59, 60].

Each immune cell subpopulation expresses different surface proteins, such as cluster of differentiation (CD), that distinguishes the different subtypes of immune cells. Th and Treg cells express in their surface CD4, while CTLs express CD8. All T cells subtypes express CD3 associated to T cell receptor (TCR), and all leukocytes express CD45. Other markers can be used to identify different population of immune cells, like transcription factors or even cytokines and cytokine receptors. The phenotypes of the different effector $CD4^+$ T cells, Th1, Th2 or Th17, depend on the expression of specific T-box transcription factor, such as TBX21 (Tbet), Gata-3 or RAR-related orphan receptor gamma (Rorγ) T, respectively, while regulatory T cells depend on transcription factor forkhead box P3 (Foxp3) [57, 59]. Concerning to DCs, whenever maturated, they express specific markers, such as CD86 and CD80, which are costimulatory factors for T cell lymphocyte activation [58, 60]. These markers are currently used in molecular biology to discriminate and analyze each subclass of immune cells [57].

4.2. Cancer immunosurveillance and immunoediting

The concept of immunosurveillance was proposed by Ehrlich in 1909 [61], defending that nascent transformed cells arise continuously in the organism and that immune system can recognize and eradicate these transformed cells. This concept was postulated by Burnet and Thomas in the mid-20^{th} century [62]. However, the controversial

studies led to abandon the immunosurveillance concept and new findings, suggesting that immune system is capable to promote or select tumor variants with reduced immunogenicity, gave rise to the **tumor immunoediting** hypothesis [63].

The immunoediting hypothesis consider dual functions for the immune system: host protection and tumor promotion [64]. This process consists in three phases: elimination, equilibrium and escape. The elimination phase, that corresponds to immunosurveillance, consists on the recognition of transformed cells by the innate and adaptive immune system, through mechanisms (production of chemokines and other cytokines) that directly kill tumor cells. If some tumor cells persist in the elimination phase, then the process can progress to equilibrium phase, in which tumor do not expand due to immune pressure. In the escape phase, the immune system is inhibited and the tumor cells become less immunogenic and thus evading anti-tumor immune response. This escape phase occurs concomitantly with the appearance of clinically detectable, progressively growing tumors (Figure 6) [63-65].

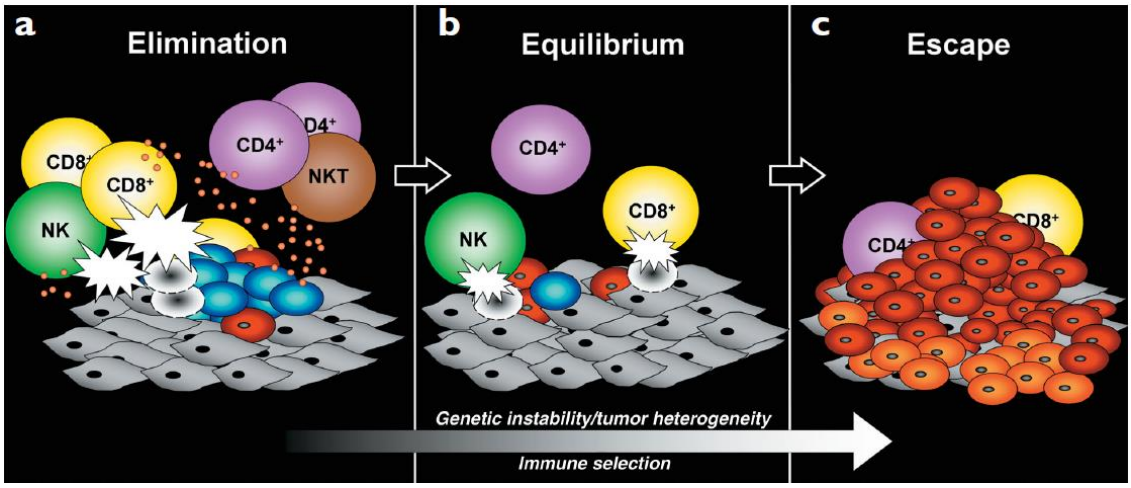

***Figure 6* | Tumor immunoediting and tumor cell selection. (a)** Elimination phase correspond to immunosurveillance, which immune system can eliminate transformed cells and eradicate tumor. **(b)** Equilibrium phase, the process where immune system selects and/or promotes the generation of tumor cell variants with increasing capacities to survive immune attack. **(c)** Escape phase is the process wherein tumor cells expand in an uncontrolled manner in the immunocompetent host. Adapted from [63].

Tumor cells adopt a diversity of mechanisms to escape from the host immune system: (1) Interference with the induction of anti-tumor immune responses, by a dysfunctional cross-talk with APC and T cells; (2) Inadequate effector cell function in tumor microenvironment, by suppression of effector T cell responses; (3) Insufficient recognition signals, through downregulation of tumor-associated antigens (TAA) or decreased expression of human leukocyte antigen (HLA, the MHC of human immune system); (4) selection of resistant cellular variants [66, 67].

4.3. Immune cells in Tumor microenvironment

The presence of inflammatory infiltrates in the tumor milieu are frequently associated with improved prognosis and better patient survival [68-71]. Controversially, other reports have indicated a lack of significant correlation between lymphocytic infiltrate and improved prognosis [72, 73].

The tissue microenvironment is modulated and dominated by the tumor. Immune cells in the tumor microenvironment include those mediating adaptive immunity, such as T lymphocytes, DCs, and occasionally B cells, as well as effectors of innate immunity, macrophages, polymorphonuclear leukocytes and rare NK cells. NK cells, mediators of innate immunity and rich in perferin- and granzyme-containing granules, are absent from most tumor infiltrates or even pre-cancerous lesions. Although NK cells mediate a potent antitumor cytotoxicity, they are infrequent in tumor, which is a mechanism of evading anti-tumor immune response [66].

Tumor-infiltrate lymphocytes (TIL) are usually a major component of the tumor microenvironment, where T cells are specific for TAAs. The accumulation of these effector $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells in the tumor might be an evidence of immunosurveillance by the host, but they are largely ineffective in arresting tumor growth [66]. Treg ($CD4^+CD25^{high}Foxp3^+$) cells are overexpressed in tumor microenvironment in comparison with their low frequency in the peripherical circulation of patients with cancer. These cells are capable of suppressing proliferation of other T cells in the microenvironment through contact-dependent mechanisms or by secretion of anti-inflammatory cytokines, such as Interleukine-10 (IL-10) and Transforming Growth Factor (TGF)-β [74, 75]. IL-10 has potent immunosuppressive effects on APCs and effector T cells [74], through different mechanisms such as: reduction of expression of type 1 cytokines; inhibition of antigen-specific T cell proliferation and by precluding the production of pro-inflammatory cytokines by macrophages [76] and APCs [77]. TGF-β is commonly overexpressed in many tumors and has important immunosuppressive effects, including the inhibition of T cell proliferation and their development into CTLs and T-helper cells [78].

The functions of Th1-type cells within a tumor context is the secretion of cytokines, such as IFN-γ, which play essential roles in antitumor immunity. IFN-γ can substantially increase the levels of IL-12 production by stimulated DCs, that in turn polarize naïve T cells into Th1 phenotype [79]. IFN-γ also regulates various biological programs that could participate in tumor rejection, including the capacity to inhibit cellular proliferation, induction of apoptosis, and inhibition of angiogenesis. The effects of IFN-γ is essentially the promotion of an effective antitumor immune responses, together with the inhibition and activation of Treg cells [80]. However, IFN-γ secretion can also lead to suppression of the

immune response indirectly through the upregulation of IFN-γ-inducible genes. Indoleamine 2,3-dioxygenase (IDO) is an IFN-γ-inducible enzyme that catalyzes tryptophan catabolism, an essential amino acid to T cells, and causes proliferation arrest of T lymphocytes [81]. On the other hand, Th2 cells are crucial in recruiting eosinophils to the tumor site, which can kill tumor cells via secretion their cytotoxic products [79]. The predominance of Th1 and Th2 cells can be variable depending on the tissue or tumor stage, whereas the presence of one subtype discourages the other. Accordingly, gastric cancer [82] and colorectal cancer [83] patients with higher Th1:Th2 ratio have a better prognosis and longer disease-free survival, while patients of non-small cell lung cancer with higher Th1:Th2 ratio in the peripheral blood mononuclear cells predict shorter survival [84].

Macrophages present in tumor, known as tumor-associated macrophages (TAMs), are re-programed to inhibit lymphocyte functions through release of inhibitory cytokines, such as IL-10, prostaglandins and reactive oxygen species (ROS) [85]. Immunoinhibitory activities of ROS are mediated by the NF-κB pathway, which in turn is regulated by hypoxia and/or re-oxygenation [86]. The tumor microenvironment is a hypoxic environment and it determines the nature of inflammatory infiltrates. It is created early in the tumor development through activation of hypoxia-responsive genes in tumor cells [87]. NF-κB activation in cancer cells, as well as in leukocytes lead to secretion of Tumor necrosis factor (TNF)-α and other pro-inflammatory cytokines, which can promote tumor development and growth [88].

Regarding this, cell proliferation and cell differentiation, matrix remodeling, blood vessel growth and cell migration/recruitment are all re-programmed to the benefit of the tumor. The immune cells present in tumor milieu try, unsuccessfully, to fight tumor development, but the tumor immunoediting process modulates immune responses and evade anti-tumor immune response.

4.4. Glycosylation in Immune Responses

As described before, glycans have a key role in cancer development and progression, being implicated in various biological processes, as well as in immune responses and tumor microenvironment [32].

Immune cells have glycans-binding receptors (GBR), known as lectins, involving in glycans recognition, which can be secreted or found on the cell surface of immune cells. These receptors can be divided in several families and types, such as galectins, C-type lectins and Sialic acid-binding immunoglobulin-like lectins (Siglecs). All these GBR carry

one or more carbohydrate recognition domains (CRD) that coordinate the interaction with a specific glycan [89-92].

Galectins are a family of soluble lectins and recognize glycans containing the disaccharide N-acetyllactosamine (Gal-β1,3-GlcNAc or Gal-β1,4- GlcNAc) [93]. In immune system, galectins are expressed in activated T and B cells, as well as in activated macrophages and Treg cells. Galectin-1 and galectin-3 have a varied tissue distribution, while others have preferential localization like galectin-10 on eosinophils and Treg cells [94].

C-type lectins receptors (CLR) are calcium- dependent carbohydrate-binding proteins and consist of a large family of receptors (60-80) that can be divided in two categories based on amino acid motif involved in glycans recognition and coordination of the Ca^{2+} ion [92]. C-type lectins that contain an Glu-Pro-Asn amino acid motif, such as DC-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN), mannose receptor (MR), Langerin and L-SIGN/DC-SIGNR, have specificity for mannose- and/or fucose-terminated glycans. In contrast, galactose lectin, that contain the Gln-Pro-Asp like macrophage galactose lectin (MGL) and DC-asialoglycoprotein receptor (DC-ASGPR) recognize galactose-terminated or GalNAc-terminated glycan structures [95]. The type II subfamily of CLRs is mainly restricted to APCs, such as macrophages and DCs, whereas some of them have also been identified on NK cells or endothelial cells [96].

Siglecs can convey regulatory signals that positively or negatively control immune responses, though most siglecs are known to be negative regulators of cell signaling by the presence of immune receptor tyrosine-based inhibitory motifs (ITIMs). Siglec-1, or sialoadhesin, is preferentially expressed on macrophages, siglec-2 on B cells, siglec-8 on eosinophils and siglec-9 are expresses on myeloid-derived DCs, while siglec-5 on plasmocytic DCs [97].

As examples, TAAs like Carcinoembryonic antigen (CEA) and Mucin 1 (MUC-1), displaying increased expression of O-linked glycans Le^x and Tn in cancer [97] are specific ligands for CLRs expressed in DCs, DC-SIGN and MGL, that do not interact with normal tissue. Tn glycans in MUC-1 bind to MGL and instruct DC to drive Th2-mediated responses, which, unlike Th1 responses, do not contribute to tumor eradication [98]. In addition to altered glycosylation, tumor cells can secrete Galectin-1 that has been shown to contribute to immunosuppression of several types of cancer by selectively modulating T cell and DC responses [95]. The mechanisms underlying this immunoregulatory effect involve a bias toward a Th2-dominant cytokine profile and activation of tolerogenic circuits mediated by IL-27-producing DCs and IL-10-producing Treg cells [99]. Moreover, overexpression of galectin-9 results in increased granulocytic myeloid suppressor cells

and inhibition of antitumor responses [100], while presence of galectin-3 control the anergic state T cells [101]. This mechanism is represented in *Figure 7*.

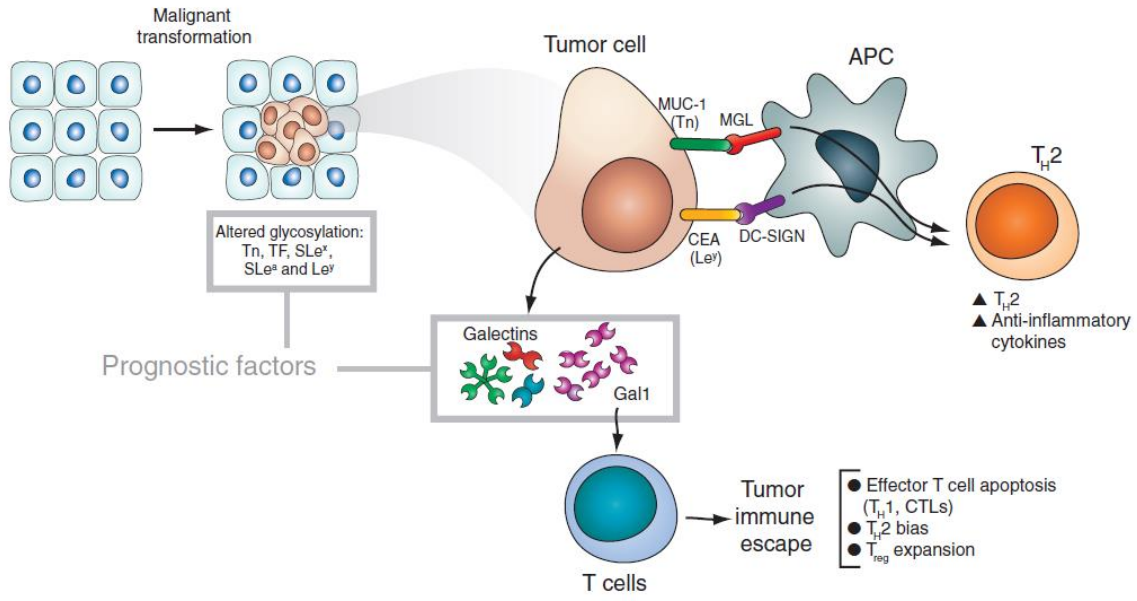

***Figure 7* | Immune responses to altered glycosylation.** Glycan modifications in MUC-1 and CEA during malignant transformation results in selective recognition by lectins and modulation of immune responses. These glycans can be detected by C-type lectins (MGL and DC-SIGN), which affect APC function, leading to anti-inflammatory responses. Moreover, T cell identification of galectins secreted by tumor cells promote tumor immune escape induce Th2 and Treg expansion and Th1 and CTL apoptosis. Galectins secreted by tumor and altered glycans can be used as prognostic markers. Adapted from [97].

However, little is known about the relationship between aberrant N-glycans present in cancer cells, namely complex β1,6-GlcNAc branched N-Glycans, and tumor immune response. In vitro evidences, supporting our hypothesis, showed that downregulation of MGAT5 with shRNA in breast cancer cell lines can enhanced activation and proliferation of $CD4^+$ T cells and macrophages and the production of Th1 cytokines in splenocytes from animals injected with shRNA-Mgat5-transfected MA782 tumor cells [102]. In addition, our results on showed that in T cell from patients with inflammatory bower disease (IBD), an autoimmune disease, have a deficit of β1,6-GlcNAc branched N-Glycans in TCR, positively associated with disease severity [103]. Accordingly, *in vivo* studies using $Mgat5^{-/-}$ mice shown an identical result, where negative regulation of TCR signaling by GnT-V-derived branched N-glycans promotes development of Th2 over Th1 responses, enhances polarization of Th2 cells [104].

Therefore, and given the current relevance of cancer immunotherapy strategies it is important to understand how cancer cells aberrantly glycosylated impact in cancer immunoediting.

Aim

The goal of this thesis project was to study the impact of expression of aberrant N-glycans tumor cells on the immune response and tumor progression, aiming to clarify how glycans modulate tumor immunoediting. Therefore, we propose to address two specific aims:

1. To characterize the glycosylation profile *in situ* during gastrointestinal carcinogenesis and its relation with local T cell frequencies.

2. To evaluate the mechanistic impact of abnormal N-glycans expression on cancer immunoediting, using a gastric cancer cell line overexpressing aberrant branched glycans and assessing its impact on T cells and DCs immune response.

