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GlycoMAPP - Exploring the Impact of **Glyco**sylation on **Ma**chrophage **P**olarization in the Context of Tumour **P**rogression

Camila Adriana Gonçalves Lourenço

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M.ICBAS **2024**

INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR

CAMILA ADRIANA GONÇALVES LOURENÇO

GLYCOMAPP – EXPORING THE IMPACT OF **GLYCO**SYLATION ON **M**ACROPHAGE **P**OLARIZATION ON THE CONTEXT OF TUMOUR **P**ROGRESSION

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“You do not just wake up
and become the butterfly”

- rupi kaur **-**

PRELIMINARY NOTE

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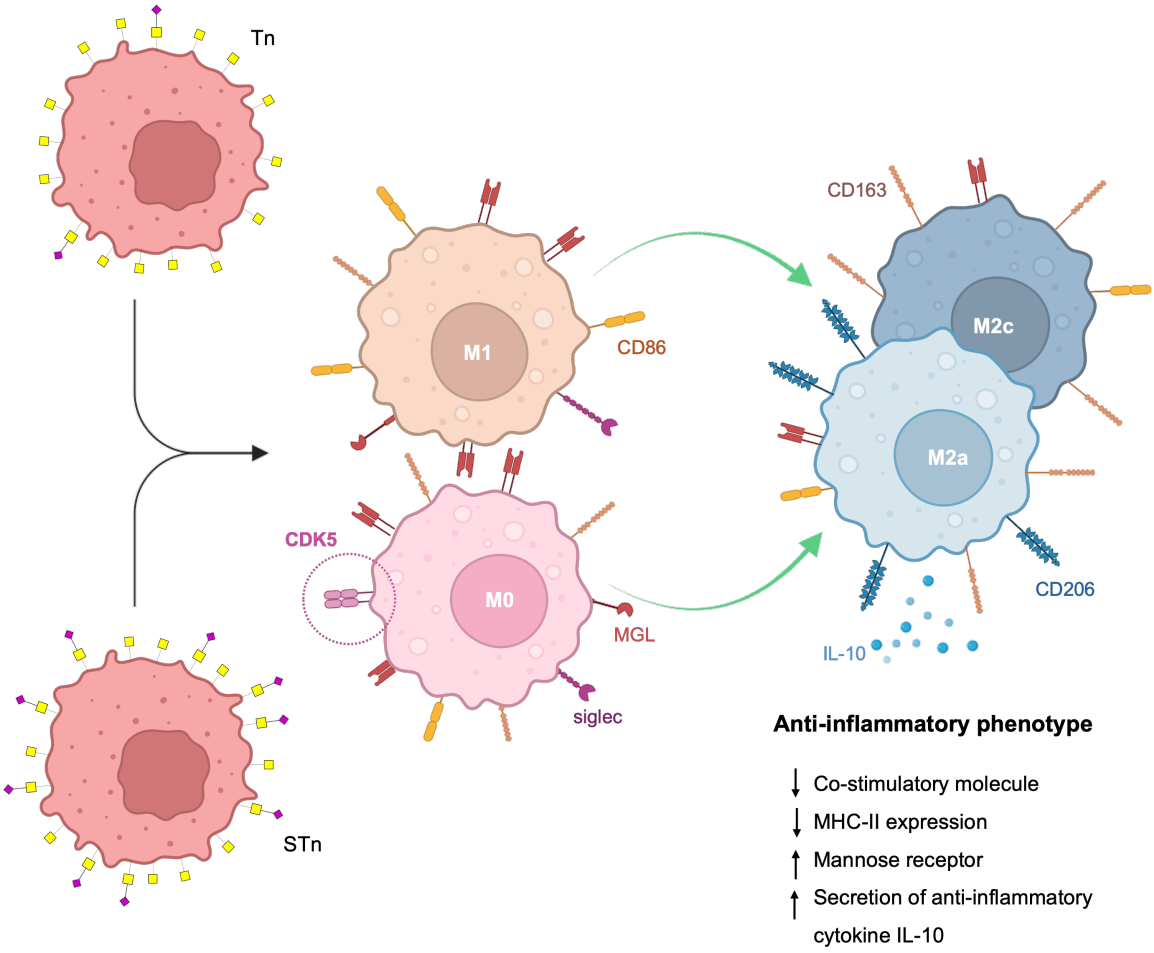
GRAPHICAL ABSTRACT



ABSTRACT

Cancer is a multifaceted disease strongly associated with a wide range of cellular alterations, including early-stage disruptions in glycosylation during carcinogenesis. Among the most prevalent changes in the *O*-glycosylation of cancer proteins is the production of truncated *O*-glycans, such as Tn and sialyl-Tn (STn), which have been implicated in significantly dampening the immune response by altering antigen presentation, thereby compromising the orchestration of an efficient adaptive anti-tumoral immune response. Considering the essential role that macrophages play in immune defence and tissue homeostasis, this study seeks to unravel how glycosylation modifications in cancer cells influence macrophage polarization state and uncover key mediators involved in immune suppression and evasion within the tumour microenvironment.

To explore this, glycoengineered cellular models of bladder (T24) and gastric cancer (AGS) overexpressing Tn and STn were cultured with human monocyte-derived macrophages in different co-culturing settings. The study first established a clear characterization of the pro-inflammatory (M1) and anti-inflammatory (M2a and M2c) phenotypes. Using these established models, immature macrophages (M0) were co-cultured with Tn and STn overexpressing cells, which led to their polarization toward an M2a-like anti-inflammatory phenotype, compared to the controls of non-edited tumour cells cultured with immature macrophages. This shift was evidenced by a marked increment in CD206 mannose receptor expression and a decrease in CD86 co-stimulatory molecule across both bladder and gastric cancer models. Likewise, interactions with glycoengineered tumour cells reprogrammed M1 macrophages to an anti-inflammatory phenotype, characterized by reduced levels of pro-inflammatory markers and significant increases in CD206 and CD163 receptors. The role of Tn and STn glycans in modulating macrophage polarization and reprogramming towards an anti-inflammatory state was further supported by elevated levels of the inhibitory cytokine IL-10 in the conditioned media, which suppresses the activation of cytotoxic T cells and dendritic cells, while enhancing the recruitment of regulatory T cells, leading to an immunosuppressive tumour microenvironment that facilitates tumour growth. In addition, human gastric and bladder tumour tissues positive for short-chain *O*-glycans showed a low infiltration of $CD68^+$ and $CD163^+$ cells compared to tumours without altered glycosylation.

To further elucidate the molecular players involved in cancer cell-macrophage crosstalk, this work developed a novel proteomics-based approach using paramagnetic beads coated with enriched Tn/STn membrane glycoproteins from edited tumour cell lines (T24 and AGS), with wild-type cells serving as controls. Following interaction with macrophages, the proteins involved in the immune synapse were specifically and robustly extracted for

subsequent mass spectrometry analysis. Among the proteins identified, only CDK5 emerged as a significant factor present simultaneously in both macrophage-T24 KO and macrophage-AGS KO interactions. Given its crucial role in cell migration and immune responses, CDK5 could be a pivotal protein in the immune synapse between *O*-glycosylated tumour cells and immature macrophages.

In summary, the present work has deepened our understanding of the immunomodulatory roles of Tn and STn glycans, revealing that the suppression of *O*-glycan chain extension allows tumour cells to evade adaptative immune response by impairing proper antigen presentation and establishing an immunosuppressive microenvironment. Additionally, the research has unveiled a key molecule involved in the interactions between tumour cells with aberrant glycosylation and macrophages through a novel proteomics-based approach, offering a valuable tool for identifying promising therapeutic targets for immunotherapy that could be leveraged to restore effective immune surveillance against cancer.

Keywords: cancer cells, glycosylation, Tn, STn, macrophages, glycan-binding proteins

RESUMO

O cancro é uma doença multifacetada fortemente associada a uma gama de alterações celulares, incluindo alterações precoces na glicosilação durante a carcinogénese. Entre as alterações mais prevalentes na *O*-glicosilação de proteínas cancerígenas está a produção de *O*-glicanos truncados, como o Tn e sialil-Tn (STn), que têm implicações na diminuição significativa da resposta imunológica ao alterar a apresentação de antigénios, comprometendo assim a orquestração de uma resposta imunológica adaptativa anti-tumoral eficiente. Considerando o papel essencial que os macrófagos desempenham na defesa imunológica e na homeostase tecidual, este estudo pretende desvendar como as modificações de glicosilação nas células cancerígenas influenciam o estado de polarização dos macrófagos e descobrir mediadores-chave envolvidos na supressão e evasão imunológica dentro do microambiente tumoral.

Para explorar este aspeto, modelos celulares geneticamente editados de cancro de bexiga (T24) e gástrico (AGS), que sobreexpressam Tn e STn, foram cultivados com macrófagos derivados de monócitos humanos em diferentes contextos de co-cultura. O estudo começou por estabelecer uma clara caracterização dos fenótipos pró-inflamatórios (M1) e anti-inflamatórios (M2a e M2c). Utilizando estes modelos, os macrófagos imaturos (M0) foram co-cultivados com células que sobreexpressão Tn e STn, o que levou à sua polarização para um fenótipo anti-inflamatório do tipo M2a, em comparação com os controlos de células tumorais não editadas cultivadas com macrófagos imaturos. Esta mudança foi evidenciada por um aumento acentuado na expressão do recetor de manose CD206 e uma diminuição na molécula co-estimuladora CD86 em ambos os modelos de cancro. Da mesma forma, as interações com células tumorais geneticamente editadas reprogramaram os macrófagos M1 para um fenótipo anti-inflamatório, caracterizado por níveis reduzidos de marcadores pró-inflamatórios e aumentos significativos nos recetores CD206 e CD163. O papel dos glicanos Tn e STn na modulação da polarização dos macrófagos e reprogramação em direção a um estado anti-inflamatório foi ainda apoiado por níveis elevados da citocina inibitória IL-10 nos meios condicionados, que suprimem a ativação de células T citotóxicas e células dendríticas, enquanto aumentam o recrutamento de células T reguladoras, levando a um microambiente tumoral imunossupressor que facilita o crescimento tumoral. Além disso, os tecidos de tumores gástricos e da bexiga humanos positivos para *O*-glicanos de cadeia curta apresentaram uma baixa infiltração de células $CD68^+$ e $CD163^+$ em comparação aos tumores sem glicosilação alterada.

Para melhor elucidar os intervenientes moleculares envolvidos na interação entre células cancerígenas e macrófagos, este trabalho desenvolveu uma nova abordagem baseada na proteómica, utilizando esferas paramagnéticas revestidas com glicoproteínas

de membrana Tn/STn enriquecidas de linhas celulares tumorais editadas (T24 e AGS), com células de tipo WT como controlo. Após interação com macrófagos, as proteínas envolvidas na sinapse imunitária foram extraídas para posterior análise por espetrometria de massa. Entre as proteínas identificadas, apenas a CDK5 surgiu como um marcador presente simultaneamente nas interações macrófago-T24 KO e macrófago-AGS KO. Dado o seu papel crucial na migração celular e nas respostas imunitárias, a CDK5 poderá ser uma proteína fundamental na sinapse imunitária entre células tumorais *O*-glicosiladas e macrófagos imaturos.

Em resumo, o presente trabalho aprofundou a nossa compreensão dos papéis imunomoduladores dos glicanos Tn e STn, revelando que a supressão da extensão da cadeia de *O*-glicanos permite que as células tumorais evitem a resposta imunitária adaptativa, prejudicando a apresentação adequada de antigénios e estabelecendo um microambiente imunossupressor. Além disso, a investigação revelou uma molécula-chave envolvida nas interações entre as células tumorais com glicosilação aberrante e os macrófagos através de uma nova abordagem baseada em proteómica, oferecendo uma ferramenta valiosa para a identificação de alvos terapêuticos promissores para a imunoterapia que poderiam ser aproveitados para restabelecer uma vigilância imunitária eficaz contra o cancro.

Palavras-chave: células cancerígenas, glicosilação, Tn, STn, macrófagos, proteínas de ligação a glicanos

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ABBREVIATIONS & ACRONYMS

APCs Antigen-presenting cells
ACN acetonitrile
Asn Asparagine
BSA bovine serum albumin
CARMA Macrophages with chimeric antigen receptors
CAR-T Engineered chimeric antigen receptors
CDG Congenital disorders of glycosylation
CDK5 Cyclin-dependent Kinase 5
CSK buffer cytoskeletal buffer
CR3 Complement receptor 3
CTCs Circulating tumour cells
CTLA-4 Cytotoxic T-lymphocyte-associated antigen 4
DAMPs damage-associated molecular patterns
DCs Dendritic cells
DC-SIGN Dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin
dST disialyl-T
ECM Extracellular matrix
ER Endoplasmic reticulum
FFPE Formalin-fixed paraffin-embedded
FoxM1 Forkead protein M1
GA Golgi apparatus
GalNAc N-acetylgalactosamine
GalNAc-Ts Polypeptides GalNAc transferases
GBPs Glycan-binding proteins
GlcNAc N-acetylglucosamine
GO gene ontology
HLA-ABC class I histocompatibility complex
HGF Hepatocyte growth factor
ICB Immune checkpoint blockade
iNKT Natural killer T cells
irAEs Immune-related adverse effects
Le Lewis
MHCs Major Histocompatibility Complexes
MIBC muscle-invasive bladder cancer

NK Natural killer

PAMPs pathogen-associated molecular patterns

PD-1 Death programmed 1

PBMCs peripheral blood mononuclear cells

PNGaseF Peptide-*N*-glycosidase F

PTM Post-translational modification

ROS Reactive oxygen species

SAMPs Self-associated molecular patterns

SDS sodium dodecyl sulfate

Ser Serine

Siglecs sialic acid-binding immunoglobulin-like lectins

SLe^A Sialyl lewis A

SLe^X Sialyl lewis X

ST Sialyl-T

STn Sialyl-Tn

TAMs Tumour-associated macrophages

TBS Tris-buffered saline

TCR-T Gene-modified T-cell receptors

TIICs - tumour-infiltrating immune cells

Thr Threonine

TME Tumour microenvironment

Tregs Regulatory T cells

Tyr Tyrosine

VVA *Vicia Villosa*

CHAPTER I

INTRODUCTION

1. OVERVIEW

Recognized as a highly complex and dynamic disease originating in an abnormal cell, distinguished by uncontrolled cell growth and characterized by an inherent ability to invade tissues, culminating in metastatic spread to different organs, it is possible to define the term cancer(1, 2). Consequently, cancer is acknowledged as one of the main contributors to global mortality, accounting for around 9.7 million deaths and 20 million new cases in the year 2022, with an expected increase to 35 million cases in the year 2050(3). These high numbers are reflected not only in mortality but also in cancer incidence rates **(Figure 1)**, which tend to increase exponentially with the growth and ageing of the population, mostly driven by non-intrinsic and extrinsic risk factors such as physical inactivity, excessive alcohol consumption, unhealthy diet, obesity, exposure to infectious agents and prolonged exposure to the sun(4, 5).

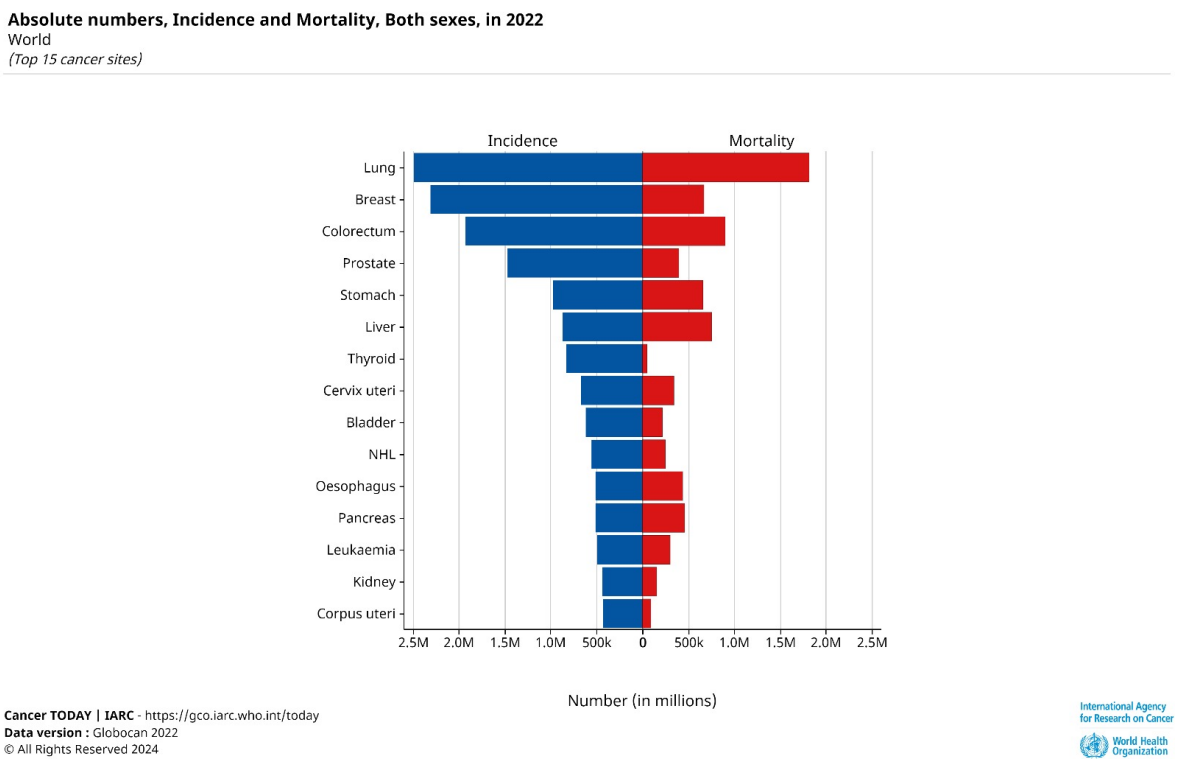


Figure 1 - Worldwide incidence and mortality of all cancers. Data Source: Globocan 2022 Graph production: Global Cancer Observatory.

To combat this lethal disease, it has been necessary to rely on certain forms of treatment conventionally applied in clinical practice: surgery, chemotherapy, and radiotherapy. These therapeutic approaches can be used jointly or independently, depending on a series of criteria, such as the type and stage of the cancer under consideration and the patient's individual and limiting conditions(6, 7). Nevertheless, the majority of patients are diagnosed

late, creating a significant obstacle when it comes to the application of the most common interventions and curative treatments, causing a series of serious consequences for the patient, including their death(8). In this way, advances in modern diagnostic and molecular characterization technologies have led to the development of new therapeutic approaches intended to increase patient survival time and combat mortality rates(9, 10). Indeed, immunotherapy has emerged as one of the main innovations in research and clinical practice, distinguished by its ability to produce prolonged responses and durable remissions in several types of cancer, making it the first line of treatment in some cancers(11). Nowadays, it is possible to classify the current immunotherapy therapeutics into various classes based on their clinical application: cancer vaccines, adoptive cell therapy, monoclonal antibodies, and small-molecule inhibitors(12, 13). While these therapies hold significant promise, their effectiveness varies widely, with only a minority of patients experiencing substantial benefits and some enduring severe immune-related side effects(14, 15). The variable efficacy and response rates are influenced by multiple factors, including cancer type, tumour heterogeneity, the microenvironment, immunogenicity, the patient's immune system strength, and the potential development of resistance to treatment(16). Therefore, more research is urgently needed in this field to increase the survival of more cancer patients and achieve longer-lasting responses in both early and late stages, developing more specific and effective therapeutic strategies to eliminate malignant cells without causing immune-mediated toxicity or autoimmunity(17).

Targeting tumour-specific molecular abnormalities with tailored therapies is the current concept underlying precision oncology, aiming for effective treatment with potentially reduced toxicity. Particularly, aberrantly expressed proteins resulting from these abnormalities underlying oncogenic transformation, such as aberrant glycoproteins overexpressed on the surface of malignant cells, have been intensively explored, due to their potential as molecular targets for a clinically relevant therapeutic strategy in the directed fight against cancer(18). In fact, during the process of tumorigenesis and progression, changes occur in the glycosylation patterns of proteins present in the cell membrane that reflect genetic, transcriptomic, and epigenetic instability, which together promote all the malignant characteristics essential for tumour survival, growth, and metastasis(19, 20). Therefore, the mechanisms associated with tumour-related glycosylation changes involve glycan branching, truncation structures, sialylation and fucosylation processes(21). These cancer-associated glycans have the distinct advantage of being predominantly linked to tumours, with minimal expression in healthy tissues(22), while also playing a crucial role in immunity(23). In fact, the innate immune system can recognize patterns indicative of tissue damage, known as damage-associated molecular

patterns (DAMPs) and pathogen-associated molecular patterns (PAMPs), which are primarily composed of mis localized or foreign glycans and glycoconjugates that trigger the release of pro-inflammatory cytokines and activate immune cells. However, cancer cells exhibit self-associated molecular patterns (SAMPs) that attenuate these pro-inflammatory signals through a mechanism driven by specific glycan epitopes. Consequently, immune cells interact with these specific glycan epitopes present on tumour cells via glycan-binding proteins (GBPs), leading to suppression or evasion of an effective immune response, ultimately inducing immune tolerance(24, 25). Therefore, given the pivotal role of glycans in immune modulation, it is imperative to consider both the proteome of the patient's tumour cells and the GBPs present in immune cells, as they represent promising targets for immunotherapy(26).

2. GLYCOSYLATION

2.1. Fundamental insights into glycosylation

Glycosylation is a post-translational modification (PTM) that can be defined as an enzymatic process in which glycosidic chains are added to proteins, lipids or saccharides, involving sequential steps in the endoplasmic reticulum (ER) and Golgi apparatus (GA) through the action of multiple glycosyltransferases and glycosidases(27-29). In mammals, glycans are synthesized through complex biosynthetic pathways involving the diverse coupling of the following monosaccharide units: glucose, galactose, fucose, xylose, mannose, sialic acid, iduronic acid, glucuronic acid, *N*-acetylgalactosamine (GalNAc) and *N*-acetylglucosamine (GlcNAc)(30-32). Therefore, glycosylation results in the formation of a variety of glycoconjugates, such as glycoproteins with *N*-/*O*-linked glycans, glycosaminoglycans, and glycosylphosphatidylinositol anchors, and glycolipids such as glycosphingolipids(33, 34).

Remarkably, the cell’s surface is covered by a layer replete with glycans called the glycocalyx(35). Given their location, it is natural that they play important biological and physiological roles such as cell-cell and cell-matrix adhesion(21, 36), cell and tissue growth and differentiation(37, 38), mediation of cell signalling(39, 40), mediation of host-pathogen interactions(41, 42), and modelling of immune responses(43, 44). Nonetheless, glycans are also integral in the regulation of protein folding, stability, trafficking, and renewal(40, 45, 46).

Among the diverse glycoproteins present on the cellular membrane, *N*-glycans and *O*-glycans are particularly noteworthy as they represent the most common and well-studied

forms of glycosylation. Indeed, several proteins are modified by *N*-glycosylation, a process initiated within the cytoplasmic compartment of the ER. Here, an oligosaccharide is linked to a dolichol pyrophosphate (Dol-P) by sequential enzymatic steps, generating a precursor of 14 sugar residues, $Glc_3Man_9GlcNAc_2$, which is in turn transferred to an asparagine residue of an Asn-X-Ser-Thr sequence (X can be any amino acid except proline) by an oligosaccharyltransferase(34, 47-49). This is followed by enzymatic processing in the lumen of the ER, where remodelling of the *N*-glycans takes place, and continues in the secretory pathway in the GA, where further modification of these proteins occurs catalysed by glycosidases and glycosyltransferases, forming the diverse glycan profile found all over the cell(21, 50, 51). This allows the types of *N*-glycans to be grouped into three major categories according to the branching of the side chains: oligomannose *N*-glycans, glycans composed mainly of mannose residues; hybrid *N*-glycans; and complex *N*-glycans, glycans that elongate the main chain by the addition of GlcNAc in the GA(52, 53).

O-glycosylation, commonly referred to as mucin-type glycosylation due to the high percentage of *O*-glycans in the mucin mass, is abundantly present in the glycocalyx of cells and, therefore, plays an essential role in biological processes(54, 55). In turn, *O*-glycosylation is initiated in the GA and does not require a dolichol precursor to start its biosynthesis, as in the case of *N*-glycosylation, instead it only requires a single monosaccharide, GalNAc(56). In mammalian, there are around 20 different polypeptides GalNAc transferases (GalNAc-Ts) that reside in the Golgi complex and have the ability to add GalNAc to a hydroxyl group of Ser, Thr or, less frequently, tyrosine (Tyr) residue present in the protein chain(37, 57, 58). In this way, the simplest *O*-glycan, the Tn antigen, is formed, which can then be elongated to create four core structures **(Figure 2)**. Thus, through the action of the C1GalT-1 glycosyltransferase enzyme, also known as T-synthase, supported by the COSMC chaperone, it is possible to add a Gal to the Tn antigen through a β1-3 bond, allowing the formation of the structure core 1, commonly known as the T antigen(54, 59-61). Accordingly, core 2 structure formation is catalysed by the enzyme β1-6 N-acetylglucosaminyltransferase, or more simply C2GnT, which adds the β1-6 binding of a GlcNAc residue to core 1(62, 63). The Tn antigen can also be transformed into core 3 by adding a β1,3-GlcNAc residue or core 4 by adding a β1,6-GlcNAc residue to the core 3 base structure, according to the C3GnT6 and C2GnT2 enzymes, respectively(64-66). Alternatively, both T and Tn antigens can be modified by sialyltransferases, involving the action of the ST3Gal-T and ST6GalNAc enzymes, forming sialylated antigens such as sialyl-T (ST), disialyl-T (dST) and sialyl-Tn (STn)(67, 68). Subsequently, the various core structures mentioned above can be elongated to form complex *O*-glycans, recognised by

the presence of specific antigens such as Lewis(Le) blood group antigens ABO(H) blood group determinants as terminal epitopes(45, 57).

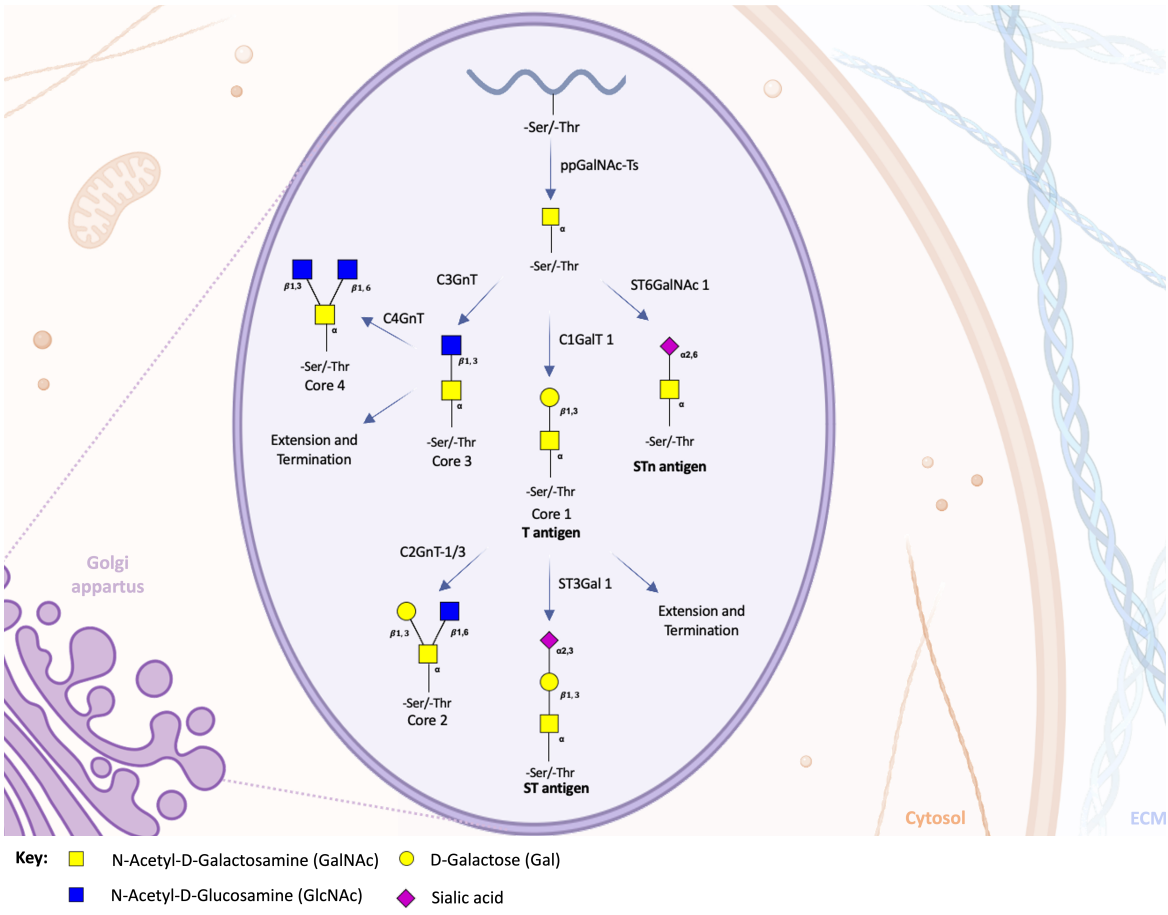


Figure 2 - **Pathways of *O*-Glycosylation.** In the Golgi apparatus, *O*-glycosylation is initiated by the addition of GalNAc residues to Ser/Thr residues on a protein, leading to the formation of the Tn antigen. This antigen can be further extended or modified by various enzymes, resulting in the generation of cores 1–4. Alternatively, Tn and T antigens can be sialylated by the sialyltransferases ST6GalNAc1 and ST3Gal1, respectively, resulting in the formation of shortened and truncated STn and ST structures.

2.2. Decoding the connection between glycosylation and cancer

The structure of glycans, particularly the composition of the glycosidic moiety, has garnered significant attention given the fundamental role of glycans in biological systems. In fact, any deviation that occurs in glycan biosynthesis will result in abnormal glycan expression, such as (i) alteration in the epitopes of the terminal groups, (ii) modification in the substitution patterns of sialic acid and fucose residues, (iii) drastic reduction in *O*-gylcans elongation by oversialylation, and (iv) changes in the core fucosylation, sialylation,

and branching of *N*-glycans(69, 70). Consequently, a variety of pathological conditions may arise from this dysregulation of glycosylation, including congenital disorders of glycosylation (CDG)(71-73), infection(74, 75), chronic inflammation(76, 77), autoimmunity(78, 79) and oncogenic transformations(28, 37, 80). In the context of cancer, aberrant glycosylation occurs with greater frequency in tumour cells compared to healthy cells and is commonly associated with various advanced tumours. This represents a major source of molecular heterogeneity because it is protein-, cell- and site-specific and may result from either incomplete synthesis, which typically occurs at an early stage of carcinogenesis, or through de novo synthesis of neoantigens, which predominates at a later stage of tumour development(28, 81, 82).

The contribution of glycosylation becomes apparent across the diverse stages of tumour development and progression, underscoring its pivotal role in the intricate processes of cancer biology. The *O*-GlcNAc modification intricately sustains the relentless proliferation of cancer cells by orchestrating the regulation of crucial transcription factors like cMYC, cyclin D1, and forkhead protein M1 (FoxM1), leading to their stabilisation and thereby propelling oncogenesis(83, 84). Moreover, cancer-associated glycans(85-87), proteoglycans(88-90), and glycosyltransferases(91) adeptly shape the signalling pathways and functions of various growth factor receptors, such as EGFR, FGFR, PDGF, MET, ERBB2/HER2, and IGFR, fuelling the cascade of cell proliferation. For cancer cells to thrive, they must evade the mechanisms that ordinarily restrict cell proliferation, many of which hinge on tumour suppressor genes like p53 and retinoblastoma. These canonical proliferation suppressors, equipped with potential glycosylation sites, experience their functions modulated by the dynamic interplay of *O*-GlcNAcylation and phosphorylation(92, 93). Actually, *O*-GlcNAcylation of p53 can enhance tumour-suppressing capabilities by inhibiting its phosphorylation but also can stabilize gain-of-function mutant forms of p53 by magnifying their pro-oncogenic potential(94). Beyond this, malignant cells masterfully circumvent apoptosis by employing their glycosylation machinery to modify glycans present on cell death receptors, like Fas(95) and TRAIL-R(96), as well as their respective ligands, affecting receptor-ligand interactions(97), the formation of signalling complexes(98), and secretion of ligands from effector cells(99), allowing tumour cells to escape programmed death and continue thriving. Nevertheless, to excel in tumour development, malignant cells must surmount cell-cell adhesion barriers and invade surrounding tissues(100). Indeed, the upregulation of sialylated glycan structures, frequently linked to malignant transformation, enables invasion and metastasis by inducing the dissociation of primary tumour cells through electrostatic repulsion of negative charges, ultimately impairing cell-cell adhesion(101, 102). Besides, aberrant glycosylation can profoundly impact proteins

involved in cell adhesion, such as E-cadherin, by altering their function and localization, thereby contributing to invasion(103, 104). Conversely, specific glycosidic modifications, particularly the overexpression of sialyl lewis A (SLe^A) and sialyl lewis X (SLeX) antigens, enhance tumour cell adhesion to the endothelium via interactions with selectins(68). This mechanism promotes the recruitment of malignant cells to blood vessels and their rolling on the endothelial surface, ultimately leading to the detection of circulating tumour cells (CTCs) in distant sites and promoting metastasis(105, 106). Concurrently, glycosylation wields a profound influence on angiogenesis by sculpting the activity of VEGF and its VEGFR receptors, which are indispensable for tumour progression(107). This nuanced modulation impacts critical aspects such as endothelial survival(108) and vascular permeability(109), sculpting the tumour's growth landscape. Ultimately, transformed cells must overcome immune surveillance through interactions involving tumour-associated glycans and molecules present in immune cells to significantly undermine the immune system's antitumor response and foster the emergence of resistant cancer cells(110), a topic we will explore in greater depth further.

In this way, malignant cells acquire an invasive phenotype following changes in glycosylation that confer the ability to influence cell-cell and cell-extracellular matrix contact interactions, and to activate specific carbohydrate receptors that easily modify immunogenicity and antigenicity, creating an immunosuppressive environment that favours cell growth, progression, and tumour metastasis(100, 111, 112). Among the different abnormal glycan structures present in cancer cells, the short-chain *O*-glycans, namely the Tn and STn antigens stand out since they have been identified in almost all types of human carcinomas(113, 114). Hence, the following section will address the premature stop of *O*-glycan elongation to enlighten its implications in the context of pancarcinoma.

2.3. Intricate patterns of short-chain *O*-glycans in cancer

The aberrant glycophenotype frequently associated with oncogenic transformation is the expression of the most immature *O*-glycan structures, namely the Tn and STn antigens. These structures are commonly observed in the early stages of carcinogenesis in adenocarcinomas, being present in epithelial tumour cells and various epithelial preneoplastic lesions(115, 116). Accordingly, the abnormal expression of truncated *O*-glycans underlies some of the following events in the biosynthetic pathway: i) disorganization or relocation of organelles belonging to the secretory pathway of tumour cells, ii) hypermethylation or somatic mutation in Cosmc, the gene encoding the molecular chaperone T-synthase, iii) intracellular environmental effects, such as pH fluctuations, and

iv) altered or even absent expression of glycosyltransferases(63, 67, 117, 118). Therefore, it is necessary to explore the molecular mechanisms underlying the abnormal expression of these tumour-associated glycans and understand the potential biological roles that these glycans play throughout the carcinogenesis process.

