Macrophage modulation by glycoengineered colorectal cancer cell-derived extracellular vesicles

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“*We go through this life slowly but surely, just collecting these little pieces of ourselves that we can’t really live without until, eventually, we have enough of them to where we feel whole*.”

This is Us

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Abstract

Colorectal cancer is a heterogeneous disease at the genetic and molecular levels. Colorectal tumors exhibit differential gene expression patterns, activated signaling pathways, and diverse immune landscapes that determine disease progression, metastasis, and patients' response to current treatment options. It is necessary to better understand the various genetic and molecular mechanisms involved during carcinogenesis in order to devise the best possible therapeutic strategy to tackle this disease.

Protein glycosylation is a highly regulated process and the most abundant and diverse form of protein post-translational modification. Glycosylation acts as a key regulator of several molecular and cellular processes, such as cellular adhesion, cell-matrix interactions, molecular signaling and immune responses. In cancer, defects in the glycosylation machinery leads to the emergence of tumor-associated glycans involved in the crosstalk of cancer cells and their surrounding microenvironment, contributing for the acquisition of cancer hallmarks. Dysregulation of sialyltransferases leads to an upregulation of sialylated glycans, a pattern frequently found in many types of cancer. The β-galactoside α2,6-sialyltransferase 1 (ST6Gal1) catalyzes the terminal addition of α2,6-linked neuraminic acid (α2,6NeuAc) on *N*-glycans. Overexpression of ST6Gal1 was first reported in colorectal cancer and correlated with high invasion and metastatic capacities, contributing to a more aggressive phenotype. Since then, overexpression of ST6Gal1 has been reported in different cancers and its role in malignancy is being extensively studied.

Understanding the communication network within the tumor microenvironment is fundamental to unravel mechanisms that cancer cells use to invade and metastasize. Extracellular vesicles produced by cancer cells act as mediators of intercellular communication and are involved in educating target cells, such as stromal and immune cells, to support cancer onset. The surface of extracellular vesicles is enriched with diverse glycans, which affect their biogenesis, recognition and uptake by recipient cells. Cancer cells produce extracellular vesicles that carry the same aberrant glycosylation patterns as their parent cells and it has been reported that tumor-associated glycans carried by extracellular vesicles are also involved in supporting tumorigenesis. Additionally, tumor-associated glycans present in extracellular vesicles might unveil new biomarkers of disease.

In this study, the immunomodulatory role of ST6Gal1 present in extracellular vesicles produced by colorectal cancer cells is addressed. Extracellular vesicles derived from a previously established glycoengineered model of CRC with differential *ST6GAL1* expression levels were isolated and characterized in our group. Our results demonstrate that ST6Gal1-mediated sialylation of EVs affects their uptake by human monocyte-derived macrophages. Additionally, we observed that the presence of ST6Gal1 in extracellular vesicles seems to negatively affect macrophage viability. However, the expression of ST6Gal1 in extracellular vesicles produced by colorectal cancer cells does not seem to affect the polarization of macrophages. Overall, these results show a role of ST6Gal1 in the uptake capacity and survival of macrophages. Further

studies are needed to disclose the immunomodulatory impact of ST6Gal1 and its role in colorectal cancer progression.

Keywords: Colorectal cancer, glycosylation, sialylation, ST6Gal1, extracellular vesicles, macrophages, immune system.

Resumo

O cancro colorretal é considerado uma doença heterogénea a nível genético e molecular. Tumores colorretais apresentam padrões distintos de expressão génica, vias de sinalização ativadas e diversas populações imunes que determinam a progressão da doença, metastização e resposta dos doentes às atuais opções de tratamento. Desta forma, é necessário aprofundar o conhecimento dos vários mecanismos genéticos e moleculares envolvidos na carcinogénese para delinear a melhor estratégia terapêutica para combater esta doença.

A glicosilação de proteínas é um processo altamente regulado e constitui a forma mais abundante e diversa de modificação pós-traducional de proteínas. A glicosilação atua como um regulador-chave de vários processos moleculares e celulares, tais como adesão celular, interações célula-matriz, sinalização molecular e respostas imunes. No cancro, defeitos na maquinaria de glicosilação geram glicanos aberrantes, os quais estão envolvidos na comunicação das células tumorais com o seu microambiente e na aquisição de características malignas. A desregulação de sialiltransferases conduz a um aumento de glicanos sialilados, um padrão frequentemente observado em vários tipos de cancro. A sialiltransferase ST6Gal1 catalisa a adição terminal de ácido neuramínico em ligações α2,6 (α2,6NeuAc) a *N*-glicanos. A sobreexpressão de ST6Gal1 foi descrita pela primeira vez no cancro colorretal tendo sido correlacionada com maior capacidade invasiva e metastática, contribuindo para um fenótipo mais agressivo. Desde então, a sobreexpressão de ST6Gal1 foi reportada em vários tipos de cancros e o seu papel na promoção da carcinogénese tem sido extensivamente estudado.

Compreender a complexa rede de comunicação dentro do microambiente tumoral é fundamental para desvendar os mecanismos usados pelas células tumorais para invadir e metastizar. As vesículas extracelulares produzidas por células tumorais atuam como mediadores da comunicação intercelular e estão envolvidas na educação de células-alvo, nomeadamente, células estromais e imunes, para iniciar o desenvolvimento do cancro. A superfície das vesículas extracelulares é enriquecida em diversos glicanos, o que afeta a sua biogénese, o seu reconhecimento e a sua captação pelas células recetoras. As células tumorais produzem vesículas extracelulares que carregam os mesmos padrões de glicosilação aberrantes das suas células progenitoras, estando estes glicanos aberrantes transportados por vesículas extracelulares envolvidos no processo de carcinogénese e a serem usados como novos biomarcadores tumorais. Além disso, glicanos presentes em vesículas extracelulares produzidas por células tumorais podem ser usados como novos biomarcadores de cancro.

Neste estudo, abordámos o papel imunomodulatório da ST6Gal1 presente em vesículas extracelulares produzidas por células de cancro colorretal. Vesículas extracelulares derivadas de um modelo celular de cancro colorretal com níveis de expressão distintos de *ST6GAL1* foram isoladas e caracterizadas. Os nossos resultados demonstram que a sialilação de vesículas extracelulares mediada pela ST6Gal1 afeta a sua internalização por macrófagos derivados de monócitos humanos. Adicionalmente, observamos que a presença de ST6Gal1 em vesículas extracelulares parece afetar negativamente a viabilidade dos macrófagos. No entanto, a

expressão de ST6Gal1 em vesículas extracelulares não parece ter impacto na polarização dos macrófagos. Tendo em conta os resultados obtidos, identificamos um potencial papel da enzima ST6Gal1 presente em vesículas extracelulares na capacidade de internalização e na sobrevivência de macrófagos, sendo necessário novos estudos que sustentem o impacto imunomodulatório da ST6Gal1 e o seu papel na progressão de cancro colorretal.

Palavras-chave: cancro colorretal, glicosilação, sialilação, ST6Gal1, vesículas extracelulares, sistema imune, macrófagos.

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Scientific outputs

MSc student of the following project: “GLYCOTARGET. A new frontier in extracellular vesicle-mediated cancer immunomodulation"

During my work at the Glycobiology in Cancer group, I had the opportunity to participate in additional projects that allowed me to consolidate a set of skills that were crucial for the development of the project presented in this dissertation. The projects I have collaborated on are listed below:

1. PhD project of Joana Rodrigues "Glyco-Immune Checkpoint in Cancer: role of glycosylation in tumour immune response modulation".

2. PhD project of Álvaro Martins “A novel tool for cancer immunotherapy: ex vivo manipulation of cancer derived extracellular vesicles”.

My participation in ongoing projects in our group allowed me to be co-author of the following publications:

1. Francisca Diniz, **Pedro Coelho**, Henrique O. Duarte, Bruno Sarmento, Celso A. Reis and Joana Gomes. “Glycans as targets for drug delivery in cancer”. *Cancers (Basel)*. 2022;14(4):911. doi:10.3390/cancers14040911

2. Joana G. Rodrigues, Catarina Moreira-Barbosa, Rita Matos, **Pedro Coelho**, Filipe Pinto, Maria J. Oliveira, Joana Gomes, Sandra J. van Vliet, Celso A. Reis. “Sialylation by ST6Gal1 protects colorectal cancer cells from macrophage-mediated phagocytosis through interactions with the sialic acid receptor Siglec-10" (manuscript in preparation).

Abbreviations

α2,3NeuAc	α2,3-linked neuraminic acid
α2,6NeuAc	α2,6-linked neuraminic acid
Alix	ALG-2-interacting protein X
APC	Antigen presenting cell
AREG	Amphiregulin
ARF6	ADP-ribosylation factor 6
BCR	B cell receptor
BMDC	Bone marrow-derived cells
BSA	Bovine serum albumin
CAFs	Cancer-associated fibroblasts
CCL22	C-C motif chemokine 22
CD	Cluster of differentiation
CIMP	CpG-island methylator phenotype
CIN	Chromosomal instability
CMS	Consensus molecular subtype
CRC	Colorectal cancer
CRD	Carbohydrate-recognition domain
CSF-1	Colony stimulating factor 1
CTLA-4	Cytotoxic T lymphocyte-associated protein 4
DC-SIGN	Dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin
ECM	Extracellular matrix
EGFR	Epidermal growth factor receptor
EMT	Epithelial-mesenchymal transition
ER	Endoplasmic reticulum
ESCRT	Endosomal sorting complex required for transport
EV	Extracellular vesicle
FAP	Familial adenomatous polyposis
FasL	Fas ligand
FasR	Fas receptor
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
Fuc	Fucose
GAG	Glycosaminoglycan
Gal	Galactose
GalNAc	*N*-acetylgalactosamine
GBP	Glycan-binding protein
GC	Golgi complex
GlcNAc	*N*-acetylglucosamine
GSL	Glycosphingolipid
HDI	Human development index
HIF1	Hypoxic inducible factor 1
HLA	Human leukocyte antigen
ICI	Immune checkpoint inhibitor
IFN-γ	Interferon-γ
IL	Interleukin
ITIM	Immunoreceptor tyrosine-based inhibitory
KO	Knockout
LeY	Lewis Y
LPS	Lipopolysaccharide
mAb	Monoclonal antibody
MAL-I	*Maackia amurensis* lectin 1

Man	Mannose
MCP-1	Monocyte chemoattractant protein 1
M-CSF	Macrophage colony-stimulating factor
MFI	Median fluorescence intensity
MHC	Major histocompatibility complex
MIF	Migration inhibitory factor
miR	Micro RNA
MMP	Metalloprotein
MMR	Mismatch repair
MSI	Microsatellite instability
MSS	Microsatellite stability
MVB	Multivesicular body
MVE	Multivesicular endosome
NK	Natural killer cells
NO	Nitric oxide
NTA	Nanoparticle tracking analysis
OAA	*Oscillatoria Agardhii* agglutinin
ON	Overnight
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PD-1	Programmed death 1
PDAC	Pancreatic ductal adenocarcinoma cancer
PDGF	Platelet-derived growth factor
PD-L1	Programmed death ligand 1
PenStrep	Penicillin/streptomycin
PFA	Paraformaldehyde
PVP	Polyvinylpyrrolidone
rMFI	Relative median fluorescence intensity
ROS	Reactive oxygen species
RT	Room temperature
RTK	Receptor tyrosine kinase
Siglec	Sialic acid-binding immunoglobulin-type lectin
SIRPα	Signal regulatory protein α
SLe^a	Sialyl Lewis a
SLe^X	Sialyl Lewis X
SNA	*Sambucus nigra* agglutinin
ST3Gal4	β-galactoside α2,3-sialyltransferase 4
ST6Gal1	β-galactoside α2,6-sialyltransferase 1
STn	Sialyl Tn
T	Thomsen-Friedenreich antigen
TAM	Tumor-associated macrophage
TBS	Tris-buffered saline
TCGA	The Cancer Genome Atlas
TEM	Transmission electron microscopy
TGFβ	Transforming growth factor β
TME	Tumor microenvironment
TNF	Tumor necrosis factor
TNFR1	Tumor necrosis factor receptor 1
Treg	T regulatory lymphocytes
TSG101	Tumor susceptibility gene 101
VEGF	Vascular endothelial growth factor
WT	Wild-type