For the first aim, we have selected paraffin embedded clinical samples from normal tissue, pre-malignant lesions/conditions and carcinoma, both from intestinal-type gastric and sporadic colorectal carcinogenesis. We have performed histochemistry using specific lectins that recognize high-mannose N-glycans and branched N-glycans, as well as immunohistochemistry for detection of Tbet and Foxp3 expression. Furthermore, RNA was extracted and Real-time PCR was performed to evaluate MGAT5 transcription levels.

For the second aim, blood samples from healthy donors were used and peripheral blood mononuclear cells (PBMCs) were obtained and co-cultured with gastric cancer cells, which overexpressed GnT-V-mediated branched N-glycans. The impact of these aberrant glycans on T cell differentiation, cytokine production, and moDC´s activation was analyzed by flow cytometry. Cytokines released to the culture medium were also evaluated by flow cytometry and Enzyme-linked Immunosorbent assay (ELISA).

Materials and Methods

1. Materials and reagents

Formalin-fixed paraffin-embedded (FFPE) tissues section were cut with microtome blades S35, FEATHER®, performed in Paraffin microtome *Microm HM335E* and adhered to silane coated microscope slides APTACA® or KLINPATH®.

For histochemistry were used: *Biotinylated Phaseolus Vulgaris Leucoagglutinin* (L-PHA, 2 mg), *Biotinylated Galanthus Nivalis Lectin* (GNA, 2 mg) and VECTASTAIN® Elite ABC HRP Kit from *Vector Laboratories*; Xylene, Ethanol ≥99.8%, Methanol, Oxygen peroxide (H_2O_2) 30% and *UltraVision* Quanto Detection System HRP DAB kit from *Thermo Fisher Scientific*; Bovine serum albumin (BSA) ≥98%, Tween 20 0.06 mg/mL and 3,3′-Diaminobenzidine tetrahydrochloride (DAB) 10 mg/tablet from *Sigma-Aldrich*; Gill's Hematoxylin n.3 from *Bio-Optica*; the antibodies anti-human (mAbs) anti-Foxp3 (236A/E7, 0.5 mg/mL), Anti-Tbet (eBio4B10, 0.5 mg/mL) from ***eBioscience™****, Thermo Fisher Scientific*; Entellan® New, mounting medium from *VWR;* Cover glasses, from Marienfield.

For RNA extraction and Real-time Polymerase Chain Reaction (RT-PCR) were used: ***RecoverAll™*** Total Nucleic Acid Isolation Kit, Reverse Transcriptase SuperScript II, dNTP Set 100 mM Solutions, RNase OUT (40units/µL) Random Primers 9 A_{260} units 3 µg/µL, MicroAmp™ Fast Optical 96-Well Reaction Plate, prove MGAT5 TaqMan™ Hs00159136_m1, prove Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) TaqMan™ Hs02758991_g1, prove CD3E TaqMan™ Hs01062241_m1, prove T-box transcription factor (TBX21) TaqMan™ Hs00203436_m1, TaqMan™ Universal PCR Master Mix from *Thermo Fisher Scientific*; prove 18S ribosomal RNA (18S) Custom_Human (custom by P.Oliveira) from *Integrated DNA Technologies* (*IDT*); Chloroform ≥99.8% contains 0.5-1.0% ethanol as stabilizer, 2-Propanol ≥99.5%, for molecular biology, TRI reagent®, for RNA extraction from *Sigma-Aldrich*.

For cell culture were used: Culture medium *Roswell Park Memorial Institute* (RPMI) 1640 GlutaMAX™ medium, Fetal bovine serum (FBS), Penicillin-Streptomycin (Pen-Strep) 10,000 U/mL, Trypsin-EDTA 0.05%, Trypan Blue Solution 0.4% from *Thermo Fisher Scientific*; Geneticin (G418) 100 mg/ml from *InvivoGen*; 75 cm^3 cell culture flasks with filter cap (T75 flasks), and 25 cm^3 cell culture flasks with vent cap (T25 flasks), from Orange Scientific; Dimethyl sulfoxide (DMSO) cell culture grade from PanReac AppliChem.

For PBMCs isolation and monocyte-derived DCs differentiation were used: Lymphoprep™ from *Stemcell™ Technologies*, CD14 Microbeads Human, MACS cell separation from *Miltenyi Biotec*; recombinant proteins IL-4 and GM-CSF (*E. coli*) from

Peprotech; Lipopolysaccharides (LPS) from *Sigma-Aldrich*; 6 Flat Test Plate from *Orange Scientific.*

For cocultures were used: Anti-human CD3 1,0 mg/mL (OKT3), Anti-human CD28 1,0 mg/mL (CD28.2) from *eBioscience™, Thermo Fisher Scientific;* X-VIVO15^{TM} serum-free medium from *Lonza.* Brefeldin A 10 mg/mL, Phorbol 12-myristate 13-acetate (PMA), Ionomycin from *Sigma-Aldrich;* 96 U Test Plate from *Orange Scientific.*

For Flow cytometry staining were used: Foxp3/Transcription Factor Staining Buffer Set from eBioscience^{TM} Thermo Fisher Scientific; L-PHA- fluorescein isothiocyanate (FITC) from Vector Laboratories; the following anti-human (mAb):

***Table 1* | Monoclonal anti-human Antibodies used in flow cytometry analysis, the respective clones and conjugation fluorescence molecules, as well as their supplier**.

mAb	Clone	Conjugation	Supplier
FVD	-	APC-eFluor 780	eBioscience
CD45	HI30	Brilliant Violet 510	Biolegend
CD4	RPA-T4	eFluor450	eBioscience
Gata3	TWAJ	Alexa Fluor 488	eBioscience
Tbet	eBio4B10	PerCP-Cy5.5	eBioscience
RorγT	AKJS-9	APC	eBioscience
Foxp3	PCH101	PE	eBioscience
CD4	RPA-T4	PerCP-Cy5.5	Biolegend
IL-17A	eBio64DEC17	eFluor450	eBioscience
IFN-γ	4S.B3	APC	eBioscience
IL-10	JES3-9D7	PE-Cy7	eBioscience
TNF-α	MAb11	Alexa Fluor 488	eBioscience
HLA-DR	G46-6	PE-Cy7	BD Pharmingen
CD86	IT2.2	PE-Cy5	eBioscience
CD14	MEM-18	PE	Immunotools
CD11c	BU15	FITC	Immunotools

FITC: fluorescein isothiocyanate; **PE:** phycoerythrin; **APC:** allophycocyanin; **PerCP:** peridinin chlorophyll protein; **Cy5 or 7**: Cyanine 5 or cyanine 7.

For cytokine analyses were used two kits for Flow Cytometry analysis: BD™ Cytometric Bead Array Human Th1/Th2/Th17 Kit and BD™ Cytometric Bead Array Human Inflammatory Cytokine Kit from *BD Bioscience*; and two ELISAs: Human IL-23 DuoSet ELISA and Human TGF-beta 1 DuoSet ELISA from *R&D systems*.

2. Patients and samples

The tissues samples were collected in routinely medical exams or as part of patient's treatment, with ethical approved by the local research and ethics committee and all patients donating tissue had given full prior consent.

The biopsies of normal colon and the samples of colon high and low dysplasia were assigned by *Centro Hospitalar Universitário do Porto* (CHUP). The colon adenocarcinoma samples were assigned by *Instituto Português de Oncologia* (IPO) of Porto. In the follow up, the biopsies of normal, gastritis, atrophy/metaplasia lesions of stomach were assigned by CHUP and the gastric adenocarcinoma intestinal type by *Centro Hospitalar do São João* (CHSJ).

Together with blocks or slices, it is providing the hematoxylin/eosin slice for each sample with a delimitation and classification of colon carcinomas and adenomas, analyzed by a pathologist.

3. Lectin histochemistry and Immunohistochemistry

The glycan profile was evaluated by lectin histochemistry using biotinylated lectins *L-PHA* and *GNA* that recognize β1,6-GlcNAc branched and terminal α1,3 linked mannose structures, respectively (Figure 8).

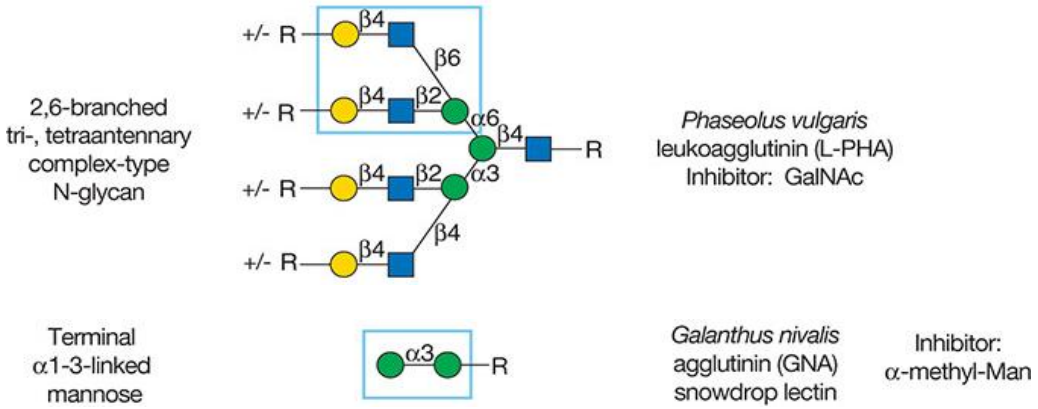

***Figure 8* | Lectin recognition structures**. Complex structure β1,6-GlcNAc branched recognized by L-PHA, and terminal α1,3 linked mannose recognized by GNA. Adapted from [34].

The 3 µm sections from formalin-fixed paraffin-embedded (FFPE) tissues from distinct stages of colon or gastric carcinogenesis were deparaffinized twice, using xylene for 10 min. The slides were hydrated in a series of ethanol solutions (100% twice and 70%, 5 min each) and water (10 min). To block endogenous peroxidase, H_2O_2 3% in methanol solution was used for 10 min followed by washing with Phosphate Buffer Saline (PBS,

pH=7,4) twice for 5 min each. Before staining, inespecific protein was blocked with BSA 10% in PBS for 30 min. The sections were incubated for 60 min with biotinylated lectin (L-PHA or GNA diluted to 13,3 µg/mL (1:150) in PBS) and washed with PBS Tween 20 0,05%. To provide the detection, the sections were incubated with ABC HRP solution (1:100) in PBS for 30 min followed by DAB substrate (0,67 mg/mL) activated with fresh H_2O_2 30%.

Foxp3 and Tbet immunohistochemistry was performed in the sequencial sections from same samples. The slices were deparaffinized and hydrated as before, and antigen retrieval was performed using vapor heat during 40 min (1M Citrate Buffer, pH=6) of. At this time, all steps before and after the staining were performed using solutions provided by *UltraVision Quanto Detection System HRP DAB* kit according to the manufacturer's recommended protocol. The sections were incubated with primary mAb anti-Foxp3 (1:75, 6,7 µg/mL) or anti-Tbet (1:100, 5 µg/mL) diluted in BSA 5% overnight at 4ºC for colon samples or 2 hours at room temperature for stomach samples. The sections were contrasted with hematoxylin, dehydrated and preserved with appropriated mounting medium and cover glasses.

The results were analyzed by 3 independent observers following the same classification. In case of lectin-histochemistry, a semi-quantitative scale was used: < 25%; 25-50%; 50-75%; >75% of staining, and for immunohistochemistry, the classification was qualitative for Tbet and Foxp3 staining: +/-; +; ++; +++.

4. Cell lines and cell cultures

The *in vitro* studies were performed in the MKN45 gastric cancer cell line stably transfected with empty vector (MKN45 Mock) or with MGAT5 cDNA (MKN45 T5). These cells were cultured in RPMI medium supplemented with 10% FBS, Pen-strep (100 units/ml), under the selection pressure of G418 (500 µg/ml) at 37ºC in 5% CO_2. To subculture cells, the cell monolayer was washed with sterile PBS and 3 mL of Trypsin-EDTA were added for T75 flask (1 mL for T25 flask) during 5-10 min at 37ºC in 5% CO_2. Trypsin action was inactivated with the double volume of RPMI supplemented with FBS. The suspension was transferred to falcons and centrifuged at 1200 rpm for 5 min, the supernatant was discarded, and fresh culture medium was added. In general, ~1/3 of cells were dispensed into new culture flasks. To preserve cells, these were resuspended in 3 mL freezing medium (90% FBS + 10% DMSO), divided 1 mL cells per vial and preserved at -80ºC. If needed, cells were counted with Trypan blue solution before new procedures.

5. RNA Extraction and Real-Time PCR

Using the same samples of the histochemistry and immunohistochemistry, the RNA was extracted with *RecoverAll*™ Total Nucleic Acid Isolation Kit from FFEP 10 µm sections according to the manufacturer's recommended protocol. In the case of colon carcinomas and adenomas, only the selected and identified lesion tissue was processed. The RNA was extracted by FFEP tissue of 5 cases of normal colon, 7 cases of low grade dysplasia (LGD), 6 cases of high grade dysplasia (HGD) and 12 of colon carcinoma, 9 of them were stage III of disease.

The RNA of MKN45 cell pellets, from 80% confluent cell T75 flasks, were extracted with TRI reagent according to the manufacturer's recommended protocol.

The total RNA was quantified in *Nanodrop* system and the cDNA synthesis was performed using Reverse transcriptase (150 units), RNase OUT (8 units), Random Primers (0,1µg/µL) and dNTP (1mM) during 1 hour at 37ºC. The Real-Time PCR were performed in 96-well reaction plates and cDNA was amplified using the respective TaqMan proves to quantify MGAT5, GAPDH,18S TBX21 and CD3. Amplification data were acquired with 7500 Software v2.3 using the following method: Holding stage 50,0ºC for 20 sec and 95,0ºC for 10 min; Cycling stage 95,0ºC for 15 sec and 60,0ºC for 1 min during 40 cycles.

The mRNA expression of the genes of interest was normalized using the internal control GADPH and 18S mRNA levels (ΔCt). In clinical samples, RQ values were calculated by the equation $2^{-(\Delta Ct)}$ to compare gene expression in the normal tissue with their expression in pre-malignant tissue or in cancer. In the case of cell lines, RQ values were calculated by the equation $2^{-(\Delta\Delta Ct)}$, where ΔΔCT represents the relative expression of ΔCT of the gene in comparison with MKN45 Mock.

6. PBMCs isolation and Monocytes-derived DCs Differentiation

The PBMCs were isolated from peripheral blood diluted 1:2 with PBS. To isolate PBMCs, 1 volume of *Lymphoprep*™ was used for 2 of diluted whole blood, which was carefully pipetted on the top of the density gradient medium and was centrifuged for 30 min at 800 g with brake off. After this step, the separation of the different layers is achieved as represented in *Figure 9* and buffy coat interphase, that contain PBMCs, was isolated by aspiration. The cells were washed twice with PBS, and resuspended in the appropriate medium.

The CD14 positive monocytes were purified from PBMCs with CD14 MACS Microbeads Human using magnetic separation with MS columns according to manufacturer's instructions.

The enriched $CD14^+$ cells were cultured in 6-well flat plates with 3mL of complete RPMI at a concentration of $1x10^6$ cells/mL and supplemented with 50 ng/ml of recombinant proteins IL-4 and GM-CSF to drive differentiation into monocyte-derived DCs (moDCs). On the 3rd day of culture, the medium was replaced and the cells were in cultured for 3 more days, in a total of 6 days. At that time, monocytes were differentiated into immature moDCs and in half the wells 100 ng/mL of LPS were added to the medium to mature DCs. 48 hours later, the moDCs were maturated and were washed with PBS twice before being resuspended in X-VIVO 15™ serum-free medium for posterior co-cultures with cancer cells.

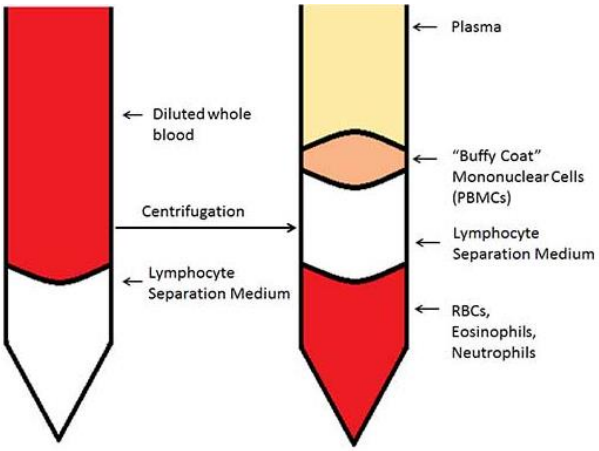

***Figure 9* | Schematic representation of blood separation with Lymphoprep™.** The separation of the different layers occurs after density gradient centrifugation, represented on the right, showing the mononuclear cell layer which was recovered by aspiration.

7. Co-cultures

Initially, PBMCs were co-cultured with MKN45 Mock or MKN45 T5 cells during different time points, from 18 h to a maximum of 96 h. The results indicated that brief time cultures were the best to analyze T lymphocyte cell differentiation and cytokine release. Hereupon, the sequent experiments were performed 18 h after co-culture.