The Tn antigen, also recognised as the simplest *O*-glycan, has a rare and restricted expression in normal tissues but is highly expressed in several solid epithelial tumour tissues, around 70-90%, and can be found in colorectal, pancreatic, breast, bladder, cervix, lung, gastric, ovarian and prostate cancers(40, 119, 120). Additionally, the overexpression of this antigen is intricately associated with a substantial decline in patient survival rates and a notably adverse clinical prognosis(119, 121). Although the impact of the Tn antigen on the carcinogenic process and its underlying mechanisms is still unclear, recent data suggests that this antigen is involved in various hallmarks of cancer, thereby playing a contributory role in the metastatic cascade. Indeed, some studies into colorectal cancer have demonstrated that the overexpression of this simple short-chain *O*-glycan can induce the epithelial-mesenchymal transition of cancer cells, thereby facilitating metastasis(122). Moreover, both *in vitro* and *in vivo* studies have revealed a marked increase in cell proliferation in hepatocellular carcinoma linked to the overexpression of the C1GalT-1 enzyme. Subsequently, in breast cancer, elevated Tn antigen expression has been found to promote cell migration and invasion when GalNAc-Ts are relocated from the Golgi to the ER(91). Likewise, in pancreatic cancer, hypermethylation of COSMC, leading to aberrant Tn antigen expression, has been linked to increased invasiveness and migration of tumour cells by inducing epithelial-mesenchymal transition and acquiring stem cell-like properties(123).

Similarly, the expression of the STn antigen in healthy tissues is low or practically non-existent, in contrast to the high expression seen in more than 80% of human carcinomas, including oesophageal, pancreatic, breast, lung, gastric, ovary, colon, and endometrium(124, 125). It is also well-known that the high tumoral expression of this particular antigen is strongly involved in tumour progression, contributing to metastasis and, consequently, to the poor prognosis and worse overall survival of patients diagnosed with these diverse types of cancer(60, 125-127). Furthermore, STn has also been aberrantly reported in a variety of benign epithelial lesions regarded as a predisposing of malignancy, including gastric intestinal metaplasia, atypical oedematous hyperplasia of the lung, dysphasic squamous epithelia of the oesophagus, ductal hyperplasia of the breast and apocrine metaplasia or moderate dysplasia of the colon, suggesting that it commonly occurs in the early stages of carcinogenesis(128). The overexpression of STn has demonstrating

to be pivotal in the acquisition of a more malignant phenotype, accompanied by numerous profound modifications in cell behaviour associated with the process of carcinogenesis. In line with this, studies focusing on gastric cancer showed that aberrant expression of STn can induce phenotypic alterations in tumour cells, enhancing their motility and invasive potential(129). Following this, the overexpression of the STn antigen in gastric tumour cells was linked to a marked reduction in homotypic cell-cell aggregation, coupled with enhanced adhesion to extracellular matrix (ECM) components such as collagen and fibronectin, thereby fostering the transition of the primary tumour by increasing the dissemination of cancer cells(130). Consistently, aberrant STn expression increases migration and invasion in bladder(103) and breast(131) carcinomas in an ST6GalNAc-I-dependent manner while reducing cell adhesion in prostate cancer(132). Owing to these acquired aggressive traits, STn-overexpressing cells are often situated at the leading edges of invasion, facilitating their entry into blood or lymphatic vessels and, consequently, driving the establishment of metastases(133, 134). Indeed, recent findings indicate that CTCs in the blood of patients with metastatic bladder cancer predominantly display an undifferentiated, aggressive basal phenotype coupled with elevated STn antigen expression(134). This characteristic suggests that the STn antigen may provide bladder cancer cells with a noteworthy advantage, enhancing both their invasive potential and the mechanisms crucial for effective cancer dissemination. On top of this, the STn present in tumour cells is recognized to play a crucial role in establishing tolerogenic phenotypes of innate and adaptive immunity cells, namely for jeopardizing the maturation of dendritic cells and, in turn, impacting the response triggered by anti-tumour T cells, leading concomitantly to the secretion of anti-inflammatory cytokines in the tumour microenvironment(135). In summary, the expression of STn contributes to reduced cell-cell aggregation, enhanced adhesion to the ECM, and increased tumour cells' invasion and migration (45), all contributing to metastasis.

The collective insights from these studies elevate the glycome from a mere participant to a central orchestrator in cancer progression and dissemination, intricately interwoven with the established hallmarks of cancer. These revelations not only enrich our understanding of the intricate nature of the disease but also highlight the critical role of the glycome in driving the aggressive behaviour of tumours. However, to fully comprehend the scope of glycosylation influence, it is necessary to shift our focus toward the immune system, the primary defence against malignancy. In this way, it becomes imperative to explore how glycosylation interacts with immune components within the landscape of tumour progression.

3. UNVEILING THE DYNAMICS BETWEEN GLYCANS AND THE IMMUNE SYSTEM IN CANCER

Throughout the preceding years, research has demonstrated that the distribution, density, and changes in the composition of glycans present in secreted proteins or on the cell surface of tumour cells are responsible for the existence of molecular fingerprints unique to cancer(70, 136-138). Despite the considerable focus on these glycoproteins due to their extreme role in tumour aggressiveness, it is important not to neglect their interaction with the immune system. Given the ability of innate and adaptive immune cells to infiltrate the tumour microenvironment (TME), the immune system plays a decisive role in immunosurveillance and in guiding tumour progression(139-141). Immune cells, particularly antigen-presenting cells (APCs), possess the ability to detect aberrant glycan structures expressed by tumour cells through GBPs, including galectins, C-type lectins, and sialic acid-binding immunoglobulin-like lectins (siglecs), thereby triggering immunomodulatory or immunostimulatory responses that ultimately favour tumour progression(142, 143). Particularly, galectins, soluble molecules secreted via non-classical pathways(144), are crucial in orchestrating both innate and adaptive immunity through their interaction with glycosylated ligands(145). Found across a wide range of immune cells, they exert profound tolerogenic effects on effector T cells, such as $CD8^+$ T cells, leading to a cytokine imbalance that can induce T cell anergy or enhance the activity of regulatory T cells (Tregs)(146, 147). Beyond modulating adaptive immunity, galectins are also implicated in the apoptosis of circulating tumour lymphocytes and in undermining the anti-tumour functions of natural killer (NK) cells, thereby contributing to immune evasion and tumour progression(148). Meanwhile, C-type lectins, a class of calcium-dependent receptors distinguished by their carbohydrate recognition domains, are pivotal in immune regulation(149). Particularly notable as standardized receptors for dendritic cells (DCs) and macrophages, these lectins are responsible not only for promoting leukocyte adhesion to the vascular endothelium but also for inducing cellular cytotoxicity within the TME(150-152). This is primarily achieved through the modulation of cytokine profiles, where a reduction in IL-12 production and an elevation in IL-10 lead to compromised DCs competencies(153). Consequently, this impairment manifests through spontaneous apoptosis or defective antigen presentation, thereby diminishing the efficacy of the immune response against cancer cells(154). In turn, siglecs, a unique class of immunoglobulin-type lectins, exhibit a strong affinity to recognise glycans that specifically have sialic acid attached to immunoregulatory sequences and are broadly expressed across immune and hematopoietic systems(155, 156). When interacting with glycans overexpressed in tumour cells, siglecs play an immunosuppressive role,

leading to a significant reduction in the protective immune responses orchestrated by neutrophils and NK cells(157, 158), hindering the expansion of $CD8^+$ and $CD4^+$ effector T cells and fostering the proliferation of Tregs and tumour-associated macrophages (TAMs), and controlling over the tolerogenic functions of DCs, further facilitating immune evasion and tumour progression(159, 160).

Recent research has extensively explored and documented the interactions between glycans and various immune cells, especially T cells(161). However, there remains a significant gap in fully understand how altered glycans on tumour cells interact with certain immune cells, especially macrophages given their vital function in immunological response. In the forthcoming section, we will delve deeply into the role of macrophages in this context, aiming to uncover critical insights that could pave the way for enhancing therapeutic strategies and advancing cancer treatment.

3.1. Macrophages-glycans interplay driving suppressive microenvironment

Within the context of the tumour microenvironment, macrophages stand out for playing an indispensable role in innate immunity recognised for their involvement in the body's homeostasis and in various physiological processes, including recognition and elimination of external threats, tissue repair and remodelling, elimination of damaged, exhausted, or senescent cells, and metabolic function(162-164). Notably, macrophages exhibit remarkable plasticity and can be categorised into two main subpopulations: classically activated macrophage or M1 and alternatively activated macrophage or M2(165, 166). Based on their involved role in the various functions of the biological system, both M1 and M2 macrophages can infiltrate tumour tissues or populate the microenvironment of solid tumours, being defined as TAMs, thereby affecting tumour growth, metastasis, angiogenesis, chemoresistance, and immune regulation(167, 168).

Given these premises, the task of detecting and responding to the glycosignatures present in the environment by the GBPs has been recognised as an essential element in shaping the development, function, and immune dynamics of macrophages(169) **(Figure 3)**. Part of the C-type lectin family, the macrophage galactose lectin (MGL) is widely expressed in macrophages and dendritic cells with the ability to recognize glycoconjugates, such as the Tn and STn antigens, as mentioned above(170, 171). Indeed, studies on pancreatic ductal adenocarcinomas have elucidated the involvement of the Tn antigen in

the activation of this receptor, along with the activation of the dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin (DC-SIGN) receptor by fucosylated antigens in TAMs, resulting in elevated IL-10 production and reduced IL-6 levels, orchestrating a tolerogenic tumour microenvironment that impairs the initiation a robust anti-tumour response(172). Meanwhile, emerging research has identified Dectin-1, a C-type lectin receptor expressed in macrophages, as a key player in reprogramming TAMs to an M1 phenotype, thereby boosting the activation of effector T cells and affecting the metabolic reprogramming of macrophages(173). Additionally, Dectin-1 activation via the Syk-JNK-AP-1 signaling pathway and complement receptor 3 (CR3) triggers inflammatory responses involving TNF and IL-6(174) while also promoting phagocytosis, antigen presentation, and the production of reactive oxygen species (ROS) and IL-1β(175). In turn, the C-type lectin mannose receptor, more commonly known as CD206, an anti-inflammatory marker typical of M2 macrophages, participates in phagocytosis, cytokine production, cross-presentation of antigens, and endocytosis in macrophages. The research underscores the central role of CD206 in binding to carbohydrates, such as STn and mucins present in the TME, ultimately fostering tumour growth and immunosuppression(176, 177).

Regarding galectins, these molecules hold the power to reprogramme the destiny and capabilities of myeloid cells by activating various tolerogenic pathways, such as galectin-1, which can promote the induction of M2-type macrophages(178), and galectin-9, which facilitates the expansion of myeloid-derived suppressor cells(179). Besides, in a pathological scenario, galectins can also favour pro-inflammatory functions, exemplified by galectin-3's role in modulating the differentiation, survival, and recruitment and effector functions of mast cells, macrophages, neutrophils, and T cells, through broad pro-inflammatory effects(180). Accordingly, the harmonious collaboration between the various members of the galectin family, capable of transmitting both pro- and anti-inflammatory signals, may be essential to guide the inflammatory cascade in different pathophysiological conditions.

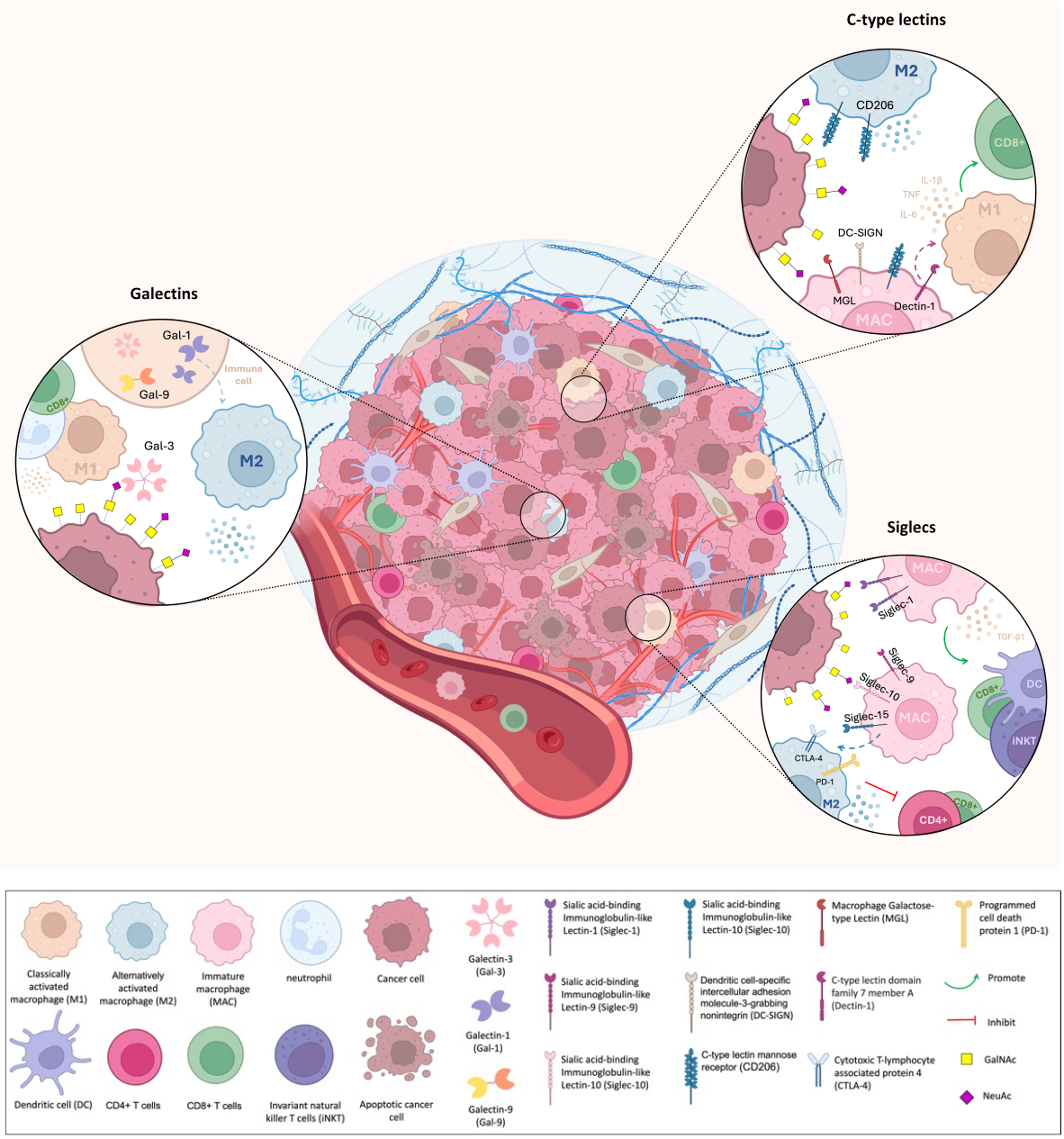


Figure 3 – Mechanisms of immune recognition mediated by glycan-binding receptors. Glycans exposed on the surface of cancer cells can modulate macrophage states through engagement with GBPs. C-type lectins (CD206, DC-SIGN, MGL, Dectin-1) recognize specific glycans, influencing macrophage activation towards M1, which regulates inflammatory responses and activates effector T cells, or M2, which conversely enhances inhibitory signalling (such as anti-inflammatory cytokine production like IL-10) promoting a tolerogenic environment. Galectins (Gal-1, Gal-3, Gal-9) bind to β-galactoside glycans, modulating macrophage polarization and effector immune cell functions, and are essential in directing the inflammatory cascade under various pathophysiological conditions. Siglecs (Siglec-1, -9, -10, -15) recognize sialic acid-containing glycans, modulating macrophage activity, potentially promoting a robust adaptive immune response by activating iNKT and $CD8^+$ cells, or suppressing T cell responses, thus enabling immune evasion by cancer cells. This network of interactions mediated by GBPs on macrophages coordinates immune response regulation in the tumour microenvironment, influencing both immune responses and cancer progression.

Lastly, the focus shifts to the family of siglecs present on the surface of immune cells, when interacting with sialoglycans via cell-cell contact, can trigger changes in the adhesion properties and activation level of these immune cells(181). Recent studies suggest that activation of Siglec-1 can attenuate the innate immune response by enhancing the production of TGF-β1, stimulating the inflammatory responses of macrophages and augmenting phagocytosis(182-184). Moreover, it is established that Siglec-1 activation in macrophages facilitates the transfer of exogenous antigens to DCs, thereby promoting robust activation of invariant natural killer T cells (iNKT) and cytotoxic T cells ($CD8^+$), ultimately eliciting robust adaptive immune responses(185-187). Meanwhile, several studies have focused on Siglec-9, highlighting its ability to induce macrophage polarization towards an M2 phenotype and increased production of anti-inflammatory cytokines(188-191). Furthermore, a study on hepatocellular carcinoma revealed that, in samples from patients with poor survival outcomes, Siglec-10 was predominantly expressed in the majority of the macrophage population, which also exhibited anti-inflammatory and immunosuppressive markers such as cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) and death programmed 1 (PD-1)(192). More recently, a study demonstrated that TAMs in pancreatic ductal adenocarcinoma expressing Siglec-15, by binding to the STn antigen, not only promote the immunosuppressive polarization of M2 macrophages but also act as predominant suppressors of antigen-specific T cell responses mediated by macrophages, inhibiting the increase of $CD4^+$ T cells and the activity of $CD8^+$ T cells, thereby representing a key regulator of tumour progression(193).

To summarize, the intricate interaction between glycans covering tumour cells and GBPs in macrophages, and other immune cells, often generates responses that promote tumour progression, fostering the creation of a typically immunosuppressive microenvironment. Consequently, given the intricate nexus between immune cells and glycobiology, these GBPs constitute promising therapeutic targets, especially for patients who do not respond to current immunotherapy.

4. IMMUNOTHERAPY CHALLENGES AND NEW TARGET OPPORTUNITIES

The immune system engages in a complex and multifaceted manner with cancer cells, playing a dual role in safeguarding the host from uncontrolled cellular proliferation and, contrariwise, potentially contributing to malignancy. Understanding the protective functions of the immune system in its interplay with cancer cells is crucial for developing new and alternative therapeutic strategies. Within this framework, cancer immunotherapy has arisen as an anchor of oncological treatment, distinguished by its personalized, targeted, and safer approach compared to traditional methods(194) **(Figure 4)**. Accordingly, tumour vaccines have emerged as a promising therapy as they contain pre-defined or unknown antigens designed to control tumour growth, a process mediated by activated T cells through the direct destruction of cancer cells and cytokine-mediated processes. In reality, this process involves the uptake of antigens by APCs, which are activated and then migrate to the lymph nodes where they interact with T cells, which in turn migrate to the tumour site after being activated to perform their expected function(195, 196). However, despite the progress made with this therapy, the high cost combined with limited availability and non-significant results in ongoing clinical trials limit the number of patients who can benefit(197). As an alternative to traditional chemotherapy, small molecule drugs have emerged as a promising approach, designed to specifically target tumour cells while preserving normal cells and offering the advantages of low toxicity and high potency(198). Currently, eighty-nine anti-cancer small molecules have been developed, addressing various targets, including those associated with macrophages(198, 199). However, despite these advances, the use of small molecule inhibitors in cancer therapy continues to encounter significant challenges, such as low response rates, short duration of action, toxicity, and the emergence of resistance(200). Moreover, another promising therapeutic approach under extensive investigation is adoptive cell therapy, which aims to induce tumour regression by transferring specific immune cells to the tumour-bearing host(201). The strategy of this therapy consists of using cells from the patient or a donor, which are manipulated *in vitro* to enhance their effector functions, such as gene-modified T-cell receptors (TCR-Ts) and engineered chimeric antigen receptors (CAR-Ts)(201-203). Regardless of its potential, this T-cell-based therapy faces significant challenges, such as inadequate infiltration into solid tumours and the risk of severe cytokine release syndrome, which in some cases can be fatal, thus constituting critical limitations and must be carefully considered(204). To overcome these challenges, researchers are exploring an alternative approach involving modified macrophages with chimeric antigen receptors (CARMA), which has surprisingly demonstrated potent antitumor

activity in preclinical models(205). To counteract tumour development, the immune checkpoint blockade (ICB) stands out as a particularly significant strategy(206, 207). This therapy has arisen due to the relevance of immune checkpoints, including PD-1 and CTLA-4 expressed on T cells infiltrating the tumour microenvironment, proving to be ideal targets given their role as negative regulatory molecules of the adaptive immune response(208-211). Accordingly, immune checkpoint inhibitors (ICIs), composed of monoclonal antibodies directed against CTLA-4, the PD-1, and its ligand PD-L1, have greatly improved the treatment of various tumour types by re-establish an effective anti-tumour response of cytotoxic T cells(207, 208, 212, 213). Despite their effectiveness, the eligibility for ICIs remains limited among cancer patients, with a response rate of around 15% to 30% for the majority of solid tumours(214, 215). Besides, certain patients may develop immune-related adverse effects (irAEs) such as inflammation and autoimmune responses able to damage healthy tissues and organs, regardless of the effectiveness of the treatment against the tumour(216, 217). Notwithstanding, primary innate resistance to this type of treatment remains a significant obstacle that appears to be linked to the difficulty of T-cell infiltration or the inability to generate T-cells within the tumour microenvironment(218). Commonly termed cold tumours, these malignancies are characterized by their interference with the antigen presentation mechanism, APCs deficiency, difficulty in activating T cells, and even blocking anti-tumour T lymphocytes from entering the tumour microenvironment, alongside the absence of tumour antigens and impaired infiltration into the tumour site(219, 220). These adversities are intrinsically dependent on the tumour microenvironment(221) and probably amplified by the aberrant glycosylation of the tumour(222), a process known to profoundly influence cellular interactions, immune evasion, and overall tumorigenic behaviour, as described above.

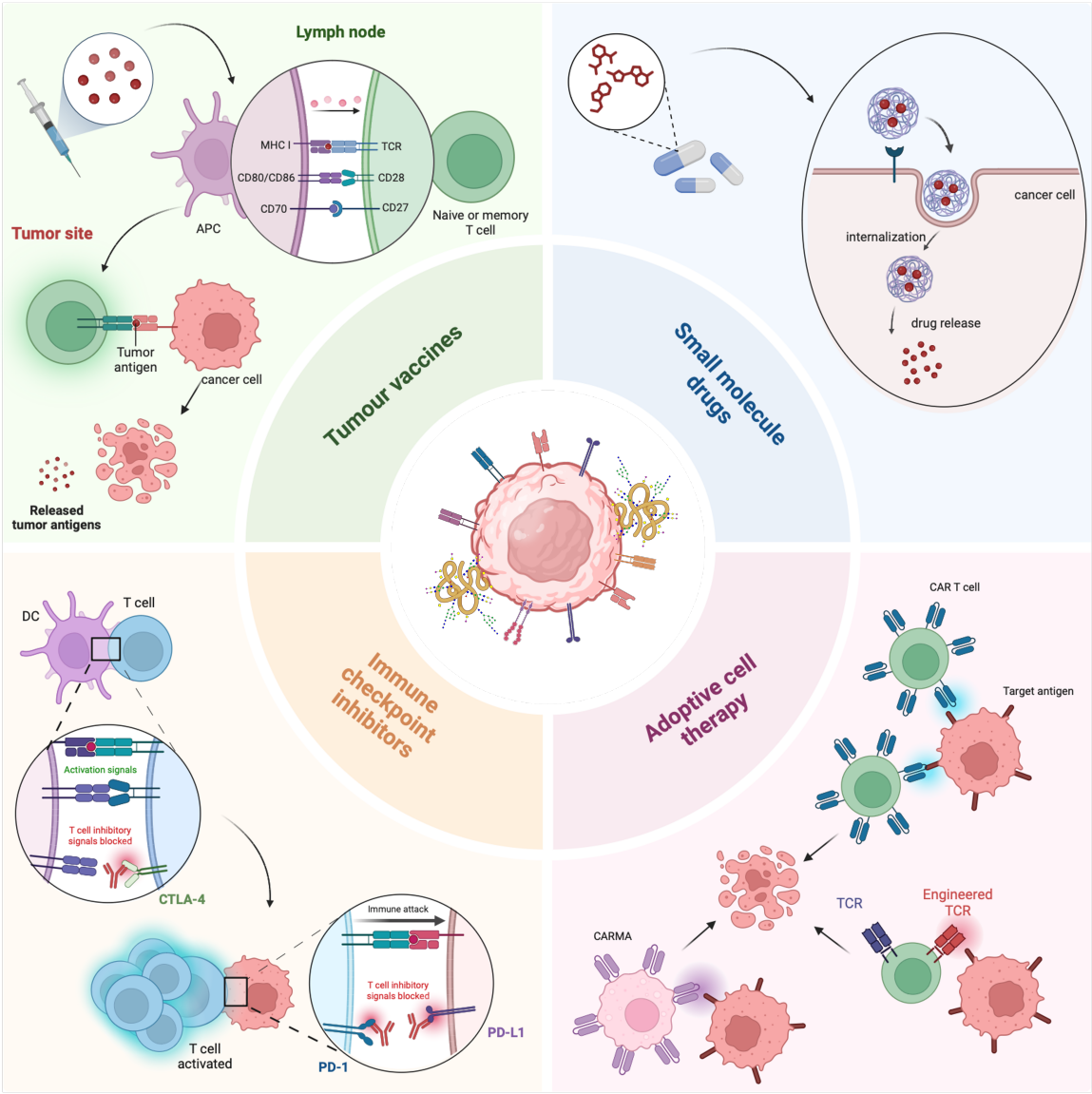


Figure 4 - Schematic representation of immunotherapies approaches. Currently, four modalities of immunotherapies have been developed to recognise specific proteins of cancer cells that serve as therapeutic targets in the treatment of cancer, demonstrating a way to overcome the immune evasion of the tumour. Tumour vaccines have been developed to release tumour antigens that are recognised by APCs and presented to naïve or memory T cells in the lymph nodes, transforming these T cells into cytotoxic T cells capable of recognising tumour antigens and inducing cancer cell apoptosis. Small molecule drugs are internalised by the tumour cells once they have been recognised, releasing a cytotoxic load that targets specific tumour survival and proliferation pathways. Adoptive cell therapy has been developed to take advantage of patient's lymphocytes, such as T cells and NK cells, and more recently macrophages, through in vitro engineering, to induce the expression of specific TCRs or CARs that trigger a persistent anti-tumour response. In turn, immune checkpoint inhibitors focus on blocking immune checkpoints, such as CTLA-4 and PD-1/PD-L1, through an antibody directed at these targets, thus preventing the tumour from suppressing immune responses and allowing T cells to remain active and attack tumour cells.

In this context, identifying new reliable targets and developing targeted therapies against tumours is imperative. Indeed, exploring targets within glycobiology appears to be a promising direction, given their pivotal role in tumour development(21). Specifically, the interactions between glycans and GBPs present in various immune cells warrant particular attention, as they represent central mechanisms in carcinogenesis, notably in the formation of immunosuppressive networks within the tumour microenvironment(223), as discussed previously. Regarding Siglecs, targeting Siglec-2 has been linked to markedly improved survival rates in human B-cell lymphoma(224). Additionally, Siglec-7 and Siglec-9 have emerged as promising candidates for cancer immunotherapy, with antibody-mediated blockade of these lectins resulting in significant reductions in tumour burden in both lung and ovarian cancers(225). Moreover, Siglec-15 has emerged as a noteworthy target for tumour-selective immunotherapy due to its role in indirectly inhibiting T cell activation at the tumour site by modulating IL-10 production from TAMs(193). Its selective up-regulation in human non-small cell lung cancer cells further underscores its potential as an ideal candidate for targeted therapeutic strategies. Likewise, certain galectins, such as galectin-1 and galectin-9, have emerged as promising therapeutic targets. Inhibiting galectin-1 has been shown to reduce tumour progression and mast cell death in breast and colon carcinomas while enhancing the infiltration of tumour-infiltrating lymphocytes across various cancers, including pancreatic adenocarcinoma, glioblastoma, lung cancer, melanoma, and breast cancer(226-228). Additionally, targeting galectin-9 with specific antibodies has demonstrated potential in preclinical models, as this galectin is linked to T-cell apoptosis and poor prognosis in several human malignancies(229). These insights collectively underscore the importance of delving deeply into the interactions between glycans and the immune system to pave the way for the next generation of immunotherapies. It is crucial to select suitable experimental models to unravel this complex interplay fully, as this will inform the development of targeted therapies and guide preclinical research efforts.

Previous research has highlighted the critical role of aberrant glycosylation in immune evasion, particularly the impact of tumour-expressed antigens on immune cell populations, such as dendritic cells, which can lead to an immunosuppressive tumour microenvironment characterized by the persistence of antigen-presenting cells in their immature state. Building on this, this project attempts to explore how abnormal expression of cancer-associated glycans affects other antigen-presenting cells, notably macrophages, and identify a potential key receptor involved in this crosstalk that could serve as potential therapeutic target in future treatments.

5. REFERENCES

1. Stratton MR, Campbell PJ, Futreal PA. The cancer genome. Nature. 2009;458(7239):719-24.
2. Brown JS, Amend SR, Austin RH, Gatenby RA, Hammarlund EU, Pienta KJ. Updating the Definition of Cancer. Mol Cancer Res. 2023;21(11):1142-7.
3. Bray F, Laversanne M, Sung H, Ferlay J, Siegel RL, Soerjomataram I, et al. Global cancer statistics 2022: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin. 2024;74(3):229-63.
4. Schwartz SM. Epidemiology of Cancer. Clin Chem. 2024;70(1):140-9.
5. Wu S, Zhu W, Thompson P, Hannun YA. Evaluating intrinsic and non-intrinsic cancer risk factors. Nat Commun. 2018;9(1):3490.
6. Cavalcanti IDL, Soares JCS. Conventional Cancer Treatment. Advances in Cancer Treatment: From Systemic Chemotherapy to Targeted Therapy. Cham: Springer International Publishing; 2021. p. 29-56.
7. Krzyszczyk P, Acevedo A, Davidoff EJ, Timmins LM, Marrero-Berrios I, Patel M, et al. The growing role of precision and personalized medicine for cancer treatment. Technology (Singap World Sci). 2018;6(3-4):79-100.
8. Crosby D, Bhatia S, Brindle KM, Coussens LM, Dive C, Emberton M, et al. Early detection of cancer. Science. 2022;375(6586):eaay9040.
9. Debela DT, Muzazu SG, Heraro KD, Ndalama MT, Mesele BW, Haile DC, et al. New approaches and procedures for cancer treatment: Current perspectives. SAGE Open Med. 2021;9:20503121211034366.
10. Advancing Cancer Therapy. Nature Cancer. 2021;2(3):245-6.
11. Szeto GL, Finley SD. Integrative Approaches to Cancer Immunotherapy. Trends Cancer. 2019;5(7):400-10.
12. Wang X, Fan S, Pan H, Chen W, Wang H. Cancer immunotherapy for metastasis: past, present and future. Brief Funct Genomics. 2019;18(2):140-6.
13. Duan Z, Luo Y. Targeting macrophages in cancer immunotherapy. Signal Transduction and Targeted Therapy. 2021;6(1):127.
14. Liu Y-H, Zang X-Y, Wang J-C, Huang S-S, Xu J, Zhang P. Diagnosis and Management of Immune Related Adverse Events (irAEs) in Cancer Immunotherapy. Biomedicine & Pharmacotherapy. 2019;120:109437.
15. Kong J, Ha D, Lee J, Kim I, Park M, Im S-H, et al. Network-based machine learning approach to predict immunotherapy response in cancer patients. Nature Communications. 2022;13(1):3703.

16. The expanding palette of immunotherapy research. Nature Cancer. 2022;3(6):651-.
17. Hegde PS, Chen DS. Top 10 Challenges in Cancer Immunotherapy. Immunity. 2020;52(1):17-35.
18. Diniz F, Coelho P, Duarte HO, Sarmento B, Reis CA, Gomes J. Glycans as Targets for Drug Delivery in Cancer. Cancers (Basel). 2022;14(4).
19. Peixoto A, Ferreira D, Azevedo R, Freitas R, Fernandes E, Relvas-Santos M, et al. Glycoproteomics identifies HOMER3 as a potentially targetable biomarker triggered by hypoxia and glucose deprivation in bladder cancer. J Exp Clin Cancer Res. 2021;40(1):191.
20. Munkley J, Elliott DJ. Hallmarks of glycosylation in cancer. Oncotarget. 2016;7(23):35478-89.
21. Thomas D, Rathinavel AK, Radhakrishnan P. Altered glycosylation in cancer: A promising target for biomarkers and therapeutics. Biochim Biophys Acta Rev Cancer. 2021;1875(1):188464.
22. Saad AA. Targeting cancer-associated glycans as a therapeutic strategy in leukemia. All Life. 2022;15(1):378-433.
23. Thurin M. Tumor-Associated Glycans as Targets for Immunotherapy: The Wistar Institute Experience/Legacy. Monoclon Antib Immunodiagn Immunother. 2021;40(3):89-100.
24. Mereiter S, Balmaña M, Campos D, Gomes J, Reis CA. Glycosylation in the Era of Cancer-Targeted Therapy: Where Are We Heading? Cancer Cell. 2019;36(1):6-16.
25. Choi Y, Kim J, Chae J, Hong J, Park J, Jeong E, et al. Surface glycan targeting for cancer nano-immunotherapy. J Control Release. 2022;342:321-36.
26. Scott E, Elliott DJ, Munkley J. Tumour associated glycans: A route to boost immunotherapy? Clinica Chimica Acta. 2020;502:167-73.
27. Silsirivanit A. Chapter Five - Glycosylation markers in cancer. In: Makowski GS, editor. Advances in Clinical Chemistry. 89: Elsevier; 2019. p. 189-213.
28. Pinho SS, Reis CA. Glycosylation in cancer: mechanisms and clinical implications. Nat Rev Cancer. 2015;15(9):540-55.
29. Schjoldager KT, Narimatsu Y, Joshi HJ, Clausen H. Global view of human protein glycosylation pathways and functions. Nat Rev Mol Cell Biol. 2020;21(12):729-49.
30. Illiano A, Pinto G, Melchiorre C, Carpentieri A, Faraco V, Amoresano A. Protein Glycosylation Investigated by Mass Spectrometry: An Overview. Cells. 2020;9(9).
31. Moremen KW, Tiemeyer M, Nairn AV. Vertebrate protein glycosylation: diversity, synthesis and function. Nat Rev Mol Cell Biol. 2012;13(7):448-62.
32. Smith BAH, Bertozzi CR. The clinical impact of glycobiology: targeting selectins, Siglecs and mammalian glycans. Nature Reviews Drug Discovery. 2021;20(3):217-43.