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1. INTRODUCTION

1.1. Colorectal cancer

Cancer incidence and mortality are rapidly increasing worldwide, with cancer ranking as the second leading cause of death and an important barrier to increase life expectancy in every country of the world. Amongst the various cancers, colorectal cancer (CRC) is reported to be the third most incident (10%) and the second deadliest (9.4%) cancer worldwide [1]. Considered a highly heterogeneous cancer at the genetic and molecular level, most CRCs (>90%) are adenocarcinomas developed from colon or rectal glandular epithelial cells. Other types, although rare, include squamous cells carcinoma, adenosquamous carcinoma, spindle cell carcinoma and undifferentiated carcinoma [1, 2]. The vast majority of CRC cases (60-65%) are sporadic, emerging in individuals without a family history of CRC or acquired mutations that increase the risk of CRC. These sporadic occurrences are due to accumulation of genetic and epigenetic mutations potentially acquired through modifiable risk factors. However, CRC also exhibits a hereditary component, with an estimated hereditability of 35-40%. In about 25% of CRC cases, although no clear genetic cancer syndrome is present, there is a family history. Only 5% of CRC are attributable to hereditary cancer syndromes, such as familial adenomatous polyposis (FAP) or hereditary nonpolyposis colorectal cancer, which are driven by inherited germline mutations in rare but high-penetrance susceptibility genes [3].

The increasing rates of CRC reflect the westernization of countries, with incidence rates being higher in economically developed countries with high human development indexes (HDI) [3]. This is inextricably linked to the lifestyle choices made by western countries, such as increased consumption of processed foods and red meat, decreased physical exercise, and increasing sedentarism, all of which are significant CRC risk factors. Additionally, heavy alcohol use and cigarette smoking are modifiable risk factors associated to CRC [3]. Furthermore, rising incidence rates are being observed in transitioning countries that were formerly low-risk and had lower HDI but are now undergoing industrialization, economic growth, and shifting towards a more western lifestyle [1]. As such, CRC is frequently described as an indicator of socioeconomic development [1, 3]. Since cancer is a disease with a propensity to develop as people age, 90% of CRC cases are reported to occur after age 50, with age-adjusted rates being superior in men than in women. However, in some countries there has been an increasing pattern of CRC incidence in people under 50 years of age. These early-onset CRCs are mostly sporadic, and are usually detected in advanced clinical stages [1, 3].

Colorectal malignancies are frequently detected at late stages, with some individuals already exhibiting metastatic disease at the time of diagnosis, while patients with initial localized disease often develop metastasis [4]. Metastatic colorectal disease offers a worse prognosis and is associated with the high mortality rates observed for CRC. Surgery remains the primary form of treatment for CRC and can be combined with chemotherapy and radiation therapy before and/or

after surgery to prevent recurrence [5, 6]. Patients that develop unresectable metastatic CRC can be treated with systemic therapy, such as cytotoxic chemotherapy, monoclonal antibodies (mAbs) that target mitogenic or angiogenic receptors, and combinations of these therapies [5]. Mitogenic receptors like the epidermal growth factor receptor (EGFR) are frequently overexpressed in CRC and support accelerated tumor growth. Cetuximab and Panitumumab are two mAbs clinically used to treat metastatic CRC, by targeting the extracellular domain of EGFR and blocking EGFR-mediated downstream signaling pathways, thus preventing tumor growth [7, 8]. However, these mAbs are only eligible for treatment of tumors without mutations in the *RAS* oncogene, as patients bearing activating mutations in the gene coding for *RAS* do not benefit from Cetuximab or Panitumumab treatment. Furthermore, only a subset of patients without other known activating mutations in EGFR-downstream pathways respond to mAb therapy [4, 9-11]. Bevacizumab, a mAb that targets the vascular endothelial growth factor (VEGF), causes the existing tumor vasculature to regress and prevents the development of new blood vessels, hence limiting tumor growth. A variety of cancers, including metastatic CRC, can be treated with Bevacizumab, which can be combined with standard chemotherapeutics to improve survival [12]. However, despite demonstrating a moderate clinical outcome when combined with standard chemotherapy, there are resistance mechanisms that can be developed upon anti-angiogenic therapy [13]. As such, anti-angiogenic treatment with Bevacizumab may select hypoxia-resistant tumor cells, while also inducing the upregulation of different pro-angiogenic factors, such as the fibroblast growth factor (FGF) and platelet-derived growth factor (PDGF), all of which induce tumor angiogenesis [14].

1.1.1. The CRC tumor microenvironment

The tumor microenvironment (TME) is a fundamental concept consisting of a specialized environment developed by tumor cells. It is in this microenvironment that tumor cells establish intricate interactions with stromal cells, endothelial cells, infiltrating immune cells and with the extracellular matrix (ECM) itself (Fig.1) [15].

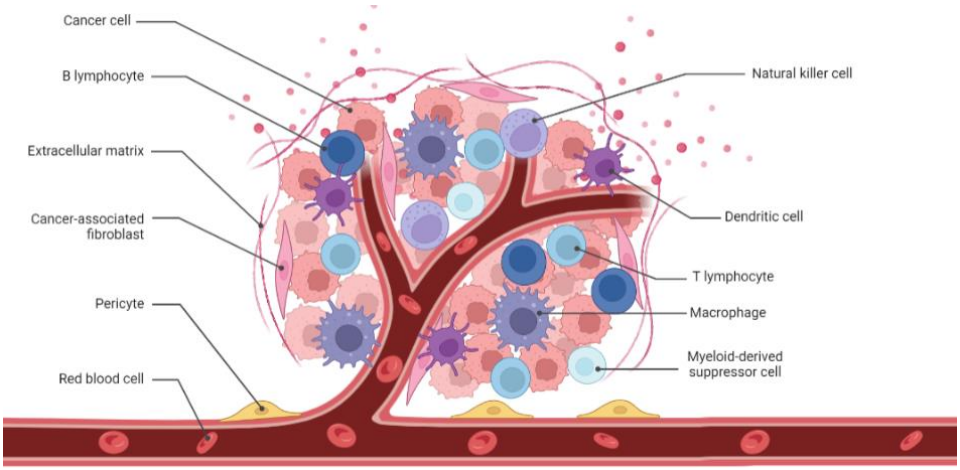

Figure 1. Schematic representation of the tumor microenvironment (image adapted from BioRender.com freeware image and icon banks).

Accordingly, these interactions between the different cellular and structural components present at the TME are often described as promoters of tumor progression, allowing tumor cells to become more invasive and able to spread from primary sites to more distant locations to metastasize [16]. The TME is essential in the therapeutic context. In fact, recent works have highlighted the role of the TME in modulating therapeutic responses, with many approaches being developed with the aim of targeting not only tumor cells, but also the various components that constitute the TME [16]. The role of immune cells present in the TME has been studied over the years. As cancer cells are antigenically different from healthy cells in our tissues, immune cells are able to detect and eliminate initial neoplastic cells, preventing tumor growth due to the effects of strong inflammatory responses [17]. However, tumor cells that aren't eliminated enter in a phase of equilibrium with the immune system, in which they are neither detected nor destroyed, but are rather in a dormant state [17]. During this phase, there is enough time for the generation of new variants with different mutations that give cancer cells the ability to resist and evade the immune system [17]. Consequently, new tumor cell variants that have acquired resistance to immune cells begin to expand uncontrollably [17]. The three phases described above - elimination, equilibrium and escape - are described as the 3Es of cancer immunoediting and demonstrate the dual role of the immune system in suppressing or promoting tumor progression [17, 18]. Immune cells are controlled by regulatory networks that finely tune the immune response and are crucial for self-tolerance, preventing immune cells from reacting indiscriminately against healthy cells. Immune checkpoints therefore constitute a family of stimulatory or inhibitory proteins that control the activation of immune cells and ensure homeostasis [19]. Immune checkpoints as the programmed cell death 1 (PD-1) and the cytotoxic T lymphocyte-associated protein 4 (CTLA-4), expressed mainly by T lymphocytes, play a pivotal role in downregulating T cell activation, suppressing their inflammatory activity [19]. Cancer cells exploit these immunosuppressive networks by overexpressing ligands for the corresponding immune checkpoint receptors expressed on immune cells [20]. A well-known example is the marked upregulation of the ligand for PD-1, the programmed death ligand 1 (PD-L1), observed in a variety of malignancies and correlated with poor clinical outcomes. By overexpressing PD-L1, tumor cells are able to induce cell death of T lymphocytes, thus avoiding being destroyed by cytotoxic T cells [21]. Ligands for CTLA-4 are typically expressed by antigen presenting cells (APCs), such as dendritic cells and macrophages. The recognition of these ligands by CTLA-4 activates inhibitory signaling cascades that downregulate the activation status of T lymphocytes [19]. Similar to PD-1 and PD-L1, several cancers upregulate ligands for CTLA-4 with a consequent marked downregulation of T lymphocytes cytotoxic activity towards cancer cells [22]. These examples constitute one of several mechanisms that tumor cells use to evade the immune system [20]. As a result, the dynamic crosstalk between tumor cells and immune cells at the TME laid the groundwork for the introduction of immune checkpoint inhibitors (ICI) into the clinic as a form of immunotherapy for cancer treatment [23]. ICIs were developed to target CTLA-4 and PD-1, aiming to block the interaction between tumor and immune cells through these immune checkpoints, thus enhancing native antitumor immune responses [23, 24]. Examples of ICIs approved for the treatment of

different cancers include Ipilimumab, a mAb that targets CTLA-4, and Pembrolizumab, which is directed against PD-1 [23]. In several studies, patients treated with anti-PD-1 and anti-CTLA-4 ICIs demonstrated increased survival rates when compared to those treated with chemotherapy [23, 25]. Thus, the introduction of ICIs in the clinic led to a significant improvement in the treatment of various cancers, with an increase in the average life expectancy of patients [25]. Unfortunately, despite the improvements brought by ICIs therapy, many patients who initially responded positively to treatment, eventually relapse and develop a new tumor, reflecting, once again, the importance and complexity of the TME [24-26]. Treatment with these ICIs naturally exerts selective pressure, with the consequent emergence of tumor cells capable of resisting the immune system through mechanisms independent of PD-1 and CTLA-4 [24]. The notion that tumor cells are able to develop alternative mechanisms to escape immune surveillance stresses the need to develop new therapeutic strategies able to modulate antitumor immune responses [26].