The co-cultures were done in 96-well U plates, that were coated with mAb anti-CD3 (0,1 µg/well) 2 hours before. The wells were washed twice with PBS and $1x10^5$ PBMCs were co-cultured with $1x10^5$ MKN45 Mock or MKN45 T5 cells in complete RPMI medium supplemented with G418 and mAb anti-CD28 (0,1 µg/well; 0,5 µg/mL) to induce T cell activation. At the selected time point, the supernatants were collected for cytokine analysis and the cells were labeled to allow CD4 T lymphocytes transcription factors

analysis by flow cytometry. Three hours before the selected time point, Brefeldin A (10 ng/mL) was added to the wells designated for intracellular cytokines expression analysis by flow cytometry. Single cultures of PBMCs, MKN45 Mock or MKN45 T5 cells were used as controls. In last time points, it was necessary added 20 ng/mL PMA and 200 ng/mL ionomycin concomitant to brefeldin A.

Similar to PBMC co-cultures, $1x10^5$ immature or mature moDCs were co-cultured with $1x10^5$ MKN45 Mock or MKN45 T5 in X-VIVO 15 medium supplemented with G418. After 18 h, the supernatants were collected for cytokine analysis and the moDCs were labeled to analyze their differentiation and activation by flow cytometry. Single cultures of moDCs, MKN45 Mock or MKN45 T5 cells were used as controls.

8. Flow Cytometry

To evaluate the expression of transcription factors in CD4 T cells, cells from co-cultures and controls were collected and washed with PBS, followed by dead cell staining with Fixable viability dye (FVD) (1:1000). Cells were surface stained against CD4 and CD45 (mAb anti-CD4-eFluor450 (1:100) and anti-CD45 (1:400)) before being washed, fixed for 30 min and permeabilized for 10 min, using the solutions from Foxp3/Transcription Factor Staining Buffer Set according to the manufacturer's instructions. After blocking with 2% mouse serum, the staining of the transcription factors Gata3 (1:100), Tbet (1:300) RorγT (1:200) and Foxp3 (1:100) was performed.

Intracellular cytokine analysis was done upon Golgi blockade with Brefeldin A. Cells were collected, washed and stained with FVD, followed by CD45 (1:400) and CD4-PercP-Cy5.5 (1:200) staining. Cells were washed and fixed with formaldehyde 2%. Permeabilization was done with FACS buffer containing 0,1% saponin, and staining of IL-17A (1:100), IFN-γ (1:200), IL-10 (1:200), and TNF-α (1:100) was performed.

The procedure used to analyze moDCs phenotype upon co-culture with cancer cells was similar to PBMCs staining. Cells were collected, washed and dead cells stained with FVD (1:1000). Cells were stained with mAb anti- CD45 (1:400), HLA-DR (1:100), CD86 (1:100), CD11c (1:25) and CD14 (1:25) after blocking with 2% mouse serum.

Cellsells were washed and resuspended in FACS buffer for analysis by flow cytometry. Lymphocytes and single cells were selected by light scatter characteristics, and cells negative for FVD, were considered alive. Next, only cells $CD45^+$ were selected to exclude tumor cells from analysis and CD4 cells were gated within $CD45^+CD4^+$ cells. moDCs phenotype was analyzed following an approach similar to that of PBMCs. Cells were gated based on their light scatter characteristics and bright CD45 expression.

$CD11c^{+}CD14^{-}$ cells were selected and the surface expression of the activation/maturation markers CD86 and HLA-DR (MHC class II) was analyzed .

Data acquisition was performed in a FACS Canto v.2 flow cytometer (BDBioscience), using the FACSDiva software (BDBioscience). The collected data files were analyzed with FlowJo software version vX.10.0.

Moreover, the tumor cells MKN45 Mock and MKN45 T5 were stained with L-PHA (1:1000) to check overexpression of branched N-glycans on the surface of the cells by cytometry prior and after each experiment.

All results were expressed as the Mean Fluorescence Intensity (MFI) due to antigen staining or L-PHA staining.

9. Cytokine secreted analysis

The cytokines present in culture supernatants were analyzed by the BD™ Cytometric Bead Array Human Th1/Th2/Th17 Kit, in the case of PBMCs co-cultures, and by the BD™ Cytometric Bead Array Human Inflammatory Cytokine, in the case of moDCs co-cultures, according to manufacturer's recommended protocols. This analysis was achieved in collaboration with the Hematology Department from CHUP.

To complement the DCs cytokine analysis, IL-23 and TGF-β quantification was done by ELISA according to the manufacturer's recommendations.

10. Statistical analysis

RQ values were used to compare transcription rates and the statistical significance of results was determined by unpaired or pared Student t-test. MFI and cytokine concentrations ratio with MKN45 Mock co-cultures were used and statistical significance of results was determined by unpaired Student t-test. When suitable, ANOVA analysis was also performed. All data were analyzed using the GraphPad Prism 4 Software (GraphPad Software). Results were considered statistically significant with P values of less than 0.05.

Results

1. Colorectal carcinogenesis

1.1. Increased expression of branched N-glycans along colorectal carcinogenesis

The results of lectin histochemistry in colorectal carcinogenesis were analyzed by 3 independent observers and showed an increase of β1,6-GlcNAc branched N-Glycans expression along progression to malignancy that was accompanied in the carcinoma stage by a decreased expression of high-mannose glycans. Interestingly, a higher expression of high-mannose N-linked glycans was observed on the pre-malignant condition, dysplasia (low and high).

In normal colon, low expression of branched glycans was observed, that was accompanied by a high expression of mannosylated glycans. As expected, in colon carcinoma a higher expression of β1,6-GlcNAc branched N-linked Glycans was observed when compared to normal colon. However, L-PHA binding in large adenoma is as high as in carcinoma (Figure 10A). On the other hand, the high-mannose N-linked glycans were more extensively expressed in adenomas compared with normal colon and colon carcinoma. In colon carcinoma, the expression of these glycans decrease (Figure 10A).

In general, branched N-glycans appear to be more expressed along carcinogenesis, while high-mannose structures are frequently observed in inflammatory infiltrate, as well as in normal and premalignant conditions.

This data clearly shows an alteration in the glycans profile along colorectal carcinogenesis.

1.2. Effector Th1 cell frequency was associated with high-mannose glycans expression, while regulatory T cell frequency accompanied the branched N-glycans profile

Our results point toward that alterations in glycan expression may somehow influence the frequency of different T cells present in the tissue milieu, regulating thereby tumour immune response. The *Figure 10B*, represent the expression profile of glycans, characterized by lectins, and effector Th1 and regulatory T cells, characterized by the expression of the transcription factors Tbet and Foxp3. Normal colon was classified with an expression of branched structures <25% and an expression of high-mannose structures ≥ 50%, while the frequency of Tbet and Foxp3 was balanced. With the

appearance of adenoma, the branched structures increased, as well as high-mannose structures, whereas in large adenoma, both are expressed in more than 75% of the tissue. This glycan profile was accompanied by increased frequencies of Foxp3- and Tbet-expressing cells. In colon carcinoma, the decrease of high-mannose N-glycans expression was accompanied by an increase in β1,6 branched N-glycans as well as with an high frequency of Foxp3-expressing cells (Figure 10B). On the opposite, at this stage of the disease, the frequency of Tbet-expressing cells was clearly reduced.

The similarity between the kinetics of branched N-glycans and frequency of Foxp3-expressing cells, as well as between high-mannose glycans and Tbet-expressing cells suggests a potential relationship between these glycans expression and the immune microenvironment. Altogether, this data shows that differentiation and recruitment of adaptive immune cells, such as T cells, may be dependent on cell surface's glycans expression.

To validate these preliminary observations, we performed a RT-PCR to MGAT5 mRNA, that encodes GnT-V, the enzyme that catalyze the addition of β1,6-GlcNAc branched glycans. The results showed a clear tendency of increased expression of MGAT5 transcription, from normal colon to HGD, suffering a slight decrease in colon carcinoma. When considering only colon carcinoma of stage III, the expression is not altered, thought the relative expression of MGAT5 slightly increased when compared with all general colon carcinomas (Figure 10C).

Preliminary evidences in one case of normal colon and colon carcinoma stage III, showed an interesting increase of CD3 mRNA in carcinoma, consistent with increased T cell infiltration in these lesions. However, when TBX21 mRNA (encoding Tbet) expression was normalized to CD3 mRNA, a decrease of TBX21 expression, normalized to CD3 expression, was observed when compared to normal colon, supporting a decreased frequency of effector Th1-type cells in cancer compared with normal contexts (Figure 10D).

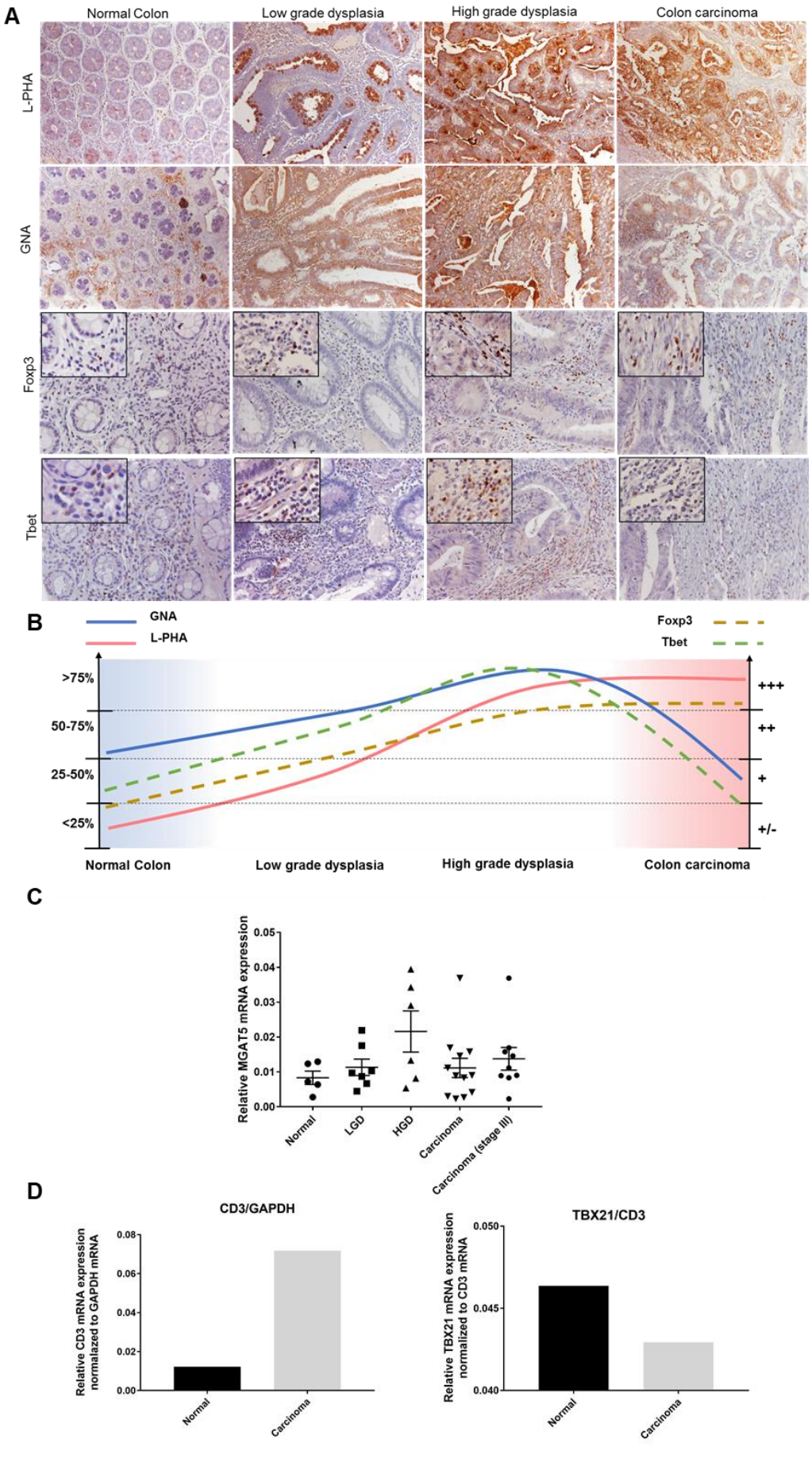
A
Normal Colon
Low grade dysplasia
High grade dysplasia
Colon carcinoma
L-PHA
GNA
Foxp3
Tbet
B
GNA
L-PHA
Foxp3
Tbet
>75%
50-75%
25-50%
<25%
+++
++
+
+/-
Normal Colon
Low grade dysplasia
High grade dysplasia
Colon carcinoma
C
Relative MGAT5 mRNA expression
0.05
0.04
0.03
0.02
0.01
0.00
Normal
LGD
HGD
Carcinoma
Carcinoma (stage III)
D
CD3/GAPDH
Relative CD3 mRNA expression normalized to GAPDH mRNA
0.08
0.06
0.04
0.02
0.00
Normal
Carcinoma
TBX21/CD3
Relative TBX21 mRNA expression normalized to CD3 mRNA
0.050
0.045
0.040
Normal
Carcinoma

Figure 10* | *Colorectal carcinogenesis: alterations in glycans profile and evaluation of T lymphocytes differential expression. (A) L-PHA histochemistry detecting the β1,6GlcNAc-branched N-glycans showed an increase of branched-structures during development of malignancy accompanied by increase of Foxp3 frequency, representing Treg cell differentiation/recruitment. On the contrary, Tbet frequency, which is expressed mainly by Th1-type cells, was high in dysplasia lesions showing a kinetic profile similar to that of high-mannose N-glycans expression, detected by GNA. **(B)** Schematic representation of histochemistry staining, that translate the profile of glycans and T cells present in the tissue microenvironment showed in A. The characterization of lectin histochemistry follows the Y axis on the left, with a semi-quantitative classification, and immunohistochemistry for Tbet and Foxp3 follows a qualitative classification represented in Y axis on the right. The similarity between glycan expression profile and frequency of specific T cell types present was evident. An increase of high-mannose N-glycans denoted high Th1-type cell recruitment and branched N-glycans were better related with Treg frequency. **(C)** Relative MGAT5 mRNA expression in colon carcinogenesis. We can see an increase of MGAT5 expression from normal to HGD, but a decrease in colon carcinoma for levels like LGD. However, when only advanced carcinomas, in stage III, were selected, the scenario did not change considerably, though de mean of MGAT5 expression slightly increased. The MGAT5 was normalized by the mean of 2 house keepings, 18S and GAPDH, for all cases. The results were submitted to ANOVA test and t-test, but without statistical significance in any analysis performed(significant P≤0,05). **(D)** Relative CD3 mRNA expression in normal colon compared to carcinoma. An increase of CD3 expression, which represent T lymphocytes on colon carcinoma was observed, however a decreased expression of TBX21 (Th1-type response) was observed when normalized to CD3 mRNA expression, suggesting a decreased frequency of Th1-type cells within the T cell population in carcinoma compared with normal colon.

2. Gastric carcinogenesis

2.1. Increase of branched N-glycans expression with malignant transformation

In gastric carcinogenesis, the glycan profile was similar to the profile found in colon carcinogenesis. In normal stomach, the expression of high-mannose N-glycans was high, whereas the staining of L-PHA was very weak or negative, even in gastritis, where mannose expression keeps high. The β1,6-GlcNAc branched N-glycans start increasing in atrophy or metaplasia precancerous conditions. At this stage, high-mannose N-glycans were also increased, keeping the expression levels in gastric carcinoma, and branched N-glycans expression increased gradually in atrophy or metaplasia to gastric carcinoma (Figure 11A).

The abnormal glycans, such as branched N-glycans, in the stomach, begin to appear with the emergence of premalignant conditions, such as atrophic intestinal metaplasia, displaying an higher expression in gastric carcinoma. On the other hand, the origin of this carcinogenesis cascade is predominantly inflammatory, and thereby associated with an high expression of high-mannose N-glycans, as demonstrated by increased staining with GNA.

2.2. Both effector Th1-type and regulatory T cells exhibit a peak in gastritis, with Foxp3-expressing cells prevailing in gastric cancer, concomitantly with a decrease of Tbet-expressing cells.

In normal tissue, Tbet staining was rare, but a little more frequent than that of Foxp3. In an inflammatory context, such as gastritis, both Th1- and Treg-associated transcription factors expression were high, with frequency of Tbet-expressing cells decreasing drastically in gastric carcinoma. Treg frequency decreased in atrophy/metaplasia and was maintained at similar levels in gastric carcinoma (Figure 11A).

The schematic *Figure 11B*, that summarizes the results, show that L-PHA staining was close to negative (<25%) in normal stomach and progressively increase. It was kept <25% in gastritis and increased up to 25-50% in intestinal atrophy/metaplasia, being highly increased in gastric carcinoma (>75%). GNA staining started high in normal stomach, close to 75% of tissue stained, keeping high in gastritis and increasing in metaplasia and gastric carcinoma to more than 75%. Like previously described, Tbet- and Foxp3-expressing cells had a peak of frequency in gastritis, as expected due to high inflammatory infiltrates, and both Th1- and Treg-associated transcription factors expression decreased in metaplasia, though frequency of Tbet-expressing cell decreased to levels lower than those observed for Foxp3 in gastric carcinoma (Figure 11B).