33. Reily C, Stewart TJ, Renfrow MB, Novak J. Glycosylation in health and disease. Nat Rev Nephrol. 2019;15(6):346-66.
34. Shivatare SS, Shivatare VS, Wong CH. Glycoconjugates: Synthesis, Functional Studies, and Therapeutic Developments. Chem Rev. 2022;122(20):15603-71.
35. Rodrigues JG, Balmaña M, Macedo JA, Poças J, Fernandes Â, de-Freitas-Junior JCM, et al. Glycosylation in cancer: Selected roles in tumour progression, immune modulation and metastasis. Cell Immunol. 2018;333:46-57.
36. Azevedo R, Silva AMN, Reis CA, Santos LL, Ferreira JA. In silico approaches for unveiling novel glycobiomarkers in cancer. J Proteomics. 2018;171:95-106.
37. Magalhães A, Duarte HO, Reis CA. The role of O-glycosylation in human disease. Mol Aspects Med. 2021;79:100964.
38. Varki A. Biological roles of glycans. Glycobiology. 2016;27(1):3-49.
39. Ohtsubo K, Marth JD. Glycosylation in cellular mechanisms of health and disease. Cell. 2006;126(5):855-67.
40. Chia J, Goh G, Bard F. Short O-GalNAc glycans: regulation and role in tumor development and clinical perspectives. Biochim Biophys Acta. 2016;1860(8):1623-39.
41. Lin B, Qing X, Liao J, Zhuo K. Role of Protein Glycosylation in Host-Pathogen Interaction. Cells. 2020;9(4).
42. Thompson AJ, de Vries RP, Paulson JC. Virus recognition of glycan receptors. Curr Opin Virol. 2019;34:117-29.
43. Marth JD, Grewal PK. Mammalian glycosylation in immunity. Nature Reviews Immunology. 2008;8(11):874-87.
44. Pinho SS, Alves I, Gaifem J, Rabinovich GA. Immune regulatory networks coordinated by glycans and glycan-binding proteins in autoimmunity and infection. Cellular & Molecular Immunology. 2023;20(10):1101-13.
45. Ferreira JA, Magalhães A, Gomes J, Peixoto A, Gaiteiro C, Fernandes E, et al. Protein glycosylation in gastric and colorectal cancers: Toward cancer detection and targeted therapeutics. Cancer Lett. 2017;387:32-45.
46. Shental-Bechor D, Levy Y. Effect of glycosylation on protein folding: a close look at thermodynamic stabilization. Proc Natl Acad Sci U S A. 2008;105(24):8256-61.
47. Stanley P, Moremen KW, Lewis NE, Taniguchi N, Aebi M. N-Glycans. In: Varki A, Cummings RD, Esko JD, Stanley P, Hart GW, Aebi M, et al., editors. Essentials of Glycobiology. Cold Spring Harbor (NY): Cold Spring Harbor Laboratory Press
Copyright © 2022 The Consortium of Glycobiology Editors, La Jolla, California; published by Cold Spring Harbor Laboratory Press; doi:10.1101/glycobiology.4e.9. All rights reserved.; 2022. p. 103-16.
48. Hirata T, Kizuka Y. N-Glycosylation. Adv Exp Med Biol. 2021;1325:3-24.

49. Mikolajczyk K, Kaczmarek R, Czerwinski M. How glycosylation affects glycosylation: the role of N-glycans in glycosyltransferase activity. Glycobiology. 2020;30(12):941-69.
50. Bieberich E. Synthesis, Processing, and Function of N-glycans in N-glycoproteins. Adv Neurobiol. 2014;9:47-70.
51. Fisher P, Thomas-Oates J, Wood AJ, Ungar D. The N-Glycosylation Processing Potential of the Mammalian Golgi Apparatus. Front Cell Dev Biol. 2019;7:157.
52. Lin Y, Lubman DM. The role of N-glycosylation in cancer. Acta Pharm Sin B. 2024;14(3):1098-110.
53. Hu M, Zhang R, Yang J, Zhao C, Liu W, Huang Y, et al. The role of N-glycosylation modification in the pathogenesis of liver cancer. Cell Death & Disease. 2023;14(3):222.
54. Wang S, Chen C, Gadi MR, Saikam V, Liu D, Zhu H, et al. Chemoenzymatic modular assembly of O-GalNAc glycans for functional glycomics. Nature Communications. 2021;12(1):3573.
55. Fu C, Zhao H, Wang Y, Cai H, Xiao Y, Zeng Y, et al. Tumor-associated antigens: Tn antigen, sTn antigen, and T antigen. Hla. 2016;88(6):275-86.
56. Van den Steen P, Rudd PM, Dwek RA, Opdenakker G. Concepts and principles of O-linked glycosylation. Crit Rev Biochem Mol Biol. 1998;33(3):151-208.
57. Mehta AY, Heimburg-Molinaro J, Cummings RD, Goth CK. Emerging patterns of tyrosine sulfation and O-glycosylation cross-talk and co-localization. Curr Opin Struct Biol. 2020;62:102-11.
58. de Las Rivas M, Lira-Navarrete E, Gerken TA, Hurtado-Guerrero R. Polypeptide GalNAc-Ts: from redundancy to specificity. Curr Opin Struct Biol. 2019;56:87-96.
59. Reis CA, Osorio H, Silva L, Gomes C, David L. Alterations in glycosylation as biomarkers for cancer detection. J Clin Pathol. 2010;63(4):322-9.
60. Ju T, Wang Y, Aryal RP, Lehoux SD, Ding X, Kudelka MR, et al. Tn and sialyl-Tn antigens, aberrant O-glycomics as human disease markers. Proteomics Clin Appl. 2013;7(9-10):618-31.
61. Oliveira-Ferrer L, Legler K, Milde-Langosch K. Role of protein glycosylation in cancer metastasis. Semin Cancer Biol. 2017;44:141-52.
62. Brockhausen I, Wandall HH, Hagen KGT, Stanley P. O-GalNAc Glycans. In: Varki A, Cummings RD, Esko JD, Stanley P, Hart GW, Aebi M, et al., editors. Essentials of Glycobiology. Cold Spring Harbor (NY): Cold Spring Harbor Laboratory Press
Copyright © 2022 The Consortium of Glycobiology Editors, La Jolla, California; published by Cold Spring Harbor Laboratory Press; doi:10.1101/glycobiology.4e.10. All rights reserved.; 2022. p. 117-28.

63. Yue S, Wang X, Ge W, Li J, Yang C, Zhou Z, et al. Deciphering Protein O-GalNAcylation: Method Development and Disease Implication. ACS Omega. 2023;8(22):19223-36.
64. Lee SH, Fukuda M. Study of the biological functions of mucin type core 3 O-glycans. Methods Mol Biol. 2013;1022:41-50.
65. Kudelka MR, Ju T, Heimburg-Molinaro J, Cummings RD. Simple sugars to complex disease--mucin-type O-glycans in cancer. Adv Cancer Res. 2015;126:53-135.
66. Thompson N, Wakarchuk W. O-glycosylation and its role in therapeutic proteins. Biosci Rep. 2022;42(10).
67. Azevedo R, Peixoto A, Gaiteiro C, Fernandes E, Neves M, Lima L, et al. Over forty years of bladder cancer glycobiology: Where do glycans stand facing precision oncology? Oncotarget. 2017;8(53):91734-64.
68. Hugonnet M, Singh P, Haas Q, von Gunten S. The Distinct Roles of Sialyltransferases in Cancer Biology and Onco-Immunology. Front Immunol. 2021;12:799861.
69. Mereiter S, Balmana M, Campos D, Gomes J, Reis CA. Glycosylation in the Era of Cancer-Targeted Therapy: Where Are We Heading? Cancer Cell. 2019;36(1):6-16.
70. Fernandes E, Sores J, Cotton S, Peixoto A, Ferreira D, Freitas R, et al. Esophageal, gastric and colorectal cancers: Looking beyond classical serological biomarkers towards glycoproteomics-assisted precision oncology. Theranostics. 2020;10(11):4903-28.
71. Francisco R, Brasil S, Poejo J, Jaeken J, Pascoal C, Videira PA, et al. Congenital disorders of glycosylation (CDG): state of the art in 2022. Orphanet J Rare Dis. 2023;18(1):329.
72. Paprocka J, Jezela-Stanek A, Tylki-Szymańska A, Grunewald S. Congenital Disorders of Glycosylation from a Neurological Perspective. Brain Sci. 2021;11(1).
73. Williams SE, Noel M, Lehoux S, Cetinbas M, Xavier RJ, Sadreyev RI, et al. Mammalian brain glycoproteins exhibit diminished glycan complexity compared to other tissues. Nature Communications. 2022;13(1):275.
74. Kreisman LS, Cobb BA. Infection, inflammation and host carbohydrates: A Glyco-Evasion Hypothesis. Glycobiology. 2012;22(8):1019-30.
75. Sackstein R, Hoffmeister KM, Stowell SR, Kinoshita T, Varki A, Freeze HH. Glycans in Acquired Human Diseases. In: Varki A, Cummings RD, Esko JD, Stanley P, Hart GW, Aebi M, et al., editors. Essentials of Glycobiology. Cold Spring Harbor (NY): Cold Spring Harbor Laboratory Press
Copyright © 2022 The Consortium of Glycobiology Editors, La Jolla, California; published by Cold Spring Harbor Laboratory Press; doi:10.1101/glycobiology.4e.46. All rights reserved.; 2022. p. 615-30.

76. Radovani B, Gudelj I. N-Glycosylation and Inflammation; the Not-So-Sweet Relation. Front Immunol. 2022;13:893365.
77. Brazil JC, Parkos CA. Finding the sweet spot: glycosylation mediated regulation of intestinal inflammation. Mucosal Immunology. 2022;15(2):211-22.
78. Pinho SS, Alves I, Gaifem J, Rabinovich GA. Immune regulatory networks coordinated by glycans and glycan-binding proteins in autoimmunity and infection. Cell Mol Immunol. 2023;20(10):1101-13.
79. Maverakis E, Kim K, Shimoda M, Gershwin ME, Patel F, Wilken R, et al. Glycans in the immune system and The Altered Glycan Theory of Autoimmunity: A critical review. Journal of Autoimmunity. 2015;57:1-13.
80. Wang M, Zhu J, Lubman DM, Gao C. Aberrant glycosylation and cancer biomarker discovery: a promising and thorny journey. Clin Chem Lab Med. 2019;57(4):407-16.
81. Hakomori S, Kannagi R. Glycosphingolipids as tumor-associated and differentiation markers. J Natl Cancer Inst. 1983;71(2):231-51.
82. Peixoto A, Relvas-Santos M, Azevedo R, Santos LL, Ferreira JA. Protein Glycosylation and Tumor Microenvironment Alterations Driving Cancer Hallmarks. Frontiers in Oncology. 2019;9.
83. Caldwell SA, Jackson SR, Shahriari KS, Lynch TP, Sethi G, Walker S, et al. Nutrient sensor O-GlcNAc transferase regulates breast cancer tumorigenesis through targeting of the oncogenic transcription factor FoxM1. Oncogene. 2010;29(19):2831-42.
84. Itkonen HM, Minner S, Guldvik IJ, Sandmann MJ, Tsourlakis MC, Berge V, et al. O-GlcNAc Transferase Integrates Metabolic Pathways to Regulate the Stability of c-MYC in Human Prostate Cancer Cells. Cancer Research. 2013;73(16):5277-87.
85. Rebbaa A, Yamamoto H, Saito T, Meuillet E, Kim P, Kersey DS, et al. Gene transfection-mediated overexpression of beta1,4-N-acetylglucosamine bisecting oligosaccharides in glioma cell line U373 MG inhibits epidermal growth factor receptor function. J Biol Chem. 1997;272(14):9275-9.
86. Yokoe S, Takahashi M, Asahi M, Lee SH, Li W, Osumi D, et al. The Asn418-Linked N-Glycan of ErbB3 Plays a Crucial Role in Preventing Spontaneous Heterodimerization and Tumor Promotion. Cancer Research. 2007;67(5):1935-42.
87. Liu Y-C, Yen H-Y, Chen C-Y, Chen C-H, Cheng P-F, Juan Y-H, et al. Sialylation and fucosylation of epidermal growth factor receptor suppress its dimerization and activation in lung cancer cells. Proceedings of the National Academy of Sciences. 2011;108(28):11332-7.
88. Iozzo RV, Sanderson RD. Proteoglycans in cancer biology, tumour microenvironment and angiogenesis. Journal of Cellular and Molecular Medicine. 2011;15(5):1013-31.

89. Kim S-H, Turnbull J, Guimond S. Extracellular matrix and cell signalling: the dynamic cooperation of integrin, proteoglycan and growth factor receptor. J Endocrinol. 2011;209(2):139-51.
90. Neill T, Schaefer L, Iozzo RV. Decoding the Matrix: Instructive Roles of Proteoglycan Receptors. Biochemistry. 2015;54(30):4583-98.
91. Wu Y-M, Liu C-H, Huang M-J, Lai H-S, Lee P-H, Hu R-H, et al. C1GALT1 Enhances Proliferation of Hepatocellular Carcinoma Cells via Modulating MET Glycosylation and Dimerization. Cancer Research. 2013;73(17):5580-90.
92. Wells L, Slawson C, Hart GW. The E2F-1 associated retinoblastoma-susceptibility gene product is modified by O-GlcNAc. Amino Acids. 2011;40(3):877-83.
93. Yang WH, Kim JE, Nam HW, Ju JW, Kim HS, Kim YS, et al. Modification of p53 with O-linked N-acetylglucosamine regulates p53 activity and stability. Nat Cell Biol. 2006;8(10):1074-83.
94. Fardini Y, Dehennaut V, Lefebvre T, Issad T. O-GlcNAcylation: A New Cancer Hallmark? Front Endocrinol (Lausanne). 2013;4:99.
95. Shatnyeva OM, Kubarenko AV, Weber CE, Pappa A, Schwartz-Albiez R, Weber AN, et al. Modulation of the CD95-induced apoptosis: the role of CD95 N-glycosylation. PLoS One. 2011;6(5):e19927.
96. Wagner KW, Punnoose EA, Januario T, Lawrence DA, Pitti RM, Lancaster K, et al. Death-receptor O-glycosylation controls tumor-cell sensitivity to the proapoptotic ligand Apo2L/TRAIL. Nat Med. 2007;13(9):1070-7.
97. Keppler OT, Peter ME, Hinderlich S, Moldenhauer G, Stehling P, Schmitz I, et al. Differential sialylation of cell surface glycoconjugates in a human B lymphoma cell line regulates susceptibility for CD95 (APO-1/Fas)-mediated apoptosis and for infection by a lymphotropic virus. Glycobiology. 1999;9(6):557-69.
98. Swindall AF, Bellis SL. Sialylation of the Fas death receptor by ST6Gal-I provides protection against Fas-mediated apoptosis in colon carcinoma cells. J Biol Chem. 2011;286(26):22982-90.
99. Schneider P, Bodmer JL, Holler N, Mattmann C, Scuderi P, Terskikh A, et al. Characterization of Fas (Apo-1, CD95)-Fas ligand interaction. J Biol Chem. 1997;272(30):18827-33.
100. Häuselmann I, Borsig L. Altered tumor-cell glycosylation promotes metastasis. Front Oncol. 2014;4:28.
101. Seidenfaden R, Krauter A, Schertzinger F, Gerardy-Schahn R, Hildebrandt H. Polysialic acid directs tumor cell growth by controlling heterophilic neural cell adhesion molecule interactions. Mol Cell Biol. 2003;23(16):5908-18.

102. Schultz MJ, Swindall AF, Bellis SL. Regulation of the metastatic cell phenotype by sialylated glycans. Cancer and Metastasis Reviews. 2012;31(3):501-18.
103. Peixoto A, Fernandes E, Gaiteiro C, Lima L, Azevedo R, Soares J, et al. Hypoxia enhances the malignant nature of bladder cancer cells and concomitantly antagonizes protein O-glycosylation extension. Oncotarget. 2016;7(39):63138-57.
104. Pinho SS, Reis CA, Paredes J, Magalhães AM, Ferreira AC, Figueiredo J, et al. The role of N-acetylglucosaminyltransferase III and V in the post-transcriptional modifications of E-cadherin. Human Molecular Genetics. 2009;18(14):2599-608.
105. Burdick MM, Henson KA, Delgadillo LF, Choi YE, Goetz DJ, Tees DF, et al. Expression of E-selectin ligands on circulating tumor cells: cross-regulation with cancer stem cell regulatory pathways? Frontiers in Oncology. 2012;2.
106. Reymond N, d'Água BB, Ridley AJ. Crossing the endothelial barrier during metastasis. Nature Reviews Cancer. 2013;13(12):858-70.
107. Lynch TP, Ferrer CM, Jackson SR, Shahriari KS, Vosseller K, Reginato MJ. Critical role of O-Linked β-N-acetylglucosamine transferase in prostate cancer invasion, angiogenesis, and metastasis. J Biol Chem. 2012;287(14):11070-81.
108. Kitazume S, Imamaki R, Ogawa K, Komi Y, Futakawa S, Kojima S, et al. Alpha2,6-sialic acid on platelet endothelial cell adhesion molecule (PECAM) regulates its homophilic interactions and downstream antiapoptotic signaling. J Biol Chem. 2010;285(9):6515-21.
109. Xu D, Fuster MM, Lawrence R, Esko JD. Heparan sulfate regulates VEGF165- and VEGF121-mediated vascular hyperpermeability. J Biol Chem. 2011;286(1):737-45.
110. Cadena AP, Cushman TR, Welsh JW. Chapter Four - Glycosylation and Antitumor Immunity. In: Galluzzi L, editor. International Review of Cell and Molecular Biology. 343: Academic Press; 2019. p. 111-27.
111. Grzesik K, Janik M, Hoja-Łukowicz D. The hidden potential of glycomarkers: Glycosylation studies in the service of cancer diagnosis and treatment. Biochim Biophys Acta Rev Cancer. 2023;1878(3):188889.
112. Freitas R, Peixoto A, Ferreira E, Miranda A, Santos LL, Ferreira JA. Immunomodulatory glycomedicine: Introducing next generation cancer glycovaccines. Biotechnol Adv. 2023;65:108144.
113. Munkley J, Elliott DJ. Hallmarks of glycosylation in cancer. Oncotarget. 2016;7(23).
114. Kudelka MR, Ju T, Heimburg-Molinaro J, Cummings RD. Chapter Three - Simple Sugars to Complex Disease—Mucin-Type O-Glycans in Cancer. In: Drake RR, Ball LE, editors. Advances in Cancer Research. 126: Academic Press; 2015. p. 53-135.
115. Radhakrishnan P, Dabelsteen S, Madsen FB, Francavilla C, Kopp KL, Steentoft C, et al. Immature truncated O-glycophenotype of cancer directly induces oncogenic features. Proc Natl Acad Sci U S A. 2014;111(39):E4066-75.

116. Festari MF, Jara E, Costa M, Iriarte A, Freire T. Truncated O-glycosylation in metastatic triple-negative breast cancer reveals a gene expression signature associated with extracellular matrix and proteolysis. Scientific Reports. 2024;14(1):1809.
117. Zeng J, Mi R, Wang Y, Li Y, Lin L, Yao B, et al. Promoters of Human Cosmc and T-synthase Genes Are Similar in Structure, Yet Different in Epigenetic Regulation. J Biol Chem. 2015;290(31):19018-33.
118. Pearce OMT. Cancer glycan epitopes: biosynthesis, structure and function. Glycobiology. 2018;28(9):670-96.
119. Matsumoto Y, Jia N, Heimburg-Molinaro J, Cummings RD. Targeting Tn-positive tumors with an afucosylated recombinant anti-Tn IgG. Scientific Reports. 2023;13(1):5027.
120. Zhang Y, Sun L, Lei C, Li W, Han J, Zhang J, et al. A Sweet Warning: Mucin-Type O-Glycans in Cancer. Cells. 2022;11(22).
121. Pinto D, Parameswaran R. Role of Truncated O-GalNAc Glycans in Cancer Progression and Metastasis in Endocrine Cancers. Cancers. 2023;15(13):3266.
122. Liu Z, Liu J, Dong X, Hu X, Jiang Y, Li L, et al. Tn antigen promotes human colorectal cancer metastasis via H-Ras mediated epithelial-mesenchymal transition activation. Journal of Cellular and Molecular Medicine. 2019;23(3):2083-92.
123. Thomas D, Sagar S, Caffrey T, Grandgenett PM, Radhakrishnan P. Truncated O-glycans promote epithelial-to-mesenchymal transition and stemness properties of pancreatic cancer cells. Journal of Cellular and Molecular Medicine. 2019;23(10):6885-96.
124. Ferreira JA, Videira PA, Lima L, Pereira S, Silva M, Carrascal M, et al. Overexpression of tumour-associated carbohydrate antigen sialyl-Tn in advanced bladder tumours. Mol Oncol. 2013;7(3):719-31.
125. Munkley J. The Role of Sialyl-Tn in Cancer. Int J Mol Sci. 2016;17(3):275.
126. Ito R, Nagao K, Ohtsubo K. Role of Sialyl-Tn Antigen in Cancer Metastasis. In: Furukawa K, Fukuda M, editors. Glycosignals in Cancer: Molecular Assembly and Recognition. Singapore: Springer Nature Singapore; 2023. p. 53-78.
127. Lugo R, Ávila-Nava A, García-Pérez R, Herrera-Escalante S, De la Cruz-Acosta J, Gutiérrez-Solis AL. Systematic review and meta-analysis of the clinical survival significance of Sialyl-Tn expression in histological tissues from cancer patients. Transl Cancer Res. 2020;9(2):547-55.
128. Julien S, Videira PA, Delannoy P. Sialyl-Tn in Cancer: (How) Did We Miss the Target? Biomolecules. 2012;2(4):435-66.
129. Freitas D, Campos D, Gomes J, Pinto F, Macedo JA, Matos R, et al. O-glycans truncation modulates gastric cancer cell signaling and transcription leading to a more aggressive phenotype. EBioMedicine. 2019;40:349-62.

130. Pinho S, Marcos NT, Ferreira B, Carvalho AS, Oliveira MJ, Santos-Silva F, et al. Biological significance of cancer-associated sialyl-Tn antigen: modulation of malignant phenotype in gastric carcinoma cells. Cancer Lett. 2007;249(2):157-70.
131. Julien S, Adriaenssens E, Ottenberg K, Furlan A, Courtand G, Vercoutter-Edouart AS, et al. ST6GalNAc I expression in MDA-MB-231 breast cancer cells greatly modifies their O-glycosylation pattern and enhances their tumourigenicity. Glycobiology. 2006;16(1):54-64.
132. Munkley J, Oltean S, Vodák D, Wilson BT, Livermore KE, Zhou Y, et al. The androgen receptor controls expression of the cancer-associated sTn antigen and cell adhesion through induction of ST6GalNAc1 in prostate cancer. Oncotarget. 2015;6(33):34358-74.
133. Neves M, Azevedo R, Lima L, Oliveira MI, Peixoto A, Ferreira D, et al. Exploring sialyl-Tn expression in microfluidic-isolated circulating tumour cells: A novel biomarker and an analytical tool for precision oncology applications. New Biotechnology. 2019;49:77-87.
134. Lima L, Neves M, Oliveira MI, Dieguez L, Freitas R, Azevedo R, et al. Sialyl-Tn identifies muscle-invasive bladder cancer basal and luminal subtypes facing decreased survival, being expressed by circulating tumor cells and metastases. Urologic Oncology: Seminars and Original Investigations. 2017;35(12):675.e1-.e8.
135. Carrascal MA, Severino PF, Guadalupe Cabral M, Silva M, Ferreira JA, Calais F, et al. Sialyl Tn-expressing bladder cancer cells induce a tolerogenic phenotype in innate and adaptive immune cells. Mol Oncol. 2014;8(3):753-65.
136. He K, Baniasad M, Kwon H, Caval T, Xu G, Lebrilla C, et al. Decoding the glycoproteome: a new frontier for biomarker discovery in cancer. Journal of Hematology & Oncology. 2024;17(1):12.
137. Cotton S, Ferreira D, Soares J, Peixoto A, Relvas-Santos M, Azevedo R, et al. Target Score-A Proteomics Data Selection Tool Applied to Esophageal Cancer Identifies GLUT1-Sialyl Tn Glycoforms as Biomarkers of Cancer Aggressiveness. Int J Mol Sci. 2021;22(4).
138. Fernandes E, Freitas R, Ferreira D, Soares J, Azevedo R, Gaiteiro C, et al. Nucleolin-Sle A Glycoforms as E-Selectin Ligands and Potentially Targetable Biomarkers at the Cell Surface of Gastric Cancer Cells. Cancers (Basel). 2020;12(4).
139. Zhang Y, Zhang Z. The history and advances in cancer immunotherapy: understanding the characteristics of tumor-infiltrating immune cells and their therapeutic implications. Cell Mol Immunol. 2020;17(8):807-21.
140. Ribatti D. The concept of immune surveillance against tumors. The first theories. Oncotarget. 2017;8(4):7175-80.

141. Kallingal A, Olszewski M, Maciejewska N, Brankiewicz W, Baginski M. Cancer immune escape: the role of antigen presentation machinery. Journal of Cancer Research and Clinical Oncology. 2023;149(10):8131-41.
142. Alves I, Fernandes Â, Santos-Pereira B, Azevedo CM, Pinho SS. Glycans as a key factor in self and nonself discrimination: impact on the breach of immune tolerance. FEBS Letters. 2022;596(12):1485-502.
143. Schnaar RL. Glycans and glycan-binding proteins in immune regulation: A concise introduction to glycobiology for the allergist. J Allergy Clin Immunol. 2015;135(3):609-15.
144. Popa SJ, Stewart SE, Moreau K. Unconventional secretion of annexins and galectins. Seminars in Cell & Developmental Biology. 2018;83:42-50.
145. Liu FT, Stowell SR. The role of galectins in immunity and infection. Nat Rev Immunol. 2023;23(8):479-94.
146. Rabinovich GA, Gabrilovich D, Sotomayor EM. Immunosuppressive Strategies that are Mediated by Tumor Cells. Annual Review of Immunology. 2007;25(Volume 25, 2007):267-96.
147. Liu F-T, Rabinovich GA. Galectins as modulators of tumour progression. Nature Reviews Cancer. 2005;5(1):29-41.
148. Rabinovich GA, Toscano MA. Turning 'sweet' on immunity: galectin–glycan interactions in immune tolerance and inflammation. Nature Reviews Immunology. 2009;9(5):338-52.
149. Drouin M, Saenz J, Chiffoleau E. C-Type Lectin-Like Receptors: Head or Tail in Cell Death Immunity. Frontiers in Immunology. 2020;11.
150. Raz A, Nakahara S. Biological Modulation by Lectins and Their Ligands in Tumor Progression and Metastasis. Anti-Cancer Agents in Medicinal Chemistry. 2008;8(1):22-36.
151. Fukuda M, Hiraoka N, Yeh J-C. C-Type Lectins and Sialyl Lewis X Oligosaccharides: Versatile Roles in Cell–Cell Interaction. Journal of Cell Biology. 1999;147(3):467-70.
152. Lavrsen K, Madsen CB, Rasch MG, Woetmann A, Ødum N, Mandel U, et al. Aberrantly glycosylated MUC1 is expressed on the surface of breast cancer cells and a target for antibody-dependent cell-mediated cytotoxicity. Glycoconjugate Journal. 2013;30(3):227-36.
153. RodrÍguez E, Schetters ST, van Kooyk Y. The tumour glyco-code as a novel immune checkpoint for immunotherapy. Nature Reviews Immunology. 2018;18(3):204-11.
154. Rughetti A, Pellicciotta I, Biffoni M, Bäckström M, Link T, Bennet EP, et al. Recombinant tumor-associated MUC1 glycoprotein impairs the differentiation and function of dendritic cells. The journal of Immunology. 2005;174(12):7764-72.

155. Läubli H, Nalle SC, Maslyar D. Targeting the Siglec-Sialic Acid Immune Axis in Cancer: Current and Future Approaches. Cancer Immunol Res. 2022;10(12):1423-32.
156. Daly J, Carlsten M, O'Dwyer M. Sugar Free: Novel Immunotherapeutic Approaches Targeting Siglecs and Sialic Acids to Enhance Natural Killer Cell Cytotoxicity Against Cancer. Frontiers in Immunology. 2019;10.
157. Jandus C, Boligan KF, Chijioke O, Liu H, Dahlhaus M, Démoulins T, et al. Interactions between Siglec-7/9 receptors and ligands influence NK cell–dependent tumor immunosurveillance. The Journal of clinical investigation. 2014;124(4):1810-20.
158. Läubli H, Pearce OM, Schwarz F, Siddiqui SS, Deng L, Stanczak MA, et al. Engagement of myelomonocytic Siglecs by tumor-associated ligands modulates the innate immune response to cancer. Proceedings of the National Academy of Sciences. 2014;111(39):14211-6.
159. Perdicchio M, Ilarregui JM, Verstege MI, Cornelissen LA, Schetters ST, Engels S, et al. Sialic acid-modified antigens impose tolerance via inhibition of T-cell proliferation and de novo induction of regulatory T cells. Proc Natl Acad Sci U S A. 2016;113(12):3329-34.
160. Jiang KY, Qi LL, Kang FB, Wang L. The intriguing roles of Siglec family members in the tumor microenvironment. Biomark Res. 2022;10(1):22.
161. Fernandes Â, Azevedo CM, Silva MC, Faria G, Dantas CS, Vicente MM, et al. Glycans as shapers of tumour microenvironment: A sweet driver of T-cell-mediated anti-tumour immune response. Immunology. 2023;168(2):217-32.
162. Wynn TA, Chawla A, Pollard JW. Macrophage biology in development, homeostasis and disease. Nature. 2013;496(7446):445-55.
163. Katkar G, Ghosh P. Macrophage states: there's a method in the madness. Trends in Immunology. 2023;44(12):954-64.
164. Zhang C, Yang M, Ericsson AC. Function of Macrophages in Disease: Current Understanding on Molecular Mechanisms. Front Immunol. 2021;12:620510.
165. Shapouri-Moghaddam A, Mohammadian S, Vazini H, Taghadosi M, Esmaeili SA, Mardani F, et al. Macrophage plasticity, polarization, and function in health and disease. J Cell Physiol. 2018;233(9):6425-40.
166. Long KB, Collier AI, Beatty GL. Macrophages: Key orchestrators of a tumor microenvironment defined by therapeutic resistance. Mol Immunol. 2019;110:3-12.
167. Lin Y, Xu J, Lan H. Tumor-associated macrophages in tumor metastasis: biological roles and clinical therapeutic applications. Journal of Hematology & Oncology. 2019;12(1):76.
168. Sadhukhan P, Seiwert TY. The role of macrophages in the tumor microenvironment and tumor metabolism. Seminars in Immunopathology. 2023;45(2):187-201.

169. Park DD, Chen J, Kudelka MR, Jia N, Haller CA, Kosaraju R, et al. Resident and elicited murine macrophages differ in expression of their glycomes and glycan-binding proteins. Cell Chem Biol. 2021;28(4):567-82.e4.
170. Tumoglu B, Keelaghan A, Avci FY. Tn antigen interactions of macrophage galactose-type lectin (MGL) in immune function and disease. Glycobiology. 2023;33(11):879-87.
171. Sletmoen M, Gerken TA, Stokke BT, Burchell J, Brewer CF. Tn and STn are members of a family of carbohydrate tumor antigens that possess carbohydrate-carbohydrate interactions. Glycobiology. 2018;28(7):437-42.
172. Rodriguez E, Boelaars K, Brown K, Madunić K, van Ee T, Dijk F, et al. Analysis of the glyco-code in pancreatic ductal adenocarcinoma identifies glycan-mediated immune regulatory circuits. Communications Biology. 2022;5(1):41.
173. Liu M, Luo F, Ding C, Albeituni S, Hu X, Ma Y, et al. Dectin-1 Activation by a Natural Product β-Glucan Converts Immunosuppressive Macrophages into an M1-like Phenotype. The Journal of Immunology. 2015;195(10):5055-65.
174. Huang J-H, Lin C-Y, Wu S-Y, Chen W-Y, Chu C-L, Brown GD, et al. CR3 and Dectin-1 Collaborate in Macrophage Cytokine Response through Association on Lipid Rafts and Activation of Syk-JNK-AP-1 Pathway. PLOS Pathogens. 2015;11(7):e1004985.
175. Goodridge HS, Reyes CN, Becker CA, Katsumoto TR, Ma J, Wolf AJ, et al. Activation of the innate immune receptor Dectin-1 upon formation of a 'phagocytic synapse'. Nature. 2011;472(7344):471-5.
176. Yang D, Yang L, Cai J, Hu X, Li H, Zhang X, et al. A sweet spot for macrophages: Focusing on polarization. Pharmacological Research. 2021;167:105576.
177. Allavena P, Chieppa M, Bianchi G, Solinas G, Fabbri M, Laskarin G, et al. Engagement of the mannose receptor by tumoral mucins activates an immune suppressive phenotype in human tumor-associated macrophages. Clin Dev Immunol. 2010;2010:547179.
178. Yu X, Qian J, Ding L, Yin S, Zhou L, Zheng S. Galectin-1: A Traditionally Immunosuppressive Protein Displays Context-Dependent Capacities. Int J Mol Sci. 2023;24(7).
179. Zhang CX, Huang DJ, Baloche V, Zhang L, Xu JX, Li BW, et al. Galectin-9 promotes a suppressive microenvironment in human cancer by enhancing STING degradation. Oncogenesis. 2020;9(7):65.
180. Liu F-T, Stowell SR. The role of galectins in immunity and infection. Nature Reviews Immunology. 2023;23(8):479-94.
181. Fraschilla I, Pillai S. Viewing Siglecs through the lens of tumor immunology. Immunological Reviews. 2017;276(1):178-91.

182. Wu Y, Lan C, Ren D, Chen GY. Induction of Siglec-1 by Endotoxin Tolerance Suppresses the Innate Immune Response by Promoting TGF-β1 Production. J Biol Chem. 2016;291(23):12370-82.
183. Phongsisay V. The immunobiology of Campylobacter jejuni: Innate immunity and autoimmune diseases. Immunobiology. 2016;221(4):535-43.
184. Karmakar J, Mukherjee K, Mandal C. Siglecs Modulate Activities of Immune Cells Through Positive and Negative Regulation of ROS Generation. Front Immunol. 2021;12:758588.
185. van Dinther D, Veninga H, Iborra S, Borg EGF, Hoogterp L, Olesek K, et al. Functional CD169 on Macrophages Mediates Interaction with Dendritic Cells for CD8(+) T Cell Cross-Priming. Cell Rep. 2018;22(6):1484-95.
186. Grabowska J, Lopez-Venegas MA, Affandi AJ, den Haan JMM. CD169+ Macrophages Capture and Dendritic Cells Instruct: The Interplay of the Gatekeeper and the General of the Immune System. Frontiers in Immunology. 2018;9.
187. Kawasaki N, Vela JL, Nycholat CM, Rademacher C, Khurana A, van Rooijen N, et al. Targeted delivery of lipid antigen to macrophages via the CD169/sialoadhesin endocytic pathway induces robust invariant natural killer T cell activation. Proc Natl Acad Sci U S A. 2013;110(19):7826-31.
188. Wang Y, He M, Zhang C, Cao K, Zhang G, Yang M, et al. Siglec-9(+) tumor-associated macrophages delineate an immunosuppressive subset with therapeutic vulnerability in patients with high-grade serous ovarian cancer. J Immunother Cancer. 2023;11(9).
189. Wu Y, Huang W, Xie Y, Wang C, Luo N, Chen Y, et al. Siglec-9, a Putative Immune Checkpoint Marker for Cancer Progression Across Multiple Cancer Types. Frontiers in Molecular Biosciences. 2022;9.
190. Beatson R, Tajadura-Ortega V, Achkova D, Picco G, Tsourouktsoglou T-D, Klausing S, et al. The mucin MUC1 modulates the tumor immunological microenvironment through engagement of the lectin Siglec-9. Nature Immunology. 2016;17(11):1273-81.
191. Beatson R, Graham R, Grundland Freile F, Cozzetto D, Kannambath S, Pfeifer E, et al. Cancer-associated hypersialylated MUC1 drives the differentiation of human monocytes into macrophages with a pathogenic phenotype. Communications Biology. 2020;3(1):644.
192. Xiao N, Zhu X, Li K, Chen Y, Liu X, Xu B, et al. Blocking siglec-10hi tumor-associated macrophages improves anti-tumor immunity and enhances immunotherapy for hepatocellular carcinoma. Experimental Hematology & Oncology. 2021;10(1):36.