The CRC TME is generally characterized by having a stiff ECM and diverse populations of immune cells that infiltrate the tumor stroma, namely infiltrating T cells, dendritic cells and macrophages. In addition, other cell components include cancer-associated fibroblasts (CAFs) and endothelial cells [27, 28]. All cellular components of the TME, together with the ECM, establish a complex communication network that support tumor progression and metastasis [27]. The immune populations present at the TME of CRC play a determinant role in tumor development [2]. Tumor infiltrating $CD4^+$ and $CD8^+$ T lymphocytes elicit strong inflammatory responses that are crucial to prevent tumor growth. In addition, B lymphocytes and natural killer (NK) cells help eliminating tumor cells, and their presence at the TME generally correlates with a better outcome for patients [27]. However, as the tumor progresses, tumor cells develop escape mechanisms from inflammatory immune cells and begin to secrete mediators that recruit immunosuppressive immune cells, such as T regulatory (Treg) lymphocytes, leading to the establishment of a gradually immunosuppressive TME and, consequently, more permissive to tumor progression and invasion [2, 17, 20]. Populations of myeloid immune cells are frequently found in the TME of CRC, which play dual roles in tumor development [29]. For example, dendritic cells, by presenting antigens to T cells, promote strong antitumor responses that suppress tumor growth. Macrophages are also active players in shaping the various intercellular dynamic circuits occurring in the TME of CRC [27, 29]. Numerous studies have already reported the pivotal role of macrophages in promoting immune evasion and tumor progression and, recently, several strategies have been explored to target macrophages present in the TME of CRC [30].

1.1.1.1. The role of tumor-associated macrophages in the CRC TME

Macrophages are innate immune cells belonging to the mononuclear phagocytic system. Macrophages can be derived from circulating monocytes that originate in the bone marrow and differentiate upon leaving the bloodstream. In turn, tissue-resident macrophages originate from precursors present during embryonic development [31]. Macrophages exhibit enormous phenotypic plasticity, since they present different phenotypes according to the stimuli received

from the surrounding tissue microenvironment in which they are inserted [32]. As such, macrophage polarization describes the different functional phenotypes that these cells exhibit and their specialized cellular functions. The cytokine-induced M1 and M2 phenotypes consist of two contrasting macrophage polarization states, thus representing the two extremes of the polarization axis [31, 32]. M1 macrophages are commonly classified as classically activated macrophages, being involved in pro-inflammatory responses and phagocytosis of pathogens. During acute inflammation, M1 macrophages can be stimulated by bacterial products, such as lipopolysaccharide (LPS), and by increasing local levels of pro-inflammatory cytokines, such as interferon-γ (IFN-γ) and tumor necrosis factor α (TNFα). In response, M1 macrophages begin to secrete high levels of pro-inflammatory cytokines, such as interleukins IL-1β, IL-6 and IL-12, and chemokines that recruit adaptive immune cells to the inflammatory site, namely T and B lymphocytes, to develop an inflammatory response. Additionally, M1 macrophages present antigens to lymphocytes via major histocompatibility complex II (MHC-II). The production of reactive oxygen species (ROS) and nitric oxide (NO) is another characteristic of M1 macrophages [31, 32]. M2 macrophages are alternatively activated and exhibit an anti-inflammatory profile, being normally involved in the resolution phase of inflammation and in repair/regeneration processes. This macrophage subset is stimulated by anti-inflammatory cytokines, such as IL-4, IL-10, transforming growth factor β (TGFβ), as well as by glucocorticoids. Consequently, M2 macrophages produce the anti-inflammatory cytokines IL-10 and TGFβ. By producing high levels of metalloproteins (MMPs), M2 macrophages are actively involved in ECM remodeling. Additionally, they promote angiogenesis by secreting angiogenic factors such as VEGF [31, 32]. Furthermore, the M2 phenotype can be subclassified into M2a, M2b, M2c, or M2d, according to the different stimuli macrophages receive and their specific cellular functions [32].

Tumor-associated macrophages (TAMs) actively participate in the intercellular dynamics of the TME, playing a crucial role in promoting immune system evasion, invasion and metastasis [16, 33]. TAMs are recruited to the tumor site via chemokines secreted within the TME, such as monocyte chemoattractant protein 1 (MCP-1) and colony-stimulating factor 1 (CSF-1) [34]. Since macrophages are very sensitive to the molecular cues received from their surrounding microenvironment, the TME has a very evident role in educating infiltrating TAMs, resulting in an ongoing transition between pro- and anti-inflammatory phenotypes [34, 35]. In CRC, TAMs have been reported to promote migration, motility and invasion of tumor cells through diverse mechanisms, including phosphorylation of EGFR expressed on CRC cells, with a consequent increase in the activity of the small GTPases RhoA and Cdc42 [36]. Additionally, TAMs have been widely described as regulators of immunity in the CRC TME, being able to directly suppress $CD8^+$ T lymphocytes through L-arginine metabolism catalyzed by arginase 1 and oxygen or nitrogen radicals. By producing cytokines such as CCL22, TAMs recruit Treg lymphocytes to the TME, which inhibit antitumor responses of effector T cells [27, 34]. CRC cells have also been shown to evade macrophages by overexpressing CD47, a cell surface glycoprotein commonly known as a "don't eat me" signal [37]. CD47 binds to signal regulatory protein α (SIRPα) expressed on macrophages, resulting in the inhibition of phagocytosis [37, 38]. By overexpressing CD47, tumor

cells are able to avoid being eliminated by macrophage-mediated phagocytosis [38, 39]. TAMs are therefore recognized as key players in tumor development, including in CRC, and increased number of TAMs localized at the TME is correlated with poor clinical outcomes [40]. Several strategies have been designed to target TAMs, such as drugs that block the inhibitory CD47-SIRPα axis, allowing phagocytosis of tumor cells, and reprogramming of TAMs to a more M1-like phenotype in order to elicit potent pro-inflammatory and antitumor responses [35, 41, 42].

1.1.2. CRC consensus molecular subtypes: molecular phenotypes and immune profiles

Cancer immunotherapy has been prominent as a potential approach for CRC treatment. Even though some patients respond well to these treatments and have positive clinical outcomes, many CRC patients still do not benefit from immunotherapy and maintain their poor prognosis. CRC is a highly heterogeneous disease, being classified in different subtypes according to the identified mutations and immune populations present within the TME of CRC, all of which determine patients response to immunotherapy [2]. The acquisition of genomic instability is an essential process for the development of CRC, which can be obtained through chromosomal instability (CIN), microsatellite instability (MSI), or through a CpG-island methylator phenotype (CIMP) [43]. Colorectal CIN tumors account for 85% of the sporadic cases and are characterized by losses or gains of portions of chromosomes or entire chromosomes, resulting in an abnormal number of chromosomes and a loss of heterozygosity. Furthermore, CIN tumors possess mutations in tumor suppressor genes and in proto-oncogenes, such as *KRAS* [44]. CIMP tumors exhibit hypermethylation of CpG islands present in the promotor of genes, leading to silencing of tumor suppressor genes [45]. Defects in the DNA mismatch repair (MMR) system increases the accumulation of genetic errors in repetitive sequences, ultimately culminating in the development of MSI colorectal tumors that are hypermutated and represent around 15% of CRC cases [46]. MSI tumors harbor a high tumor mutational burden and neoantigen load, which favors infiltration of highly inflammatory immune cells that elicit strong antitumor immune responses [2, 43, 46, 47]. However, most CRCs are microsatellite-stable (MSS), a subset associated with lower infiltration of immune cells [43, 48]. Thus, in view of the high infiltration of inflammatory immune cell populations in the microenvironment of MSI CRC tumors, it is hypothesized that immunotherapies are more efficient within this subset of CRC [2, 48]. For instance, Pembrolizumab treatment in CRC patients with MSI status has been shown to be significantly beneficial, as these patients have a higher progression-free survival rate when compared to patients with MSS colorectal tumors. Furthermore, treatment with both Ipilimumab and the anti-PD-1 Nivolumab antibody was also shown to be efficient in metastatic colorectal MSI tumors, demonstrating the potential of combining different ICIs for treatment of CRC patients with MSI status [49].

The different CRC subtypes exhibit several genetic and molecular features, which led to the establishment of four molecular consensus subtypes (CMS) that categorize colorectal tumors according to their gene signature, activated molecular pathways and TME composition (Fig.2)

[50]. The CMS1 group is enriched in MSI and CIMP tumors that are highly infiltrated with activated $CD4^+$ and $CD8^+$ T cells, while also exhibiting high expression of human leukocyte antigen (HLA) and immune checkpoint molecules, as well as high neoantigen loads, all of which favor strong antitumor responses. Therefore, CMS1 tumors are commonly named as “immune activated” tumors and are considered the ideal candidates for immunotherapies [2, 50]. The CMS4 group also assembles tumors with high immune infiltration, however, most of the immune cells that infiltrate the tumor have immunosuppressive features, such as Treg lymphocytes and M2 macrophages [50]. Furthermore, tumors of this subtype exhibit activation of epithelial-mesenchymal transition (EMT) programs, matrix remodeling, TGFβ secretion, and induce angiogenesis by secreting high levels of VEGF, all of which support tumor progression and metastasis [2]. Contrary to the CMS1 and CMS4 groups, the tumors from the CMS2 (“immune desert”) and CMS3 (“immune excluded”) groups commonly exhibit poor infiltration of immune cells [2, 50]. Ultimately, CRC molecular heterogeneity and the immune landscape of each subtype offers a new prognostic factor in CRC, allowing better patient stratification and helping the selection of the most appropriate therapeutic strategy for each CRC patient.

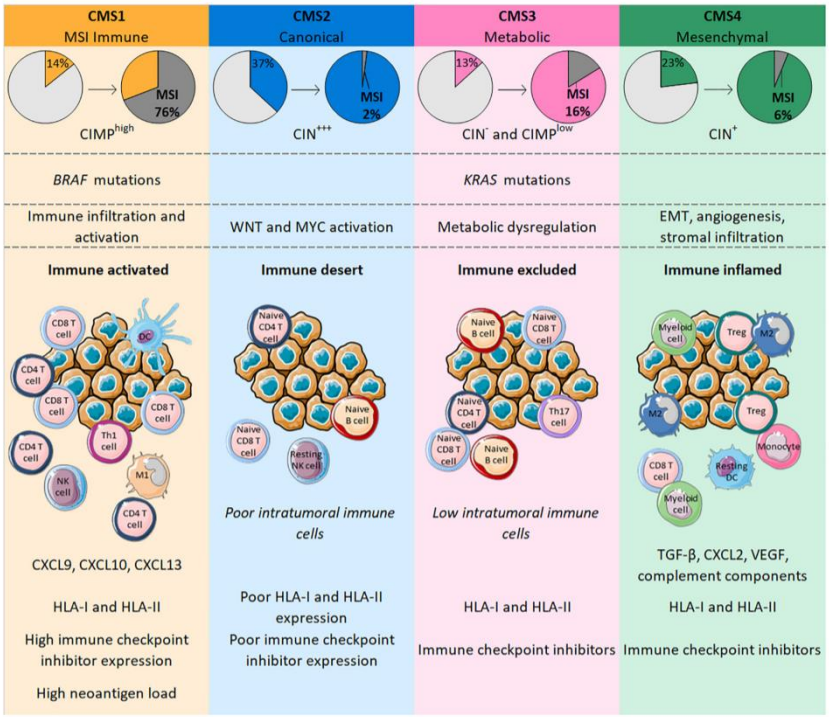

Figure 2. CMS classification of CRC according to gene signatures, activated molecular pathways and infiltrating immune cell subsets. Adapted from Picard et al. (2020) [2].