Therefore, an inverse relationship between Th1 differentiation/recruitment and β1,6-GlcNAc-branched N-linked glycans is suggested.

Overall, these results show that alterations in the glycan profile may be involved in altered differentiation and/or recruitment of different T cells subsets. In colon, the increase of β1,6-GlcNAc branched N-glycans was accompanied by increased Treg recruitment, and likewise in gastric carcinogenesis, the increase of this abnormal glycans were associated with a decline of Th1-type cell differentiation/recruitment.

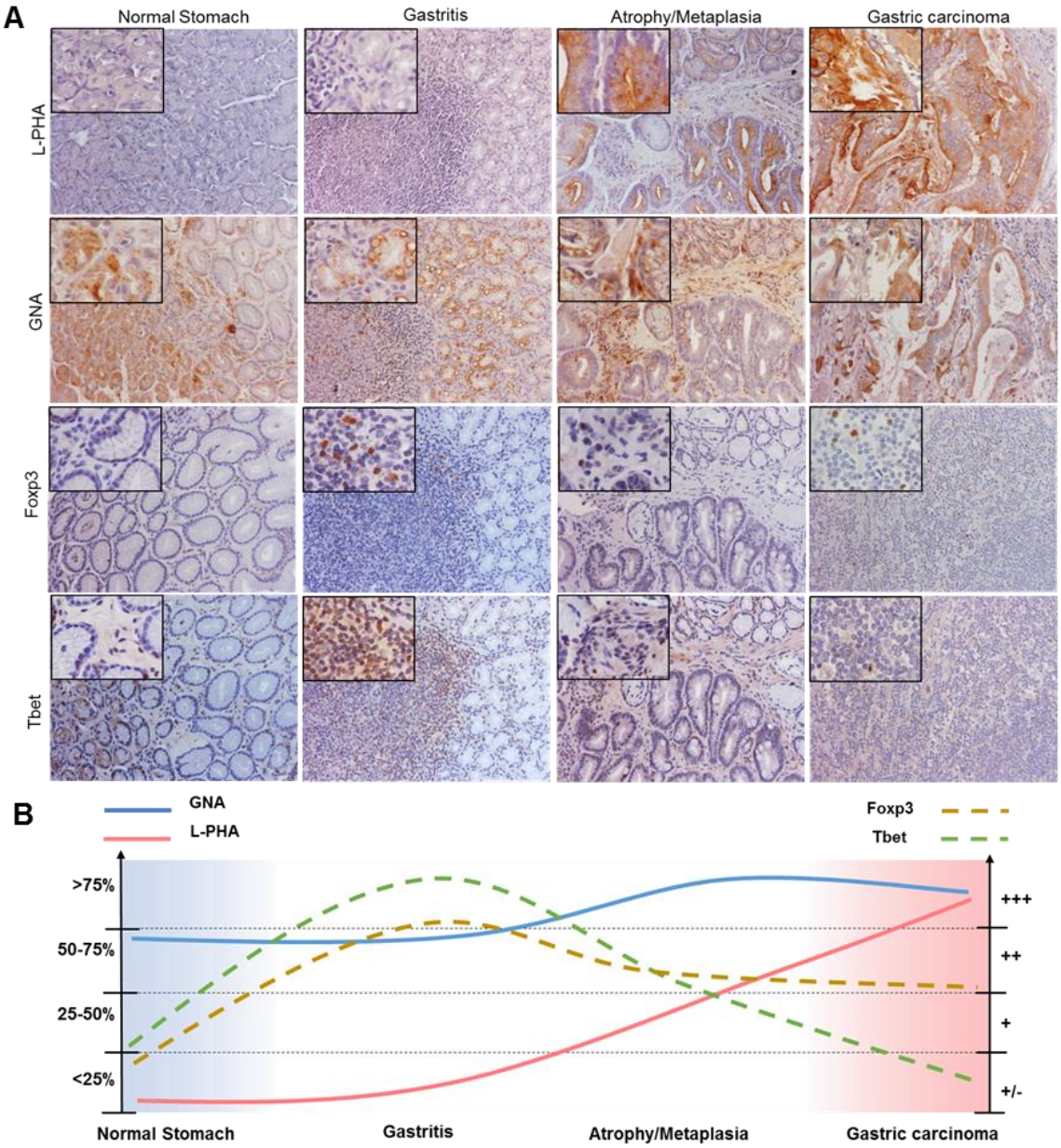

***Figure 11* | Gastric carcinogenesis: alterations in glycan profile and evaluation of T lymphocytes differential expression. (A)** L-PHA histochemistry detecting the β1,6-GlcNAc-branched N-glycans showed an increase of branched structures during development of malignancy accompanied by decreased Tbet-expressing cells frequency, representing Th1-type cells differentiation/recruitment. Foxp3 and Tbet expression peaked in gastritis, but in gastric carcinoma Treg cells kept present, while Th1-type cells were mostly absent. A high staining of GNA, that translate high-mannose glycans, was observed in all carcinogenesis, with an increment in transformed atrophic and metaplastic tissue and gastric carcinoma. **(B)** Schematic representation of histochemistry staining, that translate the profile of glycans and T cells present in the tissue microenvironment showed in A. The characterization of lectin histochemistry follows the Y axis on the left, with a semi-quantitative classification, and immunohistochemistry for Tbet and Foxp3 follows a qualitative classification represented in Y axis on the right. In the case of gastric carcinogenesis, an negative association is seen between branched N-glycans profile and Th1-type immune cell presence.

3. Functional impact of tumor-associated glycans on immune responses

3.1. Overexpression of β1,6-GlcNAc branched N-linked glycans in gastric cancer cells affects $CD4^+$ T cell response

The MKN45 gastric cancer cell line stably transfected with MGAT5 (MKN45 T5) and transfected with an empty vector (MKN45 Mock) were used to evaluate the impact of the overexpression of β1,6-GlcNAc branched N-linked glycans on *in vitro* immune response. These cell lines display morphological differences, in which MKN45 T5 are more fibroblastoid and disperse, as well as phenotypically more invasive, but no differences wee observed in proliferation as previously described [28, 56] (Figure 12A). The levels of MGAT5 expression were confirmed by RT-PCR in various serial passages of the cultures and a significantly higher expression of MGAT5 was consistently observed in MKN45 T5 when compared to MKN45 Mock (Figure 12B). The overexpression of β1,6-GlcNAc branched N-linked glycans was confirmed by flow cytometry, using the L-PHA lectin conjugated with FITC. As expected, Mock cells had a MFI, due to L-PHA binding, lower than T5 cells (Figure 12C).

Using these cell lines as a model of cancer cells that exhibit distinct branched glycans levels, we performed co-cultures of these tumor cells with PBMCs from healthy donors and evaluated the signature of T cell transcription factors by flow cytometry (Figure 13A and B). A kinetic analysis was performed and the time point that displayed the major differences in T cell differentiation and cytokine production between the co-culture conditions -18h (short-time cultures) - was chosen for further studies (Figure 13C).

The expression of the transcription factors Tbet, Gata3, Roryt, and Foxp3 was assessed to evaluate Th1, Th2, Th17 and Treg cells, respectively, among $CD4^+$ cells from co-cultures of PBMCs with MKN45 T5 and MKN45 Mock. The MFI values of each transcription factor within $CD4^+$ cells in co-cultures of PBMCs and MKN45 T5 were normalized to the correspondent ones in the co-cultures of PBMCs and MKN45 Mock (Figure 13D). $CD4^+$ cells from co-cultures of PBMCs and MKN45 T5 showed decreased expression of all transcription factors when compared with $CD4^+$ from PBMCs co-cultures with MKN45 Mock cells, being Tbet and Foxp3 expression significantly lower. However, when Th1:Th2 ratio in MKN45 Mock co-cultures were compared with T5 co-cultures, no significant differences were observed (Figure 13E).

This data shows that overexpression of branched N-linked glycans impact in transcription factors expression, with a significant effect on Th1 differentiation, through a decrease in Tbet expression. The expression of Foxp3 also slightly decreased.

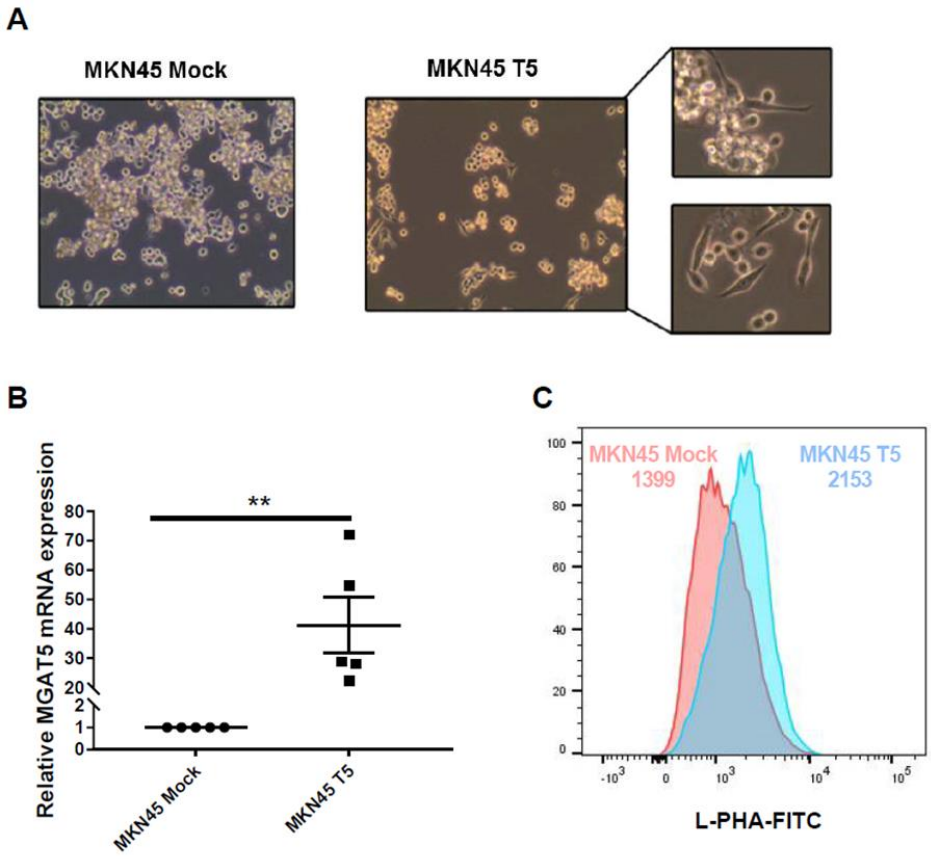

***Figure 12* | MGAT5 and β1,6-GlcNAc branched glycans expression. (A)** MKN45 Mock and T5 cells in culture, showing that MKN45 T5 cells were morphologically different, with fibroblastoid appearance. **(B)** Relative MGAT5 mRNA expression evaluated by RT-PCR, using mRNA extracted from MKN45 Mock and T5 cells, normalized to GAPDH or 18S. The relative mRNA expression of T5 cells was also normalized against the MGAT5 expression of Mock cells. The expression of MGAT5 was significantly higher in MKN45 T5 cells compared to the correspondent Mock cells (nearly 50 times more expressed in T5 than in Mock cells), in sequentially passaged cell cultures. **(C)** β1,6-GlcNAc branched N-glycans abundance on the surface of Mock and T5 cells detected upon incubation with L-PHA-FITC by flow cytometry. A positive shift in L-PHA-FITC fluorescence was observed in MKN45 T5 cells. Numbers inside the histogram overlay correspond to the mean fluorescence intensity (MFI) due to L-PHA-FITC staining. All data was submitted to paired t-tests. P≤0,01 (**).

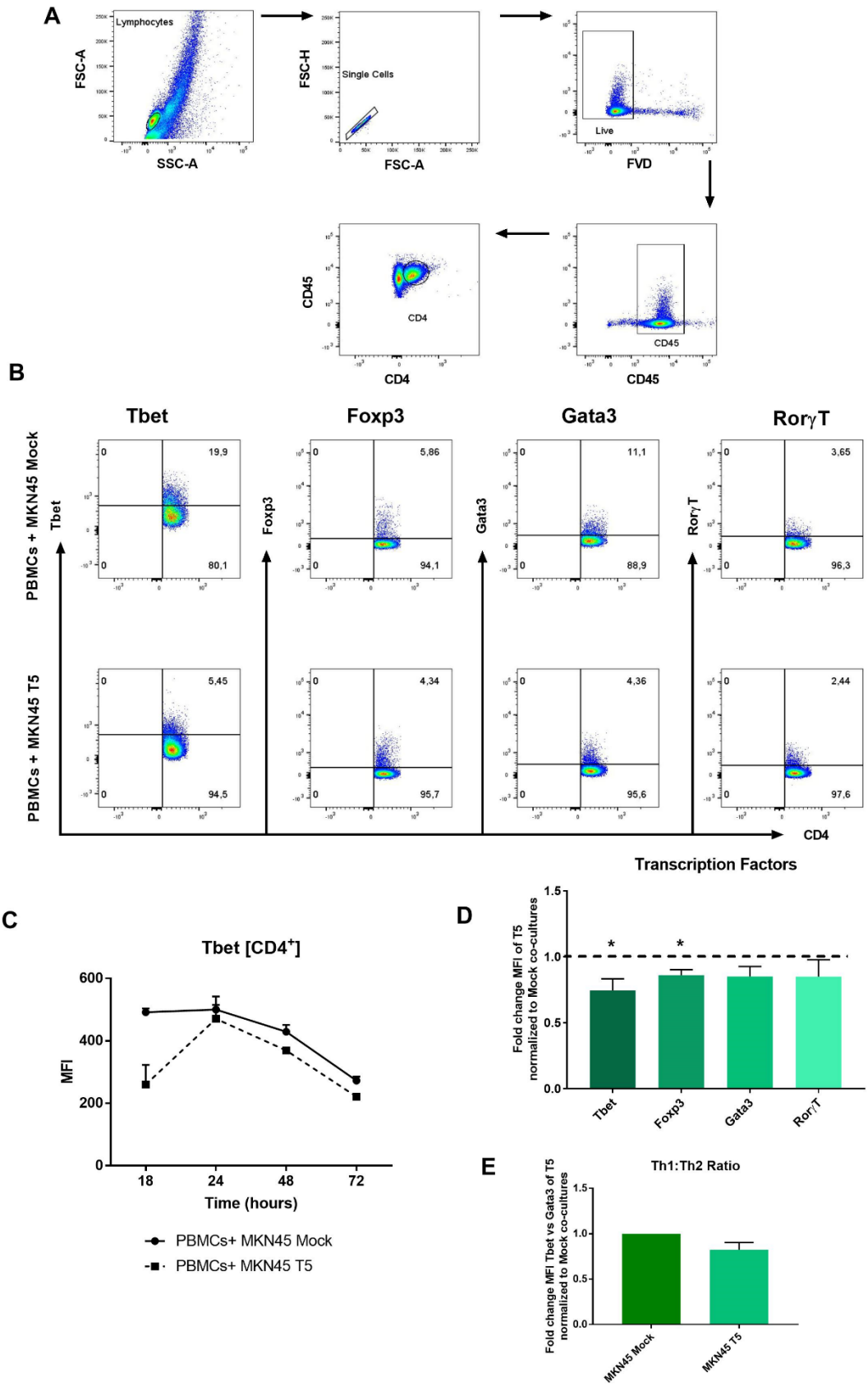
A
Lymphocytes
FSC-A
SSC-A
Single Cells
FSC-H
FSC-A
Live
FVD
CD45
CD4
CD4
CD45
B
Tbet
Foxp3
Gata3
RorγT
PBMCs + MKN45 Mock
PBMCs + MKN45 T5
19,9
80,1
5,86
94,1
11,1
88,9
3,65
96,3
5,45
94,5
4,34
95,7
4,36
95,6
2,44
97,6
CD4
Transcription Factors
C
Tbet [CD4+]
MFI
Time (hours)
PBMCs+ MKN45 Mock
PBMCs+ MKN45 T5
D
Fold change MFI of T5 normalized to Mock co-cultures
Tbet
Foxp3
Gata3
RorγT
E
Th1:Th2 Ratio
Fold change MFI Tbet vs Gata3 of T5 normalized to Mock co-cultures
MKN45 Mock
MKN45 T5

***Figure 13* | Transcription factors expression in PBMCs co-cultured with MKN45 Mock or T5 cell lines. (A)** Representative example of the gating strategy used to define CD4 T lymphocytes and evaluate transcription factors expression and cytokine production by flow cytometry analysis. Lymphocytes and single cells were selected based on their light scatter characteristics, and alive cells were selected by non-incorporation of the fixable viability dye (FVD). $CD45^+$ cells were selected to exclude tumor cells. **(B)** Representative examples of dotplots showing Tbet, Foxp3, Gata3 and RorγT expression within gated $CD4^+$ cells, selected from PBMCs co-cultured with MKN45 Mock and MKN45 T5 cells for 18 h. Numbers in the graphs correspond to the percentage of cells in each quadrant. **(C)** Tbet expression (MFI) in gated CD4 T cells at different time points upon co-culture of PBMCs and MKN45 Mock or MKN45 T5 cells, n=3. **(D)** Fold change in the described transcription factors MFI in $CD4^+$ cells gated within PBMCs co-cultured with MKN45 T5 cells. The MFI of $CD4^+$ cells from PBMC cultures with T5 cells were normalized to the ones of CD4 from PBMCs cultured with Mock cells. The dashed line of y=1 represents the MFI fold change of Mock when normalized with itself. The normalized expression of Tbet and Foxp3 was significantly lower in CD4 lymphocytes cultured with T5 cells than in CD4 lymphocytes cultured with Mock cells. **(E)** Th1:Th2 ratio calculated as the ratio of the MFI of Tbet and Gata3 in gated CD4 lymphocytes from PBMC co-cultures with MKN45 Mock or T5, normalized to the Mock co-cultures. Bars represent mean + S.E.M. of pooled results from four independent experiments (n=4), each with 2 to 3 technical replicates. Data were submitted to unpaired t-test. P≤0,05 (*).