193. Wang J, Sun J, Liu LN, Flies DB, Nie X, Toki M, et al. Siglec-15 as an immune suppressor and potential target for normalization cancer immunotherapy. Nature Medicine. 2019;25(4):656-66.
194. Gupta SL, Basu S, Soni V, Jaiswal RK. Immunotherapy: an alternative promising therapeutic approach against cancers. Mol Biol Rep. 2022;49(10):9903-13.
195. Saxena M, van der Burg SH, Melief CJM, Bhardwaj N. Therapeutic cancer vaccines. Nature Reviews Cancer. 2021;21(6):360-78.
196. Sellars MC, Wu CJ, Fritsch EF. Cancer vaccines: Building a bridge over troubled waters. Cell. 2022;185(15):2770-88.
197. Lin MJ, Svensson-Arvelund J, Lubitz GS, Marabelle A, Melero I, Brown BD, et al. Cancer vaccines: the next immunotherapy frontier. Nature Cancer. 2022;3(8):911-26.
198. Zhong L, Li Y, Xiong L, Wang W, Wu M, Yuan T, et al. Small molecules in targeted cancer therapy: advances, challenges, and future perspectives. Signal Transduction and Targeted Therapy. 2021;6(1):201.
199. Cabrales P. RRx-001 Acts as a Dual Small Molecule Checkpoint Inhibitor by Downregulating CD47 on Cancer Cells and SIRP-α on Monocytes/Macrophages. Translational Oncology. 2019;12(4):626-32.
200. Liu GH, Chen T, Zhang X, Ma XL, Shi HS. Small molecule inhibitors targeting the cancers. MedComm (2020). 2022;3(4):e181.
201. Rosenberg SA, Restifo NP. Adoptive cell transfer as personalized immunotherapy for human cancer. Science. 2015;348(6230):62-8.
202. Shao W, Yao Y, Yang L, Li X, Ge T, Zheng Y, et al. Novel insights into TCR-T cell therapy in solid neoplasms: optimizing adoptive immunotherapy. Experimental Hematology & Oncology. 2024;13(1):37.
203. Cappell KM, Kochenderfer JN. Long-term outcomes following CAR T cell therapy: what we know so far. Nature Reviews Clinical Oncology. 2023;20(6):359-71.
204. Sterner RC, Sterner RM. CAR-T cell therapy: current limitations and potential strategies. Blood Cancer Journal. 2021;11(4):69.
205. Klichinsky M, Ruella M, Shestova O, Lu XM, Best A, Zeeman M, et al. Human chimeric antigen receptor macrophages for cancer immunotherapy. Nature Biotechnology. 2020;38(8):947-53.
206. Bonifant CL, Burns WR. Chapter 10 - The major clinical components of cancer immunotherapy (modulating cell-mediated immune mechanisms). In: Niederhuber JE, editor. Clinical Immuno-Oncology. New Delhi: Elsevier; 2024. p. 195-222.e7.
207. Robert C. A decade of immune-checkpoint inhibitors in cancer therapy. Nature Communications. 2020;11(1):3801.

208. Kiyotani K, Toyoshima Y, Nakamura Y. Personalized immunotherapy in cancer precision medicine. Cancer Biol Med. 2021;18(4):955-65.
209. Meng L, Wu H, Wu J, Ding Pa, He J, Sang M, et al. Mechanisms of immune checkpoint inhibitors: insights into the regulation of circular RNAS involved in cancer hallmarks. Cell Death & Disease. 2024;15(1):3.
210. Babamohamadi M, Mohammadi N, Faryadi E, Haddadi M, Merati A, Ghobadinezhad F, et al. Anti-CTLA-4 nanobody as a promising approach in cancer immunotherapy. Cell Death & Disease. 2024;15(1):17.
211. Lin X, Kang K, Chen P, Zeng Z, Li G, Xiong W, et al. Regulatory mechanisms of PD-1/PD-L1 in cancers. Molecular Cancer. 2024;23(1):108.
212. Li T, Liu T, Zhu W, Xie S, Zhao Z, Feng B, et al. Targeting MDSC for Immune-Checkpoint Blockade in Cancer Immunotherapy: Current Progress and New Prospects. Clin Med Insights Oncol. 2021;15:11795549211035540.
213. Sangro B, Sarobe P, Hervás-Stubbs S, Melero I. Advances in immunotherapy for hepatocellular carcinoma. Nature Reviews Gastroenterology & Hepatology. 2021;18(8):525-43.
214. Pilard C, Ancion M, Delvenne P, Jerusalem G, Hubert P, Herfs M. Cancer immunotherapy: it's time to better predict patients' response. British Journal of Cancer. 2021;125(7):927-38.
215. Das S, Johnson DB. Immune-related adverse events and anti-tumor efficacy of immune checkpoint inhibitors. Journal for ImmunoTherapy of Cancer. 2019;7(1):306.
216. Conroy M, Naidoo J. Immune-related adverse events and the balancing act of immunotherapy. Nature Communications. 2022;13(1):392.
217. Ibis B, Aliazis K, Cao C, Yenyuwadee S, Boussiotis VA. Immune-related adverse effects of checkpoint immunotherapy and implications for the treatment of patients with cancer and autoimmune diseases. Front Immunol. 2023;14:1197364.
218. Wang MM, Coupland SE, Aittokallio T, Figueiredo CR. Resistance to immune checkpoint therapies by tumour-induced T-cell desertification and exclusion: key mechanisms, prognostication and new therapeutic opportunities. British Journal of Cancer. 2023;129(8):1212-24.
219. Cao LL, Kagan JC. Targeting innate immune pathways for cancer immunotherapy. Immunity. 2023;56(10):2206-17.
220. Haibe Y, El Husseini Z, El Sayed R, Shamseddine A. Resisting Resistance to Immune Checkpoint Therapy: A Systematic Review. Int J Mol Sci. 2020;21(17).
221. Wang L, Geng H, Liu Y, Liu L, Chen Y, Wu F, et al. Hot and cold tumors: Immunological features and the therapeutic strategies. MedComm (2020). 2023;4(5):e343.

222. Peixoto A, Relvas-Santos M, Azevedo R, Santos LL, Ferreira JA. Protein Glycosylation and Tumor Microenvironment Alterations Driving Cancer Hallmarks. Front Oncol. 2019;9:380.
223. RodrÍguez E, Schetters STT, van Kooyk Y. The tumour glyco-code as a novel immune checkpoint for immunotherapy. Nature Reviews Immunology. 2018;18(3):204-11.
224. Chen WC, Completo GC, Sigal DS, Crocker PR, Saven A, Paulson JC. In vivo targeting of B-cell lymphoma with glycan ligands of CD22. Blood. 2010;115(23):4778-86.
225. Ibarlucea-Benitez I, Weitzenfeld P, Smith P, Ravetch JV. Siglecs-7/9 function as inhibitory immune checkpoints in vivo and can be targeted to enhance therapeutic antitumor immunity. Proceedings of the National Academy of Sciences. 2021;118(26):e2107424118.
226. Jandus C, Boligan KF, Chijioke O, Liu H, Dahlhaus M, Démoulins T, et al. Interactions between Siglec-7/9 receptors and ligands influence NK cell-dependent tumor immunosurveillance. Journal of Clinical Investigation. 2014;124(4):1810-20.
227. Rubinstein N, Alvarez M, Zwirner NW, Toscano MA, Ilarregui JM, Bravo A, et al. Targeted inhibition of galectin-1 gene expression in tumor cells results in heightened T cell-mediated rejection: A potential mechanism of tumor-immune privilege. Cancer Cell. 2004;5(3):241-51.
228. Sundblad V, Morosi LG, Geffner JR, Rabinovich GA. Galectin-1: A Jack-of-All-Trades in the Resolution of Acute and Chronic Inflammation. The Journal of Immunology. 2017;199(11):3721-30.
229. Yang R, Sun L, Li C-F, Wang Y-H, Yao J, Li H, et al. Galectin-9 interacts with PD-1 and TIM-3 to regulate T cell death and is a target for cancer immunotherapy. Nature Communications. 2021;12(1):832.

CHAPTER II

AIMS AND SCOPES

Cancer remains a significant global health challenge, urging for constant and significant advances in its treatment. Over the last few decades, new and effective treatments have emerged, including the introduction of target therapies and immunotherapies with immune checkpoint inhibitors, enhancing the prospects of success, either alone or in combination with conventional therapeutic lines. However, the proportion of patients who benefit from this therapeutic scheme is relatively low. This emphasises the importance of identifying new viable targets and creating new therapeutic strategies that aim to overcome these challenges. Tumour cells face profound restructuring of the glycoproteome with the loss of *O*-glycosylation extension and overexpression of short-chain structures, resulting in the formation of unique molecular profiles on the cell surface. Given the absence or low abundance of these molecular fingerprints in virtually all normal tissues, a unique opportunity arises for designing highly precise and targeted therapeutic approaches against cancer. Indeed, the remarkable potential of cancer glycoepitopes essentially encompasses their immunosuppressive or low immunogenic nature, often culminating in adverse outcomes in most solid tumours. Notably, short-chain *O*-glycans, particularly the Tn and sialyl-Tn antigens, appear to be key regulators of the immune balance, capable of modulating immune responses in favour of tumour progression. This modulation is facilitated through several mechanisms, particularly the interaction between glycans and glycan-binding proteins on the extracellular surface of APCs, which triggers signalling pathways that promote an immunosuppressive environment. In light of these considerations, this work aims to elucidate how alterations in the glycosylation pattern of cancer cell proteins influence immune modulation, particularly in co-cultures with macrophages, assessing their polarization and reprogramming capabilities, as well as the profile of cytokines/chemokines produced. This work is also dedicated to exploring and identifying the receptors involved in the interaction between cancer cells and macrophages. Thus, the main goal is to expand knowledge about the immunomodulatory impact of short-chain *O*-glycans on APCs, especially macrophages, including the identification of the mediators involved in immune evasion, which will contribute to the development of future innovative immunotherapies. In line with these aims, this work comprehends the following specific objectives:

I. Decode the effect of Tn and STn overexpression by tumour cells on macrophage immune signature *in vitro* and tissue;
II. Explore the cytokine/chemokines mediated crosstalk between glycoengineered cancer cells and macrophages;
III. Identify receptors involved in the interaction between macrophage and glycoengineered cell lines by proteomic analysis.

These objectives are addressed in chapters III and IV of this dissertation. The chapters follow a research paper format, including an abstract, introduction, materials and methods, results, discussion, and conclusions. Chapter V provides concluding remarks and explores future perspectives.

CHAPTER III

DECODING THE IMPACT OF CANCER-ASSOCIATED SHORT-CHAIN O-GYLCANS ON MACROPRAGE IMMUNE SIGNATURE

ABSTRACT

One of the greatest challenges facing science remains in the field of oncology, where constant innovation is required to achieve better therapeutic results. In fact, the introduction of immunotherapies and checkpoint inhibitors has demonstrated considerable progress; however, many patients continue to face significant limitations or do not respond at all to these therapies, highlighting the urgent need to explore alternative targets to enhance cancer immune surveillance. In this way, tumour glycobiology has been explored in the overexpression of truncated short-chain *O*-glycans, namely the Tn and sialyl-Tn antigens, which create unique molecular profiles on the cell surface, frequently described as contributors to immune evasion and tumour progression.

In an attempt to better understand the role of short-chain *O*-glycan expression in cancer, we investigated the interaction between immature and differentiated macrophages, derived from peripheral blood mononuclear cells of healthy donors, and glycoengineered tumour models, including T24 *C1GALT1 KO*, AGS *C1GALT1 KO*, and T24 *C1GALT1 KO/ST6GALNAC1 KI*, which overexpress the Tn and STn glycans. Following a well-defined characterization of macrophage phenotypes through cell surface markers and cytokine production, it was possible to identify a shift towards an anti-inflammatory M2a/M2c phenotype after co-cultures, indicated by the high expression of CD206 and CD163 receptors, coupled with a marked reduction of antigen-presenting molecules (MHC-II) and costimulatory molecules (CD86). These findings were further supported by the presence of high levels of the inhibitory cytokine IL-10 in the conditioned media from the co-cultures. Additionally, human bladder and gastric cancer tissues Tn/STn-positive exhibited less macrophage infiltration. Collectively, the results suggest that Tn and STn glycans have the potential to polarize and reprogramme macrophages towards a pro-tumour phenotype, increasing the presence of a subset of immunosuppressive cells and impeding the recruitment of cytotoxic cells to the microenvironment.

Keywords: cancer**,** Tn, STn, macrophages, immunosuppressive microenvironment

1. INTRODUCTION

Cancer is recognized as a highly heterogeneous disease characterized by intricate interactions between malignant cells and TME(1). The process of tumorigenesis is profoundly influenced by the dynamic interplay between cancer cells and this complex microenvironment, composed of a diversity of immune cells, a heterogeneous set of tissue cells, the extracellular matrix, and secreted factors(2, 3). The immune component of the TME stands out due to its dual nature, as it can either suppress or promote tumour development(4, 5). Indeed, tumour-infiltrating immune cells (TIICs) are key players, not only affecting cancer growth and progression but also influencing therapeutic outcomes and patient prognosis(6-8). One of the main types of TIICs are TAMs, which represent the most abundant immune population within the TME and play a central role in this intricate intercellular network(9, 10). Moreover, it is known that a high level of TAMs infiltration within most human tumours is strongly correlated with a poor prognosis(11), underscoring the critical need to focus our research and therapeutic strategies on these cells within the oncological landscape. Macrophages are the most important tissue patrol cells recognizing diverse molecular patterns, including PAMPs and DAMPs, through receptors on their extracellular surface, acting as APCs and transmitting signals to helper T cells, thereby activating the adaptive immune system(12). These immune cells exhibit heterogeneous properties, which can align with either an anti-tumoral phenotype, known as pro-inflammatory macrophages, or a pro-tumoral phenotype, known as anti-inflammatory macrophages, depending on the surrounding microenvironment and influencing factors(13). During tumour progression, pro-tumorigenic TAMs, predominantly composed of the M2-like phenotype, become dominant by secreting anti-inflammatory molecules that effectively suppress anti-tumour T cell responses. This suppression occurs through the inhibition of Th1 cells, CTLs and the reduction of NK cell cytolytic activity(14-16). However, under the influence of other factors, including inflammation, local hypoxia, and secretory molecules, the M1 population may also be present exhibiting pro-tumour properties(14, 17). Additionally, TAMs prominently respond to "don't eat me" signals, leading to a reduced capacity for phagocytosis and cross-presentation of antigens, processes that are critical for the eradication of malignant cells(18). Given their pivotal role in tumour progression and their intricate interactions with the immune system, TAMs have emerged as an attractive therapeutic target in the development of novel cancer treatments.

Glycans, including cancer-associated glycans such as Tn and STn, represent key molecular patterns on malignant cells. These glycans, when recognized by APCs, can trigger pro-inflammatory responses or, conversely, induce immune suppression, leading to immune tolerance(19-21). Exploring the interactions between immune cells and cancer-

associated glycans offers valuable insights into how aberrantly glycosylated malignant cells influence the surrounding immune environment, thus paving the way for the development of novel glycan-based therapies. This chapter aims to elucidate the role of truncated *O*-glycans in the immunomodulation of macrophages, through macrophage and glycoengineered cell lines co-cultures and subsequently validate these findings in human bladder and gastric cancer tissues.

2. MATERIALS AND METHODS

2.1. Cell lines and culture conditions

In this experiment, T24 human bladder cancer cell lines and AGS human gastric cancer cell lines, both sourced from the American Type Culture Collection (ATCC), were subjected to glycoengineering via CRISPR/Cas9 technology to overexpress truncated *O*-glycans typical of advanced-stage tumours. Briefly, the *C1GALT1* gene was knocked out, resulting in Tn antigen overexpression, followed by the knock-in of the *ST6GALNAC1* gene, leading to the overexpression of STn antigen, consequently suppressing the formation of more complex *O*-glycan chains. The cells were maintained in RPMI Medium 1640 + GlutaMAXTM-I (Gibco™, Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (FBS) (Gibco™, Thermo Fisher Scientific) and 1% penicillin/streptomycin (PenStrep) (10,000 Units/mL penicillin; 10,000 mg/mL streptomycin; Gibco, Thermo Fisher Scientific) and incubated at 37°C in a humidified atmosphere with 5% CO_2.

2.2. Human monocyte isolation and macrophage differentiation

Human monocytes were isolated from buffy coats obtained from healthy blood donors at the Immunohematology Service of Hospital São João (Porto, Portugal). Procedures were approved by the Hospital ethical committee (Protocol reference 90/19). Succinctly, peripheral blood mononuclear cells (PBMCs) were collected from the turbid interface of previously centrifuged buffy coats (30 minutes, 1200xg, RT, acceleration 5, no brake). These cells were then incubated with RosetteSep Human Monocyte Enrichment Cocktail (StemCell Technologies) for 20 minutes on a roller mixer at room temperature. The resulting mixture was subsequently diluted 1:1 in phosphate-buffered saline (PBS) supplemented with 2% FBS, layered over Histopaque-1077 (Sigma-Aldrich), and centrifuged again under similar conditions as before (30 minutes, 1200xg, RT, no acceleration, no brake). The monocyte-enriched ring was collected and washed three times with PBS, with each wash followed by centrifugation (6 minutes, 1300 rpm, RT, acceleration 9, brake 9).

For in vitro differentiation of macrophages from monocytes, the isolated cells were resuspended in complete RPMI Medium 1640 GlutaMAXTM-I (supplemented with 10% FBS and 1% Pen Strep), further enriched with 50 ng/mL M-CSF (ImmunoTools), and seeded onto glass coverslips placed in 6-well plates at a density of $1.5x10^6$ monocytes/well, for 7 days at 37°C in a humidified atmosphere with 5% CO_2. Following the differentiation period, the macrophages were polarized into M1, M2a, or M2c phenotypes by a 3-day stimulation with IFN-γ (25 ng/mL) (ImmunoTools) + LPS (10 ng/mL) (Sigma), IL-4 (20 ng/mL) (ImmunoTools), or IL-10 (10 ng/mL) (ImmunoTools), respectively, or left unstimulated to maintain the immature M0 profile.

2.3. Co-cultures of macrophages and glycoengineered cancer cell lines

After 72h of macrophage polarization, the cancer cell models were added to the 6-well plates containing macrophages at a 1:3 cancer cells/macrophage ratio for a 24-hour or 72-hour incubation period at 37°C in a humidified atmosphere with 5% CO_2. Following this co-culture period, the conditioned media were collected for subsequent cytokine analysis, and the cells were detached by adding Accutase (Thermo Fisher Scientific) to the wells, followed by a 30-minute incubation on ice. After centrifugation (7 minutes, 1500xg, 4°C), the cells were resuspended in FACS buffer (PBS, 0.5-1% BSA or 5-10% FBS, 0.1% NaN3 sodium azide) and immune stained for flow cytometry analysis.

2.4. Flow cytometry

For macrophage phenotype characterization, immunolabeling was performed using FITC anti-human CD86 (21480863, Immunotools), PE anti-human HLA-DR (21278994, Immunotools), PerCP5.5 anti-human CD14 (325632, BioLegend), and APC-H7 anti-human CD206 (321120, BioLegend) at a 1:25 dilution in FACS buffer, APC anti-human CD163 (333610, BioLegend) at a 1:50 dilution in FACS buffer, and PE-Cy7 anti-human CD45 (368532, BioLegend) at a 1:100 dilution in FACS buffer. Additionally, cell viability was assessed using DAPI staining (750 ng/mL). Cell acquisition was conducted using a Beckman Coulter Navios flow cytometer, and the extracted data were analysed with Kaluza C analysis software (version 2.1, Beckman Coulter) to discriminate distinct cell populations, determine the percentage of positive and negative cells, and calculate the mean fluorescence intensity (MFI).

2.5. ELISA of IL-10 cytokine

Cytokine levels of IL-10 were quantified from the conditioned media of all co-culture conditions using ELISA, following the manufacturer's instructions (BioLegend). In brief, 100 µL of undiluted samples were added to a 96-well plate pre-coated with a capture antibody. After incubation and subsequent washing, an enzyme-linked polyclonal antibody, specific to the cytokine in question, was introduced into the wells and incubated for an additional 2 hours. Lastly, the unbound antibody-enzyme complex was washed away, and a substrate solution was added to induce colour development proportional to the amount of cytokine bound. The absorbance was measured using the iMarkTM Bio-Rad fluorescence microplate reader and correlated with the cytokine concentration (pg/mL).

2.6. Immunohistochemistry of FFPE Tn^+/STn^+ and Tn^-/STn^- tumour tissues

Formalin-fixed paraffin-embedded (FFPE) bladder and gastric cancer tissues, sourced from the IPO-Porto biobank, were analyzed for macrophage detection using the CD68 antibody and for the identification of M1 and M2-like phenotypes using CD163 and CD206 antibodies. All procedures involving the manipulation of tumour tissues were performed with the patient's informed consent and following the approval of the IPO-Porto ethics committee (CES-IPOFG-EPE-86R1/017 for bladder cancer and 87/2017 for gastric cancer). In brief, the FFPE tumour sections underwent initial deparaffinization with xylene, followed by rehydration through successive washes with increasing percentages of alcohol, and incubation for 20 minutes in boiling citrate buffer (10 mM citric acid, 0.05% Tween 20, pH 6.0) or EDTA pH 8. Subsequently, the tissues were exposed to 3-4% hydrogen peroxide (Leica) for 5 minutes. After blocking with 5% bovine serum albumin (BSA) for 1 hour, macrophages were detected and identified using the Novolink Max Polymer DS Kit (Leica), according to the manufacturer's instructions, followed by incubation with 3,3'-diaminobenzidine (DAB, Leica). Additionally, hematoxylin was applied to the slides for 5 minutes to counterstain the nuclei. After dehydration, the slides were immersed in xylene and subsequently mounted using Entellan. The results were evaluated in a double-blind manner by two independent observers and validated by an experienced pathologist. In cases of disagreement, the slides were re-reviewed to reach a consensus. Table 1 summarizes the experimental conditions for antibody analysis.

Table 1 - Experimental conditions used in this immunohistochemistry technique

Antigen	Antigen retrieval	Primary antibody dilution	Incubation
CD68	EDTA pH 8	1:100	1h, RT
CD206	Citrate buffer	1:500	ON 4ºC
CD163	EDTA pH 8	1:120	1h, RT

2.7. Statistical analysis

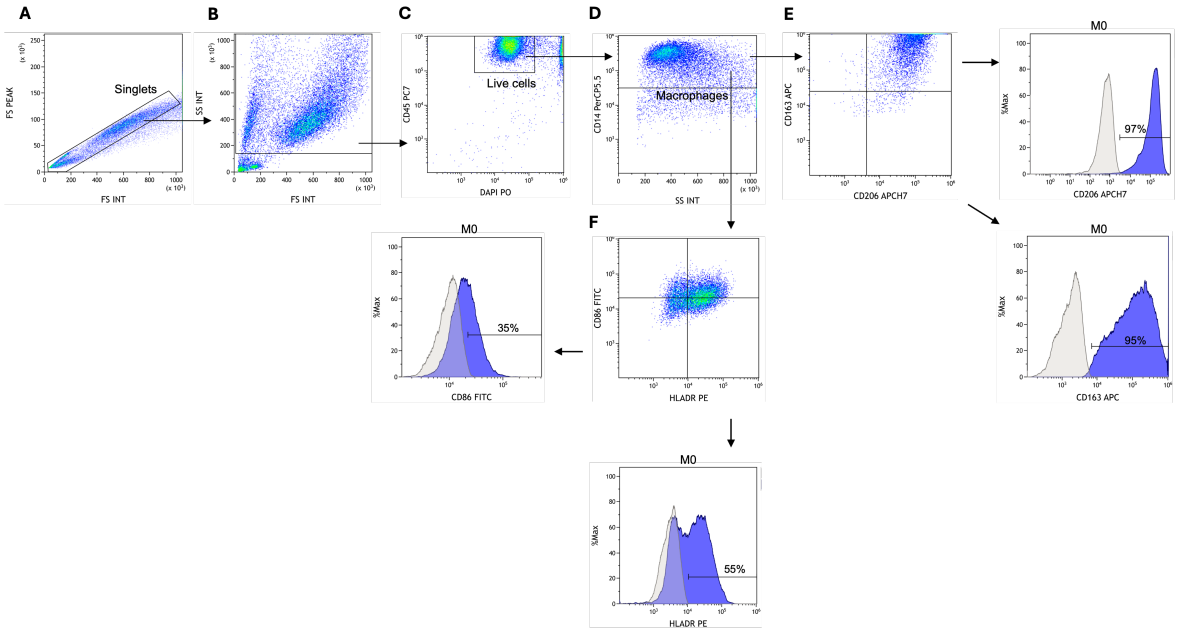
Statistical analysis was performed using GraphPad version 10.0 software (GraphPad Software Inc., La Jolla, CA, USA). Data was checked for normal distribution and homogeneity of variances using the Shapiro-Wilk's and Levene's tests, respectively. Therefore, paired one-way ANOVA (CD86, HLA-DR, CD206, CD163 expression levels) was performed among groups with Tukey's correction to compare different conditions. Statistical significance was determined as $p < 0.05$.

3. RESULTS AND DISCUSSION

The most common alterations in protein glycosylation patterns in cancer are the formation of truncated *O*-glycans, such as Tn and STn, which have been associated with tumour progression and poor survival outcomes(22, 23). Additionally, these antigens have been shown to significantly impact immune response suppression. Notably, macrophages are highlighted as they regulate immune homeostasis through inhibitory receptors, which are essential for preventing excessive inflammation(24, 25). However, cancer cells exploit these receptors to create immunosuppressive microenvironments, promoting immune evasion and facilitating cancer progression(26, 27). In light of this, this work studied the interaction between tumour cells overexpressing Tn/STn glycans and immune cells, focusing specifically on macrophages.

3.1. Flow cytometry gating strategy for macrophage phenotyping

Macrophages differentiated from monocytes of healthy donors via M-CSF stimulation were polarized into distinct phenotypes (M1, M2a, and M2c) or maintained in a naïve M0 state, and subsequently co-cultured with glycoengineered T24 or AGS cells overexpressing the cancer-associated short-chain *O*-glycans Tn and STn. The anti-inflammatory or pro-inflammatory phenotype was consecutively determined through flow cytometry. The macrophage gating strategy was meticulously designed, first focusing on the exclusion of cell aggregates **(Figure 1A)**, followed by cellular debris removal **(Figure 1B)** to ensure the accurate normalization of median fluorescence intensities at the single-cell level. Successively, the viable macrophage population was selected based on DAPI negativity and CD45 positivity, which is present in leukocytes and absent in epithelial cells, thereby allowing the exclusion of dead cells and the co-cultivated tumour cells **(Figure 1C).** Finally, macrophage identification was achieved through CD14 positivity **(Figure 1D)**, which enabled the analysis of the mannose receptor CD206, the scavenger receptor CD163 **(Figure 1E)**, the co-stimulatory molecule CD86, and the antigen-presenting molecule HLA-DR (MHC-II) **(Figure 1F)**, thus providing an effective panel for macrophage phenotype recognition. To refine the assessment of signalling thresholds for these immunomodulatory molecules, isotype controls were additionally utilized.



PB	PO	FITC	PE	PerCP-Cy5.5	PE-Cy7	APC	APC-H7
DAPI	DAPI	CD86	HLA-DR	CD14	CD45	CD63	CD206

Figure 1 - Flow cytometric gating strategy for macrophage phenotype identification and evaluation of MFI and % for CD86, HLA-DR, CD206 and CD163 expression. Cellular aggregate

(A), and cellular debris **(B)** were excluded based on forward scatter (FS) and side scatter (SS) characteristics. **(C)** Viable leukocytes were identified by applying a $CD45^+$ $DAPI^-$ gate, effectively excluding both dead $DAPI^+$ cells and $CD45^-$ tumour cells. **(D)** A $CD14^+$ cells gate was selected to identify only the macrophage population. Within the $CD14^+$ cells gate, the MFI and % of cells positive for CD206, CD163 **(E)**, CD86, and HLA-DR **(F)** were determined to characterise an anti-inflammatory (M1) or pro-inflammatory (M2) phenotype of macrophages. Isotype controls were used as negative controls.

3.2. Refining macrophage polarization for accurate phenotypic characterization

Macrophages are pivotal in various pathophysiological contexts, acting as cornerstone regulators of innate immunity by orchestrating a spectrum of homeostatic and host-defence responses, distinguished from other immune cells by their proficiency in antigen presentation, phagocytosis, and immunomodulation(28). Their functional trajectory is shaped by the surrounding microenvironment, which steers them towards either a pro-inflammatory M1 phenotype or the anti-inflammatory M2 (M2a, M2b, M2c and M2d) phenotype. These phenotypic distinctions are elegantly defined by their cytokine secretion profiles and the nuanced expression of surface markers(29).

To gain a comprehensive and representative understanding of macrophages within the immune system, we meticulously derived both classically activated macrophage (M1) and alternatively activated macrophage (M2), specifically focusing on the M2a subpopulation, known for tissue repair and cellular growth promotion(30), and M2c subpopulation, distinguished by its phagocytic activity and angiogenesis promotion(30), to acquire a broader representation of this phenotype, following a well-established methodology **(Figure 3A)**. These distinct macrophage phenotypes were initially characterized by their unique morphologies, where unpolarized macrophages (herein referred to as M0) appear round or ameboid-like **(Figure 2B)**, reflecting their undifferentiated and less specialized state, and transition to the more elongated spindle-shaped appearance of M1 **(Figure 2C)** macrophages upon activation, indicating their activated state and increased motility. Meanwhile, M2a macrophages are a heterogenous population exhibiting both round and elongated morphology **(Figure 2D)** and M2c macrophages displayed a more flattened and round shape **(Figure 2E)**.

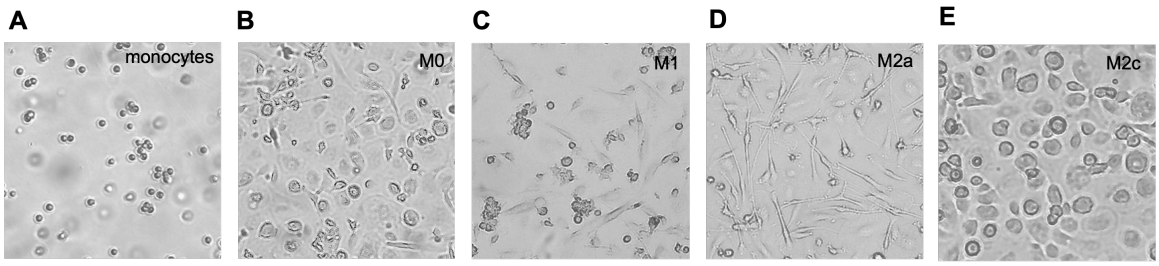


Figure 2 – Morphological characterisation of monocytes and different macrophage phenotypes. (A) Monocytes obtained from PBMCs show a small, rounded morphology typical of their undifferentiated state. In turn, unpolarized monocyte-derived macrophages **(B)** appear predominantly round or ameboid-like, indicative of their primitive phase. After stimulation, M1 macrophages **(C)** appear with an elongated shape similar to fibroblasts, reflecting their pro-inflammatory state. Alternatively, the M2a **(D)** displays a mixture of rounded and fusiform shapes and M2c **(E)** macrophages have a more uniform round shape.

In addition to discriminating macrophage types through cytokine stimulation, a comprehensive panel of immunomarkers revealed M1 macrophages exhibited higher levels of the pro-inflammatory markers histocompatibility complex class II (HLA-DR) and CD86 costimulatory molecule(31) **(Figures 3C and 3D)** coupled with lower expression of the anti-inflammatory markers CD206 and CD163 **(Figures 3E and 3F)**. In turn, M2a phenotype was distinguished by significant CD206 expression **(Figure 3E)**, whereas M2c macrophages prominently expressed CD163 **(Figure 3F)**(32). Interestingly, induction of these phenotypes resulted in higher-than-expected cell death, particularly within the M1 population, where mortality approached 40%.

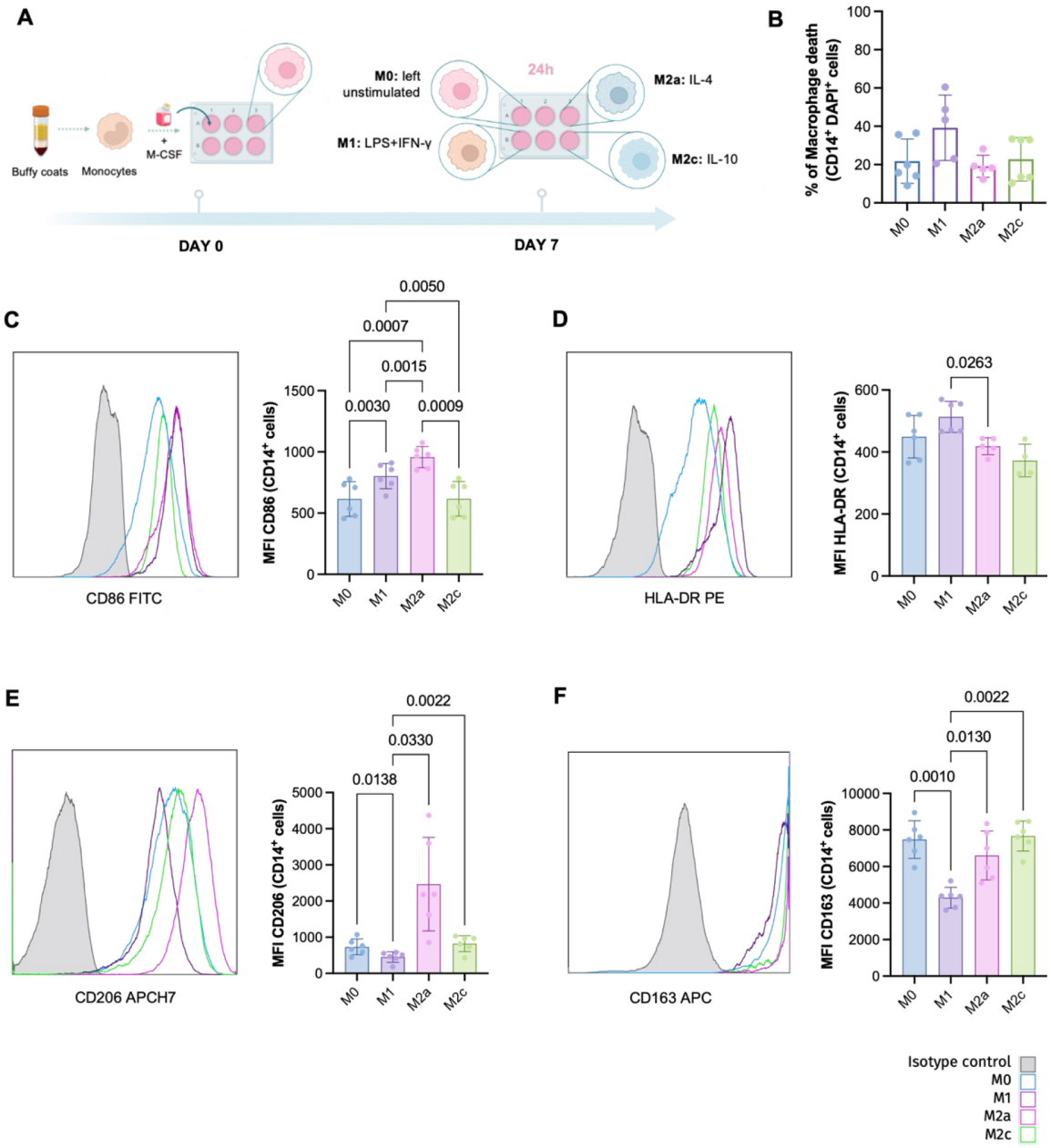


Figure 3 - Phenotypic characterization of macrophages over 24 hours of polarization through expression analysis of CD86, HLA-DR, CD206, and CD163. (A) Briefly, monocytes isolated from PBMCs were differentiated into immature macrophages over a 7-day incubation period with M-CSF cytokine. To assess phenotype plasticity, the monocyte-derived macrophages were either maintained in their immature state or stimulated for 24 hours with 50 ng/mL IFN-γ and 10 ng/mL LPS to induce a pro-inflammatory M1 phenotype, or with 20 ng/mL IL-4 and 10 ng/mL IL-10 to promote differentiation into M2a and M2c anti-inflammatory phenotypes, respectively. **(B)** Percentage of macrophage death across different phenotypes. The M1 macrophages exhibit the highest percentage of cell death. The M1 phenotype shows higher expression of CD86 **(C)** and HLA-DR **(D)** compared to the M0 and M2c phenotypes. Meanwhile, the expression of CD206 **(E)** is significantly increased in the M2a phenotype, and the expression of CD163 **(F)** is decreased in M1 compared to

M0, M2a, and M2c. Data is represented as means ± SD from 6 independent experiments, analysed by one-way ANOVA with Tukey's multiple comparisons test.