1.2. Cellular glycosylation

Glycosylation is a highly complex post-translational process that involves the covalent linkage of carbohydrates, also known as glycans, to other carbohydrates, proteins and lipids. Described as the most abundant and diverse mechanism of post-translational modification of proteins,

glycosylation is responsible for generating several glycoforms through the combined and organized action of more than 200 enzymes present in the secretory pathway (Golgi complex (GC) and endoplasmic reticulum (ER)), contributing to the amplification of the cellular proteome. Depending on the noncarbohydrate moiety to which glycans are attached, glycoconjugates can be classified as glycoproteins, glycolipids, or proteoglycans [51]. In protein glycosylation, glycan chains can establish glycosidic bonds with the polypeptide backbone through the nitrogen atom in asparagine residues, forming *N*-glycans, or the oxygen atom present in serine or threonine residues, forming *O*-glycans [52, 53]. Attachment of *N*-glycans to the asparagine residue occurs in the sequence Asn-X-Ser/Thr, where X can be any amino acid except proline. Additionally, all *N*-glycans have a common pentasaccharide core region, whose extension with additional monosaccharides generates three types of *N*-glycans: hybrid, high-mannose or complex [53]. Protein *O*-linked glycosylation is initiated by attachment of *N*-acetylgalactosamine (GalNAc) to the protein backbone. GalNAc-initiated *O*-glycans are commonly referred to as mucin-type *O*-glycans because of their high abundance in mucin glycoproteins. *O*-glycans are further extended, generating different core structures, as well as terminal structures that are frequently fucosylated or sialylated [52]. Proteoglycans are formed by a protein core heavily decorated with glycosaminoglycans (GAGs) chains, such as heparan sulfate and chondroitin sulfate. These glycoconjugates are secreted into the ECM and can also be found anchored in the plasma membrane [54]. The vast majority of glycolipids in vertebrates are glycosphingolipids (GSL), which are glycoconjugates formed by attachment of a ceramide to a hydrophilic residue, being frequently found in lipid rafts and acting as mediators of signal transduction pathways [55].

The non-templated glycan biosynthesis is highly regulated by several factors, such as substrate availability, expression levels and localization of glycosyltransferases and glycosidases within the secretory pathway, amongst others [56]. Given the position of the diverse glycoconjugates that constitute the glycocalyx, it is not surprising that glycans play crucial roles in modulating cell surface dynamics [57]. As such, glycans are often described as being regulators of cell-cell and cell-ECM interactions, signaling pathways, immune responses and host-pathogen interactions [56, 57]. In this regard, glycans play diverse biological roles, being involved in the regulation of several physiopathological conditions [56-58]. Indeed, alterations in the various mechanisms that tightly regulate the synthesis and expression of glycans result in the aberrant glycosylation of various glycoconjugates, aiding the development of various pathological conditions, including tumor onset and progression [57, 59].

Moreover, glycans interact with glycan-binding protein (GBP) partners, a vast array of human lectins that recognize specific glycan patterns at the cell surface. The interaction between glycans and different GBPs are involved in intercellular communication and subsequent cellular responses. GBPs or lectins can be classified into different groups based on similarity between carbohydrate-recognition domains (CRDs) [60]. For example, C-type lectins are mainly Ca^{2+} dependent GBPs and include selectins and endocytic receptors, such as Dectin-1 and the macrophage mannose receptor, which are very important for pathogen phagocytosis. Selectins, on the other hand, are expressed by endothelial cells (E- and P-selectin) and leukocytes (L-

selectin) and bind to sialofucosylated ligands expressed by circulating leukocytes, initiating the extravasation process during inflammatory responses [61]. I-type lectins include the vast family of sialic acid-binding immunoglobulin-type lectins (Siglecs), primarily found on the surface of immune cells and can be classified into two groups: the CD33-related Siglecs (Siglec-3, -5, - 6, -7, -8, -9, -10 and -11) and the structurally conserved ones (Siglec-1, -2 (CD22), -4 and -15). Most Siglecs have cytoplasmic immune receptor tyrosine-based inhibitory motifs (ITIMs) that suppress activating signaling of immune cells [62]. Thus, through the recognition of linkage-specific sialic acid motifs, Siglecs are able to dampen the immune response similar to the PD-1/PD-L1 axis [63].

1.2.1. Glycans as drivers of malignant transformation

Tumor-associated glycans are frequently expressed by malignant cells due to defects in the glycosylation machinery that finely regulates glycan synthesis [57]. As such, tumor cells often express altered glycan motifs that actively promote tumor onset [57-59], such as the truncated *O*-glycans Tn antigen and sialyl Tn (STn), branched *N*-glycans, terminal sialylation resulting in polysialylated structures, as well as core and terminal fucosylation (Fig.3) [57]. The emergence of these altered carbohydrate structures enriches the cell surface with glycoconjugates that affect several cellular processes, namely cell adhesion, receptor tyrosine kinases (RTKs) activation and signaling, immune activation, migration and metastasis [57, 58]. Additionally, tumor-associated glycans can be secreted and released into circulation, which has led to the establishment of serological assays that make it possible to detect and quantify the levels of these glycans in the serum of cancer patients, serving as cancer biomarkers [64].

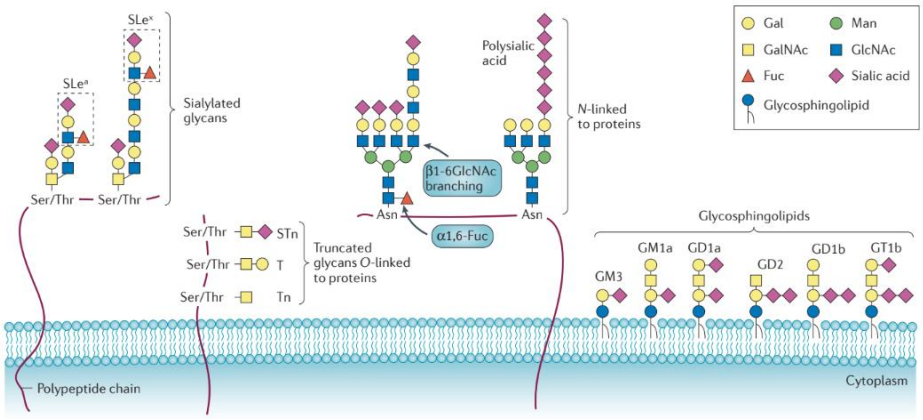

Figure 3. Aberrant glycan structures present in cancer cells. Abbreviations: Fuc-fucose; Gal-galactose; GlcNAc-*N*-acetylglucosamine; GalNAc-*N*-acetylgalactosamine; Man- mannose; STn-sialyl Tn; $SLe^{a/x}$-sialyl Lewis a/x; T-Thomsen-Friedenreich antigen. Adapted from Pinho & Reis (2015) [57].

Terminal sialylation is an important cellular glycosylation modification frequently associated with cancer. This process consists of the addition of negatively charged sialic acid (or neuraminic acid, NeuAc) residues to the terminal antennae of glycan chains through the action of sialyltransferases [65]. In the cancer setting, due to overexpression of sialyltransferases, an

overall increase in α2,3- and α2,6-sialylation is observed [57, 66]. Together with fucosyltransferases, the overexpression of sialyltransferases enriches the surface of malignant cells with sialofucosylated Lewis antigens, namely Sialyl Lewis X (SLe^X) and Sialyl Lewis a (SLe^a) [67], allowing cancer cells to interact with endothelial selectins to facilitate their extravasation from the bloodstream, consequently favoring metastasis in distant sites [67, 68]. As such, SLe^X and SLe^a are often associated with recurrence and poor patient survival [69]. Additional forms of sialylation include the terminal addition of α2,6NeuAc motifs to *N*-glycans, mediated by the β-galactoside α2,6-sialyltransferase I (ST6Gal1) glycosyltransferase [70, 71]. Several reports have already addressed the overexpression of ST6Gal1 in various human malignancies, including CRC, and its role in promoting numerous biological processes associated with tumorigenesis, such as invasion, metastasis and resistance to chemotherapy, as well as conferring stem cell-like characteristics to malignant cells [72]. The particular role of ST6Gal1 in cancer, with an emphasis on CRC, will be further elaborated in detail in the subsequent sections.

1.2.2. ST6Gal1: a key player in tumor onset

ST6Gal1 is a Golgi-residing glycosyltransferase that adds sialic acids to the terminal galactose of *N*-glycans in an α2,6-linkage [70, 71]. Synthesis of *N*-glycans initiates in the ER, where a mature glycan bound to dolichol phosphate is transferred to a protein synthesized in the respective organelle. Subsequently, the glycoprotein makes its way through the secretory pathway, transiting to the GC where it is further modified by Golgi-residing enzymes. As such, the glycoprotein is modified by trimming in the cis Golgi, it undergoes extension in the median Golgi, and, when it reaches the trans Golgi, it is terminally sialylated by sialyltransferases such as ST6Gal1. Finally, the *N*-glycoprotein is transported in vesicles and inserted into the membrane [53] (Fig. 4).

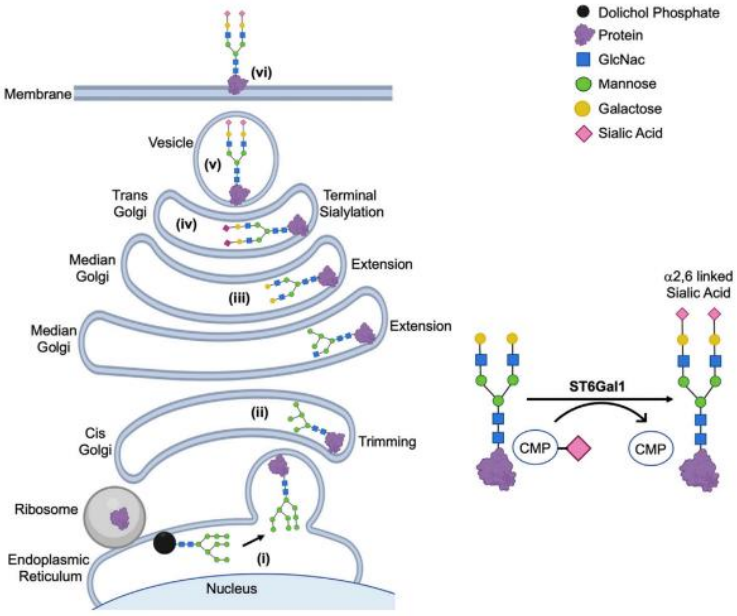

Figure 4. Biosynthesis of a *N*-glycoprotein in the secretory pathway and its terminal α2,6-sialylation catalyzed by the Golgi-residing ST6Gal1 glycosyltransferase. Adapted from Sajina et al. (2022) [73].

Overexpression of ST6Gal1 was first described in CRC as having a mechanistic role in the carcinogenesis process [74]. While upregulation of ST6Gal1 in colon cancer cells has been extensively reported, expression of this sialyltransferase is negligible in the normal differentiated colonic epithelium. However, a set of cells with ST6Gal1 expression was identified in the intestinal crypts and basal epidermal layer, two well established stem cell niches, suggesting a possible stemness-related role for this enzyme [75, 76]. Overexpression of ST6Gal1 has been reported in several carcinomas other than CRC, including gastric, pancreatic and breast cancers, and is linked to several cancer hallmarks, affecting cellular adhesion, migration, proliferative signaling, EMT, immune evasion, apoptosis and drug resistance [73]. Additionally, several reports have correlated ST6Gal1 expression with poor clinical outcomes in cancer patients [72].