3.2. Overexpression of β1,6-GlcNAc branched N-linked glycans influences cytokine production by PBMCs and $CD4^+$ lymphocytes

The production of IFN-γ, TNF-α, IL-17A and IL-10 cytokines by $CD4^+$ lymphocytes from co-cultures of PBMCs with MKN45 T5 or Mock was analyzed by intracellular flow cytometry. Cytokines released to the co-culture medium were quantified by using a Cytometric Bead Array Human Th1/Th2/Th17 Kit (by BD Biosciences), which analyzed simultaneously several cytokines.

$CD4^+$ lymphocytes from PBMC co-cultures with MKN45 T5 had, in general, lower percentage of cytokine-producing cells than $CD4^+$ lymphocytes from Mock co-cultures. Interestingly, the percentage of $CD45^+$ cells, not expressing the CD4 co-receptor, from co-cultures with MKN45 T5 cells that produced IFN-γ and TNF-α was also lower than the one observed in MKN45 Mock co-cultures (Figure 14A and B). Moreover, the percentage of $CD4^+$ T cells producing simultaneously IFN-γ and TNF-α was lower in MKN45 T5 co-cultures than in Mock co-cultures (Figure 14C). Cytokine MFI of $CD45^+$ cells or $CD4^+$ lymphocytes from MKN45 T5 co-cultures were normalized to MFI values by their counterparts in Mock co-cultures (Figure 14D). The cytokine profiles in the $CD45^+$ population and in the $CD4^+$ cells were very similar in both populations, being IFN-γ and IL-17A expression significantly decreased in MKN45 T5 co-cultures compared to Mock co-cultures (Figure 14D). The results of cytokines production within the CD45+ population indicate that the overexpression of branched glycans impacts on the production of those pro-inflammatory cytokines by $CD4^+$ lymphocytes. Suitable candidates worth exploring

would be the $CD8^+$ T cells, which are known to be important in the context of cancer immunosurveillance.

When levels of cytokine present in supernatants were normalized to Mock co-cultures, there was a significant decrease in the levels of secretion of IFN-γ, TNF, IL-10 and IL-4. The accumulated concentration of IFN-γ in co-culture supernatants was measured at different time points and the highest difference between culture conditions was observed 18 h after culture. Curiously, these differences were not maintained over time (Figure 14E). The highest decrease in normalized cytokine concentration was observed for IFN-γ in MKN45 T5 co-cultures, when compared to MKN45 Mock co-cultures, declining to half of the concentration in the 18 h co-culture supernatants (Figure 14F), suggesting a remarkable effect of branched glycans overexpression in the suppression of pro-inflammatory responses.

Altogether, these results suggest that overexpression of β1,6-GlcNAc branched N-linked glycans in cancer cell lines affect T-cell differentiation and cytokine production, demonstrating a significant impact on the suppression of effector T cell differentiation and cytokine release.

3.3. Increase of branched N-glycans impact on cytokine production by moDCs

In order to further clarify how the immune system recognizes these glycan alterations, $CD14^+$ monocytes isolated from PBMCs were differentiated for 6 days into moDCs followed by LPS stimulus for DC maturation. The Figure 15A shows the appearance of DCs before and after LPS addition, immature DCs (iDCs) and mature DCs (DCs LPS), respectively.

iDCs and DCs that were previously in contact with LPS were co-cultured with MKN45 T5 and MKN45 Mock cells during 18 h. They were then stained against the activation/maturation markers HLA-DR and CD86. DCs were gated based on CD45, and CD11c expression and excluding $CD14^+$ cells (Figure 15B). MFI of HLA-DR and CD86 were analyzed for each condition on the surface of $CD45^+CD11c^+CD14^-$ cells. Both Mock and T5 cells led to an increase in DC activation markers but no differences could be observed between DCs incubated with MKN45 T5 or Mock cells (Figure 15C).

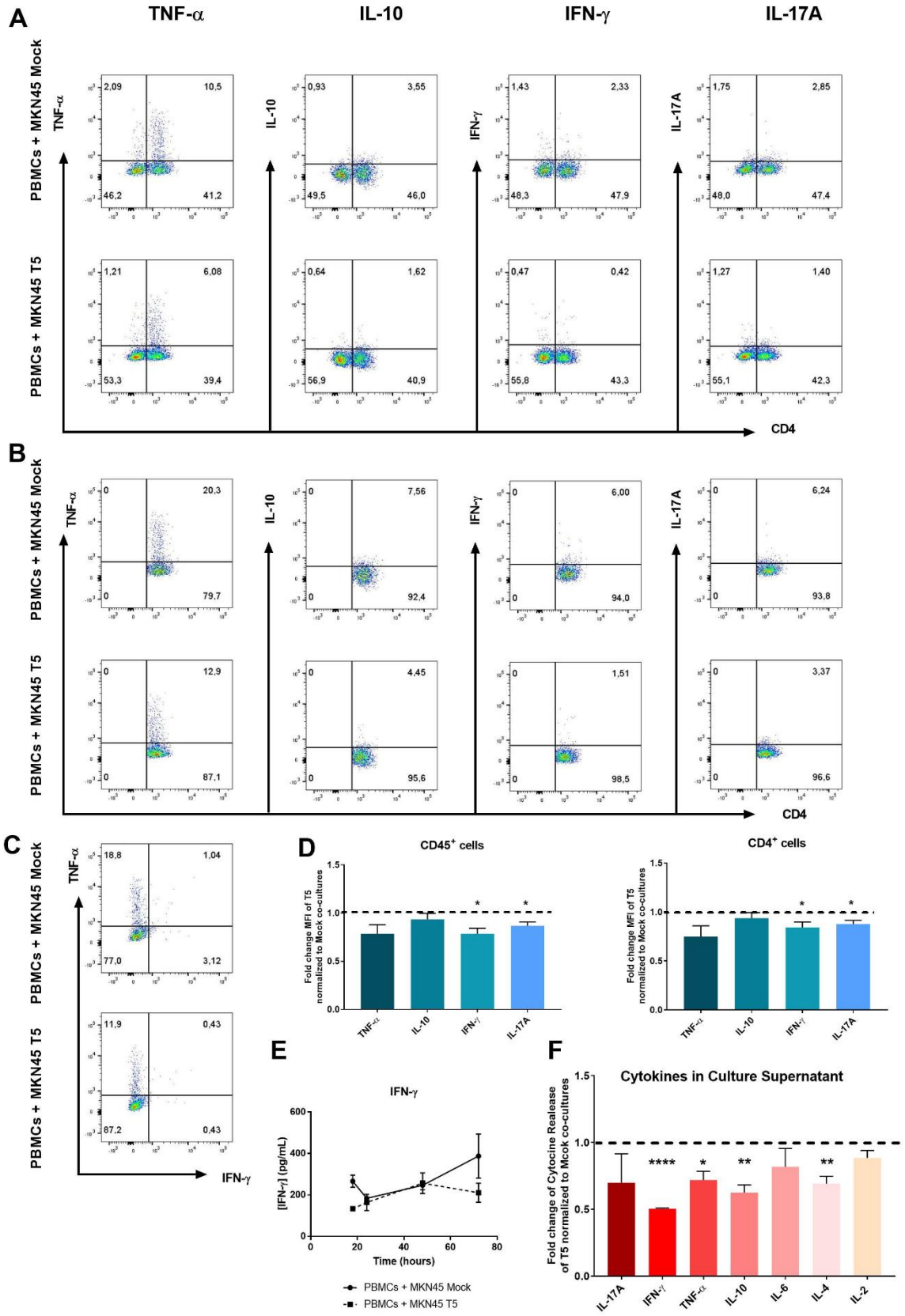

Figure 14 | **Cytokine expression in co-cultures of MKN45 Mock or T5 cell lines with PBMCs.** Intracellular TNF-α, IL-10, IFN-γ and IL-17A cytokine production **(A)** by $CD45^+$ cells from co-cultures with MKN45 Mock and MKN45 T5 cells and. **(B)** by $CD4^+$ T cells gated from the PBMC co-cultures with MKN45 Mock and MKN45 T5 cells. Numbers inside graphs

correspond to cell percentages in each quadrant. **(C)** Dot plots showing the expression of IFN-γ and TNF-α, within the $CD4^+$ population, in co-cultures of PBMCs with MKN45 Mock or MKN45 T5, evidencing single and double IFN-γ and TNF-α producing cells. Numbers inside the graphs correspond to the percentage of cells in each quadrant. **(D)** Fold change of the MFI for each cytokine in the $CD45^+$ population and in the $CD45^+CD4^+$ population of PBMCs co-cultured with T5 cells, normalized to Mock cells. The dashed line at y=1 represents normalized values in PBMCs and Mock co-cultures. **(E)** Concentration of IFN-γ in culture supernatants at different time points upon co-culture of PBMCs and MKN45 Mock or MKN45 T5 cells (n=1 experiment with 3 technical replicates). **(F)** The cytokines in the supernatants of co-cultures were analyzed for a panel of cytokines by a Cytometric Bead Array. Cytokine concentrations were normalized to the correspondent cytokine concentration in Mock co-cultures. The dashed line at y=1 represents normalized values in PBMCs and Mock co-cultures. Bars represent mean + S.E.M. All data was submitted to unpaired t-test of pooled results from three independent experiments (n=3), each with 2 to 3 technical replicates. P≤0,05 (*); P≤0,01 (**); P≤0,001 (***) and P≤0,0001 (****).

The supernatants of the co-cultures were analyzed with a Cytometric Bead Array Human Inflammatory Cytokine Kit, which analyzed the concentration of a panel of cytokines, namely IL-8, IL-1β, IL-6, IL-10, TNF, and IL-12p70. The concentration of IL-23 and TGF-β in co-culture supernatants was evaluated by sandwich ELISA. Not all cytokines were detected above the detection limit in co-culture supernatants. Therefore, only IL-12p70, IL-6, IL-8 and TGF-β concentrations are displayed. DCs activated with LPS significantly release more cytokines to the medium than did iDCs. Moreover, significant differences in the concentration of the above cited cytokines were observed between T5 and Mock co-cultures with LPS-matured DCs but not with immature DCs. When tumor cells were cultured with LPS-maturated DCs, there was an increase in the production of the pro-inflammatory cytokines IL-6 and IL-8. This increased production was significantly higher in the supernatants of LPS-DCs cultured with MKN45 Mock cells, resulting in a dramatically decrease of IL-8 whenever mature DCs were cultured with tumor cells overexpressing branched glycans. The same trend was observed in the concentrations of IL-6, which supports a role of branched glycans expression in immunosuppression. Furthermore, and validating these results, co-culture supernatants of stimulated DCs with tumor cells overexpressing branched glycans had increased concentrations of TGF-β, which further supports the impact of these structures in the suppression of effector immune responses.

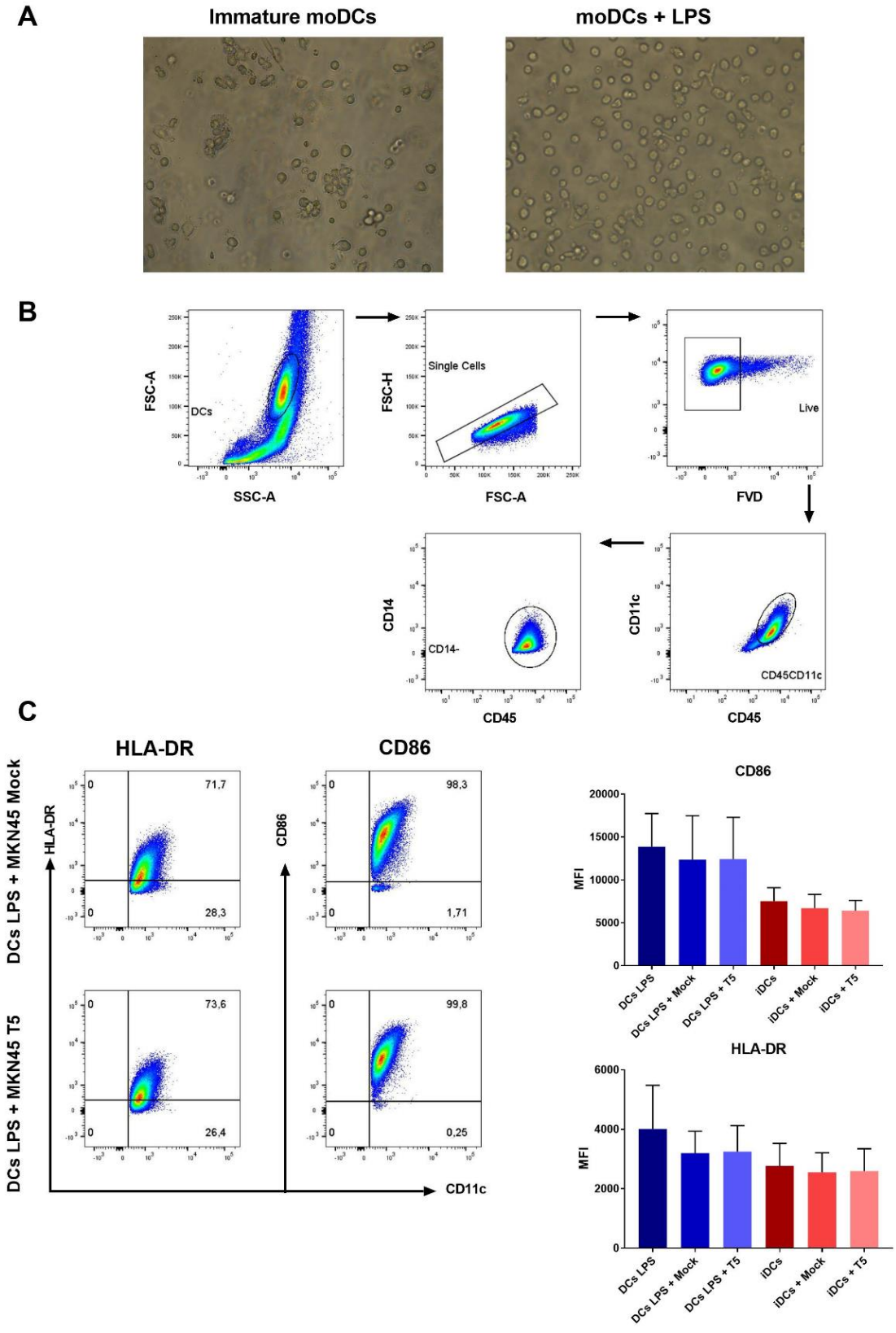

***Figure 15* | Co-cultures of MKN45 Mock or T5 cell lines with moDCs. (A)** Morphology of moDCs isolated from PBMCs before (immature DCs, iDCs) and after LPS activation (mature DCs, DCs LPS) observed under an inverted microscope (200× magnification). **(B)** Representative example showing the gating stategy used to analyze DCs activation by flow

cytometry. **(C)** Expression of the activation/maturation markers HLA-DR and CD86 on the surface of immature DCs and LPS-maturated DCs co-cultured with MKN45 Mock and T5 cells. Representative dot plots (left) and graphic representation of the MFI due to HLA-DR or CD86 specific staining (right). Numbers inside graphs correspond to the percentage of cells in each quadrant. Quadrants were set based on the autofluorescence of unstained samples. Bars correspond to mean + S.E.M. Results are from two independent experiments (n=2), each with 3 technical replicates.

These preliminary observations suggest once again, that overexpression of abnormal glycans has an active role in immunoediting, having an effect on immunosuppression. Nevertheless, these promising results need to be further confirmed and validated.