To overcome this challenge, we extended the polarization period to 72 hours and halved the IFN-γ concentration to induce the pro-inflammatory phenotype **(Figure 4A)**. Consistent with our previous findings, the M1 phenotype was marked by high CD86 and HLA-DR expression, with minimal CD206 and CD163 levels **(Figures 4C, 4D, 4E and 4F)**. Additionally, the M2a phenotype exhibited significantly elevated CD206 expression **(Figure 4E)**, while the M2c phenotype was characterized by high CD163 expression **(Figure 4F)** and lower levels of other markers. This adjustment effectively reduced macrophage mortality compared to the 24-hour stimulation **(Figure 4B)**, making the 72-hour period optimal for subsequent studies.

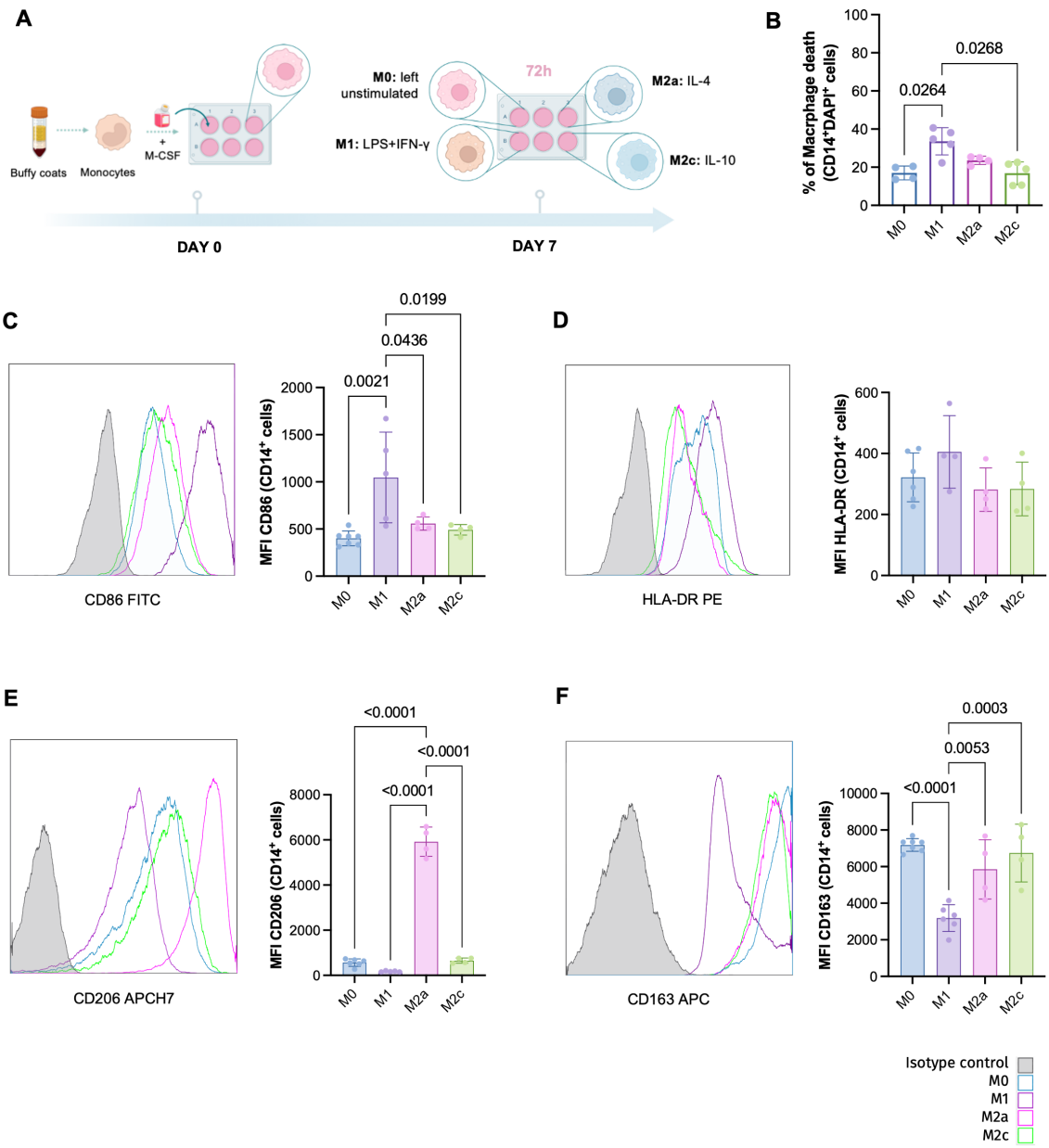


Figure 4 - Phenotypic characterization of macrophages over 72 hours of polarization through expression analysis of CD86, HLA-DR, CD206, and CD163. (A) Monocytes isolated from PBMCs were differentiated into immature macrophages over a 7-day incubation period with M-CSF cytokine. To assess phenotype plasticity, the monocyte-derived macrophages were either maintained in their immature state or stimulated for 72 hours with 25 ng/mL IFN-γ and 10 ng/mL LPS to induce a pro-inflammatory M1 phenotype, or with 20 ng/mL IL-4 and 10 ng/mL IL-10 to promote differentiation into M2a and M2c anti-inflammatory phenotypes, respectively. **(B)** Percentage of macrophage death across different phenotypes. The M1 macrophages display the highest percentage of cell death compared to M0, M2c and M2a macrophages. Additionally, M1 phenotype shows higher expression of CD86 **(C)** compared to the M0, M2a and M2c phenotypes. There were no significant differences in HLA-DR **(D)** across polarization states. Meanwhile, CD206 **(E)** expression is significantly

increased in the M2a phenotype, and CD163 **(F)** expression is decreased in M1 compared to M0, M2a, and M2c. Data is represented as means ± SD from 7 independent experiments, analysed by one-way ANOVA with Tukey's multiple comparisons test.

The flow cytometric analysis of the CD86, HLA-DR, CD163 and CD206 immune markers together with the microscopic analysis of the macrophage morphology confirms that we were able to generate, *in vitro*, the four desired macrophage phenotypes (M0, M1, M2a and M2c)(33).

3.3. Short-chain *O*-glycans induce an anti-inflammatory phenotype in immature macrophages

The activation state of macrophages is environment-dependent and underlies the tumour development and progression(34). On one hand, M1 macrophages can support a robust adaptive immune response against cancer by enhancing antigen presentation and activating adaptive immunity. On the other hand, M2 macrophages shape the TME by recruiting immunosuppressive populations, inhibiting DCs, and suppressing cytotoxic T lymphocyte responses through immune checkpoint molecules and the production of inhibitory cytokines, thereby contributing to tumour propagation(35).

To understand the immunomodulatory role of short-chain *O*-glycans Tn and STn present in tumour cells, immature macrophages derived from monocytes were co-cultured with glycoengineered bladder **(Figures 5 and 6)** or gastric **(Figure 7)** tumour cells. After 24h of co-culturing, the flow cytometric analysis of the macrophages cultured with T24 cells overexpressing Tn **(Figure 5)** and STn **(Figure 6)** revealed that both Tn and STn glycans were capable of inducing a significant downregulation of CD86 **(Figure 5A and 6A)** and upregulation of CD206 **(Figures 5C and 6C)** in comparison with the macrophages co-cultured with T24 wild-type control cells. Furthermore, the T24 overexpressing Tn induced downregulation of HLA-DR **(Figure 5B)** on macrophages in comparison with the macrophages co-cultured with normal glycosylated cells. Interestingly, both Tn- and STn-positive cells were more resistant to macrophage-induced cell death than the wild-type control T24 cells **(Figures 5F and 6F)**, suggesting that these glycans may confer a survival advantage by modulating interactions with immature macrophages. Besides, in conditioned media from the co-cultures between the macrophages and T24 overexpressing Tn and STn, the cytokine IL-10 was found significantly increased **(Figure 5G and 6G)**. Thus, the expression of short-chain *O*-glycans by bladder cancer cells T24 seems to induce

macrophage polarization into a more anti-inflammatory M2-like phenotype, more precisely an M2a phenotype. This suggests that bladder cancer cells with altered glycosylation can effectively induce the polarization of naïve macrophages towards a more pro-tumoral state.

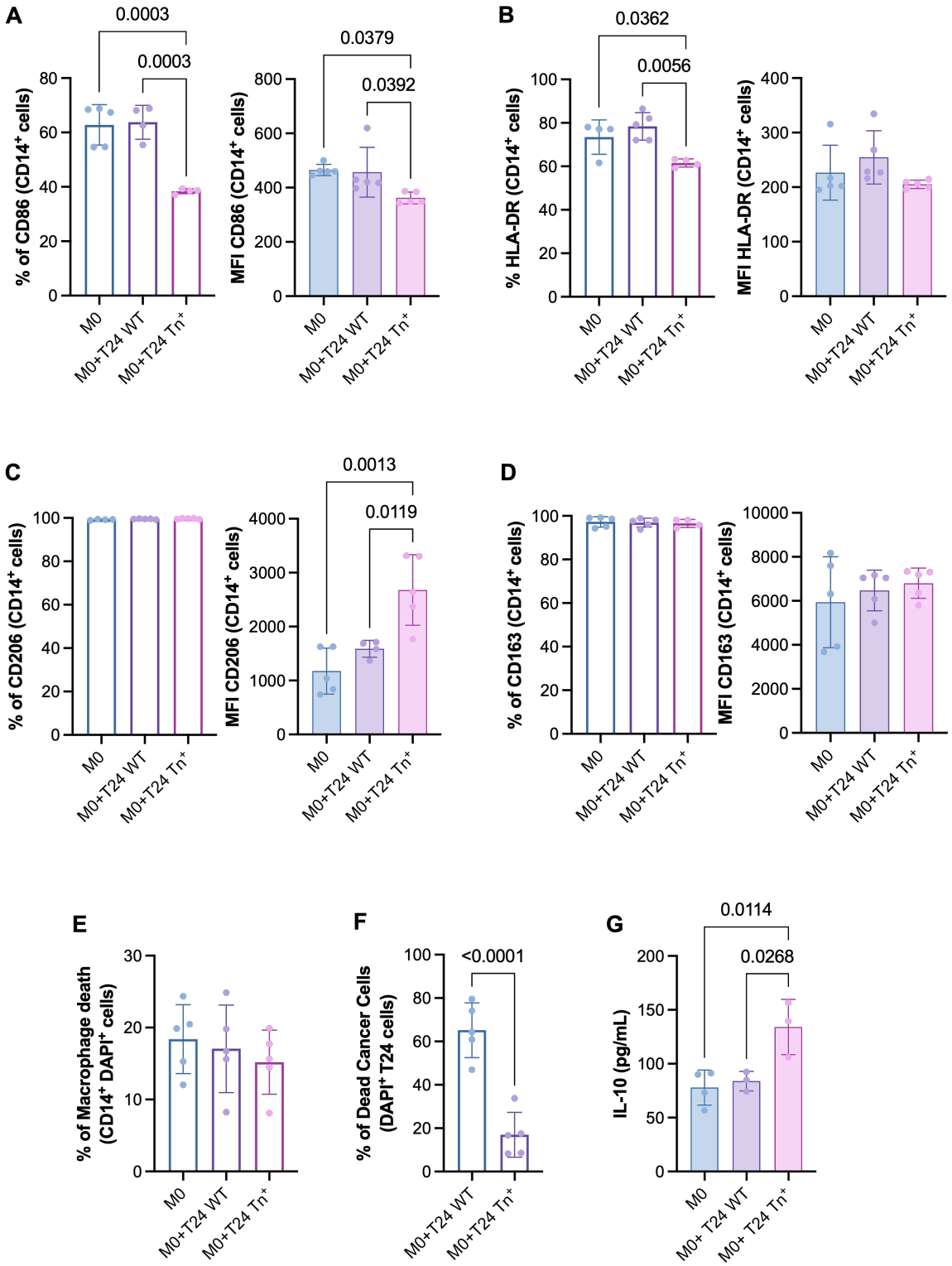


Figure 5 - Co-culture of Tn overexpressing bladder cancer cells with M0 macrophages induce an anti-inflammatory phenotype. After 24h of co-culture, the surface markers CD86, HLA-DR,

CD206 and CD163 were analysed by flow cytometry in $CD45^+$ $CD14^+$ cells. The level of antigens expression was determined by analyzing the percentage (%) and mean fluorescence intensities (MFI) of the cells. **(A)** Immature macrophages co-cultured with T24 cells glycoengineered to overexpress Tn exhibit a statistically significant reduction in CD86 expression compared to immature macrophages co-cultured with T24 WT cells and M0 alone. **(B)** Immature macrophages co-cultured with T24 C1GALT1 KO present downregulation of HLA-DR in comparison with T24 WT cells and M0 isolated. **(C)** Based on the MFI values, the Tn antigen appears to significantly influence CD206 expression in M0 macrophages co-cultured with T24 C1GALT1 KO cells, compared to other conditions, indicating a preference for an anti-inflammatory macrophage phenotype. **(D)** No significant differences in CD163 expression levels were observed across the different conditions. Cell viability was determined using DAPI staining. There were no statistical differences in macrophage death between the control and co-cultures evaluated **(E)**. $DAPI^+$ staining also revealed that tumour cells within the M0 and T24 Tn^+ co-cultures demonstrated a markedly reduced mortality rate compared to those in the M0 with T24 WT co-cultures, highlighting a potentially critical interaction influencing tumour cell survival **(F)**. Analysis of the cytokine IL-10 in the conditioned media of immature macrophages co-cultured with T24 Tn^+ cells revealed that Tn can induce upregulation of IL-10 compared to co-cultures M0 with T24 control cells and M0 **(G)**. Data is represented as mean ± SD and the statistical analysis was carried out using one-way ANOVA followed by Tukey's multiple comparisons test.

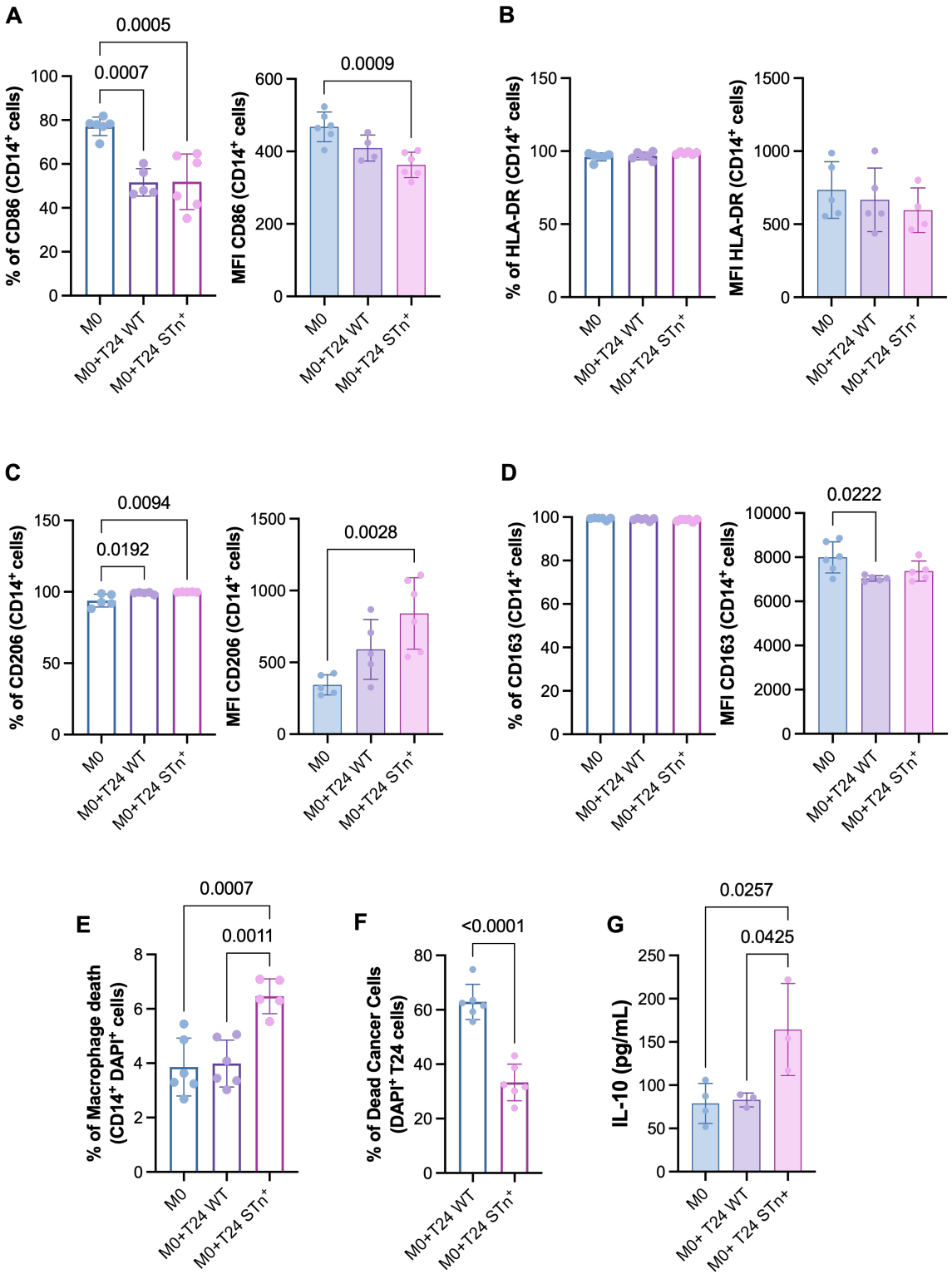


Figure 6 - Co-culture of STn overexpressing bladder cancer cells with M0 macrophages induce an anti-inflammatory phenotype. After 24h of co-culture, the surface markers CD86, HLA-DR, CD206 and CD163 were analysed by flow cytometry in $CD45^+$ $CD14^+$ cells. The level of antigen expression was determined by analyzing the percentage (%) and mean fluorescence intensities (MFI) of the cells. **(A)** Immature macrophages co-cultured with T24 cells glycoengineered to overexpress STn display a statistically significant reduction in CD86 expression compared to M0

control. **(B)** No significant differences in HLA-DR expression levels were observed across the different conditions. **(C)** M0 macrophages co-cultured with T24 *C1GALT1 KO/ST6 GALNAC1 KI* present upregulation of CD206 in comparison with T24 control cells and M0 isolated. **(D)** Based on the MFI values, the M0 co-cultured with T24 WT cells present a slightly reduction of CD163 expression compared to the co-cultures M0 with T24 STn^+. Cell viability was determined using DAPI staining. A notable increase in macrophage mortality was observed when exposed T24 cells overexpressing the STn antigen compared to the other two conditions **(E)**. $DAPI^+$ staining also revealed that tumour cells within the M0 and T24 STn^+ co-cultures demonstrated a markedly reduced mortality rate compared to those in the M0 with T24 WT co-cultures, highlighting a potentially critical interaction influencing tumour cell survival **(F)**. Analysis of the cytokine IL-10 in the conditioned media of immature macrophages co-cultured with T24 STn^+ cells revealed that STn can induce upregulation of IL-10 compared to co-cultures M0 with T24 control cells and M0, reinforcing the acquisition of an anti-inflammatory phenotype in macrophages **(G)**. Data is represented as mean ± SD and the statistical analysis was carried out using one-way ANOVA followed by Tukey's multiple comparisons test.

Similar results showed that the Tn glycan associated with gastric cancer (**Figure 7)** was also able to polarize immature macrophages towards an immunosuppressive profile through a significant increase in CD206 **(Figure 7C)** and a decrease in molecules typically characteristic of an M1 phenotype **(Figures 7A and 7B)**. These findings were further corroborated by elevated levels of the anti-inflammatory cytokine IL-10 **(Figures 7G)** in the conditioned media resulting from co-cultures of macrophages with AGS cells overexpressing the Tn and STn antigens, compared to conditioned media from macrophages cultured with control AGS cells and isolated immature macrophages.

In addition to significantly influencing macrophage polarization, the co-culture experiments revealed a striking increase in macrophage mortality when exposed to bladder cancer cells overexpressing the STn antigen **(Figure 6E)**, compared to the respective controls. In contrast, Tn glycans associated with both bladder and gastric cancer cells did not result in a significant impact on macrophage death upon exposure **(Figures 5E and 7E)**. This observed discrepancy between the effects of STn and Tn may indicate a critical functional distinction in these glycans within the tumour microenvironment. While STn appears to exert a more aggressive role, fostering a tumour microenvironment even more permissive for tumour growth through enhancing immunosuppressive milieu and directly compromising macrophage viability, Tn might be more involved in immune evasion mechanisms that do not directly induce macrophage cytotoxicity. Conversely, the overexpression of Tn in gastric cancer cells **(Figure 7F)** did not result in changes in cellular viability, indicating that the impact of these glycans is cell-line dependent.

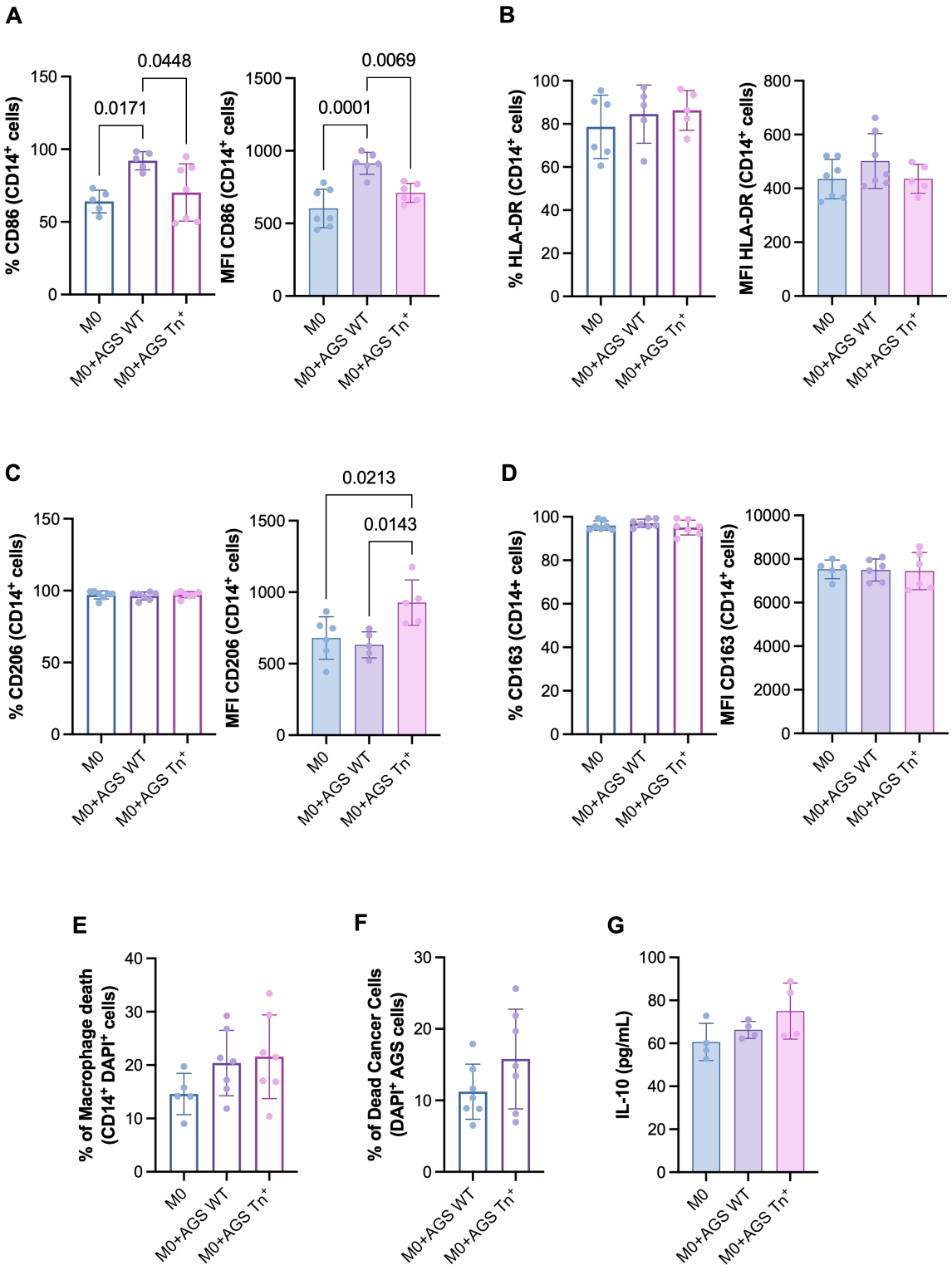


Figure 7 - Co-culture of Tn overexpressing gastric cancer cells with M0 macrophages induce an anti-inflammatory phenotype. After 24h of co-culture, the surface markers CD86, HLA-DR, CD206 and CD163 were analysed by flow cytometry in $CD45^+$ $CD14^+$ cells. The level of antigens expression was determined by analyzing the percentage (%) and mean fluorescence intensities (MFI) of the cells. **(A)** Immature macrophages co-cultured with AGS cells glycoengineered to

overexpress Tn exhibits a statistically significant reduction in CD86 expression compared to immature macrophages co-cultured with AGS control cells. **(B)** There were no significant differences in HLA-DR expression levels across the different conditions. **(C)** Considered on the MFI values, the Tn antigen appears to significantly influence CD206 expression in M0 macrophages co-cultured with AGS *C1GALT1 KO* cells, compared to other conditions, indicating a preference for an anti-inflammatory macrophage phenotype. **(D)** No significant differences in CD163 expression levels were observed between the various conditions. Cell viability was determined using DAPI staining. There were no statistical differences in macrophage death between the control and co-cultures evaluated **(E)**. In turn, no statistical differences in tumour cells death were observed between the control and co-cultures evaluated **(F)**. Analysis of the cytokine IL-10 in the conditioned media of immature macrophages co-cultured with AGS Tn^+ cells revealed a slight increase in IL-10, although this was not statistically significant compared to M0 co-cultures with AGS and M0 control cells **(G)**. Data is represented as mean ± SD and the statistical analysis was carried out using one-way ANOVA followed by Tukey's multiple comparisons test.

In summary, both the Tn and STn antigens equally contribute to the acquisition of an M2a-type anti-inflammatory phenotype, characterized by the high expression of the CD206 receptor, which promotes tumour growth, invasion, and metastasis(36, 37) through various secreted factors, including immunosuppressive cytokines like IL-10 that inhibit the activation and proliferation of NK cells and T cells, thus preventing an effective host anti-tumour immune response(36, 38). Furthermore, malignant cells can secrete these anti-inflammatory cytokines, such as IL-10, while concurrently suppressing the production of pro-inflammatory cytokines like IL-12, IL-6, TNF-α, and IFN-γ, and downregulating co-stimulatory molecules CD80/CD86, enabling the recruitment of additional immature macrophages to the tumour site, promoting polarization towards a pro-tumorigenic M2 phenotype, and ultimately allowing tumour cells to evade immune surveillance(32, 39).

3.4. Short-chain *O*-glycans induce an anti-inflammatory phenotype in pro-inflammatory macrophages

Short-chain *O*-glycans, particularly the Tn and STn antigens, demonstrated the ability to induce the polarization of immature macrophages towards an anti-inflammatory phenotype, playing a crucial role in establishing an immunosuppressive microenvironment that supports the survival and progression of various cancer types. It is well known that within tumours, immature macrophages (M0) arriving via the bloodstream rapidly undergo polarization to either M1 or M2 phenotypes, depending on the signals present in the tumour microenvironment(40). However, as the malignancy advances, there is a predominance of

differentiated macrophages which can be reprogrammed and adapted according to the microenvironmental cues(41). Given this dynamic, an important question arises as to whether these antigens exert the same immunomodulatory effect on macrophages that already possess an established phenotype.

In this way, to explore the immunomodulatory role of short-chain *O*-glycans on differentiated macrophages, we conducted 72-hour co-culture experiments involving M1 macrophages and genetically engineered bladder **(Figure 8)** or gastric **(Figure 9)** cancer cell lines. After 72 hours of culture, the macrophages were properly detached and flow cytometric analysed. Both T24 Tn and STn overexpressing cells induced macrophage reprograming by the downregulation of the co-stimulatory molecule CD86 **(Figure 8A)** and the antigen-presenting molecule HLA-DR **(Figure 8B)**, while simultaneously enhancing the expression of the CD206 **(Figure 8C)** and CD163 molecules **(Figure 8D)**, compared to mono-cultured M1 macrophages and M1 macrophages co-cultured with wild-type T24 cells. Additionally, these results were supported by the elevated levels of the anti-inflammatory cytokine IL-10 **(Figure 8G)** found in the conditioned media from co-cultures of M1 macrophages with T24 overexpressing Tn and STn antigens. Interestingly, in this experiment, wild-type bladder cancer cells exhibited a significantly higher percentage of cell death compared to cells overexpressing the short-chain *O*-glycan Tn **(Figure 8F)** when exposed to pro-inflammatory macrophages, a pattern similar to that observed with immature macrophages **(Figures 5F and 6F)**.

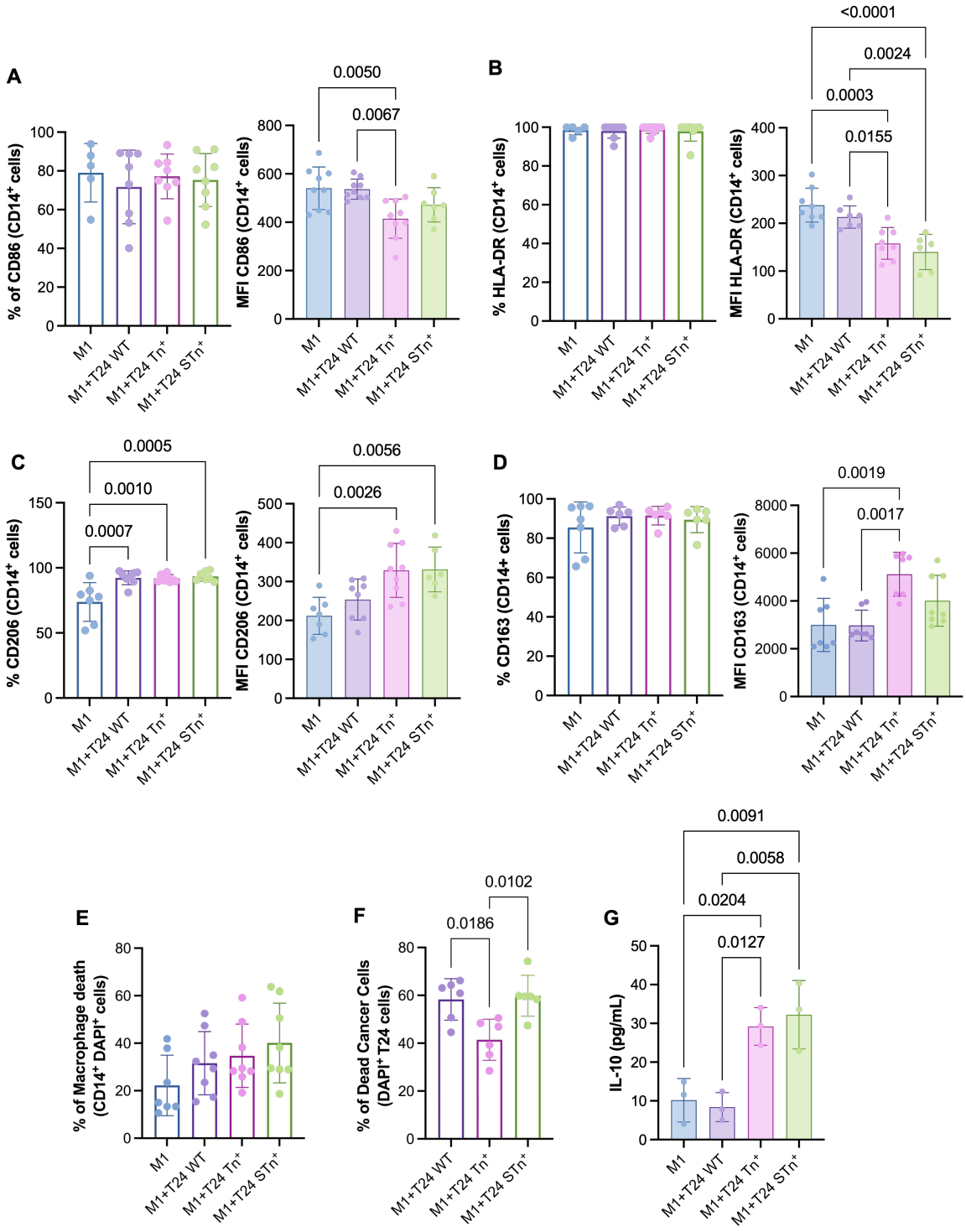


Figure 8 - Co-culture of Tn or STn overexpressing bladder cancer cells with M1 macrophages induce an anti-inflammatory phenotype. After 72h of co-culture, the surface markers CD86, HLA-DR, CD206 and CD163 were analysed by flow cytometry in CD45$^+$ CD14$^+$ cells. The level of antigens expression was determined by analyzing the percentage (%) and mean fluorescence intensities (MFI) of the cells. **(A)** CD86 expression is significantly reduced in pro-inflammatory macrophages co-cultured with T24 cells engineered to overexpress the Tn antigen, compared to those co-cultured with T24 WT cells and isolated M1. **(B)** M1 co-culture with T24 *C1GALT1 KO* and T24 *C1GALT1*

KO/ST6 GALNAC1 KI induced a reduction in HLA-DR expression compared to T24 control cells and M1 conditions. **(C)** CD206 expression levels were elevated in the presence of Tn and STn antigens in co-cultures with M1 macrophages, compared to the isolated control of pro-inflammatory macrophages. **(D)** Based on the MFI values, the M1 co-cultured with T24 Tn^+ cells present an increase of CD163 expression compared to the co-cultures M1 with T24 WT cells and M1 alone. Cell viability was determined using DAPI staining. There were no statistical differences in macrophage death between the control and co-cultures evaluated **(E)**. $DAPI^+$ staining also indicated that tumour cells within the M1 and T24 Tn^+ co-cultures had a significantly lower mortality rate than those in co-cultures with T24 WT or T24 STn^+ cells, suggesting a critical interaction that may promote tumour cell survival **(F)**. Analysis of the cytokine IL-10 in the conditioned media of pro-inflammatory macrophages co-cultured with T24 Tn^+ cells or T24 STn^+ cells revealed an upregulation of IL-10 compared to co-cultures M1 with T24 control cells and M1, reinforcing the acquisition of an anti-inflammatory phenotype in macrophages **(G)**. Data is displayed in bars as mean ± SD and the statistical analysis was carried out using one-way ANOVA followed by Tukey's multiple comparisons test.