1.2.2.1. The pro-survival effect of ST6Gal1 in malignant cells

A well-established cancer hallmark is the ability of tumor cells to evade apoptosis. The apoptotic machinery is composed of a complex network that involves the action of regulatory components and effectors that receive or sense death signals that can trigger programmed cell death. This network can be divided into two pathways, namely the extrinsic pathway, which involves the tumor necrosis factor (TNF) family, and the intrinsic pathway modulated by the mitochondria [15, 77]. Of note, aberrant glycans expressed in malignant cells are able to modulate tumor cell apoptosis [58]. The TNF superfamily is composed of cell death receptors, namely the Fas receptor (FasR), also known as CD95, and the tumor necrosis factor receptor 1 (TNFR1). After being activated by their respective ligands, these receptors initiate effector signaling pathways that culminate in cell death [77]. ST6Gal1-mediated sialylation was reported to modulate the activation of these apoptotic receptors, preventing their internalization and consequent apoptotic signaling [73]. In colon cancer cells, Fas sialylation by ST6Gal1 prevents the assembly of the cell death complex and consequent internalization of FasR, consequently hindering the apoptotic signaling mediated by Fas ligand (FasL) [78, 79]. ST6Gal1-mediated α2,6-sialylation of TNFR1 has also been reported in rectal cancer, as well as in pancreatic and ovarian cancer, preventing receptor internalization and activation of apoptosis [80, 81]. Additionally, TNFR1 sialylation has been observed in macrophages, preventing the induction of macrophage apoptosis by TNFα stimuli [82].

As previously described, CRC patients can be treated with mAbs that target growth factor receptors such as EGFR [5]. However, resistance to anti-EGFR mAb treatment is quite frequent in CRC [11]. Rodrigues et al. previously reported that ST6Gal1 overexpression in CRC cells promotes resistance to Cetuximab-induced cytotoxicity and abrogation of ST6Gal1 sensitized malignant colorectal cells to the mAb cytotoxic effect. Additionally, overexpression of ST6Gal1 was shown to downregulate EGFR expression, revealing a possible ST6Gal1-mediated mechanism of Cetuximab resistance [83]. A similar effect was also described by Duarte et al. in gastric cancer, where abrogation of ST6Gal1 sensitized gastric cancer cells to the cytotoxicity induced by the mAb Trastuzumab, which targets the ErbB2 mitogenic receptor often

overexpressed in gastric carcinoma [84]. Both of these studies describe the role of ST6Gal1 in malignant cells resistance to the direct effect of mAbs used clinically for cancer treatment [83-85].

1.2.2.2. The role of ST6Gal1 in cancer cell invasion and metastasis

Extensive studies demonstrate the effect of ST6Gal1-mediated sialylation in cellular mechanisms that promote invasion and metastasis of malignant cells [72, 86]. Tumor cells need to overcome cell-cell adhesions established between different cells in the primary tumor in order to migrate and invade the surrounding tissue. E-cadherin is the main adhesion molecule expressed in epithelial tissues that ensures close adhesion between cells, and the loss of expression of this protein is a classic hallmark of tumorigenesis, conferring increased migratory and invasive capacities to malignant cells [15]. The α2,6-sialylation of adhesion molecules such as cadherins, integrins and selectins plays a pivotal role in migration and invasion, as well as in the activation of EMT programs of cancer cells, which in turn promotes metastasis [73]. Seales et al. demonstrated that expression of the *Ras* oncogene in colon cancer cells led to an overexpression of ST6Gal1 with a concomitant increase in α2,6-sialylation of β1 integrins [87]. Additionally, they showed that α2,6-sialylation of β1 integrin stimulated adhesion and migration of colon cells onto collagen I fibers, reflecting a more migratory and invasive phenotype of colonic malignant cells [88]. The α2,6-sialylation also appears to affect cell adhesion in breast carcinoma cells, facilitating the detachment of tumor cells from the primary tumor, and greater attachment to basement membrane components, such as collagen IV, which in turn facilitates extravasation and invasion into the surrounding tissue [89]. Activation of EMT programs facilitates tumor cells to lose their epithelial characteristics, as well as their polarity, becoming more mesenchymal-like cells. This process facilitates the loss of cell-cell adhesion through the downregulation of E-cadherin and the acquisition of enhanced migratory capacity. Additionally, EMT confers stem-like properties to malignant cells, possibly giving rise to cancer stem cells [15]. In breast, lung and pancreatic cancer, overexpression of ST6Gal1 was reported to downregulate E-cadherin (epithelial marker) and upregulate N-cadherin (mesenchymal marker), supporting an ST6Gal1-mediated EMT process [90-92]. TGFβ has a preponderant role in inducing EMT, facilitating the invasion and metastasis of tumor cells [15, 93]. A link between ST6Gal1 and TGFβ expression has been shown to promote EMT, as TGFβ sialylation affected the EMT process, and the overexpression of ST6Gal1 upregulated TGFβ expression [73, 90, 94]. A study by Lu et al. demonstrated that overexpression of ST6Gal1 in breast cancer cells promoted TGFβ-dependent EMT through downregulation of E-cadherin. Moreover, ST6Gal1 abrogation reversed the mesenchymal phenotype of breast cancer cells, with a consequent increase in E-cadherin expression. In the same study, it was demonstrated that overexpression of the β-galactoside α2,3-sialyltransferase 4 (ST3Gal4), a sialyltransferase whose upregulation leads to overexpression of SLe^X in various cancers, had a neglectable effect on the induction of EMT, supporting the necessity of ST6Gal1 for the EMT process of neoplastic cells [90], ultimately affecting the invasion and metastasis of cancer cells.

ST6Gal1 also plays a key role in malignant-associated angiogenesis, as the ligands for the VEGF receptors are targets of sialylation [57, 95, 96]. A study conducted by Imamaki et al. demonstrated that ST6Gal1$^{-/-}$ mice exhibited impaired tumor-associated angiogenesis due to an increase in endothelial cell apoptosis. Furthermore, it revealed that ST6Gal1 is essential for endothelial cell survival and angiogenic signaling triggered by angiogenic factors [97]. VEGF expression can be induced by the hypoxia inducible factor 1 (HIF1) transcription factor under hypoxic conditions, a mechanism that hypoxic tumor cells use to induce local angiogenesis [98]. A study by Jones et al. demonstrated that tumor cells in a hypoxic environment exhibited an upregulation of ST6Gal1 which, in turn, led to an increase in HIF1 expression [99]. As such, Sajina et al. hypothesized a possible correlation between ST6Gal1, HIF1 and VEGF, a network capable of promoting neoplastic angiogenesis as a way to support tumor growth [73]. The induction of angiogenesis at the tumor site not only facilitates nutrients and oxygen supplies to tumor cells, but also provides a gateway for malignant cells to invade the bloodstream and metastasize to distant sites [98].

1.2.2.3. ST6Gal1 in anti-tumor immunity

A link between ST6Gal1 expression and immune evasion mechanisms is being extensively studied [73]. The role of TGFβ goes beyond its effect in promoting EMT and invasion of malignant cells, as this cytokine has an anti-inflammatory effect on the TME. TGFβ, in the vast majority of cases, acts as an antagonist of the innate and adaptive immune responses [100]. For example, TGFβ has been shown to polarize macrophages towards a more M2-like and pro-tumor phenotype and, additionally, it has been found to prevent NK cell maturation [101, 102]. Regarding subsets of T cells, TGFβ acts primarily by inhibiting the proliferation of effector T cells [103], while promoting the differentiation of naïve T cells into Treg lymphocytes, which suppresses CD4$^+$ and CD8$^+$ T cell responses [104]. Furthermore, Tregs secrete more TGFβ into the TME, constituting a gradually immunosuppressive microenvironment [100]. Given the aforementioned link between ST6Gal1 overexpression and TGFβ upregulation, it seems that ST6Gal1 may play a role in the creation of a TGFβ-mediated immunosuppressive TME that will support immune cell evasion [73]. Wang et al. found that ST6Gal1 not only enhanced the proliferation and migratory capacity of hepatocarcinoma cells, but also favored immune escape. By co-culturing CD8$^+$ T cells with ST6Gal1-overexpressing cancer cells, the authors observed an increase in the production of the anti-inflammatory cytokines IL-10 and TGFβ and a decrease in the levels of the pro-inflammatory cytokines IFN-γ and TNFα, along with decreased T cell proliferation [105].

Diverse populations of immune cells express receptors from the vast family of Siglecs that recognize linkage-specific sialylation [63]. The expression of these GBPs on the surface of immune cells is essential to dampen immune responses in inflammatory environments through the ITIM-bearing cytoplasmic tails of Siglecs [106]. Therefore, by recognizing and binding to specific NeuAc residues, Siglecs are able to modulate immune responses [63, 106]. Siglecs role in the TME is similar to the PD-1/PD-L1 axis, which is frequently exploited by malignant cells, thus

promoting immune evasion [107-109]. α2,6NeuAc motifs can be recognized by different Siglecs, such as Siglec-2, -3, -5, and -10 [110]. For example, Siglec-2 (CD22) is expressed on B cells and acts as negative regulator of B cell activation by binding to α2,6NeuAc motifs added to glycoconjugates by ST6Gal1. Additionally, ST6Gal1 modulates CD22-B cell receptor (BCR) signaling by regulating the colocalization of the BCR with CD22 in clathrin-rich microdomains [111, 112]. Furthermore, recent reports reveal that Siglec-10 expressed on the surface of TAMs acts as a modulator of phagocytosis of breast and ovarian cancer cells by interacting with the heavily sialylated CD24 glycoprotein [113]. CD24 is described as a carrier of both α2,3 and α2,6NeuAc motifs, which are two known Siglec-10 ligands [110, 114]. Moreover, by inhibiting phagocytosis through interactions with Siglec-10 expressed by macrophages, CD24 is described as a novel “Don’t Eat Me” molecule, similar to CD47 [113, 114]. The effects of ST6Gal1-mediated sialylation in anti-tumor immune responses must be further explored, as well as the interactions between Siglecs and the diverse sialylated glycoforms expressed by malignant cells.

1.3. Extracellular vesicles

1.3.1. Biogenesis, biodistribution and uptake of extracellular vesicles

Extracellular vesicles (EVs) are a heterogeneous group of lipid bilayer nano-sized particles released by all cell types into the extracellular space, serving as important mediators of intercellular communication [115, 116]. EVs have a diameter ranging from 35-5000 nm and can be classified into three types according to their size and biogenesis: exosomes, microvesicles and apoptotic bodies [115] (Fig. 5). Exosomes originate from the endocytic pathway through the inward budding of the endosomal membrane, initially forming multivesicular bodies (MVBs) which, after fusing with the cell membrane, form exosomes that are subsequently released. Meanwhile, MVBs can also fuse with lysosomes to degrade the cargo contained within the vesicles. Through the shedding or budding of the plasma membrane, microvesicles are formed, which are larger than exosomes. Additionally, apoptotic bodies are formed by cell membrane blebbing of an apoptotic cell [115]. EVs have different markers associated with their biogenesis [116, 117]. For example, exosomes can carry endosomal sorting complex required for transport (ESCRT) associated proteins, such as ALG-2-interacting protein X (Alix), tumor susceptibility gene 101 (TSG101) and the Hsp70 and Hsp90 chaperones [116]. There is also an ESCRT-independent mechanism for the release of exosomes, involving type II neutral sphingomyelase and the tetraspanin family proteins, such as CD9, CD63 and CD81, which can all be present in exosomes released by this route [117]. Microvesicles may contain specific cytosolic and membrane proteins, namely CD40, ADP-ribosylation factor 6 (ARF6), selectins, phosphatidylserine and Rho family proteins [116, 118]. Additionally, they may also contain the tetraspanins found in exosomes [119]. Apoptotic bodies contain fragments of DNA, and non-coding RNAs, as well as other proteins associated with the different organelles of the apoptotic cell, namely histones (nucleus), Hsp60

(mitochondria) and the chaperone GRP78 (ER). Annexin V is considered a marker of apoptotic bodies [120].