Taking all data together, these results propose a clear relationship between altered glycans expression and the type of immune cells recruited and differentiated. In this regard, N-glycan alterations on the surface of cancer cells may constitute an immunoediting strategy used by tumor to escape immune system.

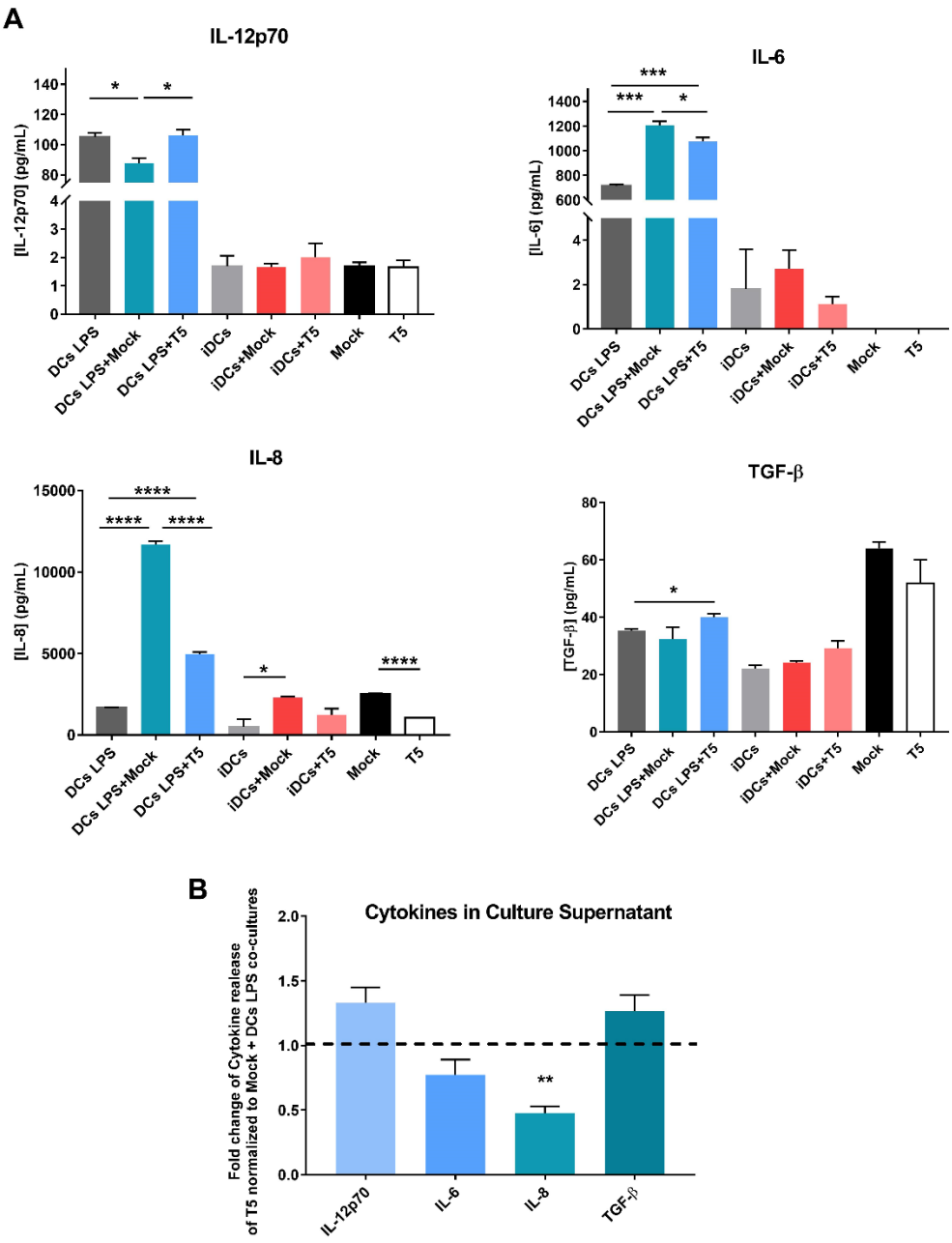

***Figure 16* | Cytokines released to the culture medium in co-cultures of moDCs and MKN45 Mock or T5 cells. (A)** Individual cytokine concentrations, in pg/mL, of IL-12p70, IL-6, IL-8 and TGF-β, in the supernatants of LPS-maturated DCs (DCs LPS) or immature DCs (iDCs) co-cultures with MKN45 Mock or T5 cells. Single cultures of DCs LPS, iDCs, Mock and T5 cells were used as controls. These results are from one experiment representative of two independent experiments for IL-12p70, Il-6 and IL-8. TGF-β was measured in a single experiment. Bars correspond to mean + S.E.M. of three technical replicates. P≤0,05 (*); P≤0,01 (**); P≤0,001 (***), P≤ 0,0001 (****). **(B)** Fold change of cytokine concentrations in the co-cultures of mature DCs with T5 cells, normalized to Mock. The dashed line at y=1 represents the normalized values in Mock plus mature DCs co-culture supernatants. Results are from two independent experiments (for IL-12p70, IL-6 and IL-8), each with 2 to 3 technical replicates. P≤0,01 (**).

Discussion

It is already known that β1,6-GlcNAc branched N-linked glycans overexpression in cancer represent an increase of malignancy, tumor development and progression in several types of cancer. This study elucidated the relevance of these altered glycans in modulating immune responses, showing that expression of branched glycans in cancer cells may be a strategy to escape to anti-cancer immune responses.

Our group has previously described that aberrant glycosylation have a key role on cell invasion and metastasis, interfering with E-cadherin stability, and consequently with cell-cell adhesion in gastric cancer [27-29, 32, 50, 105]. Furthermore, previous results described an inverse association between branched N-glycans and O-mannosylation-derived glycans in stabilization of E-cadherin and in control cancer development [50]. However, the impact of the GnT-V-mediated N-glycans as well as high-mannose N-glycans in cancer immunoediting, and immunosurveillance remains unknown.

In this study we have characterized the N-glycans profile along gastrointestinal carcinogenesis and have demonstrated a promising relationship between altered N-glycans expression on tumor cell surface and T cell differentiation and activation.

Our results demonstrate that, during colon carcinogenesis, the expression of high-mannose N-glycans is accompanied by increased frequency of Tbet-expressing cells, indicating a relationship between expression of mannose glycans and Th1-type lymphocyte differentiation/recruitment. On the contrary, the branched N-glycosylation profile was accompanied by increased Foxp3 frequency profile, since the increase of β1,6-GlcNAc branched N-glycans in colon carcinoma was associated with higher regulatory T cells frequency and a lower proportion of Tbet-expressing cells.

In gastric carcinogenesis, the expression of mannosylated glycans was predominantly observed in inflammatory conditions (gastritis), whereas the expression of branched glycans was almost negative, beginning to be positive in precancerous conditions, such as intestinal metaplasia and atrophy, and reaching high levels of expression in gastric cancer. Interestingly, we noted a tendency of Th1-type cell frequency to decrease with no changes in Treg presence in tumor microenvironment, concomitantly with an increase of abnormal glycans in carcinoma stage, suggesting that changes in N-glycans profile impact on local immune response.

In this study we demonstrated, for the first time, the glycans signature and expression profiles of branched and high-mannose N-glycans along colon and gastric carcinogenesis and their relationship with local T cell response. Therefore, we herein propose that the expression of high-mannose N-glycans appear to be associated with the

recruitment of effector Th1-type cells, whereas the expression of branched N-glycans are likely involved in the differentiation/recruitment of regulatory T cells into the lesions, and in the decreased infiltration of effector T cells subsets, such as Th1-type lymphocytes.

These *in situ* observations were further validated at the mRNA level. The levels of MGAT5 mRNA expression in different steps of colon carcinogenesis displayed a profile similar to that observed in histochemistry, with the highest levels of MGAT5 mRNA detected in high grade dysplasia. This could be due to the effects of MGAT5 expression on early steps of malignant transformation. However, the decreased MGAT5 mRNA expression in carcinoma was not correlated with β1,6-GlcNAc branched N-glycans expression, as detected by using the lectin L-PHA. Poor correlation between mRNA, protein and product expression has been frequently observed in other proteins [106, 107]. Furthermore, our preliminary results, using a single case, in which we evaluated CD3ε and TBX21 mRNA expression showed a decreased transcription of TBX21 gene in CD3 expressing T cells, which corroborates our observation at the protein level. Further biological replicates are needed to confirm these results, in all steps of the carcinogenic cascade.

The functional assays using a gastric carcinoma cell line overexpressing β1,6-GlcNAc branched N-glycans further revealed a role for these glycans in the modulation/suppression of effector T cell responses. Previous studies in E-cadherin from our group, using MKN45 Mock and MKN45 transfected with GnT-V, had already described the potential of the overexpression of these branched N-glycans in increasing invasion and metastasis [28]. Moreover, no differences in cell proliferation between both transfected cell lines were reported [56].

In this regard, when MKN45 T5 cells, that exhibit high levels of GnT-V-mediated branched N-glycans were used in co-culture with PBMCs, a significant reduction of Th1-type differentiation and a suppression of IFN-γ production was observed. Accordingly, in MA782 breast cancer cells, the knockdown of Mgat5 inhibits cell growth and activates $CD4^+$ T cells and differentiation into Th1-type cells [102].

Moreover, our results also demonstrated a decrease in Treg differentiation in PBMCs co-cultures with MKN45 T5 cell line. The literature is somehow controversial regarding the presence of regulatory T cells in tumor microenvironment and the prognosis of patient. In gastric cancer, high density of Foxp3 T lymphocytes was associated with poor prognosis, while Foxp3 expression in tumor cells was related with a better prognosis [108]. Another study described that the presence of Treg in peritumor predicted a better prognosis than when these cells were diffusely localized on the tumor [109]. Moreover, when the Th1:Th2 ratio (Tbet:Gata3 ratio) was analyzed, no differences were observed,

although there was a tendency to decrease in cells cultured with the MKN45 T5 cell line. These evidences support the evidences that overexpression of abnormal branched N-glycans have a more aggressive phenotype and poor prognosis, since decrease of Th1:Th2 ratio was previously related with worse prognosis in gastric carcinoma [82].

In fact, our data demonstrated that overexpression of branched N-glycans resulted in an interesting decrease of the pro-inflammatory IFN-γ, which reflected the modulation/suppression of Th1 differentiation. These data suggest that abnormal N-glycans expressed in tumor cells imposes an inhibition of pro-inflammatory responses.

Furthermore, in the moDCs co-cultures, we also showed that β1,6-GlcNAc branched N-glycans overexpressing cells, resulted in an increased production of TGF-β, together with a decrease of pro-inflammatory cytokines, such as IL-6 and IL-8. The cytokine TGF-β has already been described to be overexpressed in tumor microenvironment, playing essential functions in the suppression of T cell proliferation and differentiation [78]. In our data, we observed that MGAT5 overexpressing tumor cells exhibited an increased expression of TGF-β comparing with Mock cells, which further support the immunosuppressive potential of branched N-glycans expression.

IL-6 is known as a pro-inflammatory cytokine that acts on B cell differentiation and antibody production. In this study, we observed a significant increased expression of IL-6 in DCs matured with LPS compared with immature DCs. However, in MKN45 T5 co-cultures a decrease in the expression of IL-6 was detected comparatively to Mock co-cultures. IL-8 is another important pro-inflammatory cytokine that has an important role in recruiting neutrophils and is mainly produced by macrophages, epithelial cells and dendritic cells [57]. Our results indicated a dramatic increase of IL-8 production in DCs previously matured with LPS and co-cultured with tumor cells compared with single cultures of mature DCs, MKN45 Mock and MKN45 T5 cells. In DCs co-cultures without exposure to LPS with tumor cells, IL-8 production was very similar to the one evaluated in Mock and T5 cell cultures alone, suggesting that DCs have to be activated to effectively respond to the cancer cells. Nevertheless, when comparing both tumor cells co-cultures, we observed a significant decrease of IL-8 production in DCs LPS co-cultures with MKN45 that overexpressed MGAT5 compared to MKN45 Mock.

Taken together, we herein propose that alterations in glycans profile along carcinogenesis, particularly an overexpression of aberrant branched glycans have a considerable impact on immune cells response, being a potential new mechanism of cancer immunoediting.

Conclusion and future perspectives

Our findings support that β1,6-GlcNAc branched N-glycans expressed on gastrointestinal cancer cells are implicated in tumor development and malignancy, likely through immune modulation and cancer immunoediting. We have contributed to elucidate the role of these abnormal N-glycans in immunosuppression of gastrointestinal cancer.

A review discussing the impact of glycans in cancer progression was published: "de-Freitas-Junior, J.C.M.; Andrade-da-Costa, J.; Silva, M.C.; Pinho, S.S. *Glycans as Regulatory Elements of the Insulin/IGF System: Impact in Cancer Progression*. Int. J. Mol. Sci. 2017, 18, 1921".

Further studies are needed to validate and confirm these promising results.

In this regard, there is the need to increase the number of clinical cases analyzed in each stage of carcinogenesis to consolidate our results. Moreover, we aim to replicate our co-cultures assays, using different cell lines bearing distinct branched N-glycans expression and co-culture them with isolated T cells or with isolated T cells and DCs purified from the same donor. We also intend to extend the analysis to $CD8^+$ T lymphocytes. With this, we will try to understand how the cells of the immune system recognize these altered N-glycans and if APCs or T cell direct contact with those glycans is needed. Furthermore, *in vitro* and *in vivo* mechanistic assays are planned.

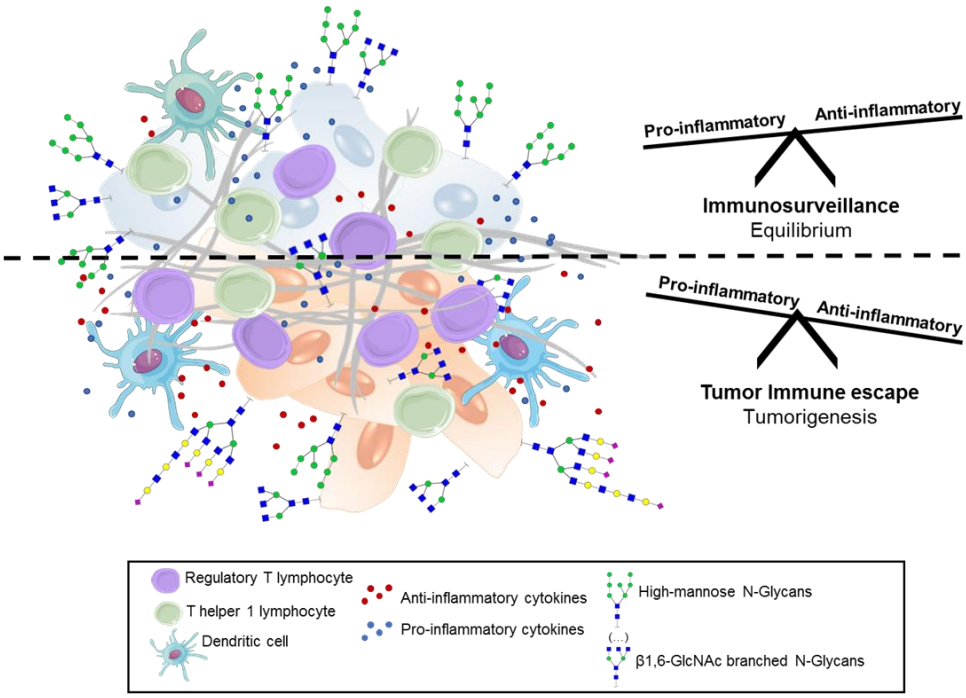

***Figure 17* | Proposed model.** Here are distinguished two different situations: Homeostasis, where the immune system is in equilibrium and pro- and anti-inflammatory responses are balanced, with predominance of high-mannose N-glycans; and Tumorigenesis situation, where anti-inflammatory response prevails due to cancer immunoediting, with alterations in glycan profile.