The capability of gastric cancer cell line AGS overexpressing Tn reprograming already polarized M1 macrophages was explored. After 72 hours of interaction activation and lineage cell surface markers on macrophages and cytokines in the conditioned medium were evaluated. As like for T24 overexpressing Tn/STn co-cultures with M1 macrophages conditions, AGS Tn-overexpressing cells also induced significantly downregulation of HLA-DR (**Figure 9B**) and upregulation of CD206 and CD163 M2-associated molecules (**Figures 9C and 9D**). Furthermore, in the conditioned media from the co-cultures with glycoengineered AGS Tn positive cells was found an increase in the IL-10 concentration in comparison with those from control conditions **(Figure 9G)**.

Conversely, Tn overexpression in gastric cancer cells **(Figure 9F)** led to a sightly decrease in cellular viability, which is the opposite resulted of T24 Tn overexpressing cells, highlighting the importance of the cell proteome and not solely the glycome. Notably, macrophage viability remained largely unchanged both in isolation and in interaction with the different cancer models tested **(Figures 8E and 9E)**. These results raise important questions regarding the potential for similar effects in glycoengineered tumour cells overexpressing Tn with other macrophage phenotypes and the specific mechanisms driving the immune evasion strategies of these cancer cells, which will be further explored in the following section.

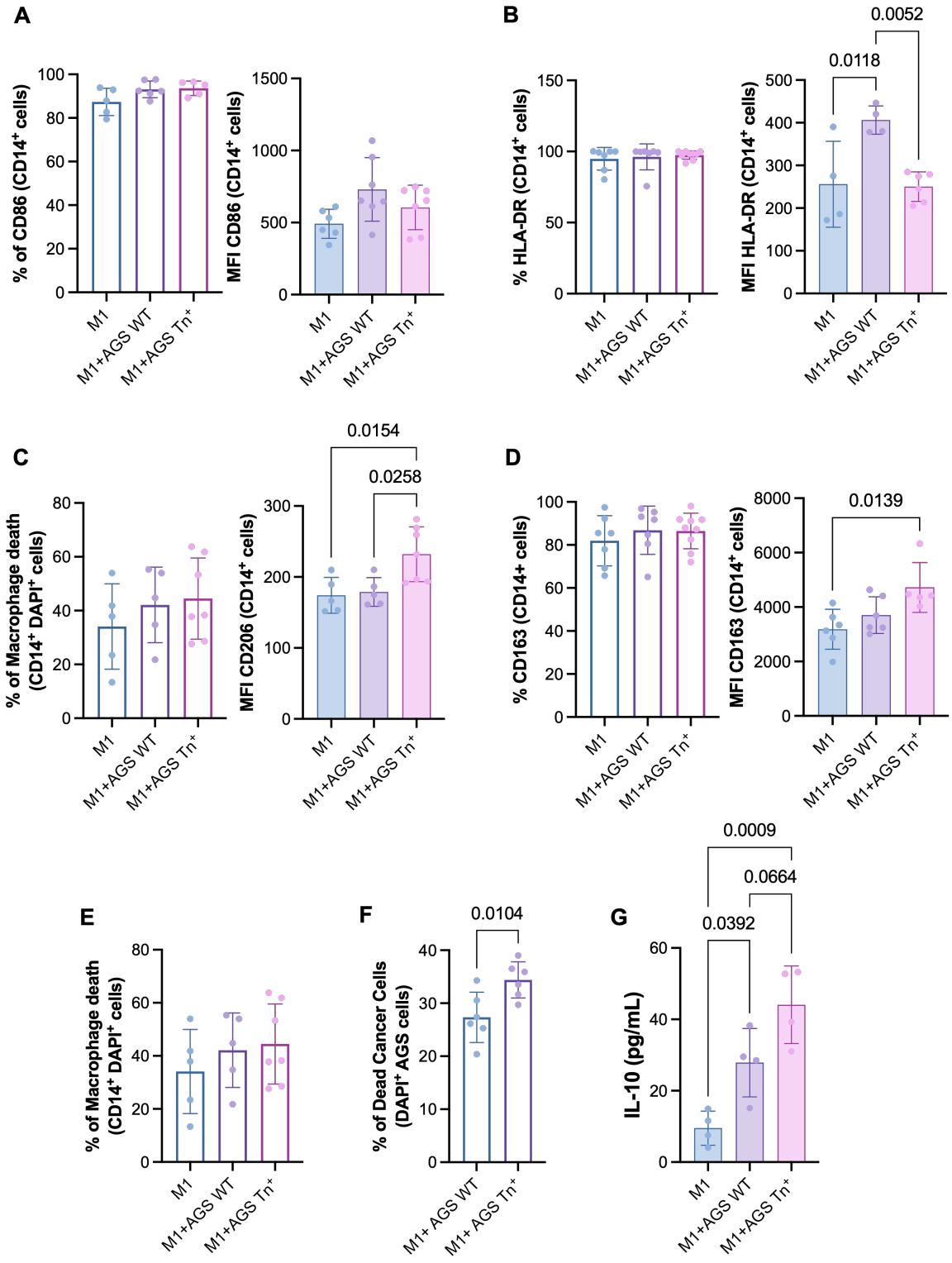


Figure 9- Co-culture of Tn overexpressing gastric cancer cells with M1 macrophages induce an anti-inflammatory phenotype. After 72h of co-culture, the surface markers CD86, HLA-DR, CD206 and CD163 were analysed by flow cytometry in $CD45^+$ $CD14^+$ cells. The level of antigens expression was determined by analyzing the percentage (%) and mean fluorescence intensities (MFI) of the cells. **(A)** No significant differences in CD86 expression levels were observed across the different conditions. **(B)** Based on the MFI values, M1 co-culture with AGS *C1GALT1 KO* induced a

reduction in HLA-DR expression compared to AGS control cells. **(C)** According to the MFI measurements, CD206 expression levels were elevated in the presence of Tn antigen in co-cultures with M1 macrophages, compared to the isolated control of pro-inflammatory macrophages and the co-cultures between M1 and AGS WT cells. **(D)** The M1 co-cultured with AGS Tn^+ cells present an increase of CD163 expression compared to M1 control condition. Cell viability was determined using DAPI staining. There were no statistical differences in macrophage death between the control and co-cultures evaluated **(E)**. $DAPI^+$ staining also suggests that tumour cells within the M1 and AGS Tn^+ co-cultures had a significantly higher mortality rate than those in co-cultures with T24 WT cells, hinting at a potential underlying factor influencing cancer cell survival **(F)**. Analysis of the cytokine IL-10 in the conditioned media of pro-inflammatory macrophages co-cultured with T24 Tn^+ cells revealed an upregulation of IL-10 compared to co-cultures M1 with T24 control cells and M1 isolated, reinforcing the acquisition of an anti-inflammatory phenotype in macrophages **(G)**. Data is represented as mean ± SD and the statistical analysis was carried out using one-way ANOVA followed by Tukey's multiple comparisons test.

These findings align with previous studies showing that the abrogation of extended *O*-glycans and the presence of short-chain and sialylated *O*-glycans promote tumour growth and reprogram macrophages into a pro-tumorigenic phenotype(42, 43). Interpreting CD206 as exclusively pro-tumorigenic may be simplistic, given that elevated levels of this receptor can trigger increased mannose-dependent endocytosis and type 1 collagen uptake, acting as modulators of the tumour matrix(44). In parallel, the high expression of CD163 on macrophages plays a critical role in the inflammation resolution phase by metabolizing pro-inflammatory components and promoting the production of anti-inflammatory agents(45). Conversely, low levels of HLA-DR and CD86 in tumorigenic conditions are linked to impaired immune responses, as these molecules are key players in antigen presentation and T-cell activation, facilitating immune evasion by tumours(46-48). Hence, short-chain *O*-glycans like Tn and STn appear to modulate macrophage polarization and reprogramming, inducing a shift from a pro-inflammatory phenotype to an anti-inflammatory one, potentially combining features of both M2a and M2c macrophages. However, the molecular mechanisms associated with this reprogramming, namely the receptors through which short-chain *O*-glycans can bind and modulate macrophages remain poorly studied and will be explored in Chapter IV.

3.5. Influence of glycosylation on tumour cell survival and immune evasion strategies

Cancer cells expressing truncated glycans can evade immune surveillance through a range of sophisticated adaptive mechanisms, including the upregulation of proteins that enhance resistance to apoptosis and promote cell survival, downregulation of class I histocompatibility complex (HLA-ABC), loss of antigens, secretion of immunosuppressive cytokines, and overexpression of "don't eat me" signals like CD47, which collectively allow them to avoid detection by defence cells such as macrophages and sustain uncontrolled proliferation(49-51).

Previous results prompted further investigation into the influence of the Tn antigen on bladder tumour cells when in contact with different types of macrophages (M0, M1, M2a and M2c). Our observations revealed that normal glycosylated bladder tumour cells exhibit higher mortality compared to bladder tumour cells overexpressing the simplest short-chain *O*-glycan **(Figure 10A)**, when cultured with both anti-inflammatory and pro-inflammatory macrophages, without directly affecting the viability of these antigen-presenting cells **(Figure 10B)**. In contrast to the findings with glycoengineered AGS tumour cells discussed in the previous section, our data shows that the expression of a simple glycophenotype, with overexpression of Tn, by T24 bladder cancer cells confers a distinct survival advantage, enhancing resistance to cell death. These differences suggested that, in addition to glycosylation, the impact on tumour cell viability after co-culturing with both naïve and effector macrophages is also dependent on the specific cancer cell line. Thus, suggesting that different mechanisms of immune evasion could be employed by glycoengineered AGS and T24 cells.

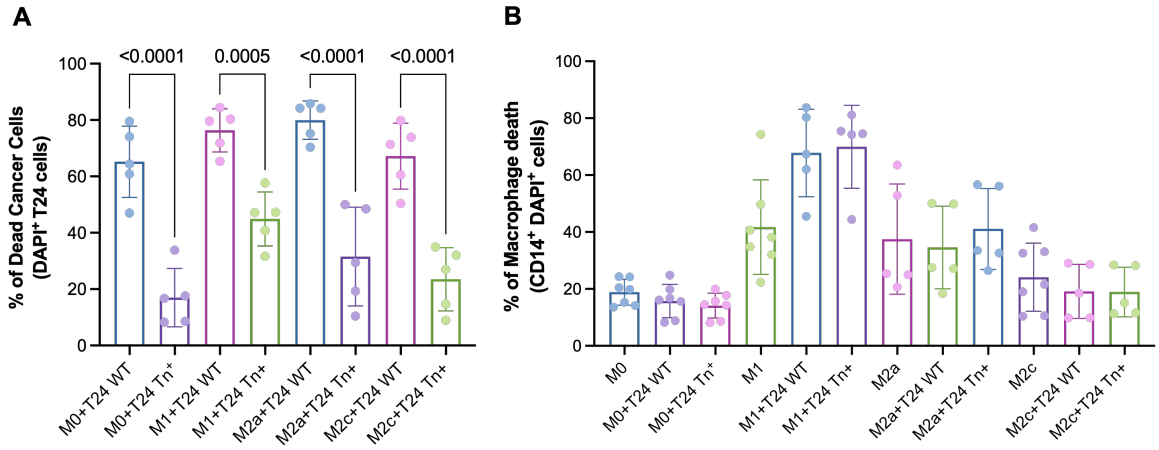


Figure 10 - Co-culture of Tn-overexpressing bladder cancer cells with distinct macrophage phenotypes confers enhanced resistance to cell death. Cell viability for both tumour cells and

macrophages was assessed using DAPI staining. **(A)** Tumour cells T24 overexpressing the Tn antigen, when co-cultured with different macrophage types (M0, M1, M2a, and M2c), showed a very low mortality rate compared to co-cultures with T24 WT control cells and various macrophage phenotypes. **(B)**, no statistically significant changes were observed between T24 Tn^+ co-cultures with the different macrophage phenotypes and their controls. Data is displayed in bars as mean ± SD and was analysed by one-way ANOVA with Tukey's multiple comparisons test.

Considering this rationale, glycoengineered T24 *C1GALT1 KO* and AGS *C1GALT1 KO* cells were analysed via flow cytometry to assess the expression of two crucial immunomodulatory molecules for immune evasion, MHC-I (HLA-ABC) and CD47 **(Figure 11)**. The analysis revealed no statistically significant differences in the expression levels of CD47 **(Figure 11A)** or HLA-ABC **(Figure 11B)** between Tn-overexpressing cells and WT CTRL cells. Consequently, the overexpression of the Tn antigen in bladder and gastric cancer cells does not appear to significantly affect classical immune checkpoint markers in these cancer models. This observation suggests that the Tn antigen present in tumour cells may mediate immune escape through alternative pathways. Therefore, it is necessary to explore more closely which glycoproteins could be involved in the lack of recognition by macrophages and resistance to apoptosis of T24 Tn overexpressing cells.

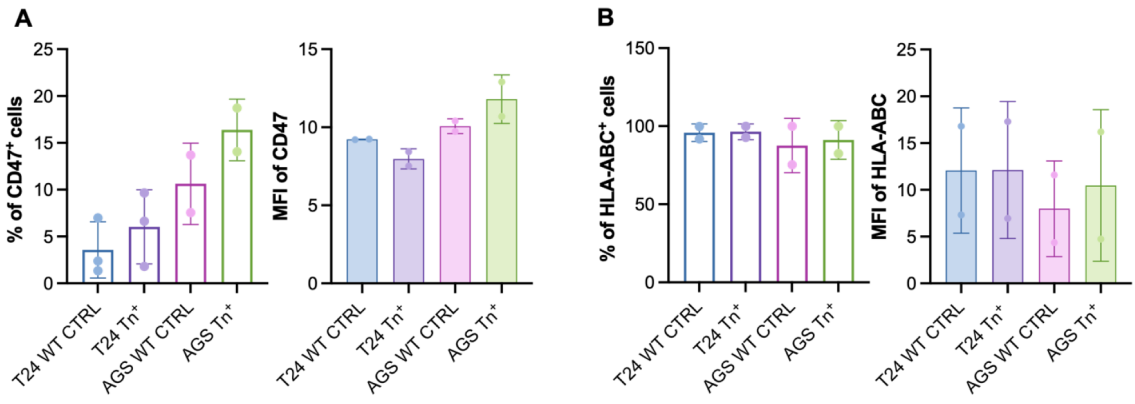


Figure 11 - Immunogenicity of glycoengineered T24 and AGS cells. A) percentage of positive cells and MFI for CD47 evaluated by flow cytometry using the T24 *C1GALT1 KO* and AGS *C1GALT1 KO* cells models and their respective control cells. **B)** percentage of positive cells and MFI for HLA-ABC (also known as MHC-I) evaluated by flow cytometry using the T24 *C1GALT1 KO* and AGS *C1GALT1 KO* cells model their respective control cells. No statistical differences between T24 *C1GALT1 KO* and AGS *C1GALT1 KO* clones and controls were found for CD47 and HLA-ABC expression. The data is represented by the mean ± SD. Statistical analysis was carried out using the Mann-Whitney test.

3.6. Analysis of Macrophage Marker Expression in Paraffin-Embedded Cancer Tissues

The presence of infiltrating anti-inflammatory macrophages in the tumour microenvironment is generally associated with a poor prognosis in various types of cancer, such as gastric cancer, breast cancer and colon cancer, suggesting their involvement in pathogenesis(52). In fact, a growing body of evidence has suggested that the expression of Tn and STn in tumours can shape the tumour immune landscape by interacting with immune receptors in tumour-infiltrating immune cells. This interaction can promote the polarization of TAM toward an M2-like phenotype, normally associated with pro-tumoral activities, and support tumour growth and metastasis(53, 54).

Based on our *in vitro* findings, we aimed to examine whether this relationship between short-chain *O*-glycans and pro-tumoral macrophage phenotypes is also observed in human cancer tissues. To do so, we analysed the CD68 marker, found in both M1 and M2 macrophages, alongside CD163 and CD206, which are more specific markers for M2 macrophages, in a cohort of 10 cases of gastric tumours and 10 cases of muscle-invasive bladder cancer (MIBC) FFPE tissues **(Figure 12)**. The FFPE tissue cases were selected based on Tn and STn expression from previous analysis. According to CD68 expression, macrophage infiltration is most significant in Tn/STn-negative MIBC tumours. We observed that 80% of the Tn/STn-positive MIBC tumours have low CD68 expression, whereas this is true for only 60% of Tn/STn-negative MIBC tumours **(Figure 12A and B)**. Regarding the CD163 expression, both Tn/STn-negative and -positive MIBC tumours have 80% low-to-medium expression of this marker. It is important to note that the expression of CD163 molecules was predominantly observed in the peritumoral area of Tn/STn-positive MIBC tumours, while the Tn/STn-negative tumours exhibit a greater expression in the intratumoral region **(Figure 12A)**. Due to the challenges associated with analysing CD206 in FFPE tissues, its expression was difficult to detect, with only one positive case identified in total, specifically in the Tn/STn-positive MIBC tumour.

For the Tn/STn-positive gastric tumours, we observed that 50% exhibited low CD68 expression, while all the Tn/STn-negative cases showed medium-to-high expression levels **(Figure 12D)**. Similarly, in the bladder tumours, CD163 expression is also lower in Tn/STn-positive gastric tumours and is predominantly localized in the peritumoral area **(Figure 12C and D)**. The relatively higher percentage of low CD163 expression cases for both Tn/STn-positive bladder and gastric tumours correlates with CD68 expression in these tumours. The scavenger receptor CD163 is primarily associated with M2 macrophages; however, it

is important to note that M1 macrophages can also express this receptor, albeit at lower levels.

Moreover, no differences were observed in CD206 expression between Tn/STn-negative and Tn/STn-positive gastric tumours, with the most prevalent phenotype (80% of cases) being the absence of this marker **(Figure 12D)**. However, these results are not conclusive regarding the preferential phenotype acquired by macrophages when interacting with tumour cells with altered glycosylation. Therefore, increasing the number of cases and including M1 phenotype markers should be considered in future studies.

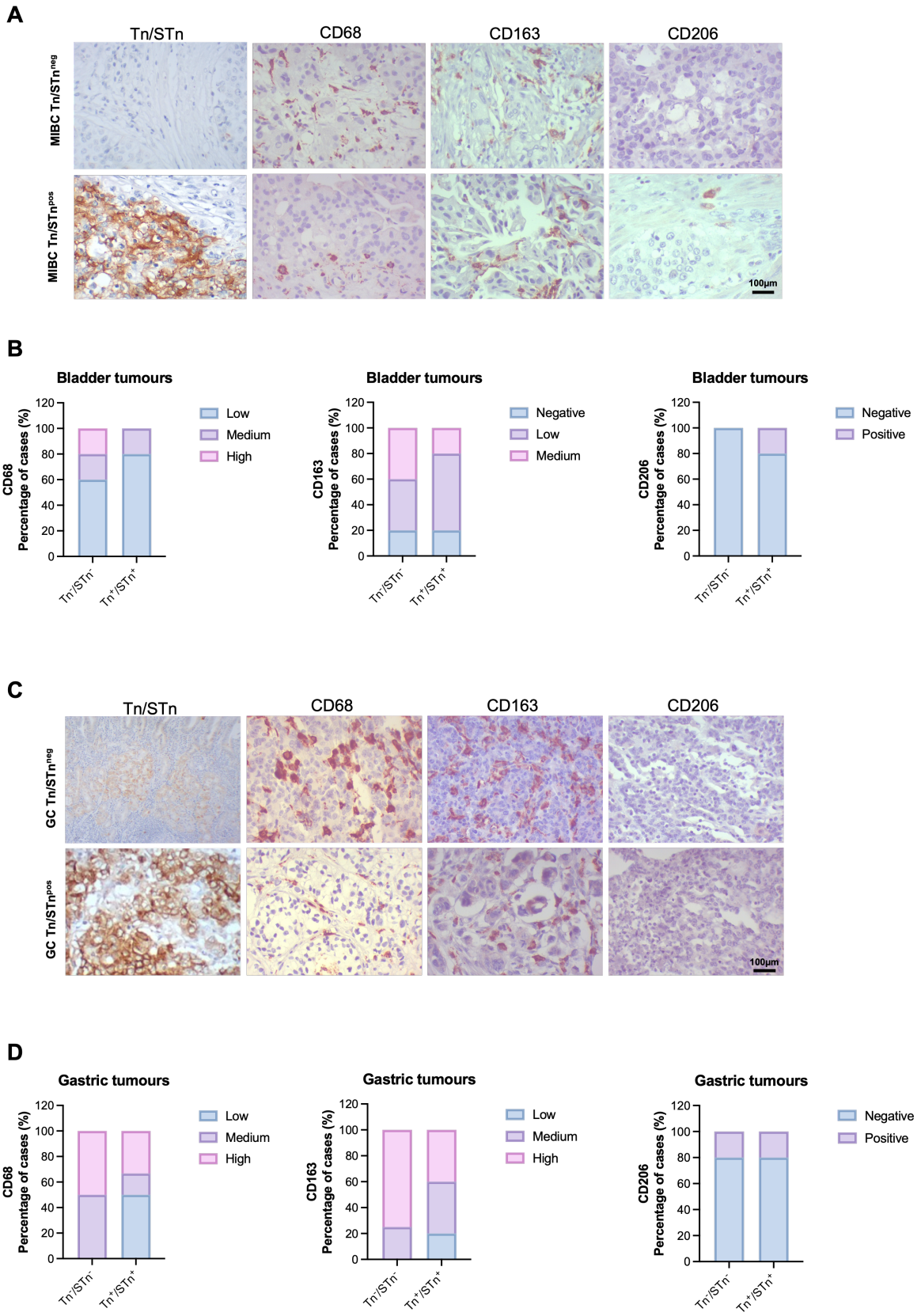


Figure 12 – Immunohistochemical analysis of macrophage phenotype in tumour tissue expressing Tn/STn antigens. (A) Immunohistochemical analysis of macrophage markers (CD68, CD163 and CD206) across muscle-invasive bladder cancer (MIBC) FFPE tissues with or without Tn/STn antigen expression (n=10). Visually, the presence of macrophages with CD68, CD163 and

CD206 expression is scarcer in tissues with altered glycosylation. **(B)** MIBC tissues with high expression of Tn/STn display lower CD68 infiltrate and decreased CD163 expression, with an even poorer population of CD206 macrophages (only 20% of positive cases). **(C)** Immunohistochemical analysis of macrophage markers (CD68, CD163 and CD206) across gastric cancer tissue samples with or without Tn/STn antigen expression (n=10). Visually, the presence of macrophages with CD68 and CD163 expression is scarcer in tissues with altered glycosylation, with an absence of CD206 macrophages in these tissues. **(D)** Gastric cancer tissues Tn/STn-positive exhibited low expression of CD68 and CD163 antigens, indicating reduced macrophage infiltrates.

4. CONCLUDING REMARKS

After decades of research in glycobiology, it is now clear that the glycome of cancer cells undergoes significant alterations, leading to a unique glycophenotype characterized primarily by an increase in truncated *O*-glycans, forming cancer-associated glycosylation patterns that differ markedly from those found in healthy cells(55-57). Despite the intense focus on their contributions to tumour aggressiveness, the way these glycosidic modifications interact with the immune system remains largely underexplored. Currently, it is believed that changes in glycosylation patterns can redefine the interaction between the immune system and tumours, promoting signals that favour an immunosuppressive response(58). These alterations in glycosylation profoundly influence antigen presentation by modifying the mechanisms through which APCs detect and internalize proteins with abnormal glycosylation patterns. This disruption affects the subsequent processing, degradation, and presentation of these proteins through Major Histocompatibility Complexes (MHCs), thereby promoting a tumour-friendly microenvironment and impacting the differentiation of T cells(59, 60). Collectively, these findings support the importance of a comprehensive understanding of the immunomodulatory role of glycosylation on crucial antigen-presenting cells within the tumour microenvironment.

This work highlights that macrophages preferentially adopt an anti-inflammatory M2a/M2c phenotype when exposed to cancer cells expressing truncated short-chain *O*-glycans. Notably, both Tn and STn antigens demonstrated their similar potential to polarize and reprogram macrophages, inducing a pro-tumoral and immunosuppressive profile. This is characterized by elevated levels of anti-inflammatory molecules (CD206 and CD163) and a significant reduction in pro-inflammatory molecules (CD86 and HLA-DR), which may facilitate immune evasion. Moreover, the induction of high levels of the inhibitory cytokine IL-10 supports these findings by promoting the recruitment of immunosuppressive cells while hindering the entry of anti-tumour cells into the tumour niche. Meanwhile, in human tumour tissues from gastric cancer and MIBC, a low expression of the CD68 and CD163

molecules was detected, indicating that the immature *O*-glycans present in the cancer cells is associated with diminished macrophage infiltration.

In summary, this chapter has provided valuable insights into the role of cancer-associated Tn and STn glycans in the polarization and reprogramming of macrophages, highlighting their tendency to adopt an anti-inflammatory profile that creates an immunosuppressive environment, particularly in the contexts of bladder and gastric cancers. However, further studies are needed to extend these findings to other glycoengineered tumour models to determine whether this immunomodulatory role is consistent across various cancer types. Additionally, a more comprehensive characterization of cytokines present in conditioned media is essential for a clearer understanding of the predominant anti-inflammatory macrophage profiles (M2a or M2c) and for more accurately validating the observed results. Future research would benefit from moving towards tri-culture systems that include T cells to better understand how these multiple cellular interactions influence tumour microenvironment dynamics and the adaptive immune response.

5. REFERENCES

1. Zuo S, Wei M, Wang S, Dong J, Wei J. Pan-Cancer Analysis of Immune Cell Infiltration Identifies a Prognostic Immune-Cell Characteristic Score (ICCS) in Lung Adenocarcinoma. Frontiers in Immunology. 2020;11.
2. Bindea G, Mlecnik B, Tosolini M, Kirilovsky A, Waldner M, Obenauf AC, et al. Spatiotemporal dynamics of intratumoral immune cells reveal the immune landscape in human cancer. Immunity. 2013;39(4):782-95.
3. Sadeghi M, Dehnavi S, Sharifat M, Amiri AM, Khodadadi A. Innate immune cells: Key players of orchestra in modulating tumor microenvironment (TME). Heliyon. 2024;10(5):e27480.
4. Peña-Romero AC, Orenes-Piñero E. Dual Effect of Immune Cells within Tumour Microenvironment: Pro- and Anti-Tumour Effects and Their Triggers. Cancers (Basel). 2022;14(7).
5. Zamarron BF, Chen W. Dual Roles of Immune Cells and Their Factors in Cancer Development and Progression. International Journal of Biological Sciences. 2011;7(5):651-8.
6. Oliver AJ, Lau PK, Unsworth AS, Loi S, Darcy PK, Kershaw MH, et al. Tissue-dependent tumor microenvironments and their impact on immunotherapy responses. Frontiers in immunology. 2018;9:70.

7. Gentles AJ, Newman AM, Liu CL, Bratman SV, Feng W, Kim D, et al. The prognostic landscape of genes and infiltrating immune cells across human cancers. Nature medicine. 2015;21(8):938-45.
8. Fridman WH, Pagès F, Sautès-Fridman C, Galon J. The immune contexture in human tumours: impact on clinical outcome. Nature Reviews Cancer. 2012;12(4):298-306.
9. Pan Y, Yu Y, Wang X, Zhang T. Tumor-Associated Macrophages in Tumor Immunity. Front Immunol. 2020;11:583084.
10. Christofides A, Strauss L, Yeo A, Cao C, Charest A, Boussiotis VA. The complex role of tumor-infiltrating macrophages. Nat Immunol. 2022;23(8):1148-56.
11. Di Ceglie I, Carnevale S, Rigatelli A, Grieco G, Molisso P, Jaillon S. Immune cell networking in solid tumors: focus on macrophages and neutrophils. Frontiers in Immunology. 2024;15.
12. Mishra AK, Banday S, Bharadwaj R, Ali A, Rashid R, Kulshreshtha A, et al. Macrophages as a Potential Immunotherapeutic Target in Solid Cancers. Vaccines (Basel). 2022;11(1).
13. Sadhukhan P, Seiwert TY. The role of macrophages in the tumor microenvironment and tumor metabolism. Seminars in Immunopathology. 2023;45(2):187-201.
14. Wang S, Wang J, Chen Z, Luo J, Guo W, Sun L, et al. Targeting M2-like tumor-associated macrophages is a potential therapeutic approach to overcome antitumor drug resistance. npj Precision Oncology. 2024;8(1):31.
15. Hao NB, Lü MH, Fan YH, Cao YL, Zhang ZR, Yang SM. Macrophages in tumor microenvironments and the progression of tumors. Clin Dev Immunol. 2012;2012:948098.
16. Basak U, Sarkar T, Mukherjee S, Chakraborty S, Dutta A, Dutta S, et al. Tumor-associated macrophages: an effective player of the tumor microenvironment. Front Immunol. 2023;14:1295257.
17. Strizova Z, Benesova I, Bartolini R, Novysedlak R, Cecrdlova E, Foley LK, et al. M1/M2 macrophages and their overlaps - myth or reality? Clin Sci (Lond). 2023;137(15):1067-93.
18. Antón IM, Wandosell F, Vicente-Manzanares M. Chapter 15 - Cancer cell development, migratory response, and the role of the tumor microenvironment in invasion and metastasis. In: Schnoor M, Yin L-M, Sun SX, editors. Cell Movement in Health and Disease: Academic Press; 2022. p. 245-70.
19. Anderluh M, Berti F, Bzducha-Wróbel A, Chiodo F, Colombo C, Compostella F, et al. Emerging glyco-based strategies to steer immune responses. Febs j. 2021;288(16):4746-72.
20. Choi Y, Kim J, Chae J, Hong J, Park J, Jeong E, et al. Surface glycan targeting for cancer nano-immunotherapy. J Control Release. 2022;342:321-36.

21. Mereiter S, Balmaña M, Campos D, Gomes J, Reis CA. Glycosylation in the Era of Cancer-Targeted Therapy: Where Are We Heading? Cancer Cell. 2019;36(1):6-16.
22. Lima L, Neves M, Oliveira MI, Dieguez L, Freitas R, Azevedo R, et al. Sialyl-Tn identifies muscle-invasive bladder cancer basal and luminal subtypes facing decreased survival, being expressed by circulating tumor cells and metastases. Urologic Oncology: Seminars and Original Investigations. 2017;35(12):675.e1-.e8.
23. Azevedo R, Peixoto A, Gaiteiro C, Fernandes E, Neves M, Lima L, et al. Over forty years of bladder cancer glycobiology: Where do glycans stand facing precision oncology? Oncotarget. 2017;8(53):91734-64.
24. Wynn TA, Chawla A, Pollard JW. Macrophage biology in development, homeostasis and disease. Nature. 2013;496(7446):445-55.
25. Mass E, Nimmerjahn F, Kierdorf K, Schlitzer A. Tissue-specific macrophages: how they develop and choreograph tissue biology. Nature Reviews Immunology. 2023;23(9):563-79.
26. Brom VC, Burger C, Wirtz DC, Schildberg FA. The Role of Immune Checkpoint Molecules on Macrophages in Cancer, Infection, and Autoimmune Pathologies. Front Immunol. 2022;13:837645.
27. Liu W, Zhou H, Lai W, Hu C, Xu R, Gu P, et al. The immunosuppressive landscape in tumor microenvironment. Immunologic Research. 2024;72(4):566-82.
28. Chen S, Saeed AFUH, Liu Q, Jiang Q, Xu H, Xiao GG, et al. Macrophages in immunoregulation and therapeutics. Signal Transduction and Targeted Therapy. 2023;8(1):207.
29. Murray PJ, Allen JE, Biswas SK, Fisher EA, Gilroy DW, Goerdt S, et al. Macrophage activation and polarization: nomenclature and experimental guidelines. Immunity. 2014;41(1):14-20.
30. Dash SP, Gupta S, Sarangi PP. Monocytes and macrophages: Origin, homing, differentiation, and functionality during inflammation. Heliyon. 2024;10(8):e29686.
31. Kerneur C, Cano CE, Olive D. Major pathways involved in macrophage polarization in cancer. Front Immunol. 2022;13:1026954.
32. Zhang Q, Sioud M. Tumor-Associated Macrophage Subsets: Shaping Polarization and Targeting. Int J Mol Sci. 2023;24(8).
33. Rostam HM, Reynolds PM, Alexander MR, Gadegaard N, Ghaemmaghami AM. Image based Machine Learning for identification of macrophage subsets. Scientific Reports. 2017;7(1):3521.
34. Kloosterman DJ, Akkari L. Macrophages at the interface of the co-evolving cancer ecosystem. Cell. 2023;186(8):1627-51.

35. Cendrowicz E, Sas Z, Bremer E, Rygiel TP. The Role of Macrophages in Cancer Development and Therapy. Cancers (Basel). 2021;13(8).
36. Sezginer O, Unver N. Dissection of pro-tumoral macrophage subtypes and immunosuppressive cells participating in M2 polarization. Inflammation Research. 2024.
37. Yuan A, Hsiao Y-J, Chen H-Y, Chen H-W, Ho C-C, Chen Y-Y, et al. Opposite Effects of M1 and M2 Macrophage Subtypes on Lung Cancer Progression. Scientific Reports. 2015;5(1):14273.
38. Zhang Q, Sioud M. Tumor-Associated Macrophage Subsets: Shaping Polarization and Targeting. International Journal of Molecular Sciences. 2023;24(8):7493.
39. Aizaz M, Khan A, Khan F, Khan M, Musad Saleh EA, Nisar M, et al. The cross-talk between macrophages and tumor cells as a target for cancer treatment. Front Oncol. 2023;13:1259034.
40. Graney PL, Ben-Shaul S, Landau S, Bajpai A, Singh B, Eager J, et al. Macrophages of diverse phenotypes drive vascularization of engineered tissues. Science Advances. 2020;6(18):eaay6391.
41. Wang N, Liang H, Zen K. Molecular mechanisms that influence the macrophage m1-m2 polarization balance. Front Immunol. 2014;5:614.
42. Stanczak MA, Rodrigues Mantuano N, Kirchhammer N, Sanin DE, Jacob F, Coelho R, et al. Targeting cancer glycosylation repolarizes tumor-associated macrophages allowing effective immune checkpoint blockade. Science Translational Medicine. 2022;14(669):eabj1270.
43. Wan Y, Adair K, Herrmann A, Shan X, Xia L, Duckworth CA, et al. C1GalT1 expression reciprocally controls tumour cell-cell and tumour-macrophage interactions mediated by galectin-3 and MGL with double impact on cancer development and progression. Cell Death & Disease. 2023;14(8):547.
44. Arlt A, von Bonin F, Rehberg T, Perez-Rubio P, Engelmann JC, Limm K, et al. High CD206 levels in Hodgkin lymphoma-educated macrophages are linked to matrix-remodeling and lymphoma dissemination. Molecular Oncology. 2020;14(3):571-89.
45. Etzerodt A, Moestrup SK. CD163 and inflammation: biological, diagnostic, and therapeutic aspects. Antioxid Redox Signal. 2013;18(17):2352-63.
46. Roerden M, Märklin M, Salih HR, Bethge WA, Klein R, Rammensee H-G, et al. Expression levels of HLA-DR in acute myeloid leukemia: implications for antigenicity and clinical outcome. Leukemia & Lymphoma. 2021;62(8):1907-19.
47. Huang A, Zhang B, Wang B, Zhang F, Fan K-X, Guo Y-J. Increased CD14+HLA-DR-/low myeloid-derived suppressor cells correlate with extrathoracic metastasis and poor response to chemotherapy in non-small cell lung cancer patients. Cancer Immunology, Immunotherapy. 2013;62(9):1439-51.