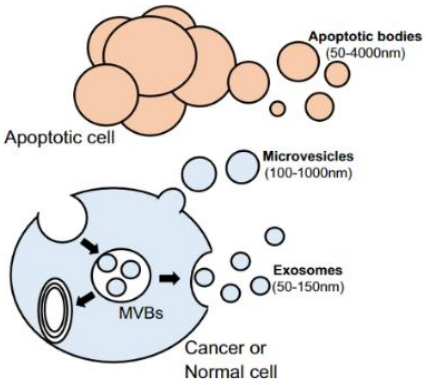

Figure 5. Schematic representation of the different type of EVs produced by cells that are secreted into the extracellular space. Adapted from Urabe et al. (2019) [118].

EVs carry an array of molecules, such as cytosolic and cytoskeletal proteins, as well as nucleic acids, all of which reflect the phenotype of the cell that originated the EVs [116, 121]. Additionally, the bilipid layer of EVs protects their cargo from degradation by nucleases and proteases present in the extracellular milieu, allowing the safe circulation of EVs until they reach the target recipient cells [122]. Moreover, the surface of EVs is frequently coated by transmembrane proteins and adhesion molecules, as well as different lipids, namely ceramide, cholesterol and phosphatidylserine, all of which support EV docking in the plasma membrane and signaling events [116].

The generation of exosomes and microvesicles involves several sorting mechanisms that promote the clustering of membrane-associated proteins and lipids into membrane microdomains. In microvesicles, lipids and proteins are clustered in specialized microdomains of the plasma membrane, while in exosomes, the clustering occurs within the limiting membrane of the endosomal MVBs. Additionally, these specialized microdomains are also involved in the recruitment of soluble components (cytosolic proteins and RNAs). It should be noted that the nature and abundance of the content loaded on EVs are dependent on the cell type, the pathophysiological state of the cell, the modulatory stimuli that the donor cell receives, as well as the mechanisms of EV biogenesis [116].

Most endosomal MVBs are destined to be fused with lysosomes for further degradation [123]. However, there are certain mechanisms that prevent the degradation of MVBs, thus allowing the secretion of exosomes by donor cells into the extracellular space [116]. The balance between exosome secretion or lysosomal degradation possibly depends on sorting mechanisms and the ESCRT machinery seems to be involved in the regulation of this balance. For example, while TSG101 appears to favor endosomal MVB degradation in lysosomes, overexpression of tetraspanin 6 appears to delay lysosomal degradation and favor exosome secretion [124, 125].

For exosome secretion to occur, endosomal MVBs have to fuse with the plasma membrane by a mechanism mediated by SNARE proteins and members of the synaptotagmin family [126]. In the case of microvesicle secretion, these have to undergo plasma membrane fission by an actin- and myosin-dependent mechanism and ATP-dependent contraction [116].

EVs are released into the extracellular space and deliver their cargo to target cells, promoting phenotypic changes and a functional response by the recipient cells [123]. Depending on the cell type, EVs can undergo membrane docking, binding to membrane receptors, such as integrins, initiating intracellular downstream signaling pathways. On the other hand, EVs can be internalized by the endocytic pathway (i.e., macropinocytosis, phagocytosis, and caveolae- or clathrin-mediated endocytosis), leading to their fusion with multivesicular endosomes (MVEs) [116]. The fusion of MVEs with lysosomes promotes EV degradation and recycling of the intraluminal contents to fuel recipient cells metabolism [127]. Additionally, EVs can fuse with the cell membrane and release their contents directly into the cytoplasm of target cells [123].

1.3.2. The role of EVs in cancer intercellular communication

Since EVs are important mediators of intercellular communication, affecting the behavior of cells at close and distant sites, the role of EVs in the modulation of cancer progression and metastasis is not surprising [128]. Cancer EVs modulate the behavior of local or recruited stromal cells in order to support a more permissive microenvironment for tumor development, namely by inducing local angiogenesis and immunosuppressive phenotypes, as well as extensive ECM remodeling, all of which support tumor onset [129]. Endothelial cells present in the TME are modulated by cancer EVs to induce angiogenesis [130]. In addition to transferring oncogenic proteins to other malignant cells in order to enhance their growth, cancer EVs have also been reported to induce and activate autocrine VEGF signaling in endothelial cells, with consequent induction of angiogenesis [131]. Additionally, EVs produced by hypoxic tumor cells are enriched in hypoxia-regulated RNA capable of stimulating endothelial cells to induce angiogenesis [132, 133]. TGFβ-containing EVs produced by cancer cells convert fibroblasts into myofibroblasts, which promote vascularization and local invasion [134]. Additionally, stromal cells secrete EVs capable of modulating the TME [129]. For example, Luga et al. reported that exosomes secreted by CAFs in breast tumors are able to promote motility, invasion and dissemination in a Wnt-dependent signaling pathway [135].

The immunomodulatory properties of cancer EVs has also been addressed, with several studies suggesting that EVs can have both pro- and anti-tumorigenic effects depending on cell type, microenvironment stimuli, and EV cargo [128, 129]. The effects of EVs on immunological surveillance may be mediated by direct signaling on immune cells, or else, EVs may transfer tumor-associated antigens to APCs, which in turn modulate the cytotoxic response of T cells [136]. Cancer EVs carrying miRNAs have also been reported to modulate macrophage polarization in the TME. For example, Zhao et al. reported that CRC-produced exosomes

containing miR-934 were able to promote M2 polarization and facilitate liver metastasis [137]. Similar findings were also reported by Shinohara et al., where it was shown that CRC-derived miR145-containing EVs were able to polarize TAMs to a M2-like phenotype and promote tumor growth in mice [138]. Liu et al. reported that miR-150 transferred to TAMs by cancer EVs were able to induce VEGF and other angiogenic factors production and support angiogenesis [139].

EVs are also involved in local invasion by inducing ECM remodeling. Enrichment of cell-ECM adhesion proteins on cancer EVs enhances adhesion, directional migration, and motility of malignant cells along ECM fibers [129]. Sung et al. demonstrated that inhibition of exosome secretion or biogenesis led to the formation of unstable protrusions and defective tumor cell migration. Moreover, it was also observed that secreted exosomes containing fibronectin enhance nascent adhesions assembly, thus promoting migration [140]. Additional studies also report the release of MMP-enriched EVs capable of promoting the proteolysis of ECM components and facilitating tumor cell migration and invasion [141, 142].

1.3.2.1. Establishment of pre-metastatic niches

The pre-metastatic niche is a specialized microenvironment prepared to receive metastatic cells that have spread from the primary tumor. It is hypothesized that the pre-metastatic niche is formed even before the primary tumor metastasizes by intercellular communication mediators released from the primary tumor site [143]. The pre-metastatic niche supports metastatic cell growth in a secondary organ that would otherwise offer a harsh environment for cells originating from a different organ [144]. Additionally, the concept of pre-metastatic niches offers keener insight into the organ tropism exhibited by metastatic cancer cells from the primary tumor [145].

The establishment of the pre-metastatic niche involves the secretion of tumor-associated factors and EVs by the primary tumor that will target specific organs where the pre-metastatic niche will develop [143]. In the secondary organ, by induction of cytokines and angiogenic factors, there is an increase in the permeability and leakiness of blood vessels, followed by the recruitment of bone-marrow derived cells (BMDCs) that actively reshape the local tissue. There is extensive remodeling of the ECM, with fibronectin deposition and collagen I cross-linking, all of which favor adhesion of BMDCs. Local and distant secretion of MMPs also enhance ECM remodeling, facilitating metastatic cells adhesion and invasion in the premetastatic niche [146]. BMDCs secrete inflammatory cytokines, growth and proangiogenic factors that support malignant cell colonization and growth [147-149]. Additionally, fibroblasts are educated to actively support the establishment of the pre-metastatic niche by producing high levels of inflammatory cytokines, MMPs and fibronectin [129, 143, 150]. The inflammatory cytokines released in the pre-metastatic microenvironment recruit immune populations, such as monocytes, macrophages, Tregs and myeloid-derived suppressor cells (MDSCs) that will induce local immunosuppression, in turn protecting malignant cells from anti-tumor immune responses [147, 151].

EVs role as long distance communication mediators is essential for the establishment of the pre-metastatic niche by reprogramming and educating target cells to adopt pro-tumor phenotypes [129, 143, 151]. Hoshino et al. demonstrated that tumor exosomes carrying distinct integrins determined organotropic metastasis. By targeting specific integrins, exosome uptake by recipient cells was abrogated, blocking lung and liver metastasis. For example, integrin $\alpha_v\beta_5$-carrying exosomes produced by CRC, gastric and pancreatic cancer cells preferentially targeted Kupffer cells, mediating liver tropism, whereas exosomal $\alpha_6\beta_4$ and $\alpha_6\beta_1$ integrins targeted lung-resident fibroblasts, favoring lung tropism [152]. Costa-Silva et al. demonstrated that pancreatic cancer-derived exosomes favored pre-metastatic niche formation in naive mice livers. These exosomes enriched in macrophage migration inhibitory factor (MIF) stimulated the production of TGFβ by Kupffer cells and the consequent deposition of fibronectin by hepatic stellate cells, supporting the establishment of a fibrotic environment capable of recruiting bone-marrow derived macrophages and neutrophils. Exosomal MIF ablation inhibited liver pre-metastatic niche formation and metastases, suggesting that MIF upregulation is an early event in liver pre-metastatic niche establishment. Additionally, the authors found that macrophage depletion in exosome-educated mice was sufficient to prevent liver metastasis, once again demonstrating the role of macrophages in establishing the pre-metastatic niche [153]. Morrissey et al. observed that exogenous administration of cancer EVs in mice educated tissue-resident macrophages to adopt an immunosuppressive phenotype that supported lung metastasis. They found that macrophages underwent metabolic reprogramming and adopted a glycolytic metabolism that supported increased expression of the PD-L1 immune checkpoint [154]. Zeng et al. reported that CRC exosomes containing miR-25-3p favored the formation of pre-metastatic niches in the liver and lungs of murine models by targeting endothelial cells and promoting angiogenesis, while also enhancing vascular permeability and leakiness [155]. These findings, along with several others, highlight the role of cancer EVs in not only determining metastatic tumor tropism, but also in educating secondary organ target cells to create their own specialized microenvironment to receive metastatic cells.

1.3.3. Impact of cancer aberrant glycosylation on EVs biological roles

The surface composition of EVs is enriched with distinct glycans that affect their biogenesis, uptake and cellular recognition [156]. Several tumor-associated glycans that arise in cancer due to defects in the glycosylation machinery are also reported to be present in cancer EVs [157]. These tumor-associated glycans found in cancer EVs are likely to control EV protein sorting and uptake by target cells, while also driving organ-tropism. Glycan composition can vary greatly between different cancer EVs, depending on the type of cancer, the cells producing the EVs, and microenvironmental stimuli [156, 158]. To date, several glycan structures have been identified in cancer EVs [156, 157, 159]. As reviewed by Martins et al., EVs can be enriched in α2,3 and α2,6NeuAc motifs, complex and core fucosylated *N*-glycans, GAGs, proteoglycans, as well as the Tn and STn antigens. These glycan structures not only were detected in EVs produced

by immortalized cell lines, but also in cancer patients samples, showcasing the potential of these glycans as circulating cancer biomarkers [156]. The presence of specific glycan structures on the surface of EVs can be used to capture and isolate EVs present in heterogeneous samples [157, 159]. The use of carbohydrate-binding lectins with high specificity can be used to isolate EVs. For example, *Maackia amurensis* lectin I (MAL-I), which recognizes α2,3NeuAc motifs in galactose or GalNAc, was efficient in isolating different sized EVs in urine samples from healthy individuals, with a higher yield and purity when compared to conventional isolation methods based on the antibody detection of the classic EV markers CD9, CD81 and CD63 [160]. Following these results, Yamamoto et al. were able to isolate and capture EVs produced by melanoma cells using the freshwater cyanobacterium *Oscillatoria Agardhii* agglutinin (OAA), which can specifically bind to high-mannose glycans. The authors demonstrated that EVs produced by melanoma cells are enriched in high-mannose-type glycans and that OAA-immobilized beads were able to capture 60% of EV particles [161]. Therefore, detailed analysis of the glycan composition present in cancer EVs can be used to devise more specific EV detection and isolation methods with the potential to be translated into the clinic [156].