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Appendix

Appendix 1: Cytokines

Cytokine	Cell source	Cellular targets and biological effects
IL-2	T cells	T cells: proliferation and differentiation into effector and memory; promotes regulatory T cell development survival, and function NK cells: proliferation and activation B cells: proliferation
IL-4	$CD4^+$ T cells (Th2)	T cells: Th2 differentiation, proliferation Macrophages: alternative activation and inhibition of IFN-γ-mediated classical activation.
IL-6	Macrophages, endothelial cells, T cells	B cells: proliferation of antibody-producing cells
IL-12	Macrophages, dendritic cells	T cells: Th1 differentiation NK cells and T cells: IFN-γ synthesis, increased cytotoxic activity
IL-17A	$CD4^+$ T cells (Th17)	Endothelial cells: increased chemokine production Macrophages: increased chemokine and cytokine production Epithelial cells: GM-CSF and G-CSF production
IL-23	Macrophages, dendritic cells	T cells: differentiation and expansion of Th17 cells
GM-CSF	T cells, macrophages, endothelial cells	Immature and committed progenitors, mature macrophages: induced maturation of granulocytes and monocytes, macrophage activation
IFN-γ	T cells (Th1, CD8+ T cells), NK cells	Macrophages: classical activation (increased microbicidal functions) T cells: Th1 differentiation Various cells: increased expression of class I and II MHC molecules, increased antigen processing and presenting to T cells
IL-10	Macrophages, T cell (mainly regulatory T cells)	Macrophages, dendritic cells: inhibition of expression IL-12, costimulators, and class II MHC

TNF	Macrophages, NK cells, T cells	Endothelial cells: activation (inflammation, coagulation) Neutrophils: activation
IL-1β	Macrophages, dendritic cells	Endothelial cells: activation (inflammation, coagulation) T cells: Th17 differentiation
TGF-β	T cells (mainly Tregs), macrophages, other type of cells	T cells: inhibition of proliferation and effector functions, differentiation of Th17 and Treg
IL-8 (CXCL8)	Macrophages, dendritic cellc, other type of cells	Neutrophils: recruitment Other leukocytes: recruitment

Appendix 2

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Review

Glycans as Regulatory Elements of the Insulin/IGF System: Impact in Cancer Progression

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Abstract: The insulin/insulin-like growth factor (IGF) system in mammals comprises a dynamic network of proteins that modulate several biological processes such as development, cell growth, metabolism, and aging. Dysregulation of the insulin/IGF system has major implications for several pathological conditions such as diabetes and cancer. Metabolic changes also culminate in aberrant glycosylation, which has been highlighted as a hallmark of cancer. Changes in glycosylation regulate every pathophysiological step of cancer progression including tumour cell-cell dissociation, cell migration, cell signaling and metastasis. This review discusses how the insulin/IGF system integrates with glycosylation alterations and impacts on cell behaviour, metabolism and drug resistance in cancer.

Keywords: insulin/IGF system; glycans; cancer; glycosylation

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1. Introduction

The insulin/insulin-like growth factor (IGF) system is known to be highly implicated in the control of glucose metabolism and glycaemia, also playing an important role in cell growth, cell differentiation and metabolic pathways [1].

In tumour progression, several studies have demonstrated the impact of the insulin/IGF system on cancer cell behaviour, particularly on the acquisition of the malignant phenotype by regulating the epithelial-mesenchymal transition (EMT) program [2–5]. Moreover, the insulin/IGF system has also been implicated in cancer metabolism, the acquisition of cancer drug resistance, as well as with cancer stem cell (CSC) phenotypes [6,7], which altogether highlight the importance of this system in the regulatory networks that occur during the processes of cancer development and progression.

Glycosylation is a frequent post-translational mechanism that is characterized by the addition of glycan structures (carbohydrates/sugar chains) to proteins/lipids through the synchronized action of different glycosyltransferases enzymes that act in a stepwise manner in the ER (endoplasmic reticulum)/Golgi compartment of essentially all cells. Glycans have been described as having a relevant impact on both homeostatic and pathological conditions such as cancer. The repertoire of glycans structures expressed in cells change during the transition from normal to malignant phenotypes as a response to genetic and/or environmental stimuli. The expression of aberrant glycans structures in cancer cells has been shown to play an instrumental role in each pathophysiological step of cancer development and progression [8]. This review describes how glycans integrate with the insulin/IGF system that include the specific ligands, receptors and signaling

pathways, addressing how this biological network affects and controls cell behaviour, drug resistance and metabolism in cancer.

2. The Insulin/Insulin-Like Growth Factor (IGF) System

2.1. Ligands and Binding Proteins

The insulin/IGF system has several components, including ligands, binding proteins, receptors and downstream proteins. Insulin is a key hormone produced by pancreatic β-cells that controls glycaemia and glucose uptake in several tissues, acting also on lipid synthesis in the liver [1]. The IGFs (IGF1 and IGF2) are peptides structurally similar to insulin, being produced in the liver and in tissues where they act in both the autocrine and paracrine pathways. The IGFs are important mediators of cell growth, differentiation and metabolism. In addition to IGF and insulin, which are responsible for triggering the signaling cascade, there is also a family of six high-affinity IGF-binding proteins (IGFBPs) that modulate the interaction between these ligands and their receptors. In biological fluids, the IGFs are frequently associated with IGFBPs and are rarely found (<1%) in their free form [9,10]. The IGFBPs are found in both precursor and mature form and their molecular structure consists of three domains (amino terminal, central, and carboxyl terminal). The central domain, also known as the binding domain, usually undergoes post-translational modifications such as glycosylation or phosphorylation [11]. IGFBPs play a key role in the availability of ligands through the regulation of their half-life, blocking the interaction with the receptor or even favoring signaling activation due to controlled release of the ligands [12,13]. Evidence has also suggested that IGFBPs may have insulin/IGF-independent functions, including during tumour progression [14].

2.2. Receptors

The three main components of the insulin/IGF system are the IGF1 receptor (IGF1R), IGF2 receptor (IGF2R), and insulin receptor (INSR). Splicing variants can also give rise to two additional isoforms of INSR—the INSR-A (short isoform) and INSR-B (long isoform) [15]. The interaction of the isoforms with the IGF1R can also generate the hybrid receptors A (HR-A) and B (HR-B). With the exception of IGF2R, which has a monomeric structure, the other five receptors form a heterotetrameric structure composed of two α and two β subunits. The α subunit is extracellular and responsible for ligand binding, whereas the β subunit has a transmembrane and an intracellular segment where the tyrosine kinase domain is located. The IGF1R autophosphorylation follows binding of the ligands and, for kinase activation, the phosphorylation of three major tyrosine residues (Tyr^{1131}, Tyr^{1135} and Tyr^{1136}) are needed [16].

Phosphorylation in an equivalent tyrosine cluster (Tyr^{1146}, Tyr^{1150}, Tyr^{1151}) is required for the full activation of INSRs [17]. These receptors can be modulated by post-translational modifications in the subunits, in which the α subunit may undergo *N*-glycosylation whereas the β subunit can be modified by both *N*- and *O*-glycosylation, with the latter being described only in INSR [18–20].The INSR ectodomain is highly glycosylated, containing in each monomer a total of 19 potential *N*-glycosylation sites of which 14 were found to be occupied by glycan moieties [18]. In adipocytes, glucose deprivation was described to lead to the expression of an aberrantly glycosylated form of the INSR, which prevents oligomerization modulating the insulin-dependent tyrosine kinase activity [21]. Furthermore, *N*-glycosylation of INSR at Asn^{397} or Asn^{418} was described as essential for its normal biosynthesis and processing [22]. Concerning the IGFRs, the α-subunit region of IGF1R also contains 11 potential *N*-glycosylation sites [23], while the extracellular domain of IGF2R exhibits 19 putative sites [24]. In addition, at least 6 mucin-type *O*-glycosylation sites were described in the INSR ectodomain [19]. These glycosylation modifications affect the receptor´s folding, activity and function and may vary accordingly with physiopathological conditions. As an example, changes in the glycosylation of placental INSR and IGF1R have been observed between the first and third trimesters of gestation in pregnant healthy women, including the decrease of fucosylation and α2,6-sialylation of INSR and IGF1R, and an increase of total fucosylation of IGF2R [25].

2.3. Downstream Proteins

The activation of the insulin/IGF receptors triggers intracellular signaling through insulin receptor substrate (IRS) proteins, a family of adaptor molecules consisting of four closely related members (IRS1–IRS4) and two distant relatives (IRS5 and IRS6, also known as docking proteins (DOK4 and DOK5 respectively)) [26], which connect receptors' activation to downstream kinase cascades, such as the RAS/MEK/ERK or PI3K/AKT1 pathways. IRS proteins have two ubiquitously expressed forms in humans, IRS1 and IRS2, which are highly regulated by both phosphorylation (Tyr, Ser and Thr) and ubiquitination [27,28]. The RAS/MEK/ERK and PI3K/AKT1 pathways, although they are generally associated with proliferation and survival respectively, are also interconnected to modulate several cellular mechanisms involved in tumour development and progression [29]. Although IRS1 and IRS2 structures are quite similar, the triggered signaling mechanisms differ from each other. Mice lacking IRS1 show insulin resistance and growth retardation, but do not develop diabetes because hyperinsulinemia possibly compensates for this resistance, whereas the disruption of IRS2 impairs insulin secretion by the pancreatic β-cells, causing type 2 diabetes [30,31]. The *IRS3* is a pseudogene in humans [32] and IRS4 induces constitutive PI3K/AKT1 pathway hyperactivation in breast cancer cells [26]. The key elements composing the insulin/IGF system, integrated with glycans alterations and the cellular outcomes, are depicted in Figure 1.

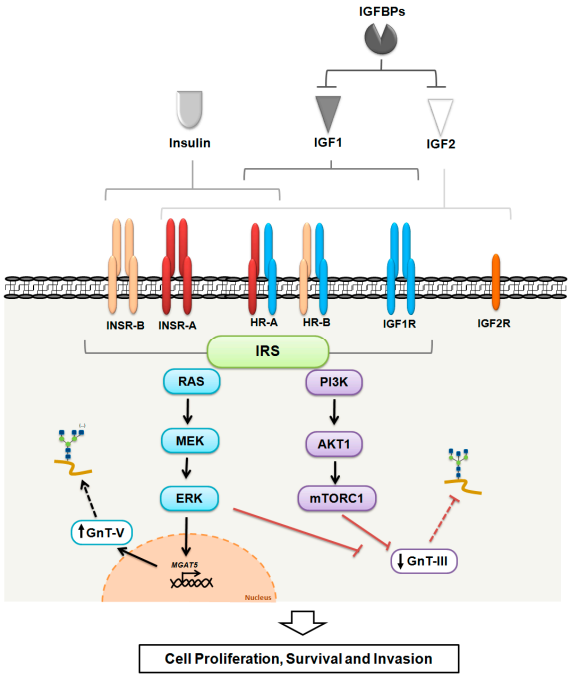

Figure 1. The insulin/insulin-like growth factor (IGF) system. The ligands (insulin, IGF1 and IGF2) bind to their specific receptors triggering downstream signaling pathways (RAS/MEK/ERK and PI3K/AKT1/mTORC1) through IRS proteins, with the exception of IGFR2 that does not transduce signaling. The IGFBPs negatively modulate both IGF1 and IGF2. The activation of RAS/MEK/ERK pathway has been shown to be involved in the upregulation of *MGAT5* gene, increasing the levels of expression of β1,6-branched *N*-glycans. On the contrary, the activation of PI3K/AKT/mTOR signaling cascade was associated with the impairment of GnT-III-mediated bisecting GlcNAc *N*-glycans expression. These signaling pathways have an impact on cell growth, survival and invasion, which favors tumour development and progression. GnT-III: *N*-acetylglucosaminyltransferase III; GnT-V: *N*-acetylglucosaminyltransferase V. GnT-III catalyzes the bisecting GlcNAc *N*-glycan structure and GnT-V catalyzes the β1,6 GlcNAc branched *N*-glycans.

3. Impact of the Insulin/IGF System in Cancer Development and Progression

3.1. Cellular Behaviour

Several mechanisms can lead to an imbalance of the insulin/IGF signaling network in cancer, including the increased bioavailability of the ligands, dysregulation of signaling proteins, and overexpression of the receptors [33]. Recently, an increasingly number of studies have revealed that insulin/IGF signaling is involved in the acquisition of the malignant phenotype by regulating epithelial-mesenchymal transition (EMT) program, with results in a negative impact on proliferation, invasion, migration, and apoptosis [2–5].

The EMT, involving the loss of cell-cell adhesion, mainly results in the acquisition of a migratory and invasive phenotype of cancer cells that accompany tumour progression. During this biological process the transcription factors ZEB1 and ZEB2 play a crucial role by binding to E-boxes of *CDH1* (E-cadherin gene) [34]. In prostate cancer cells that have the epithelial phenotype, stimulation with IGF1 upregulates ZEB1 expression in both mRNA and protein levels, leading to E-cadherin repression and upregulation of Fibronectin and *N*-cadherin [35]. Increased levels of Snail, another transcription factor that inhibits E-cadherin expression, was found to be induced by IGF1 in non-small cell lung cancer (NSCLC) cells, promoting EMT [36].

Additionally, evidence has demonstrated that the EMT process gives rise to cells with stemness features (the so-called cancer stem cells, CSC) which contribute to metastasis and drug resistance [37–39]. In breast cancer cells, the inhibition of the PI3K/AKT1/mTORC1 pathway or knockdown of IGF1R suppresses the EMT program, reducing stem cell niches [40]. Using thyrospheres models, it was observed that the thyrospheres derived from thyroid cancer have a remarkable increase of INSR-A and IGF2 when compared to normal thyrospheres. However, only IGF2 contributes to the self-renewal process, whereas the increase in the degree of differentiation was associated with the downregulation of the insulin and IGF receptors [41]. In breast cancer cells, a positive feedback mechanism was demonstrated when IGF2 binds to IGF1 receptors, triggering PI3K/AKT1 signaling and leading to the activation of DNA-binding protein inhibitor ID1, a transcriptional factor that acts not only on the maintenance of stemness but also on the positive regulation of IGF2 [42]. Interesting conclusions were also drawn in models of hepatocarcinoma in which the inhibition of Nanog-positive cells, identified to be associated with CSC resulted in down-regulation of IGF1R, influencing the self-renewal capacity of these cells. In addition, the overexpression of Nanog in Nanog-negative cells increase the expression of IGF1R, and the specific inhibition of IGF1R signaling significantly inhibit self-renewal and Nanog expression [43].

Changes in the insulin/IGF system can also be involved in the acquisition of the aggressive cancer phenotype. In hepatocellular carcinoma (HCC), decreased expression of IGFBP1 is correlated with microvascular invasion and metastasis [44]. In patients with gastric cancer, elevated IGF1R levels were associated with lymph node metastasis [45]. Interestingly, in triple-negative breast cancer cells it was also shown that overexpression of IGF1R induces migratory and invasive behaviours in a mechanism mediated by the activation of the focal adhesion kinase (FAK) signaling cascade, which can be suppressed using pharmacological inhibitors of FAKs [46].

The modulation of IGF1R signaling was further found to affect cell death programming through interplay with transforming growth factor β receptor (TGFβR) signaling. Abrogation of TGFβ/Smad3 signaling leads to increased expression and phosphorylation levels of IRS1, resulting in decreased apoptosis by increasing XIAP expression levels in FET human colon cancer cells [5]. Moreover, in triple-negative breast cancer cells the IGF1 increases the cell growth and confers a protective effect against staurosporine-induced programmed cell death [47]. Interestingly, the inhibition of *N*-glycosylation using inhibitors of *N*-glycans biosynthesis resulted in a remarkable decrease of IGF1R autophosphorylation together with its reduced expression at the cell surface, which was accompanied by a substantial decrease in the survival of Ewing's sarcoma cell lines [48].

Recently, increasing amounts of evidence have shown that microRNA may regulate insulin/IGF signaling. Increased miR-29a expression cooperates with insulin to promote the proliferation of breast cancer cells by increasing ERK phosphorylation [49]. In addition, it has been demonstrated that MicroRNA-30a, through a Src-dependent mechanism, was found to be involved in IGF1-Induced EMT in nasopharyngeal carcinoma cells [50]. In gastric cancer cells it has been shown that IGF1 is able to induce EMT through the up-regulation of ZEB2, in addition, AKT1/ERK inhibitors revert IGF1-induced EMT through up-regulation of miR-200c, suggesting the involvement of an AKT1/ERK-miR-200c-ZEB2 axis in EMT induced by IGF1 stimulation [45].

Interestingly, the integrative microRNA-insulin/IGF regulatory network seems to represent an attractive strategy for the molecular stratification of glioblastoma multiform tumours, since patients who present concomitantly low IGF1 and high miR-181d levels have a significantly longer survival rate than those with high-IGF1 and low-miR-181d [51].

3.2. Drug Resistance

Changes in the insulin/IGF system also contribute to the acquisition of resistance to chemo-, radio- and target-therapy [6,7]. A study using chemoresistant colorectal cancer cells, obtained by treatment selection with 5-fluorouracil or oxaliplatin, showed that these cells have a higher expression of CSC markers concomitantly with increased expression and activation of IGF1R. Interestingly, these cells were described to display approximately 5-fold increased responsiveness to treatment with IGF1R inhibitory monoclonal antibody compared to parental cells [52].

In ovarian cancer, the upregulation of IGF1R was associated with the early acquisition of resistance to cisplatin-paclitaxel treatment (single or in combination). In these cells, treatment with IGF1R inhibitor (in combination with cisplatin, paclitaxel or both) was able to reverse the therapy resistance at early stages [53]. Furthermore, it was also shown that the inhibition of IGF1R at early stages of therapy resistance and AKT1 inhibition at late stages were able to abrogate the CSC phenotype. Together, these data demonstrate that the IGF1R/AKT1 signaling pathway significantly impacts the acquisition of chemoresistance in cancer cells.

Experiments carried out using NSCLC cells demonstrated that the induction of EMT leads to resistance to tyrosine kinase inhibitors, however, the silencing of IGF1R (siRNA) in these cells restore their sensitivity to gefitinib or erlotinib [36].