48. Ugurel S, Uhlig D, Pföhler C, Tilgen W, Schadendorf D, Reinhold U. Down-regulation of HLA class II and costimulatory CD86/B7-2 on circulating monocytes from melanoma patients. Cancer Immunology, Immunotherapy. 2004;53(6):551-9.
49. Ward JP, Gubin MM, Schreiber RD. The Role of Neoantigens in Naturally Occurring and Therapeutically Induced Immune Responses to Cancer. Adv Immunol. 2016;130:25-74.
50. Khalaji A, Yancheshmeh FB, Farham F, Khorram A, Sheshbolouki S, Zokaei M, et al. Don't eat me/eat me signals as a novel strategy in cancer immunotherapy. Heliyon. 2023;9(10):e20507.
51. Pinto D, Parameswaran R. Role of Truncated O-GalNAc Glycans in Cancer Progression and Metastasis in Endocrine Cancers. Cancers (Basel). 2023;15(13).
52. Kvorjak M, Ahmed Y, Miller ML, Sriram R, Coronnello C, Hashash JG, et al. Cross-talk between Colon Cells and Macrophages Increases ST6GALNAC1 and MUC1-sTn Expression in Ulcerative Colitis and Colitis-Associated Colon Cancer. Cancer Immunology Research. 2020;8(2):167-78.
53. Häuselmann I, Borsig L. Altered tumor-cell glycosylation promotes metastasis. Front Oncol. 2014;4:28.
54. Matsumoto Y, Ju T. Aberrant Glycosylation as Immune Therapeutic Targets for Solid Tumors. Cancers (Basel). 2023;15(14).
55. Pinho SS, Reis CA. Glycosylation in cancer: mechanisms and clinical implications. Nature Reviews Cancer. 2015;15(9):540-55.
56. Ferreira E, Ferreira D, Relvas-Santos M, Freitas R, Soares J, Azevedo R, et al. Aberrantly Glycosylated GLUT1 as a Poor Prognosis Marker in Aggressive Bladder Cancer. Int J Mol Sci. 2024;25(6).
57. Gaiteiro C, Soares J, Relvas-Santos M, Peixoto A, Ferreira D, Paulo P, et al. Glycoproteogenomics characterizes the CD44 splicing code associated with bladder cancer invasion. Theranostics. 2022;12(7):3150-77.
58. RodrÍguez E, Schetters ST, van Kooyk Y. The tumour glyco-code as a novel immune checkpoint for immunotherapy. Nature Reviews Immunology. 2018;18(3):204-11.
59. Wolfert MA, Boons G-J. Adaptive immune activation: glycosylation does matter. Nature Chemical Biology. 2013;9(12):776-84.
60. Fernandes Â, Azevedo CM, Silva MC, Faria G, Dantas CS, Vicente MM, et al. Glycans as shapers of tumour microenvironment: A sweet driver of T-cell-mediated anti-tumour immune response. Immunology. 2023;168(2):217-32.

CHAPTER IV

SIMPLE CELL GLYCOAVATARS: BEAD-COATED MEMBRANE MODELS FOR STUDYING THE CELLULAR INTERACTOME

This chapter is part of the manuscript under the same title by Miranda A. and Lourenço C. *et al.*, currently in preparation for submission

ABSTRACT

The immune system possesses sophisticated mechanisms to recognize and respond to cellular alterations, including danger signals generated by tumours. However, cancer cells also have their own strategies to evade this surveillance, notably through alterations in the immature *O*-glycosylation of surface proteins, where glycans such as the Tn and STn antigens become prevalent in many types of advanced solid tumours. These modifications are not merely a byproduct of disease progression but can actively alter the interaction between cancer cells and the immune system, promoting a tolerogenic immune environment. Within this context, macrophages, which are key players in innate immune defence and in activating the adaptive immune response, can, upon interacting with these glycans, either inhibit or promote tumour progression. Despite this, the specific receptors involved in these interactions remain largely unidentified.

To address this gap, the present project proposes an innovative proteomic approach to identify the receptors on macrophages that recognize and interact with truncated short-chain *O*-glycans. Using "GlycoAvatars" technology, in which cell membranes from glycoengineered models with immature glycosylation are coupled to magnetic beads, a controlled interaction with macrophages is established, simulating an immune synapse. This process enables precise mapping of the protein sets involved in these interactions, identifying specific receptors and potential therapeutic targets that mediate the immune response to cells with truncated glycans.

Keywords: GlycoAvatars, CDK5, Tn, macrophages, immune synapse

1. INTRODUCTION

It is well established that glycans are integral components of cells, forming a dense layer that covers the entire extracellular surface and serving as a crucial molecular interface between the cell and its surrounding environment(1, 2). In cancer, these glycans undergo significant alterations in their composition, leading to the formation of unique molecular fingerprints on tumour cells(3, 4). The most common alterations in glycosylation patterns found in carcinogenic cells are truncated short-chain *O*-glycans, specifically Tn and STn, which can be detected by the immune system through GBPs expressed by immune cells(5, 6). These GBPs, present in both immune cells and the extracellular environment, recognise specific glycans, conveying their structural and immunoregulatory information, such as pathogen identification, leukocyte trafficking, and the activation, differentiation, and survival of immune cells, playing a critical role in immune evasion and tumour immunomodulation(7). APCs are particularly notable for expressing a wide array of carbohydrate-binding receptors, including galectins, C-type lectins, and sialic acid-binding immunoglobulin-like lectins (siglecs)(6). These specific receptors on APCs, such as macrophages—which are key components of the first line of defence and pivotal in initiating adaptive immune responses—can, upon binding to altered glycans, either amplify or suppress inflammatory responses. This modulating effect not only affects cytokine production but also influences the recruitment of immune cells to the site, thereby promoting tumour development(6, 8, 9). The far-reaching influence of glycans as facilitators of tolerogenic pathways in cancer progression highlights their role as inhibitory immune checkpoints and immunostimulatory signals, the in-depth understanding of which promises to reveal new biomarkers and therapeutic targets, driving the development of effective immunomodulatory strategies with significant clinical applications(3, 10). In this context, glycoproteomics has emerged as an essential tool for identifying molecular signatures, providing fundamental insights for understanding systems biology(11). It also plays a critical role in identifying proteins that interact with glycans, providing valuable information about the recognition of these molecules in cancer progression(11-13). For that matter, glycoproteomics is becoming an indispensable tool for personalizing cancer treatment.

In this chapter, we introduce a novel proteomics strategy designed to identify proteins within antigen-presenting cells, with a particular emphasis on macrophages, that recognize and interact with truncated short-chain *O*-glycans, specifically Tn and STn, with the goal of uncovering potential therapeutic targets.

2. MATERIALS AND METHODS

2.1. Membrane protein extraction and enrichment

A fractionation buffer was initially prepared consisting of 20 mM HEPES (pH 7.4), 10 mM KCl, 2 mM $MgCl_2$, 1 mM EDTA, and 1 mM EGTA, to which protease inhibitors were added, including 10 µL aprotinin (1 mg/mL), 10 µL leupeptin (1 mg/mL), 10 µL PMSF (35 mg/mL), 10 µL NaF (10 mM), 10 µL Na_3VO_4 (50 mM), and 10 µL $Na_4P_2O_7 \cdot 10\ H_2O$ (1 mg/mL) per mL of buffer solution. Then, the T24 and AGS glycoengineered cells models, as well as T24 WT and AGS WT cell lines, were scraped from T300 flasks using 5-7 mL of the prepared buffer and kept on ice for 15 minutes. The cell suspension was transferred to a tube and passed through a syringe with a 27-gauge needle 10 times, followed by an additional 20-minute incubation on ice. The whole-cell lysate was centrifuged (720×g, 5 min) to pellet the nuclei, and the supernatant was carefully transferred to a new tube and kept on ice. The supernatant was subsequently centrifuged (10,000×g, 5 min) to isolate the mitochondria, and the resulting supernatant was transferred to a new tube and maintained on ice. To obtain the membrane fraction, the supernatant was centrifuged one last time (100,000×g, 1h, 4°C) and the membrane pellet was resuspended in 1 mL of fractionation buffer. The remaining cell-lysate suspension was passed through a syringe with a 25-gauge needle 10 times and centrifuged at 100,000xg for 45 minutes. The final membrane pellet was resuspended in Tris-buffered saline (TBS) containing 0.3% sodium dodecyl sulfate (SDS), followed by a brief sonication (10 seconds) to homogenise the lysate. The protein content in each sample was estimated using a DC protein assay kit (Bio-Rad) and aliquots of 200 ug of protein per tube were prepared.

2.2. Treatment with PNGaseF and enrichment with VVA-agarose

After extraction and quantification of the membrane protein, each sample was adjusted to a final volume of 300 µL with TBS-0.3% SDS and 15 µL of 20% Nonidet P-40 (NP-40), a nonionic surfactant used to inactivate the SDS. Subsequently, samples were incubated overnight at 37°C with agitation using Peptide-*N*-glycosidase F (PNGaseF from *Elizabethkingia meningoseptica*; Sigma Aldrich, Missouri, USA) at 2 U per 20 µg of protein in order to ensuring efficient deglycosylation of *N*-glycans. Following incubation, PNGase F was inactivated by heating at 95°C for 20 minutes. Samples were then diluted to 400 µL with LacA buffer for optimal binding conditions. Meanwhile, the columns were prepared for affinity purification with 100 µL of *Vicia Villosa* (VVA) lectin-agarose (VectorLabs). The samples were then diluted to a final volume of 400 µL with LacA buffer and incubated in the

columns for 30 minutes with agitation, followed by a series of washes with LacA buffer and centrifugations. Successively, the bound glycoproteins were eluted with 100 µL of 3% acetic acid, and the eluates were dried using a SpeedVac concentrator. Each sample was then reconstituted in TBS-0.3% SDS solution.

2.3. Coating of the magnetic beads

For the preparation of the beads, $20x10^6$ Dynabeads™ M-450 Tosyl-activated (Thermo Fisher Scientific) were washed twice with Washing Buffer 1 (0.1M phosphate buffer, pH 7.4-8). Then, the beads were incubated with 10 µg of membrane protein from the target models for 1h30min at room temperature with agitation (1000 rpm). Following incubation, Buffer 1 containing 1% BSA (final concentration of 0.167% BSA) was added to the beads and incubated overnight at 37°C with agitation (1000 rpm) to ensure thorough protein binding. The beads were subsequently washed twice with 20 mM HEPES and incubated for 2 hours at room temperature in 25 mM bis(sulfosuccinimidyl)suberate (BS3) crosslinker (Thermo Fisher Scientific) diluted in 20 mM HEPES. After this period, the beads were washed with Buffer 3 (0.2 M Tris, 0.1% BSA, pH 8.5) and subjected to further incubation overnight at room temperature (1000 rpm) to deactivate any remaining free tosyl groups on the beads. Afterwards, the beads were washed with Buffer 2 (PBS with 0.1% BSA and 2 mM EDTA, pH 7.4), and the correct binding of Tn-enriched proteins to the beads was verified by flow cytometry using VVA. The coated beads were stored in the same buffer at 4°C for up to 1 month.

2.4. Conjugate formation and Synapse isolation

For macrophage-coated bead conjugation, macrophages were resuspended in Imaging Medium (RPMI Medium 1640, glutamine and no phenol red) and added in a 1:1 ratio with membrane protein-coated beads for 60 minutes at 37°C with agitation (1000 rpm). Following confirmation of successful conjugation via microscopy, the macrophage-bead conjugates were transferred to ice and subjected to several washes with cold cytoskeletal buffer (CSK buffer; 300 mM sucrose, 100 mM sodium chloride, 10 mM PIPES, pH 6.8, 3 mM magnesium chloride) to remove unbound cells. The conjugates were then resuspended in a fresh buffer containing CSK buffer supplemented with 0.5% Triton X-100 and protease inhibitors, followed by sonication in optimized cycles (15 seconds on, 15 seconds off for 3 cycles). Consecutively, the conjugates underwent a repetitive washing process with CSK buffer containing 0.5% Triton X-100 to eliminate any residual cellular debris. Afterwards, the proteins bound to the beads were eluted with 25 µL of Loading buffer for 30 minutes at

70°C with agitation (1000 rpm). The beads were discarded, and the eluates were either used immediately or stored at -20°C for up to one week.

2.5. In-gel digestion

The eluates were loaded into electrophoresis gel wells, and after electrophoresis, the gel was stained with a Zinc Reversible Stain Kit for protein quantification. Gel bands, excised into 1 to 2 mm pieces, were dissolved in Tris-glycine buffer (25 mM Tris, 192 mM glycine, pH 8.0) and washed three times with Milli-Q water for 10 minutes each. Thenceforth, the gel pieces underwent dehydration with acetonitrile (ACN) (LC-MS Grade, Sigma-Aldrich) for 5-10 minutes. In turn, disulfide bonds were reduced by rehydrating the gel pieces in 20 mM dithiothreitol (DTT) (Sigma-Aldrich) at 56°C for 30 minutes, followed by another dehydration step with 100% ACN. To prevent reoxidation of disulfide bonds, the gel pieces were rehydrated with 55 mM iodoacetamide (IAA) (Sigma-Aldrich) for 20 minutes in the dark at room temperature, then washed with 100 mM ammonium bicarbonate and dehydrated once more. Once the conditions were met, enzymatic digestion was carried out by adding 75 µL of 0.02 µg/µL trypsin (Promega) to the gel pieces and left to absorb on ice for 20 minutes. When all the trypsin solution had been absorbed, 40mM ammonium bicarbonate/10% ACN was added to cover the gel pieces and incubated for 18 hours at 37ºC in a humid chamber. Following this, 100% ACN was added and another incubation for 15 minutes at 37°C was performed. The supernatant was then collected into a clean tube, and the extraction process was repeated twice with 50% ACN/5% formic acid (LC-MS Grade, Sigma-Aldrich). Finally, the samples were dried using a SpeedVac concentrator for approximately 5 hours.

2.6. Mass spectrometry analysis

The nanoLC system employed in this experiment consisted of a Vanquish Neo UHPLC system coupled in-line with a QExactive Plus Hybrid Quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific), which was fitted with a nano-electrospray ion source (EASY-Spray source; Thermo Fisher Scientific). The mobile phases used were aqueous formic acid (0.1%) for eluent A and formic acid (0.1%) in acetonitrile (80%) for eluent B. A sample volume of 10 µL was directly injected into a trapping column (C18 PepMap Neo, 5 µm particle size), which then directed the sample to the analytical column (EASY-Spray C18 PepMap, 100 Å, 75 µm x 150 mm ID, and 3 µm particle size) at a flow rate of 0.25 µL/min. The temperature of the column was maintained at 35°C. Furthermore, peptide separation was achieved using the following gradient: 7 minutes from 2.5% B to

12% B, 50 minutes from 12% B to 46% B, 5 minutes from 46% B to 99% B, and 10 minutes at 99% B. For mass spectrometry, the instrument was operated in positive ion mode across the *m/z* range of 300-2000, with a spray voltage set at 1.9 kV and a transfer capillary temperature of 275°C. Full MS parameters included a resolution of 140,000, an AGC target of $3x10^6$, and a maximum injection time of 200 ms. The top 15 most intense ions were selected for high-energy collision dissociation (HCD). Data were collected and analyzed using Xcalibur software version 4.5 (Thermo Fisher Scientific).

Data was analyzed automatically using the SequestHT search engine with the Percolator algorithm for validation of protein identifications (Proteome Discoverer 3.1, Thermo Scientific). Data were searched against the human proteome obtained from the SwissProt database selecting trypsin as the enzyme and allowing for up to 2 missed cleavage sites and a precursor ion mass tolerance of 10 ppm and 0.6 Da for product ions. Carbamidomethylcysteine was selected as a fixed modification, while oxidation of methionine (+15.9) was defined as variable modification.

2.7. Bioinformatics

To generate the final curated protein list, the proteins with less than 2 unique peptides and the proteins with low or medium protein FDR confidence were removed. For the curation of plasma membrane proteins, protein annotation tables for each cell line were generated using gene ontology (GO) terms for cellular location through Proteome Discoverer 3.1. Molecular and biological functions were also explored. The plasma membrane proteins identified in the fractions Beads coated with T24/AGS WT/C1GALT1 KO proteins were used as controls. The selection of the plasma membrane proteins significantly abundant in the samples from the interactions between the macrophages and the GlycoAvatars was based on exploring Volcano Plots (VolcaNoseR; https://huygens.science.uva.nl/VolcaNoseR2/) selecting proteins with Log2 of fold change (Interactions/Coated beads) higher than 1.5 and with adjusted p-value lower than 0.05 ($-Log_{10}$p-value higher than 1.3). The subset of plasma membrane proteins identified exclusively on Macrophages-T24/AGS C1GALT1 KO samples were further analyzed regarding their protein-protein interactions, molecular and biological functions and involved KEGG pathways using the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) version 12.0 (http://string-db.org/). A network with biological processes considering only significant pathways ($p \leq 0.05$) was constructed for these common proteins using the following parameters: a GO tree interval of min. 4 and max.10, and a kappa score of 0.64 in ClueGO plugin for Cytoscape Software 3.10.2.

3. RESULTS & DISCUSSION

The surface of all living cells is adorned with carbohydrate molecules, known as glycans, which serve as ligands for several proteins(14). These interactions between GBPs and their ligands play a pivotal role in regulating various cellular functions in cancer, especially through the modulation of the immune system, with a notable impact on macrophages(6, 15). Although proteomic analyses have advanced in recent years due to technological improvements and new molecular tools(16), the identification of specific receptors involved in macrophage-glycan interactions remains poorly explored. Therefore, developing a novel method to identify GBPs that recognize glycans commonly expressed in various tumours, such as Tn and STn antigens, is essential for advancing knowledge in glycoimmunology. To address this need, we proposed a protocol involving the coating of magnetic beads with glycoproteins extracted from the plasma membrane of glycoengineered models with immature glycosylation, followed by their interaction with unpolarized macrophages in native environment to preserve the protein conformation and proper interactions ligand (coated bead) – receptor (macrophage).

As a starting point, we performed a differential cellular fractionation protocol using glycoengineered T24 and AGS tumour cell models that overexpress the Tn and STn antigens, along with their respective wild-type models for glycosylation controls. For the *C1GALT1* KO models, this step was followed by treatment with PNGase F followed by VVA-agarose enrichment to remove *N*-glycans moieties and isolate glycoproteins enriched in for Tn antigens, respectively **(Figure 1A-1)**. Afterwards, the paramagnetic beads were coated with these glycoproteins **(Figure 1A-2)**, and then validated through flow cytometry analysis, staining the beads with VVA lectin, which confirmed the success of the glycoprotein coating to the beads without aggregation **(Figure 1B)**. In turn, the plasma membrane proteins from the wild-type control models were first incubated with the magnetic beads and then treated with PNGase F. Then, the protein-coated beads were incubated with monocyte-derived macrophages isolated from healthy donors, to mimic surface tumour cell antigen interaction with antigen-presenting cells **(Figure 1A-3 and 1C)**. This approach created the conditions for assembling a network of immune cell receptors bound to their respective ligands on the beads. The magnetic properties of the beads allowed the protein collection of the specific ligand-receptor interaction after sonication and washing, and subsequently analysis by mass spectrometry of the digested peptides for protein identification **(Figure 1A-4)**.

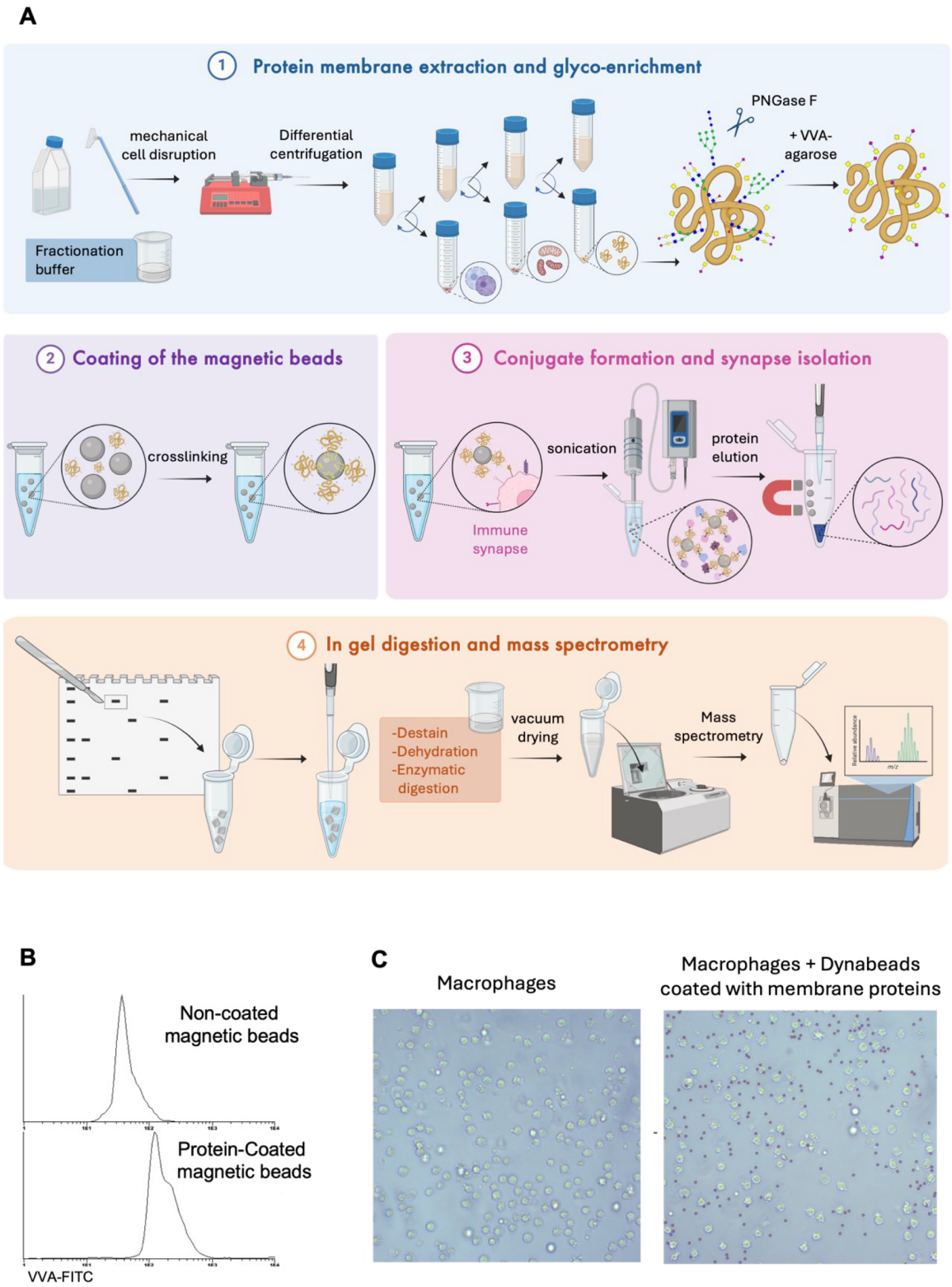


Figure 1 - Proteomic workflow for characterizing immune synapses between macrophages and Tn/STn-glycoproteins of cancer cells. (A) A roadmap for immune cell receptor identification. Briefly, extracellular membrane glycoproteins were obtained through a differential cellular fractionation protocol using the T24 *C1GALT1 KO* and AGS *C1GALT1 KO* tumour cell lines, followed by treatment with PNGase F and enrichment with VVA-agarose **(1)**. The magnetic beads were then incubated with the Tn-enriched glycoproteins, accompanied by covalent crosslinking to them **(2)**.

Once the conditions were established, an immune conjugation was formed between the glycoprotein-coated magnetic beads and the macrophages. Subsequently, the unbounded cells were washed, the macrophage membranes from the cell-bead complexes were disrupted by sonication, and then the cell debris and the free proteins were washed, followed by the protein-bead elution **(3)**. The proteins from the different conditions underwent in-gel trypsin digestion and were then ready for mass spectrometry analysis **(4)**. **(B)** The efficiency of bead coating with glycoproteins was determined by flow cytometry. Top histogram: uncoated beads stained with VVA-Lectin. Bottom histogram: coated beads stained with VVA-Lectin. **(C)** Formation of macrophage-bead conjugates using glycoprotein-coated beads. Macrophages were incubated with the coated beads for 15 to 60 minutes and analysed using inverted bright-field microscopy to confirm the occurrence of the interaction.

For all conditions, the data were searched against the human proteome and after filtering out known contaminants and low-confidence hits (characterized by having fewer than one unique peptide and a spectral count [MScount] below 5), we identified high-confidence proteins, using the Proteome Discoverer software. Since our focus was on proteins expressed in the plasma membrane, we selected only the proteins meeting these criteria, excluding all proteins that did not match the keywords "plasma membrane" for the Gene Ontology (GO) term cellular component. With this pipeline, from the 412 plasma membrane proteins (56% of the total proteins) identified in the macrophages-T24 WT protein conjugates, we constructed a Volcano plot, where we observed the presence of 195 significantly highly expressed proteins from the interaction between macrophages and beads coated with T24 WT, compared to the control beads coated with tumour proteins **(Figure 2A)**. In the interaction between macrophages and GlycoAvatars T24 *C1GALT1* KO, versus the control samples, we found approximately 97 significantly highly expressed proteins, from the 597 identified (55% of the total proteins) (**Figure 2B)**. Specifically, 161 proteins were exclusively from the macrophage-T24 WT interaction, 63 proteins were exclusively found in samples from the immune synapse between macrophages and GlycoAvatars T24 KO cell, while 34 proteins were present in both conditions **(Figure 2C)**. To gain an overview of the immunological synapse proteome involving glycoengineered bladder cancer cells, we used Cytoscape to determine the pathways associated with enriched and interaction-specific proteins, selecting GO terms related to biological processes, immune system processes, and molecular functions **(Figure 2D)**. As expected, we observed enrichment in pathways that may be involved in immunosuppression, such as “myeloid leukocyte cytokine production” and “regulation of lymphocyte apoptotic process”. These findings reflect both the modulation of macrophages towards a phenotype that supports tumour immune evasion through the secretion of anti-inflammatory cytokines(17-19) and the induction of lymphocyte death, thereby reducing the number of effector cells in

the TME(20, 21). Additionally, the pathway “regulation of protein localization to membrane” is central to the network, as accurate protein localization to the plasma membrane is essential for receptor activation, highlighting its crucial role in cellular signalling and communication(22). Nevertheless, it is still important to highlight the "p75 NTR receptor-mediated signalling" pathway due to its dual functional role depending on the recognized ligand and co-receptors, as it can lead to cell death and reduced synaptic function or, conversely, stimulate cell survival and growth(23, 24). It is also known that in tumour conditions, the p75 NTR signalling pathway may be diminished, as its overexpression impacts the modulation of the immune microenvironment by increasing the recruitment of M1 macrophages and CD8 T cells while decreasing neutrophil recruitment, significantly facilitating the death of tumour cells(25).

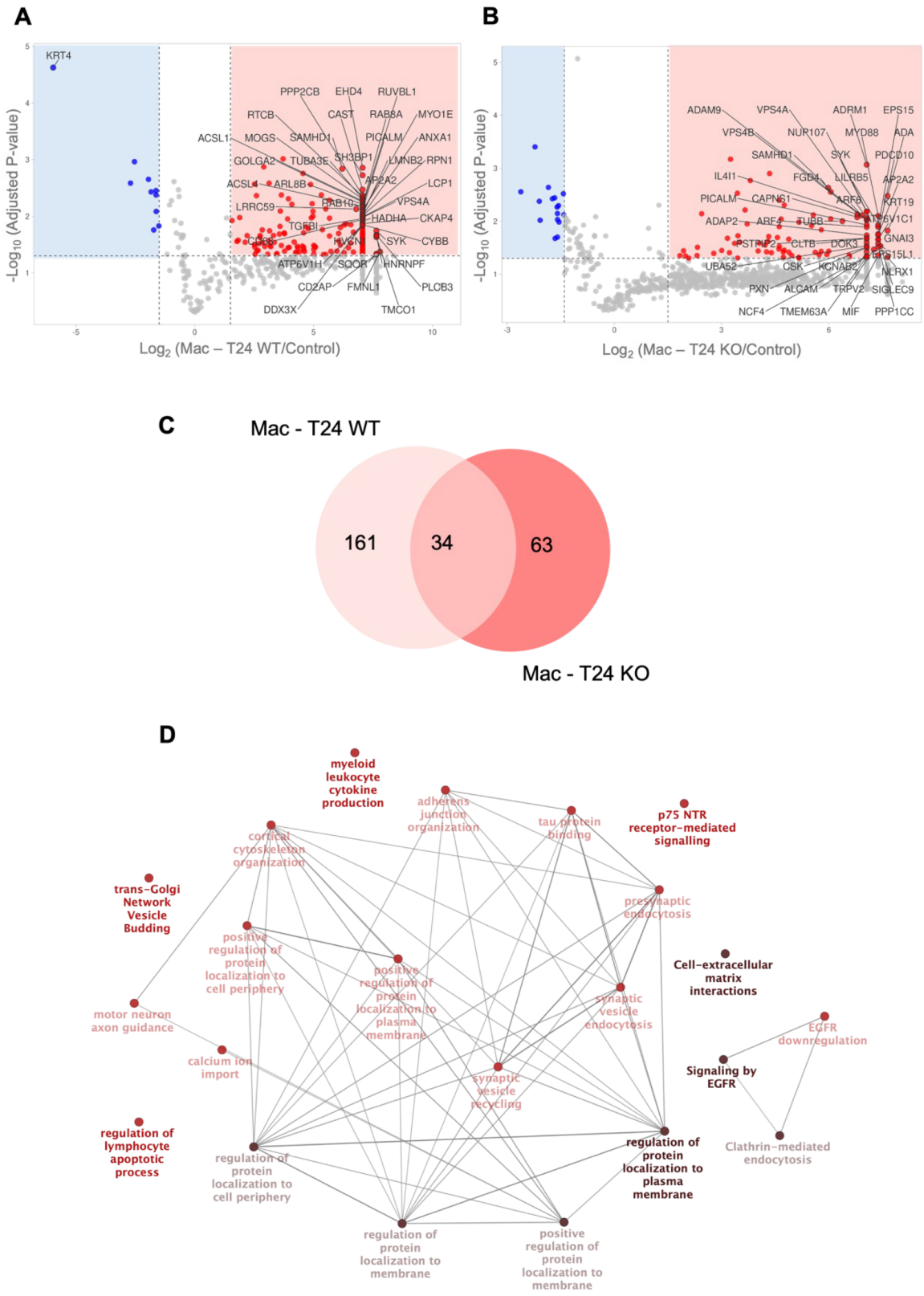


Figure 2 – Comparative proteomic analysis of macrophage interactions with GlycoAvatars representing the T24 Wild-Type and glycoengineered T24 Tn$^+$ plasma membrane proteins. (A) Volcano plot showcases global protein enrichment resulting from macrophage-bead interaction

coated with WT bladder tumour cells compared to the proteins from the T24 WT control coated beads. From 735 identified proteins with high-confidence in the three biological replicates, 412 are localized on the plasma membrane, and from those, 195 are highly abundant on the conjugate samples. **(B)** Volcano plot highlights the distribution of enriched proteins resulting from macrophage-T24 KO coated-beads interaction compared to the proteins from the T24 KO coated-beads. From 1095 identified proteins with high-confidence in the three biological replicates, 597 are localized in the plasma membrane, being 97 significantly abundant in the conjugate samples. Proteins highlighted in red are most significant abundant proteins in the conditions from macrophage-GlycoAvatars coated with either T24 WT or T24 KO cells, while those in blue are the most abundant proteins in beads. Statistical significance was determined with a p-value <0.05 and a fold change greater than 1.5. **(C)** Venn diagram elucidates the overlap of highly expressed proteins from the interactions with T24 WT and T24 KO coated beads. Out of the total proteins, 34 were shared between the two conditions, while 161 were uniquely highly expressed in MAC-T24 WT and 63 in MAC-T24 KO. **(D)** Comprehensive networks of biological processes involving the enriched proteins resulting exclusively from the isolated synapse between macrophages and T24 KO based on the GO terms biological processes, immune system processes, and molecular functions.

Following the same rationale, we identified 175 highly expressed proteins, from the 328 plasma membrane proteins (56% of total proteins), arising from the interaction between macrophages and beads coated with AGS WT proteins, compared to the control group consisting only of beads coated with tumour proteins **(Figure 3A)**. In the interaction between macrophages and GlycoAvatars AGS KO, we observed approximately 62 highly abundant proteins, from the 384 membrane proteins (62% of the total proteins) **(Figure 3B)**. Notably, 151 proteins were exclusively detected in the sample's macrophage-AGS WT interaction, 24 proteins were shared between both conditions, and only 38 proteins were specific to the immune synapse between macrophages and GlycoAvatars AGS KO **(Figure 3C)**. Afterwards, we analysed the list of most abundant proteins resulting exclusively from the interaction between macrophages and glycoengineered gastric tumour cell proteomes, focusing on their involvement in biological processes **(Figure 3D)**. This analysis revealed an enrichment of the "macrophage differentiation" pathway, suggesting that the interaction with gastric tumour cells overexpressing the Tn antigen influences macrophage behaviour, potentially promoting a phenotype associated with tumour progression(26, 27). Furthermore, the "EPH-Ephrin signalling" pathway emerged as a central node, known for

its role in cell-to-cell communication and immune response modulation(28). It may contribute to macrophage phenotypic alterations during their interaction with AGS KO cells.