Glycosylation has been described to impact the functional properties of cancer EVs. Sialylation of EVs appears to affect organ-tropism and uptake by recipient cells [156]. Royo et al. demonstrated that treatment of EVs with neuraminidase, a glycosidase that cleaves sialic acid residues, affected the *in vivo* biodistribution of EVs in mice. The authors observed a greater accumulation of treated EVs in the lung and axillary lymph nodes compared to untreated EVs [162]. Beside sialylation, other forms of glycosylation seem to influence the uptake of EVs. For example, proteoglycans anchored in the cell membrane appear to modulate the uptake of EVs. Heparan sulfate proteoglycans, such as glypican and syndecan, were shown to facilitate EV uptake by glioblastoma cells. However, disruption of their synthesis did not seem to impact EV uptake, indicating the existence of alternative uptake mechanisms independent of heparan sulfate proteoglycans [163]. Glycosylation also affects sorting mechanisms and cargo content of EVs [164]. For example, aberrant *O*-glycosylation appears to increase the amount of CD44 in EVs produced by CRC cells [165]. CD44 is a transmembrane protein that often carries truncated *O*-glycans and promotes cancer cell proliferation and invasion via RON receptor hyperactivation [58]. ST6Gal1 also controls protein sorting in cancer EVs, as it was observed that CRC cells with abrogation of ST6Gal1 produced EVs lacking the tetraspanin protein KAI1, which is described as metastasis inhibitor [166]. ST6Gal1 was also reported to be present in exomeres, a type of EV recently identified. ST6Gal1 in exomeres is transferred to recipient cells, leading to hypersialylation of cell-surface proteins, such as β1-integrin [167]. As already described in the previous sections, EVs can transfer their protein cargo to recipient cells, which will modulate the phenotype and cellular response of target cells. In this manner, glycosylation not only controls the protein cargo that is sorted into EVs, but also the type of response these EVs will elicit upon reaching their target cells. As glycosylation is an important regulator of immune responses, particularly through the interaction of glycans with their respective GBPs, it is expected that aberrant glycosylation of cancer EVs will be able to modulate anti-tumor immune responses [156].

The glycosylation profile of cancer EVs might allow to unveil new biomarkers of disease [156]. Tumor-associated glycans secreted into the circulation serve as potential cancer biomarkers and can be detected by serological assays. SLe^a can be detected serologically by CA19-9, STn by CA72-4, MUC16 by CA125 and MUC1 by CA15-3 [64]. Interestingly, exosomes isolated from the serum of ovarian cancer patients exhibited high levels of CA125. Furthermore, the levels of this carbohydrate antigen detected in the patients' serum were noticeably lower than in exosomes [168]. Exosomes obtained from patients with pancreatic cancer have also been found to contain the CA19-9 antigen. Additionally, exosomes from patients with undetectable CA19-9 serum levels revealed to be carrying this carbohydrate antigen [169].

In conclusion, the aberrant glycosylation of cancer cells has a role in promoting malignant transformation and cancer onset. Additionally, EVs produced by cancer cells reflect the altered glycosylation profile of the parental cells. The tumor-associated glycans present in EVs might play a role in promoting the dissemination of cancer cells. It is therefore crucial to understand how different glycoforms of cancer EVs are able to modulate the various intercellular dynamics in the TME to promote cancer progression.

2. Aim and objectives

Aberrant glycosylation is a frequent alteration observed in cancer. In particular, overexpression of the ST6Gal1 sialyltransferase has been reported in several malignancies, such as CRC. Cancer cells produce EVs, which act as important mediators of intercellular communication, being involved in recipient cell reprogramming, cancer progression and metastasis. Given the aforementioned role of glycosylation in the biological properties of EVs, this thesis aims to disclose the immunomodulatory effect of the oncogenic ST6Gal1 carried by EVs produced by CRC cell lines. In an ongoing project of our research group, EVs are being isolated from glycoengineered cancer cell models of CRC with differential *ST6GAL1* expression levels and are being further characterized. Our group have shown that EVs produced by cell lines with ST6Gal1 expression carry the sialyltransferase and its enzymatic product, the α2,6NeuAc glycan, while ST6Gal1 absence was observed in EVs produced by *ST6GAL1*-knockout CRC cell lines. Thus, this thesis aims to demonstrate the immunomodulatory effect of ST6Gal1-carrying EVs, using established glycoengineered CRC cell line models, on primary human macrophages.

The project presented in this thesis has the following specific objectives:

1. Assessment of ST6Gal1 expression in different subtypes of CRC:
    a. Evaluation of *ST6GAL1* gene expression levels in tumor samples of CRC patients collected from The Cancer Genome Atlas (TCGA) public database and previously classified according to their specific CMS;
    b. Analysis of ST6Gal1 expression in immortalized CRC cell lines previously classified according to their specific CMS;
2. Analysis of the immunomodulatory role of ST6Gal1-carrying EVs on human macrophages:
    a. Isolation of monocyte-derived macrophages from buffy coats of healthy blood donors;
    b. Treatment of monocyte-derived macrophages with EVs isolated from a glycoengineered cell model of CRC with differential ST6Gal1 expression;
    c. Assessment of ST6Gal1-mediated sialylation impact on EV uptake by monocyte-derived macrophages;
    d. Assessment of ST6Gal1-carrying EVs effect on macrophage polarization and survival.

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3. MATERIALS AND METHODS

3.1. Transcriptomic analysis

Data relative to *ST6GAL1* gene expression in tumor samples from CRC patients were collected from The Cancer Genome Atlas (TCGA) public database, which were previously classified by Lal et al. according to their specific CMS [170]. The levels of *ST6GAL1* mRNA expression are presented as the z-score relative to all samples. Differences in gene expression were evaluated by Kruskall-Wallis test followed by Dunn´s multiple comparisons test. All analysis and plots were performed using Prism software (GraphPad Inc.).

3.2. Cell culture

Eight human CRC cell lines (Caco-2, COLO-205, HT-29, SW48, SW480, SW620, SW837 and SW948) were cultured at 37 °C in a 5% CO_2 atmosphere. Caco-2, HT-29, SW837 and SW948 cells were cultured in DMEM, High Glucose, Pyruvate medium (Gibco). COLO-205, SW48, SW480 and SW620 cells were cultured in RPMI-1640, GlutaMAX™, HEPES medium (Gibco). The culture medium for each cell line was supplemented with 10% heat-inactivated fetal bovine serum (FBS; Biowest). The Caco-2 *ST6GAL1* knockout (KO) model established by Rodrigues et al. [83] was also used. Briefly, ST6Gal1 KO was performed on Caco-2 wild-type (WT) cells by CRISPR/Cas9-mediated abrogation of the *ST6GAL1* gene. Single cell sorting was then performed and different isogenic clones were expanded and characterized. For this study, the Caco-2 ST6Gal1 KO17 cell line was used to perform functional assays.

3.3. Western and lectin blot assays

Total protein lysates were extracted from confluent cells using Lysis Buffer 17 (R&D Systems) supplemented with a PhosSTOP phosphatase inhibitor (Sigma-Aldrich) and cOmplete™protease inhibitor (Sigma-Aldrich). The protein concentration of whole cell lysates was determined using the DC protein assay (BioRad). Total protein lysates (35 µg of protein for ST6Gal1 detection and 5 µg of protein for α2,6NeuAc detection) were separated by polyacrylamide gel electrophoresis (SDS-PAGE) and blotted onto nitrocellulose membranes (GE Healthcare). For ST6Gal1 detection, membranes were blocked for 1 hour at room temperature (RT) with 5% non-fat milk in 0.1% Tween® 20 (Sigma-Aldrich) in tris buffered saline (TBS), followed by overnight (ON) incubation with anti-ST6Gal1 primary antibody (D1/500; R&D Systems) at 4 °C. Next, membranes were incubated for 1 hour at RT with horseradish peroxidase-conjugated polyclonal anti-goat secondary antibody (D1/2000; R&D Systems). For α2,6NeuAc detection , membranes were blocked ON at 4 °C with 2% polyvinylpyrrolidone (PVP; Sigma-Aldrich) in TBS. Membranes were subsequently incubated for 1 hour at RT with biotinylated SiaFind™ α2,6-specific reagent (D1/10,000; Lectenz Bio), followed by incubation with horseradish peroxidase-conjugated

streptavidin (D1/50,000; GE Healthcare) for 1h at RT. Anti-β-actin (D1/2000; Santa Cruz Biotechnology) was used as loading control, with its respective horseradish peroxidase-conjugated polyclonal anti-mouse secondary antibody (D1/5000; Jackson Immunoresearch).

3.4. Immunofluorescence assay

Cells were seeded on 12-well microscope slides and fixed with 4% paraformaldehyde (PFA; Thermo Fisher Scientific) solution for 20 minutes at RT. Next, each well was washed with phosphate-buffered saline (PBS) and cells were permeabilized with 0.5% Triton X-100 (Sigma-Aldrich) in PBS for 10 minutes at 4 °C. Followed by additional washes, cells were blocked with animal-free blocking solution (Vector Laboratories) for 30 minutes at RT and incubated next with anti-ST6Gal1 primary antibody (D1/1000; R&D Systems) ON at 4 °C. Cells were then washed and incubated with Alexa-Fluor™ 488-conjugated anti-goat secondary antibody (D1/500; Invitrogen) for 45 minutes at RT. Nuclear counter staining was performed by incubating cells with DAPI (D1/100; Sigma-Aldrich) for 5 minutes at RT. Slides were mounted in VectaShield (Vector Laboratories) and images were acquired with Zeiss Axio Imager Z1 Apotome, using AxioVision v4.8 software.

3.5. Cell surface lectin labeling by flow cytometry

The *Sambucus nigra* (SNA) lectin was used to assess cell surface levels of α2,6NeuAc residues by flow cytometry. CRC cells were seeded in 6 well culture plates and allowed to reach confluency. Next, cells were detached by incubating with accutase at RT. Cells concentration and viability was assessed in a Neubauer chamber using trypan blue. 10^5 cells were stained with FITC-conjugated SNA (D1/2000; Vector Laboratories) for 30 minutes on ice. Prior to acquisition, 5 µl of 2 µg/ml of DAPI (Sigma-Aldrich) was added to cells and viability was assessed as the frequency of $DAPI^-$ cells. Signals were detected using a BD FACSCantoII system (BD Bioscience) and data were analyzed using FlowJo software (BD Bioscience).