Importantly, clinical studies in gastric cancer patients human epidermal growth factor receptor HER2+ but non-responders to HER2-targeted therapy (including lapatinib) [54], showed that IGF1R and INSR contribute to the acquisition of the resistant phenotype by precluding the lapatinib-induced suppression of cell motility and apoptosis by re-stimulating both AKT1 and/or ERK signaling but also EMT-related signaling [55].

In cancer cells, the absence of IGF1R-linked glycans at Asn^{913} compromised its membranous localization, being associated with insensitivity to figitumumab (a humanized anti-IGF1R antibody), suggesting that changes in the pattern of the expression of *N*-glycans attached to the growth receptor modulate the sensitivity to target therapy in cancer cells [56]. Although these results support the importance of glycans interplaying with the insulin/IGF system in cancer, further studies are required on this topic.

In addition to the mechanisms related to chemoresistance, changes in the insulin/IGF system are also involved in radioresistance. In lung cancer cells, radiation increases IGF1R expression thus triggering a downstream mechanism that leads to repression of p53-induced apoptosis through enhancement of phosphorylation of histone deacetylase-1 (HDAC1), which binds to the p53 promoter [57].

The analysis of glioma stem cells further demonstrated that fractionated radiation promotes both an increase in IGF1 secretion and a gradual upregulation of the IGF1R, which confer radioprotective effects on resistant cells. Interestingly, the treatment of tumours formed by this radioresistant glioma stem cells with picropodophyllin (an IGF1R inhibitor) increased the radiosensitivity [58].

Interestingly, the increased activity of ST6GAL1 (ST6 β-galactoside α-2,6-sialyltransferase 1) that results in increased α2,6 sialylation appears to be involved in the radiation-dependent cell migration

of carcinoma cells [59]. Accordingly, we have reported that the inhibition of *N*-glycan biosynthesis was associated with radiosensitization of undifferentiated human colorectal carcinoma HCT-116 cells [60]. However, although other evidence supports that the inhibition of *N*-glycan biosynthesis enhances the effects of radiation in cancer cells [61], it remains unclear how changes in INSR/IGF1R-linked *N*-glycans could interfere with radiosensitivity in cancer cells.

Taken together, and given the impact of the insulin/IGF pathway in cancer drugs resistance mechanisms, the modulation of this pathway might be an attractive strategy to reverse cancer therapy resistance in various types of cancer.

3.3. Cell Metabolism

The insulin/IGF system is closely related to cell metabolism. Tumour cells exhibit increased glucose uptake and most of them convert glucose to lactate even in the presence of oxygen ("aerobic glycolysis" or Warburg effect), which constitutes an advantage for growth being considered a metabolic hallmark of cancer [62]. Some of these advantages include: (1) resistance to fluctuation in oxygen tension; (2) production of lactic and bicarbonic acids that favor cell invasion suppressing the immune response; (3) protection against reactive oxygen species through the generation of NADPH; and, importantly; (4) the use of intermediates of the glycolytic pathway to fuel anabolic reactions, such as hexosamine, glycogen, ribose 5-phosphate, triacylglyceride, phospholipid, alanine and malate synthetic pathways [63]. In the case of hypoxic conditions, which is frequently found in solid tumours, cancer cells develop an adaptive program by increasing hypoxia inducible factor 1α (HIF1A), leading to increased expression of both glucose transporters (e.g., Glucose Transporter type 1, GLUT1) and key glycolytic enzymes (e.g., hexokinase (HK) and lactate dehydrogenase (LDH)) [1,64]. Activation of the PI3K/AKT1/mTORC1 signaling pathway under aerobic conditions also contributes to increased levels of HIF1A, thus generating metabolic reprogramming [65]. Accordingly, IGF1 was described to be involved in both the activation of HIF1A and in the expression of GLUT through PI3K/AKT1/mTOR signaling pathway [66,67].

Interestingly, increased HIF1A level seems to be involved in the activation of EMT under hypoxia conditions. In NSCLC cells, hypoxia-induced EMT is accompanied by an increase of IGF1, IGF1R, and IGFBP3, whereas the treatment with AEW541 (IGF1R inhibitor) reverses hypoxia-induced EMT, and the inhibition of HIF1A with YC-1 inhibitor abolishes the activation of IGF1R and reduces the expression of IGF1 and IGFBP3 in hypoxic cells [68].

Epidemiological studies have shown that the risk of developing malignant neoplasm is higher in obese or diabetic individuals, especially for those cancers whose cells exhibit aerobic glycolysis [69,70]. Consistently, a high glucose level itself induces EMT in A549 human lung carcinoma cells [71]. Hyperglycemic conditions also increase proliferation rate of several cancer cells, and this effect may be amplified when in combination with high insulin or IGF1 levels [72–74]. Paradoxically, mouse model studies have shown that genetic alterations leading to constitutive activation of the PI3K/AKT1/mTORC1 signaling pathway may promote self-sufficiency in tumour growth [75].

The insulin/IGF network is also highly affected by post-translational modifications through glycosylation. High uptake of glucose in tumour cells leads to increased levels of intracellular fructose-6-phosphate, thus fueling (with ~2–5% of a cell's glucose) the hexosamine biosynthetic pathway (HBP) by generating substrates (i.e., UDP-GlcNAc, UDP-GalNAc, CMP-Neu5Ac) for *N*-glycosylation, *O*-glycosylation, glycolipids, and *O*-GlcNAc (*O*-linked β-*N*-acetylglucosamine) modification of cytosolic proteins [76].

Glycosylation is a major post-translational mechanism occurring in essentially all mammalian cells. It is characterized by the enzymatic addition of carbohydrate structures (glycans) to secretory and membrane-anchored proteins and lipids in a very well-orchestrated process [8]. Changes in glycosylation are considered a hallmark of cancer, as cancer cells exhibit a completely different repertoire of glycans structures compared with their normal counterparts [8,77]. Glycans have been described to precisely regulate each pathophysiological step of cancer development and progression,

from the very beginning of tumour cell dissociation and invasion [77–81], to tumour growth and metastasis. Importantly, glycans was also shown to regulate the insulin/IGF signaling pathway in a cancer context [82], which highlights its importance in the regulatory circuits that integrate metabolic alterations, cancer drug resistance, and cancer cell behaviour.

4. Glycosylation as a Regulatory Mechanism of the Insulin/IGF System in Cancer

Changes in the glycosylation machinery occur during the transition from normal to malignant phenotypes giving rise to an increased diversity of glycans structures that are abnormally expressed on the cell surface that further contributes to tumour heterogeneity [8]. Several signaling pathways are known to be dysregulated in a cancer context and some of them have been found to directly impact in the activity of key glycosyltransferases. As example, RAS-RAF-MAPK signaling pathway is frequently upregulated in cancer cells and is particularly involved in the increased expression of the *MGAT5* gene that encodes human *N*-acetylglucosaminyltransferase V (also known as GnT-V) [83]. The increased activity of GnT-V results in the overexpression of β1,6 GlcNAc branching *N*-glycan structures that has been widely associated with malignant and invasive phenotypes [79,84–86].

Interestingly, mice with mammary tumours induced by the polyomavirus middle T (PyMT) oncogene (whose expression promotes increased PI3K/AKT1and RAS/MEK/ERK signaling) showed a decrease in tumour growth in *Mgat5*$^{-/-}$ mice [87,88]. Consistently, in the early-stages of these PyMT *Mgat5*$^{-/-}$ mammary tumours they also show lower levels of activation of the PI3K/AKT1signaling [87], and the cell lines derived from the PyMT *Mgat5*$^{-/-}$ are less responsive to insulin-like growth factor (IGF) [89].

The GnT-V-mediated branched *N*-glycans can be further extended giving rise to elongated poly-*N*-acetyllactosamine structures that serve as ligands for galectins, a family of conserved carbohydrate-binding proteins that form galectin-glycan structures on cell surfaces termed "lattices" [90]. The expression of branched *N*-glycans on the extracellular domain of cell surface receptors with a high number of *N*-glycosylation sites, such as on epidermal growth factor receptor (EGFR), insulin-like growth factor receptor (IGFR), and fibroblast growth factor receptor (FGFR), promote the binding to galectins forming the molecular lattice that precludes the endocytosis of glycoprotein receptor, which consequently contributes to signaling activation and increased cell proliferation, tumour growth and oncogenesis [89,91,92].

Another relevant mechanism linking glycans to cancer cell growth occurs during hypoxia. In hypoxic conditions there is inhibition of phosphofructokinase 1 (PFK1, enzyme responsible for the conversion of fructose-6-phosphate to fructose-1,6-bisphosphate-, the first irreversible reaction unique to the glycolytic pathway), which results in *O*-GlcNAcylation at Ser^{529} by OGT (*O*-linked *N*-acetylglucosamine transferase), redirecting the flux of glucose from glycolysis through the PPP and thereby conferring a proliferative advantage to cancer cells [93]. Furthermore, inhibition of *O*-GlcNAcylation at Ser^{529} was found to reduce cancer cell proliferation in vitro and impaired tumour formation in vivo [93].

As a consequence of the shift from oxidative phosphorylation to aerobic glycolysis in cancer cells, the high rates of glucose uptake potentiates the hexosamine biosynthetic pathway (HBP), culminating in the enhancement of metabolic pathways. As a response, the levels of *O*-GlcNAcylation in cancer cells increase, which has a negative impact on cancer cell behaviour [94,95]. *O*-GlcNAc has been described to modulate protein functions by regulating protein phosphorylation and thus affecting key signaling pathways in cancer [96]. Interestingly, the insulin receptor substrate-1 (IRS1) was found to be modified by *O*-GlcNAc, which modulated the effects elicited by insulin and IGF1 [97]. Moreover, the downstream effector AKT1 was also found to be regulated by *O*-GlcNAcylation [98]. Recently, it was demonstrated that high glucose concentration exacerbates colon cancer malignancy by increasing HBP flow, culminating in aberrant glycosylation with increased *O*-GlcNAc levels as well as a tendency to increase levels of branched *N*-glycans [73]. Despite this evidence, the impact of *O*-GlcNAc in the modulation of the insulin/IGF system in cancer remains poorly understood.

Interestingly, evidence points towards the existence of a regulatory circuit between glycosylation, insulin/IGF system and cancer. The interplay between E-cadherin expression—a major tumour suppressor protein in epithelial cancers—and INSR/IGF1R signaling was found to modulate the expression of bisecting *N*-glycans (complex-type *N*-glycan containing bisecting β1,4-linked GlcNAc residue attached to a β-mannose), catalyzed by *N*-acetylglucosaminyltransferase III (GnT-III), encoded by the human *MGAT3* gene [99]. The exogenous expression of E-cadherin in MDA-MB-435 epithelial carcinoma cells (which endogenously lack E-cadherin expression both at mRNA and protein levels) inhibits INSR and IGF1R phosphorylation. The stimulation of MDA-MB-435 + E-cad cells with insulin or IGF1 decreased the bisecting *N*-glycans expression on E-cadherin which consequently up-regulated mesenchymal markers with the enhancement of tumour cell invasion. These observations provide important insights into the effects of insulin/IGF1 signaling in cancer progression through glycosylation modifications [82,84].

Taken together, we might be in front of an integrated mechanism in cancer in which the interaction between the insulin/IGF system and metabolic changes might culminate in alterations of the glycosylation of cancer cells that, in turn, fine tune insulin/IGF signaling with major effects on tumour cell development and progression, a biological network that is worth exploring (Figure 2).

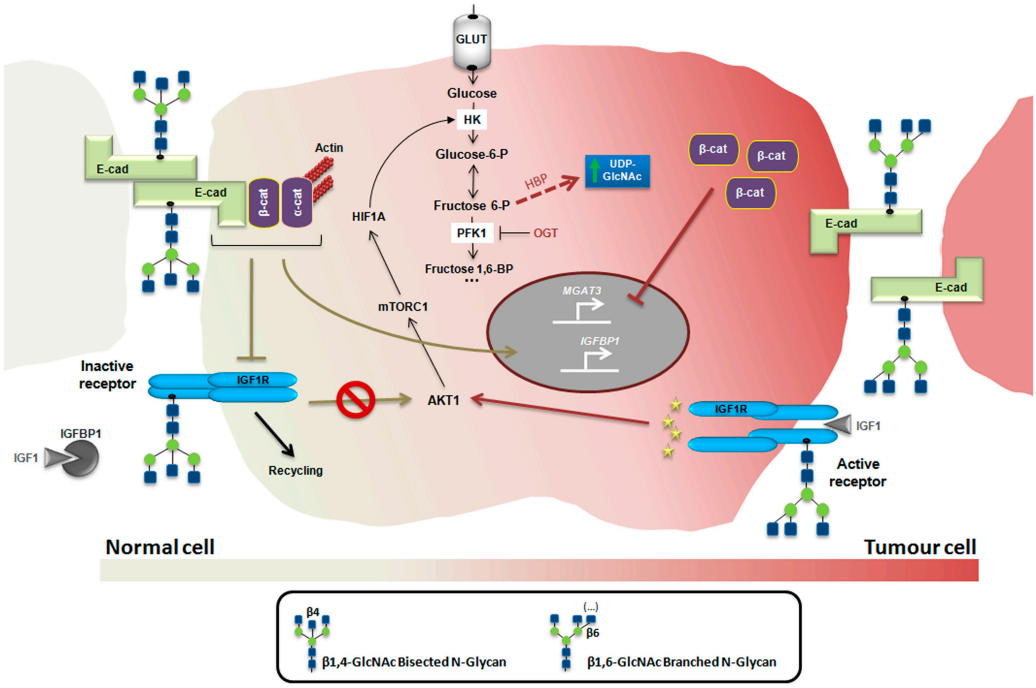

Figure 2. Glycans and insulin/IGF system in cancer: an integrated mechanism. In a normal context, high levels of bisecting GlcNAc *N*-glycans catalyzed by GnT-III favor the establishment of a stable phenotype through E-cadherin-mediated cell-cell adhesion, which in turn promotes *MGAT3* expression establishing thereby a functional feedback loop. Both the stable adherens junctions and IGFBP1 expression lead to the inhibition of IGF1R activity, which were associated with a decrease of the AKT1/mTORC1/HIF1A/HK axis. In a cancer context, the high levels of β1,6-branched *N*-glycans, catalyzed by GnT-V enzyme destabilizes E-cadherin-mediated cell-cell adhesion and favors the activation of IGF1R-mediated signaling thus increasing the AKT1/mTORC1/HIF1A/HK axis. Furthermore, as a consequence of the high HK activity, the hexosamine biosynthetic pathway (HBP) flux become higher, increasing the GlcNAc biosynthesis and the branched *N*-glycosylation. In addition, the translocation of cytoplasmic β-catenin to nucleus promotes inhibition of *MGAT3* expression, that concomitantly with the repression of *IGFBP1*results in a positive feedback mechanism on IGFR1 activity. OGT, *O*-linked *N*-acetylglucosamine transferase. GnT-III catalyzes the bisecting GlcNAc *N*-glycan structure and GnT-V catalyzes the β1,6 GlcNAc branched *N*-glycans.

Although the evidence presented herein strongly supports the existence of an integrated mechanism between glycosylation modifications and insulin/IGF system in cancer cells, it is worth mentioning that some controversial data exist due to the fact that the effects of *N*-glycans are cell/tissue/organ-specific. As examples, in rat hepatomas the enhanced expression of *MGAT3* (often associated with the suppression of metastasis) has been reported, and the progression of hepatic neoplasms is retarded in mice lacking the bisecting GlcNAc *N*-glycans [100]. Moreover, in bladder cancer the enhanced levels of *MGAT5* (widely associated with malignant and metastatic phenotypes) was associated with low malignant potential and good prognosis [101].

5. Conclusions and Future Directions

The insulin/IGF system exhibits a key role in the process of cancer development and progression. Changes in glycans expression also play a fundamental role in the cancer process. We herein propose an integrated mechanism in cancer by which glycans alterations regulate the intricated signaling pathways mediated by the insulin/IGF system with impacts on cancer cell behaviour, cancer cell metabolism, cancer drug resistance and cancer stemness. Targeting this regulatory network in cancer may constitute an interesting approach for novel cancer therapies. The understanding of this network might contribute to finding a way to contradict the biological feedback between glycans and insulin/IGF system, and consequently to control tumour development and progression.

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Abbreviations

Asn	Asparagines
CMP	Cytidine monophosphate
CSC	Cancer stem cells
EMT	Epithelial-mesenchymal transition
GlcNAc	*N*-acetylglucosamine
HBP	Hexosamine biosynthetic pathway
HCC	Hepatocellular carcinoma
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
NSCLC	Non-small cell lung cancer
PPP	Pentose phosphate pathway
PyMT	Polyomavirus middle T
RNA	Ribonucleic acid
RTK	Receptors tyrosine kinase;
Ser	Serine
Thr	Threonine
Tyr	Tyrosine
UDP	Uridine diphosphate
XIAP	Increasing x-linked inhibitor of apoptosis protein

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