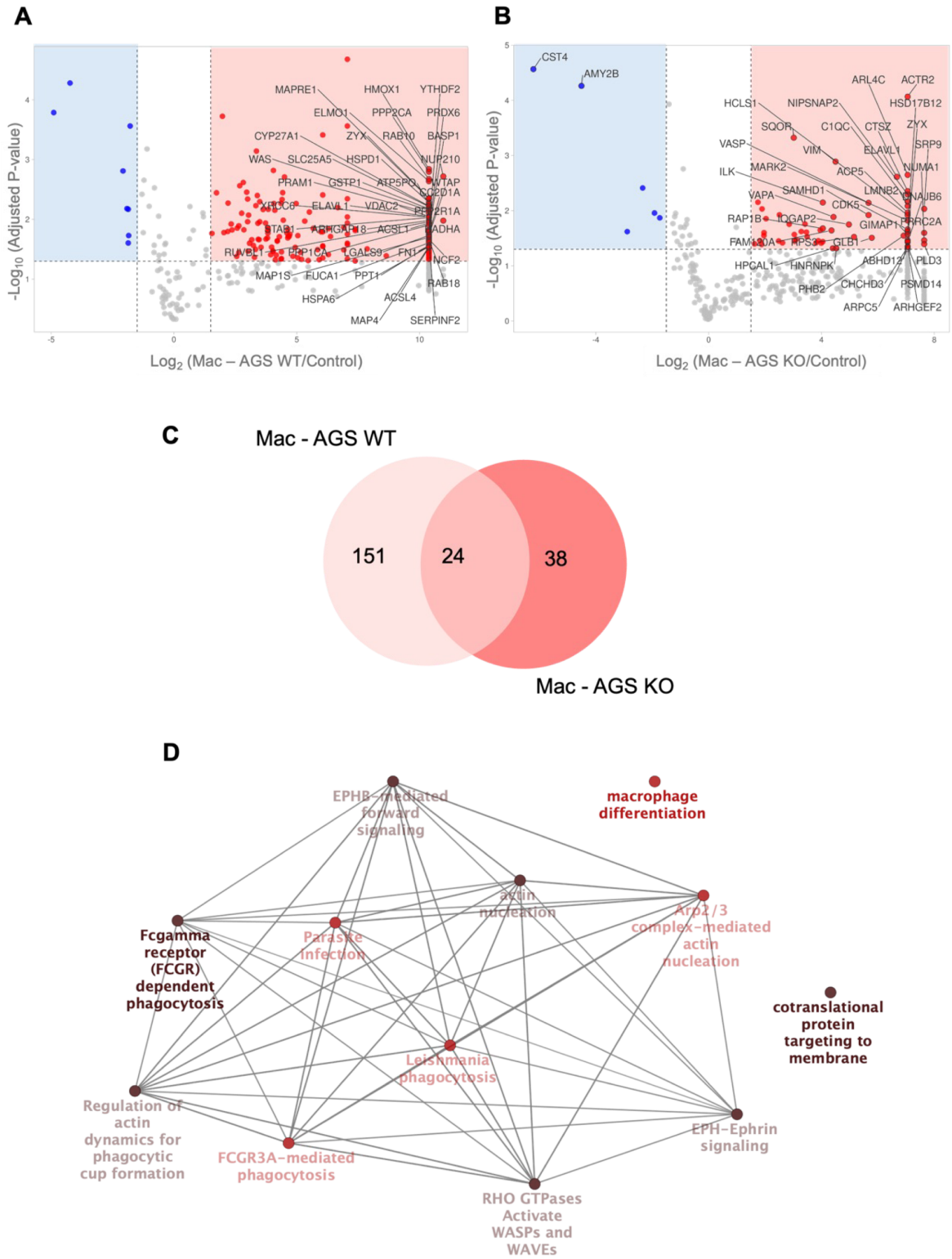


Figure 3 - Comparative proteomic analysis of macrophage interactions with GlycoAvatars representing the AGS Wild-Type and glycoengineered AGS Tn$^+$ plasma membrane proteins. **(A)** Volcano plot showcases global protein enrichment resulting from macrophage-bead interaction

coated with WT gastric tumour cells compared to the proteins from the AGS WT control beads. From 586 identified proteins with high-confidence in the three biological replicates, 328 are localized on the plasma membrane, and from those, 175 are highly abundant on the conjugate samples. **(B)** Volcano plot highlights the distribution of enriched proteins resulting from macrophage-AGS KO coated-beads interaction compared to the proteins from the AGS KO coated-beads. From 619 identified protein with high-confidence in the three biological replicates, 384 are localized in the plasma membrane, being 62 significantly abundant in the conjugate samples. Proteins highlighted in red are most significant abundant proteins in the conditions from macrophage-GlycoAvatars coated with either AGS WT or AGS KO cells, while those in blue are the most abundant proteins in beads. Statistical significance was determined with a p-value <0.05 and a fold change greater than 1.5. **(C)** Venn diagram elucidates the overlap of highly expressed proteins of the immune from the interactions with AGS WT and AGS KO coated beads. Out of the total proteins, 24 were shared between the two conditions, while 151 were uniquely highly expressed in MAC-AGS WT and 38 in MAC-AGS KO. **(D)** Comprehensive networks of biological processes involving the enriched proteins resulting exclusively from the isolated synapse between macrophages and AGS KO based on the GO terms biological processes, immune system processes, and molecular functions.

In order to identify potential targets involved in the acquisition of an immunosuppressive phenotype by macrophages upon contact with truncated short-chain *O*-glycans expressed by tumour cells, we focused our research on the proteins that are highly abundant and exclusively present in samples derived from immune synapses between macrophages and glycoengineered bladder or gastric cell models. Firstly, we analysed the interconnections among these proteins and observed that the presence of multiple interconnected nodes suggests that several proteins may be co-regulated or participate in shared pathways. For example, among the proteins from the macrophage-GlycoAvatars T24 KO interaction **(Figure 4A)**, ACTB (beta-actin) alongside VASP (Vasodilator-stimulated phosphoprotein), which modulates actin filament dynamics, plays a crucial role in phagocytic processes(29, 30), highlighting their importance in macrophage function and interaction with tumour cells, both of which are critical for determining the effectiveness or deficiency of immune responses within the TME. Meanwhile, some proteins, particularly IL4I1 (Interleukin-4 Induced 1), MRC1 (C-Type Mannose Receptor 1 or CD206), and SIGLEC-9 (Sialic Acid-Binding Ig-Like Lectin 9), stand out due to their role and subsequent impact on the immune system. IL4I1 is especially important in driving M2 macrophage polarization, while suppressing the expression of cytokines linked to the M1 phenotype and the activity of anti-tumour $CD8^+$ T cells, as well as promoting the invasion and migration of tumour cells(31, 32). In turn, MRC1, as previously mentioned, is significantly expressed in macrophages following M2a polarization, representing a crucial marker in the pro-tumorigenic environment since it can interact with glycans, leading to the production of anti-inflammatory cytokines like IL-10, and with Tn antigens on cancer cells, facilitating

immunosuppression(27). Similarly, SIGLEC-9, expressed on macrophages, can recognize sialylated structures like STn on tumour cells, promoting further M2 polarization, which can recruit immunosuppressive neutrophils and further inhibit anti-tumour T cell responses(33, 34). In this way, these proteins contribute to the formation of a macrophage phenotype that favours tumour progression, decreasing immune surveillance and promoting conditions that increase tumour survival, potentially acting as key receptors within the immunological synapse.

On the other hand, among the proteins exclusively derived from the macrophage-beads coated with AGS KO interaction **(Figure 4B)**, ARPC5 (Actin-Related Protein 2/3 Complex Subunit 5) and WASF1 (Wiskott-Aldrich Syndrome Protein Family Member 1) are involved in the dynamic of the cytoskeleton, influencing vital cellular processes such as phagocytosis, cell movement and immune synapse formation(35, 36). Moreover, the presence of the GIMAP1 (GTPase, IMAP Family Member 1) protein is linked to apoptosis, cell survival and immune homeostasis in certain immune cells, such as lymphocytes(37, 38), and in this context, its expression could potentially be disrupting macrophage polarization. By influencing the immune environment, these proteins emerge as potential regulators in the immune interaction, guiding macrophage behaviour and shaping the immunological response.

However, when we compared the expression of proteins exclusively derived from the macrophage-GlycoAvatars T24 KO interaction with those derived solely from the macrophage-GlycoAvatars AGS KO interaction, we identified only a single protein shared between these conditions **(Figure 4C)**. This protein corresponds to Cyclin-dependent Kinase 5 (CDK5), a proline-directed serine/threonine protein kinase, which is unique within its family for its primary role in cell migration rather than in cell cycle or mitosis regulation(39). The CDK5 activation does not rely on cyclins but instead depends on two specific factors: p35 (CDK5R1) or p39 (CDK5R2)(40). Beyond its well-established role in the brain, CDK5's function in immune cells, such as regulating neutrophil degranulation and T-cell activation, has gained increasing attention in recent years(41, 42). According to various studies in the literature, inhibitors of CDK5 on cancer cells show significant potential in combating invasion and metastasis in glioblastoma(43), breast(44, 45), colorectal(46, 47), and pancreatic(48, 49) cancers. However, the mechanism of action of CDK5 on macrophages remains unclear and appears to depend on its activators. CDK5 can either upregulate the PD-L1 and reduce the recruitment of $CD4^+$ T cells to the tumour site or reduce the production of anti-inflammatory cytokines, such as IL-10, while activating the NF-κB pathway(50, 51). The most widely accepted mechanism suggests that suppressing CDK5 or inhibiting the binding of its activators in macrophages increases IL-10 production,

promoting an anti-inflammatory profile that leads to immune suppression and supports tumour progression(52, 53). Based on the findings presented in Chapter 3 and the macrophage phenotype promoted by short-chain *O*-glycans, the presence of CDK5 may play a role in the immune synapse between macrophages and glycoengineered bladder and gastric cancer cells. We believe that, in addition to interacting with the well-known lectins, Tn and STn glycans may also bind to other receptors on immune cells promoting a tolerogenic phenotype. Furthermore, altered glycosylated proteins may interact with CDK5, thereby inhibiting its binding to activator molecules and contributing to the maintenance of an anti-inflammatory phenotype. In summary, CDK5 has been described as a crucial player in promoting tumour metastasis and immune modelling and its inhibition in some types of cancers, positioning it as a potential therapeutic target to restore immune surveillance and counter tumour-supportive immunosuppressive mechanisms.

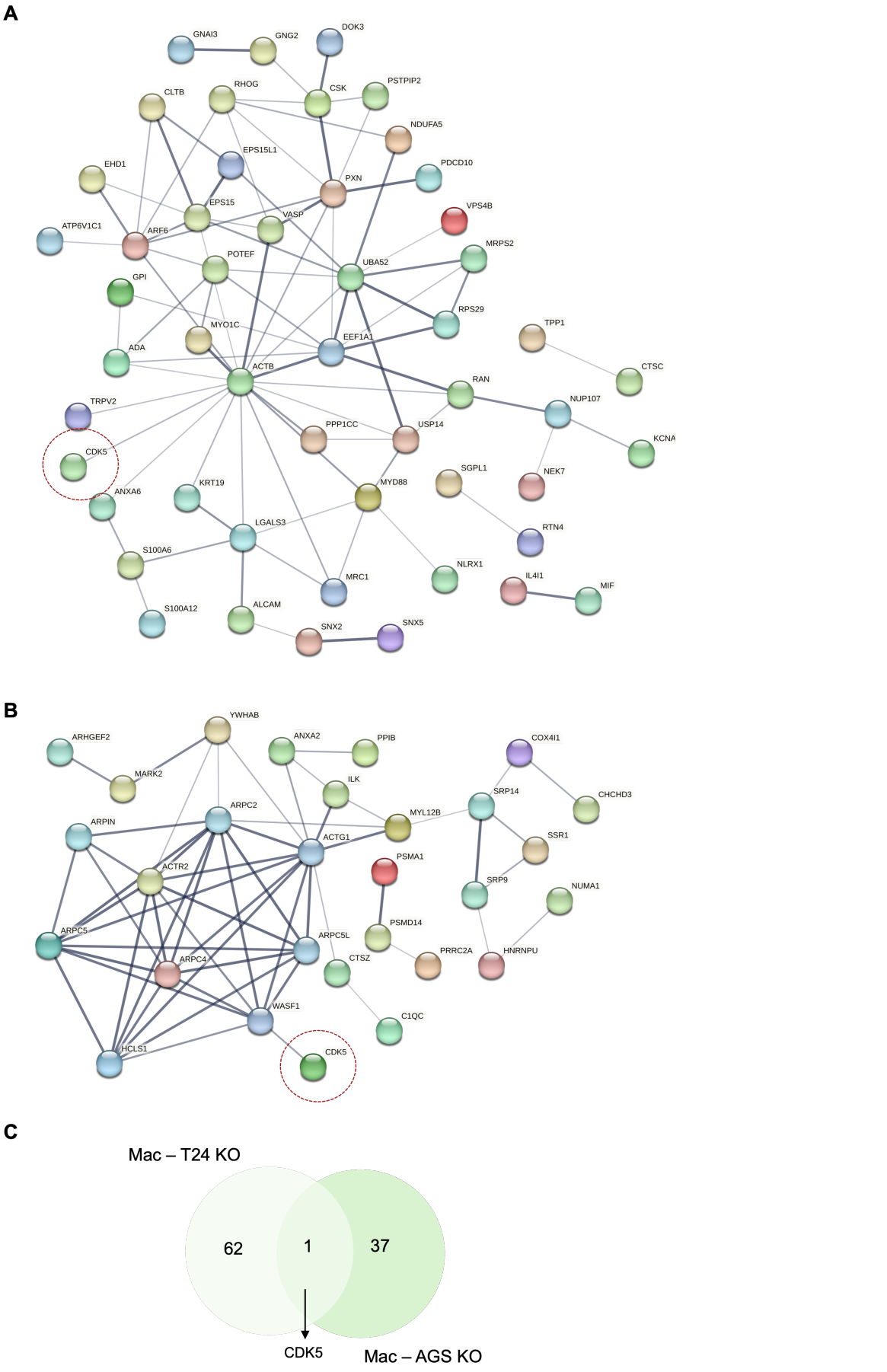


Figure 4 – Protein-protein interaction networks unique to the immune synapse between

macrophages and genetically modified gastric and bladder tumour cells. (A) Protein-protein interaction network exclusively upregulated in macrophages interacting with T24 KO. **(B)** Protein-protein interaction network exclusively upregulated in macrophages interacting with AGS KO. Each node represents a protein, and the edges linking nodes represent known interactions between the two proteins. The thickness of the edges is related to the strength of the interaction relationship. The red circle represents the protein that was found in both immunological synopses - CDK5. **(C)** Venn diagram elucidates the overlap of differentially expressed proteins of the immune synapse between the conditions T24 KO and AGS KO. Out of the total proteins, 1 was shared between the two conditions, while 62 were uniquely upregulated in MAC-T24 KO and 37 in MAC-AGS KO.

4. CONCLUDING REMARKS

Glycobiology has emerged as a crucial field in cancer research due to the complex interactions between glycans and proteins, which play a pivotal role in various aspects of tumour biology, including the modulation of the immune response(54). In fact, short-chain *O*-glycans has been emphasized in their interactions with immune cells through recognition by glycan-binding receptors, impacting immune responses and facilitating the induction of an immunosuppressive microenvironment(6, 8, 55). Over the past decade, mass spectrometry-based proteomics has been refined to enhance our understanding of protein composition, structure, expression, modification states, as well as protein interactions and connections on a global scale(13, 56). Despite these advancements, there is a notable lack of proteomic analyses specifically targeting the immune synapse between tumour cells with glycosylation modifications and immune system cells.

To address this need, this chapter outlines the development of a high-throughput proteomics-assisted strategy to characterize the immunological synapse that forms between tumour cells with altered glycosylation and antigen-presenting cells, such as macrophages. Glycoproteins were isolated from the plasma membranes of glycoengineered cell models with immature glycosylation from gastric and bladder cancers and then coated onto the surface of magnetic beads, creating “GlycoAvatars”. When exposed to macrophages, these GlycoAvatars enabled the characterization of the glycan interactome. Based on this rationale, we identified 63 plasma membrane proteins that were uniquely enriched in the interaction between macrophages and GlycoAvatars coated with proteomes from T24 *C1GALT1* KO cells, and 38 plasma membrane proteins exclusively enriched in the interaction between macrophages and GlycoAvatars coated with proteomes from AGS *C1GALT1* KO cells. Among these, only the protein CDK5 was present in both conditions, and it is closely linked to essential biological processes, including cell migration and immune responses, which are crucial in cancer development and progression. While

CDK5 shows promise as a pivotal receptor in the communication between tumour cells, which overexpress truncated short-chain *O*-glycans, and macrophages, further orthogonal validation is necessary, including blocking this protein or knocking out its gene, to determine whether the immunosuppressive characteristics persist in the co-culture settings.

5. REFERENCES

1. Colley KJ, Varki A, Haltiwanger RS, Kinoshita T. Cellular Organization of Glycosylation. In: Varki A, Cummings RD, Esko JD, Stanley P, Hart GW, Aebi M, et al., editors. Essentials of Glycobiology. Cold Spring Harbor (NY): Cold Spring Harbor Laboratory Press
Copyright © 2022 The Consortium of Glycobiology Editors, La Jolla, California; published by Cold Spring Harbor Laboratory Press; doi:10.1101/glycobiology.4e.4. All rights reserved.; 2022. p. 43-52.
2. Schnaar RL. Glycans and glycan-binding proteins in immune regulation: A concise introduction to glycobiology for the allergist. Journal of Allergy and Clinical Immunology. 2015;135(3):609-15.
3. Peixoto A, Relvas-Santos M, Azevedo R, Santos LL, Ferreira JA. Protein Glycosylation and Tumor Microenvironment Alterations Driving Cancer Hallmarks. Front Oncol. 2019;9:380.
4. Azevedo R, Peixoto A, Gaiteiro C, Fernandes E, Neves M, Lima L, et al. Over forty years of bladder cancer glycobiology: Where do glycans stand facing precision oncology? Oncotarget. 2017;8(53):91734-64.
5. Freitas R, Peixoto A, Ferreira E, Miranda A, Santos LL, Ferreira JA. Immunomodulatory glycomedicine: Introducing next generation cancer glycovaccines. Biotechnology Advances. 2023;65:108144.
6. Peixoto A, Miranda A, Santos LL, Ferreira JA. A roadmap for translational cancer glycoimmunology at single cell resolution. J Exp Clin Cancer Res. 2022;41(1):143.
7. Pinho SS, Alves I, Gaifem J, Rabinovich GA. Immune regulatory networks coordinated by glycans and glycan-binding proteins in autoimmunity and infection. Cell Mol Immunol. 2023;20(10):1101-13.
8. Cornelissen LA, Van Vliet SJ. A Bitter Sweet Symphony: Immune Responses to Altered O-glycan Epitopes in Cancer. Biomolecules. 2016;6(2).
9. Yang D, Yang L, Cai J, Hu X, Li H, Zhang X, et al. A sweet spot for macrophages: Focusing on polarization. Pharmacological Research. 2021;167:105576.

10. Pereira MS, Alves I, Vicente M, Campar A, Silva MC, Padrão NA, et al. Glycans as Key Checkpoints of T Cell Activity and Function. Front Immunol. 2018;9:2754.
11. Ferreira JA, Relvas-Santos M, Peixoto A, A MNS, Lara Santos L. Glycoproteogenomics: Setting the Course for Next-generation Cancer Neoantigen Discovery for Cancer Vaccines. Genomics Proteomics Bioinformatics. 2021;19(1):25-43.
12. He K, Baniasad M, Kwon H, Caval T, Xu G, Lebrilla C, et al. Decoding the glycoproteome: a new frontier for biomarker discovery in cancer. Journal of Hematology & Oncology. 2024;17(1):12.
13. Bagdonaite I, Malaker SA, Polasky DA, Riley NM, Schjoldager K, Vakhrushev SY, et al. Glycoproteomics. Nature Reviews Methods Primers. 2022;2(1):48.
14. Griffin ME, Hsieh-Wilson LC. Tools for mammalian glycoscience research. Cell. 2022;185(15):2657-77.
15. Lopes N, Correia VG, Palma AS, Brito C. Cracking the Breast Cancer Glyco-Code through Glycan-Lectin Interactions: Targeting Immunosuppressive Macrophages. Int J Mol Sci. 2021;22(4).
16. Nice EC. The status of proteomics as we enter the 2020s: Towards personalised/precision medicine. Analytical Biochemistry. 2022;644:113840.
17. Groth C, Hu X, Weber R, Fleming V, Altevogt P, Utikal J, et al. Immunosuppression mediated by myeloid-derived suppressor cells (MDSCs) during tumour progression. British Journal of Cancer. 2019;120(1):16-25.
18. Sezginer O, Unver N. Dissection of pro-tumoral macrophage subtypes and immunosuppressive cells participating in M2 polarization. Inflammation Research. 2024.
19. Tang L, Xu H, Wu T, Wu W, Lu Y, Gu J, et al. Advances in tumor microenvironment and underlying molecular mechanisms of bladder cancer: a systematic review. Discover Oncology. 2024;15(1):111.
20. Franzese O, Ancona P, Bianchi N, Aguiari G. Apoptosis, a Metabolic “Head-to-Head” between Tumor and T Cells: Implications for Immunotherapy. Cells. 2024;13(11):924.
21. Lu B, Finn OJ. T-cell death and cancer immune tolerance. Cell Death & Differentiation. 2008;15(1):70-9.
22. Wang ZJ, Thomson M. Localization of signaling receptors maximizes cellular information acquisition in spatially structured natural environments. Cell Systems. 2022;13(7):530-46.e12.
23. Meeker RB, Williams KS. The p75 neurotrophin receptor: at the crossroad of neural repair and death. Neural Regen Res. 2015;10(5):721-5.

24. Almeida RD, Duarte CB. p75NTR Processing and Signaling: Functional Role. In: Kostrzewa RM, editor. Handbook of Neurotoxicity. New York, NY: Springer New York; 2014. p. 1899-923.
25. Lu Q, Qu Y, Ding Y, Kang X. p75NTR/proBDNF Modulates Basal Cell Carcinoma (BCC) Immune Microenvironment via Necroptosis Signaling Pathway. J Immunol Res. 2021;2021:6652846.
26. Li J, Sun J, Zeng Z, Liu Z, Ma M, Zheng Z, et al. Tumour-associated macrophages in gastric cancer: From function and mechanism to application. Clin Transl Med. 2023;13(8):e1386.
27. Rajesh C, Radhakrishnan P. The (Sialyl) Tn antigen: Contributions to immunosuppression in gastrointestinal cancers. Front Oncol. 2022;12:1093496.
28. Arora S, Scott AM, Janes PW. Eph Receptors in Cancer. Biomedicines. 2023;11(2).
29. May RC, Machesky LM. Phagocytosis and the actin cytoskeleton. Journal of Cell Science. 2001;114(6):1061-77.
30. Mylvaganam S, Freeman SA, Grinstein S. The cytoskeleton in phagocytosis and macropinocytosis. Current Biology. 2021;31(10):R619-R32.
31. Yue Y, Huang W, Liang J, Guo J, Ji J, Yao Y, et al. IL4I1 Is a Novel Regulator of M2 Macrophage Polarization That Can Inhibit T Cell Activation via L-Tryptophan and Arginine Depletion and IL-10 Production. PLoS One. 2015;10(11):e0142979.
32. Ye F, Wang L, Li Y, Dong C, Zhou L, Xu J. IL4I1 in M2-like macrophage promotes glioma progression and is a promising target for immunotherapy. Front Immunol. 2023;14:1338244.
33. Rodriguez E, Boelaars K, Brown K, Eveline Li RJ, Kruijssen L, Bruijns SCM, et al. Sialic acids in pancreatic cancer cells drive tumour-associated macrophage differentiation via the Siglec receptors Siglec-7 and Siglec-9. Nature Communications. 2021;12(1):1270.
34. Stanczak MA, Läubli H. Siglec receptors as new immune checkpoints in cancer. Molecular Aspects of Medicine. 2023;90:101112.
35. Nunes-Santos CJ, Kuehn H, Boast B, Hwang S, Kuhns DB, Stoddard J, et al. Inherited ARPC5 mutations cause an actinopathy impairing cell motility and disrupting cytokine signaling. Nature Communications. 2023;14(1):3708.
36. Blundell MP, Worth A, Bouma G, Thrasher AJ. The Wiskott-Aldrich Syndrome: The Actin Cytoskeleton and Immune Cell Function. Disease Markers. 2010;29(3-4):781523.
37. Limoges M-A, Cloutier M, Nandi M, Ilangumaran S, Ramanathan S. The GIMAP Family Proteins: An Incomplete Puzzle. Frontiers in Immunology. 2021;12.
38. Saunders A, Lamb T, Pascall J, Hutchings A, Dion C, Carter C, et al. Expression of GIMAP1, a GTPase of the immunity-associated protein family, is not up-regulated in malaria. Malaria Journal. 2009;8(1):53.

39. Roach BL, Ngo JM, Limso C, Oloja KB, Bhandari D. Identification and characterization of a novel phosphoregulatory site on cyclin-dependent kinase 5. Biochemical and Biophysical Research Communications. 2018;504(4):753-8.
40. Liu W, Li J, Song Y-S, Li Y, Jia Y-H, Zhao H-D. Cdk5 links with DNA damage response and cancer. Molecular Cancer. 2017;16(1):60.
41. Rosales JL, Ernst JD, Hallows J, Lee K-Y. GTP-dependent Secretion from Neutrophils Is Regulated by Cdk5 *. Journal of Biological Chemistry. 2004;279(52):53932-6.
42. Pareek TK, Lam E, Zheng X, Askew D, Kulkarni AB, Chance MR, et al. Cyclin-dependent kinase 5 activity is required for T cell activation and induction of experimental autoimmune encephalomyelitis. Journal of Experimental Medicine. 2010;207(11):2507-19.
43. Tabouret E, Wang H, Amin N, Jung J, Appay R, Cui J, et al. TP5, a Peptide Inhibitor of Aberrant and Hyperactive CDK5/p25: A Novel Therapeutic Approach against Glioblastoma. Cancers. 2020;12(7):1935.
44. Goodyear S, Sharma MC. Roscovitine regulates invasive breast cancer cell (MDA-MB231) proliferation and survival through cell cycle regulatory protein cdk5. Experimental and Molecular Pathology. 2007;82(1):25-32.
45. Liang Q, Li L, Zhang J, Lei Y, Wang L, Liu D-X, et al. CDK5 is essential for TGF-β1-induced epithelial-mesenchymal transition and breast cancer progression. Scientific Reports. 2013;3(1):2932.
46. Amin N, Wang H, Song Q, Bhaskar M, Yadav SP, Gilbert MR, et al. TP5: A Novel Therapeutic Approach Targeting Aberrant and Hyperactive CDK5/p25 for the Treatment of Colorectal Carcinoma. International Journal of Molecular Sciences. 2023;24(14):11733.
47. Kim E, Chen F, Wang C-C, Harrison LE. CDK5 is a novel regulatory protein in PPARγ ligand-induced antiproliferation. Int J Oncol. 2006;28(1):191-4.
48. Feldmann G, Mishra A, Hong S-M, Bisht S, Strock CJ, Ball DW, et al. Inhibiting the Cyclin-Dependent Kinase CDK5 Blocks Pancreatic Cancer Formation and Progression through the Suppression of Ras-Ral Signaling. Cancer Research. 2010;70(11):4460-9.
49. Feldmann G, Mishra A, Bisht S, Karikari C, Garrido-Laguna I, Rasheed Z, et al. Cyclin-dependent kinase inhibitor Dinaciclib (SCH727965) inhibits pancreatic cancer growth and progression in murine xenograft models. Cancer Biology & Therapy. 2011;12(7):598-609.
50. Wang J-B, Gao Y-X, Ye Y-H, Lin T-X, Li P, Lin J-X, et al. CDK5RAP3 acts as a tumour suppressor in gastric cancer through the infiltration and polarization of tumour-associated macrophages. Cancer Gene Therapy. 2023;30(1):22-37.
51. Shupp A, Casimiro MC, Pestell RG. Biological functions of CDK5 and potential CDK5 targeted clinical treatments. Oncotarget. 2017;8(10).

52. Na YR, Jung D, Gu GJ, Jang AR, Suh Y-H, Seok SH. The early synthesis of p35 and activation of CDK5 in LPS-stimulated macrophages suppresses interleukin-10 production. Science signaling. 2015;8(404):ra121-ra.
53. Pfänder P, Fidan M, Burret U, Lipinski L, Vettorazzi S. Cdk5 Deletion Enhances the Anti-inflammatory Potential of GC-Mediated GR Activation During Inflammation. Front Immunol. 2019;10:1554.
54. Ferreira JA, Magalhães A, Gomes J, Peixoto A, Gaiteiro C, Fernandes E, et al. Protein glycosylation in gastric and colorectal cancers: Toward cancer detection and targeted therapeutics. Cancer Lett. 2017;387:32-45.
55. Fernandes Â, Azevedo CM, Silva MC, Faria G, Dantas CS, Vicente MM, et al. Glycans as shapers of tumour microenvironment: A sweet driver of T-cell-mediated anti-tumour immune response. Immunology. 2023;168(2):217-32.
56. Wilhelm M, Schlegl J, Hahne H, Gholami AM, Lieberenz M, Savitski MM, et al. Mass-spectrometry-based draft of the human proteome. Nature. 2014;509(7502):582-7.

CHAPTER V

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

The abnormal modification of proteins through glycosylation is frequently associated to malignant transformation(1-4). One of the most prominent mechanisms underlying tumour-associated chances is the shortening of *O*-glycan chains, such as the Tn and STn antigens, which give rise to unique cancer-specific glycan signatures(5-8). The overexpression of these antigens in tumours has been widely linked to therapeutic resistance, increased metastatic potential, and poor patient prognosis(9-12). Furthermore, these glycan profiles are involved in manipulating the immunological system across various types of cancer by modulating immune recognition, contributing to immune escape and fostering the development of a tolerogenic environment(13-15). However, the mechanisms by which these glycosylation modifications interact with the surrounding immune system, as well as the receptors involved, remain largely unexplored.

Taking this into consideration, this work aimed to explore the interaction between cancer-associated glycans and macrophages, which are key players in innate immune defence and adaptive immune activation, to identify potential new immune checkpoint targets. The study began by characterizing pro-inflammatory (M1) and anti-inflammatory (M2a and M2c) phenotypes based on cytokine stimulation of isolated human monocytes from healthy donors, followed by characterization of cell surface markers and cellular morphology. Once the conditions were established, we co-cultured immature (M0) or pro-inflammatory macrophages (M1) with T24 or AGS cells engineered to overexpress Tn and STn antigens. We found that both Tn and STn expression in the bladder and gastric cancer cells effectively polarized and reprogrammed macrophages into an anti-inflammatory M2a/M2c phenotype, evidenced by increased levels of the mannose receptor CD206 and decreased levels of the co-stimulatory molecule CD86 and the antigen-presenting molecule HLA-DR. Additionally, this interaction led to elevated levels of the inhibitory cytokine IL-10 in the conditioned media. Surprisingly, T24 *C1GALT1* KO cells exhibited resistance to macrophage-induced cell death compared to T24 wild-type cells. In contrast, this resistance did not occur for the AGS *C1GALT1* KO cells, emphasising the importance of the cellular proteome. Overall, these results suggest that both Tn and STn antigens induce a pro-tumoral phenotype in macrophages, facilitating immune evasion and contributing to an immunosuppressive tumour environment. Moreover, the analysis of human formalin-fixed, paraffin-embedded tumours of bladder and gastric cancer revealed that the expression of short-chain *O*-glycans by cancer cells influences macrophage tumour infiltration, as demonstrated by the significantly lower expression of CD68 and CD163 molecules in these tumours.

To pinpoint the mediator proteins on macrophage surfaces that interact with these glycans to influence immune responses, we propose a novel high-throughput proteomics-assisted strategy for characterizing the glycan interactome using magnetic bead-coated membrane models. This approach involved coating paramagnetic beads with glycoproteins

extracted from the extracellular membranes of glycoengineered bladder and gastric cancer models, leading to the creation of "GlycoAvatars" which allowed us to identify potential receptors involved by analysing their interactions with macrophages. Using this approach, we identified 63 proteins uniquely enriched in the interaction between macrophages and GlycoAvatars-T24 KO, and 38 proteins uniquely enriched in the interaction between macrophages and GlycoAvatars-AGS KO. Notably, only the protein CDK5 was present in both conditions and given its close association with important biological functions such as cell migration and immune responses, it may represent a key protein in the immune synapse between tumour cells with truncated *O*-glycosylation and immature macrophages.

Ultimately, this thesis makes a substantial contribution to the field of glycoimmunology by providing a detailed insight into the complex interactions between cancer-associated glycans and macrophages. By elucidating these intricate dynamics, the study enhances our understanding of how the altered truncated *O*-glycans on cancer cells impact macrophage phenotype. Additionally, it provides a proteomic tool for uncovering receptors that may lead to novel therapeutic opportunities.

Future research will involve analysing a detailed cytokine profile in conditioned media to clarify whether macrophage polarization shifts towards M2a or M2c phenotypes, improving our understanding of immune mechanisms and identifying potential tumour-exploited immunosuppressive pathways. We will extend this analysis to other glycoengineered cancer models to assess broader applicability, suggesting a pancarcinoma effect. However, it will be imperative to explore the molecular mechanisms that confer enhanced resistance to cell death in the T24 Tn^+ cell line when exposed to macrophages. Understanding these differences between cell lines is crucial for developing more effective and targeted therapies, preventing potential obstacles in the progression of this study. Further, orthogonal validation that CDK5 interacts with the glycans on tumour cells and promotes an anti-inflammatory and anti-tumoral environment. In subsequent stages, implementing tri-culture systems or organoid models will be crucial for mimicking the tumour microenvironment and studying interactions among cancer cells, macrophages, and other innate and adaptive immune cells, such as NK cells and T cells. These models could offer critical insights into the effectiveness of therapeutic interventions targeting our potential molecular target, evaluating whether these approaches are sufficient to restore immune surveillance and induce a robust anti-tumoral response. However, *in vivo* studies will be necessary in later stages to confirm the clinical relevance of the CDK5 targeting and evaluate the biodistribution of the blocker agent, toxicity, and therapeutic efficacy in a dynamic biological context.

REFERENCES

1. Thomas D, Rathinavel AK, Radhakrishnan P. Altered glycosylation in cancer: A promising target for biomarkers and therapeutics. Biochim Biophys Acta Rev Cancer. 2021;1875(1):188464.
2. Grzesik K, Janik M, Hoja-Łukowicz D. The hidden potential of glycomarkers: Glycosylation studies in the service of cancer diagnosis and treatment. Biochimica et Biophysica Acta (BBA) - Reviews on Cancer. 2023;1878(3):188889.
3. Rodrigues JG, Duarte HO, Reis CA, Gomes J. Aberrant protein glycosylation in cancer: implications in targeted therapy. Biochem Soc Trans. 2021;49(2):843-54.
4. Peixoto A, Relvas-Santos M, Azevedo R, Santos LL, Ferreira JA. Protein Glycosylation and Tumor Microenvironment Alterations Driving Cancer Hallmarks. Front Oncol. 2019;9:380.
5. Radhakrishnan P, Dabelsteen S, Madsen FB, Francavilla C, Kopp KL, Steentoft C, et al. Immature truncated O-glycophenotype of cancer directly induces oncogenic features. Proc Natl Acad Sci U S A. 2014;111(39):E4066-75.
6. Cotton S, Ferreira D, Soares J, Peixoto A, Relvas-Santos M, Azevedo R, et al. Target Score-A Proteomics Data Selection Tool Applied to Esophageal Cancer Identifies GLUT1-Sialyl Tn Glycoforms as Biomarkers of Cancer Aggressiveness. Int J Mol Sci. 2021;22(4).
7. Kudelka MR, Ju T, Heimburg-Molinaro J, Cummings RD. Simple sugars to complex disease--mucin-type O-glycans in cancer. Adv Cancer Res. 2015;126:53-135.
8. Rømer TB, Aasted MKM, Dabelsteen S, Groen A, Schnabel J, Tan E, et al. Mapping of truncated O-glycans in cancers of epithelial and non-epithelial origin. Br J Cancer. 2021;125(9):1239-50.
9. Lima L, Neves M, Oliveira MI, Dieguez L, Freitas R, Azevedo R, et al. Sialyl-Tn identifies muscle-invasive bladder cancer basal and luminal subtypes facing decreased survival, being expressed by circulating tumor cells and metastases. Urologic Oncology: Seminars and Original Investigations. 2017;35(12):675.e1-.e8.
10. Ferreira JA, Videira PA, Lima L, Pereira S, Silva M, Carrascal M, et al. Overexpression of tumour-associated carbohydrate antigen sialyl-Tn in advanced bladder tumours. Mol Oncol. 2013;7(3):719-31.
11. Ferreira E, Ferreira D, Relvas-Santos M, Freitas R, Soares J, Azevedo R, et al. Aberrantly Glycosylated GLUT1 as a Poor Prognosis Marker in Aggressive Bladder Cancer. Int J Mol Sci. 2024;25(6).

12. Loureiro LR, Carrascal MA, Barbas A, Ramalho JS, Novo C, Delannoy P, et al. Challenges in Antibody Development against Tn and Sialyl-Tn Antigens. Biomolecules. 2015;5(3):1783-809.
13. Pinho SS, Alves I, Gaifem J, Rabinovich GA. Immune regulatory networks coordinated by glycans and glycan-binding proteins in autoimmunity and infection. Cellular & Molecular Immunology. 2023;20(10):1101-13.
14. Carrascal MA, Severino PF, Guadalupe Cabral M, Silva M, Ferreira JA, Calais F, et al. Sialyl Tn-expressing bladder cancer cells induce a tolerogenic phenotype in innate and adaptive immune cells. Mol Oncol. 2014;8(3):753-65.
15. Cornelissen LAM, Blanas A, Zaal A, van der Horst JC, Kruijssen LJW, O'Toole T, et al. Tn Antigen Expression Contributes to an Immune Suppressive Microenvironment and Drives Tumor Growth in Colorectal Cancer. Front Oncol. 2020;10:1622.