3.6. EV isolation and characterization

Caco-2 WT and Caco-2 ST6Gal1 KO17-derived EVs were previously isolated and characterized from cell culture medium by the differential ultracentrifugation protocol, as described in [159]. Briefly, FBS-free cell culture medium was collected and centrifuged at 800g for 5 minutes, followed by centrifugation at 2,000g for 10 minutes. Supernatants were vacuum filtered using a 0.22 µm pore filtration system. Next, medium was centrifuged at 100,000g for 20 hours at 4 °C in a 70Ti rotor in a Beckman Optima XE 100 ultracentrifuge. The obtained EV pellets were resuspended in saline solution and characterized by nanoparticle tracking analysis (NTA), transmission electron microscopy (TEM), protein quantification and western blotting. For protein

quantification, EVs were lysed in RIPA buffer for 15 minutes on ice with periodic mixing. Next, EVs samples were centrifuged at 16,000g for 15 minutes at 4 °C. Protein amounts were then quantified using the BCA protein assay kit (ThermoFisher Scientific) with absorbances being read at 562 nm using Gen5.11 software (Synergy Mx). All samples were stored at -80 °C.

3.7. Human monocyte isolation and macrophage differentiation

Human monocytes were isolated by a negative selection protocol from buffy coats obtained from healthy blood donors, as previously described [36]. Briefly, the layer containing peripheral blood mononuclear cells (PBMCs) was collected from blood by centrifugation at 1200g and incubated with RosetteSep-Human Monocyte Enrichment Cocktail (StemCell Technologies) for 20 minutes under rotation. The layer was then diluted in a 1:1 ratio with PBS supplemented with 2% FBS and separated by density gradient using Ficoll-Histopaque (Sigma-Aldrich). The monocyte-enriched layer was collected and isolated cells were resuspended in DMEM, High Glucose, without Pyruvate medium (Gibco), supplemented with 10% FBS (Biowest), 1% penicillin/streptomycin (PenStrep; Gibco) and 50 ng/ml of macrophage colony-stimulating factor (M-CSF; ImmunoTools). Finally, isolated monocytes were seeded on glass coverslips in 6 well culture plates at a density of 10^6 cells/well and allowed to differentiate into macrophages for 7 days at 37 °C in a 5% CO_2 atmosphere.

3.8. Analysis of EV uptake by macrophages

3.8.1. EV uptake in indirect co-culture system

Macrophage uptake of EVs produced by Caco-2 WT cells labeled with the PKH67 Green Fluorescent Cell Linker Kit for General Membrane Labeling (1 mM; Sigma-Aldrich) was evaluated in a transwell indirect co-culture system using 0.4 µm translucent high density PET membrane inserts (Corning). A cell suspension of $2x10^7$ cells/ml of Caco-2 WT cells in Diluent C (Sigma-Aldrich) was mixed with a 4 µM PKH67 solution prepared in Diluent C. Final concentrations after mixing were $1x10^7$ cells/ml and 2 µM PKH67. Cells were incubated with the dye for 5 minutes at RT. FBS was added in an equal volume of the cell suspension to block free dye excess for 1 minute. Cells were then centrifuged three times at 400g, for 5 minutes at RT and resuspended in complete medium. $2.5x10^5$ or $5x10^5$ PKH67-labeled Caco-2 WT cells were seeded on the upper chamber of the transwell inserts and were left to grow for 24 hours. After 24 hours, cell culture medium of Caco-2 WT cells and macrophages was removed, cells were washed with PBS and culture medium containing 10% FBS previously ultracentrifuged (100,000g, ON, 4 ºC) to remove exosomes present in the serum (FBS-exosome depleted) was added. Then, inserts containing the PKH67-labeled Caco-2 WT cells were added to 6 well plates containing previously seeded 10^6 monocyte-derived macrophages/well, establishing an indirect co-culture system. After 24 hours, inserts were removed and macrophages were collected for flow cytometry analysis. Briefly,

each well was washed with PBS and macrophages were incubated with accutase at RT. After 30 minutes, macrophages were collected by gently scrapping each well. Cells were centrifuged at 400g for 5 minutes, at 4 °C and resuspended in PBS. Cells concentration and viability was assessed in a Neubauer chamber using trypan blue. 100,000 cells were incubated 30 minutes on ice with APC-conjugated anti-CD14 antibody (D1/25; ImmunoTools). As compensation controls, single stained samples of PKH67 and anti-CD14-APC were used. Unstained samples were used as basal fluorescent control. Prior to acquisition, 5 µl of 2 µg/ml DAPI (Sigma-Aldrich) was added to cells and viability was assessed as the frequency of $DAPI^-$ cells. Signals were detected using a BD FACSCantoII system (BD Bioscience) and data were analyzed using FlowJo software (BD Bioscience).

3.8.2. Uptake of fluorescently labeled EVs

Isolated EVs from Caco-2 WT and Caco-2 ST6Gal1 KO17 cells by ultracentrifugation were fluorescently labeled with the lipophilic dye PKH67 (Sigma-Aldrich) following the manufacturer's recommendations. Briefly, 90-120 µg of EVs were resuspended in Diluent C and incubated with PKH67 (D1/1000) for 5 minutes at RT. To block free dye excess, 25% of bovine serum albumin (BSA) in saline solution was added to the EV suspension and incubated for 1 minute at RT. The labeled EVs were ultracentrifuged at 100,000g for 2 hours, at 4 °C, and the obtained pellet was resuspended in saline solution. For each sample, protein was quantified using a BCA kit as detailed in 2.5.

Flow cytometric analysis was performed to assess the uptake of PKH67-labeled EVs by macrophages. After 7 days of culture, monocyte-derived macrophages were treated with 2.5 µg or 5 µg of PKH67-labeled EVs isolated from Caco-2 WT and Caco-2 ST6Gal1 KO17 cells for 30 minutes, 1 hour and 2 hours. After each timepoint, cells were collected and stained with anti-CD14-APC (ImmunoTools) as described in 3.8.1. For compensation controls, untreated cells stained with anti-CD14-APC antibody or PKH67 dye were used as single stained samples. Unstained cells served as basal fluorescence control. 5 µl of 2 µg/ml DAPI (Sigma-Aldrich) was added to cells prior to acquisition and viable cells were identified as $DAPI^-$ cells. Signals were detected using a BD FACSCantoII system (BD Bioscience) and data were analyzed using FlowJo software (BD Bioscience).

3.9. Macrophage polarization

Monocytes were seeded and allowed to differentiate into macrophages as described in 3.7. Macrophages were then stimulated for 24 hours with 100 ng/ml LPS (Sigma-Aldrich) and 20 ng/ml IFNγ (ImmunoTools) to induce a pro-inflammatory (M1) phenotype, or with 20 ng/ml IL-4 (ImmunoTools) to induce an anti-inflammatory (M2) phenotype.

To analyze the effect of Caco-2 WT and Caco-2 ST6Gal1 KO17-derived EVs on macrophage polarization, M-CSF stimulated macrophages (M0) were incubated with 5 µg of Caco-2 WT and Caco-2 ST6Gal1 KO17-derived EVs. On day 7, cell culture medium was renewed with complete medium containing 10% FBS-exosome depleted, 1% PenStrep and 5 µg of EVs. Macrophages were then incubated with the EVs for 24 or 48 hours (37 °C, 5% CO_2). After each timepoint, cells were collected as described in 3.8.1. Cells were then incubated for 30 minutes on ice with the fluorescent conjugated antibodies anti-CD14-APC (D1/25; ImmunoTools), anti-CD86-FITC (D1/25; ImmunoTools), anti-CD163-PE (D1/25; BD Biosciences), anti-HLA-DR-FITC (D1/25; ImmunoTools), anti-HLA-ABC-PE (D1/25; ImmunoTools), anti-CD40-FITC (D1/25; ImmunoTools), and anti-CD206-PE (D1/25; BioLegend). Single stained samples from each fluorescent conjugated antibody were used as compensation controls. Unstained cells served as basal fluorescence control. Prior to acquisition, 5 µl of 2 µg/ml DAPI (Sigma-Aldrich) was added to cells and viability was assessed as the frequency of $DAPI^-$ cells. Signals were detected using a BD FACSCantoII system (BD Bioscience) and data were analyzed using FlowJo software (BD Bioscience).

3.10. Statistical analysis

For assays performed with CRC cell lines, data are presented as mean ± SD of at least two independent replicates. For assays involving primary human cells, data are presented as mean ± SEM for at least two independent blood donors. EV uptake analysis was performed with two-way ANOVA, with macrophages treated with Caco-2 WT-derived EVs as control. Macrophage polarization assay results were analyzed with paired one-way ANOVA. Macrophage viability after treatment with EVs was analyzed with paired t-test. All statistical analysis were performed using Prism software (GraphPad Inc.). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.

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4. RESULTS

4.1. Expression of ST6Gal1 in CRC

ST6GAL1 transcript levels in tumor samples of CRC patients previously classified for their CMS by Lal et al. were obtained from the public TCGA database [170]. Transcriptomic analysis revealed different levels of *ST6GAL1* transcripts among the four CMS (Fig. 6a.). CMS3 tumors were shown to have the lowest *ST6GAL1* expression, followed by CMS1 tumors. The CMS2 is the most frequent subtype in CRC, followed by the CMS4 subtype [50]. In parallel, *ST6GAL1* expression levels were significantly higher in CMS2 tumors compared to the CMS4 tumors. Following these results, the expression of ST6Gal1 and its product, α2,6NeuAc motifs, were analyzed in eight CRC immortalized cell lines (Caco-2, COLO-205, HT-29, SW48, SW480, SW620, SW837 and SW948) previously classified according to their respective CMS: SW48 and COLO-205 belong to the CMS1 group; SW948 belongs to the CMS2 group; HT-29 is part of the CMS3 group; SW480, SW620, SW837 and Caco-2 belong to the CMS4 group [171, 172]. Immunofluorescent labeling of the eight CRC lines showed that only HT-29 and Caco-2 cells express ST6Gal1, evidenced by the characteristic punctuated signal (Golgi-like) in the cytoplasm, coinciding with ST6Gal1 reported residence in the Golgi apparatus (Fig. 6b.). The expression of ST6Gal1 at the protein level, as well as detection of α2,6NeuAc residues by reactivity with SiaFind™ α2,6-specific lectin, were assessed by western and lectin blots, respectively (Fig. 6c.). Only the HT-29 and Caco-2 cell lines exhibited high ST6Gal1 expression, with the remaining cell lines having neglectable expression levels of the enzyme at the protein level, corroborating the immunofluorescence results. The expression of α2,6NeuAc glycans in high molecular weight proteins was detected in Caco-2 cells, in accordance with the high levels of ST6Gal1 expressed by this cell line. Interestingly, despite expressing ST6Gal1, α2,6NeuAc motifs were not detected in HT-29 cells. Cell surface α2,6-sialylation was characterized by SNA reactivity through flow cytometry (Fig. 6d.). Caco-2 demonstrated the highest relative median fluorescence intensity (rMFI), consistent with the high expression of ST6Gal1 and detection of α2,6NeuAc residues by the lectin blot. Surprisingly, despite no detection of ST6Gal1 and α2,6NeuAc motifs by immunofluorescence and western blot, SW837 cells exhibited moderate reactivity with the SNA lectin. Following the lectin blot results, HT-29 showed lack of α2,6NeuAc epitopes, despite expressing ST6Gal1, evidencing the complex regulatory network involved in the synthesis of glycoproteins. All other cell lines exhibited negative or very low MFI values, in agreement with their neglectable ST6Gal1 expression. Among all the characterized cell lines, only Caco-2 cells exhibit high expression levels of ST6Gal1 that result in a concomitant upregulation of α2,6NeuAc motifs. This particular cell line belongs to the CMS4 subtype, in which a significant expression of *ST6GAL1* was observed in tumor samples from CRC patients. Therefore, considering the expression levels of *ST6GAL1* in tumor samples belonging to the CMS4 and the immunosuppressive nature of the TME of this subtype, the Caco-2 cell line was chosen for subsequent functional assays.

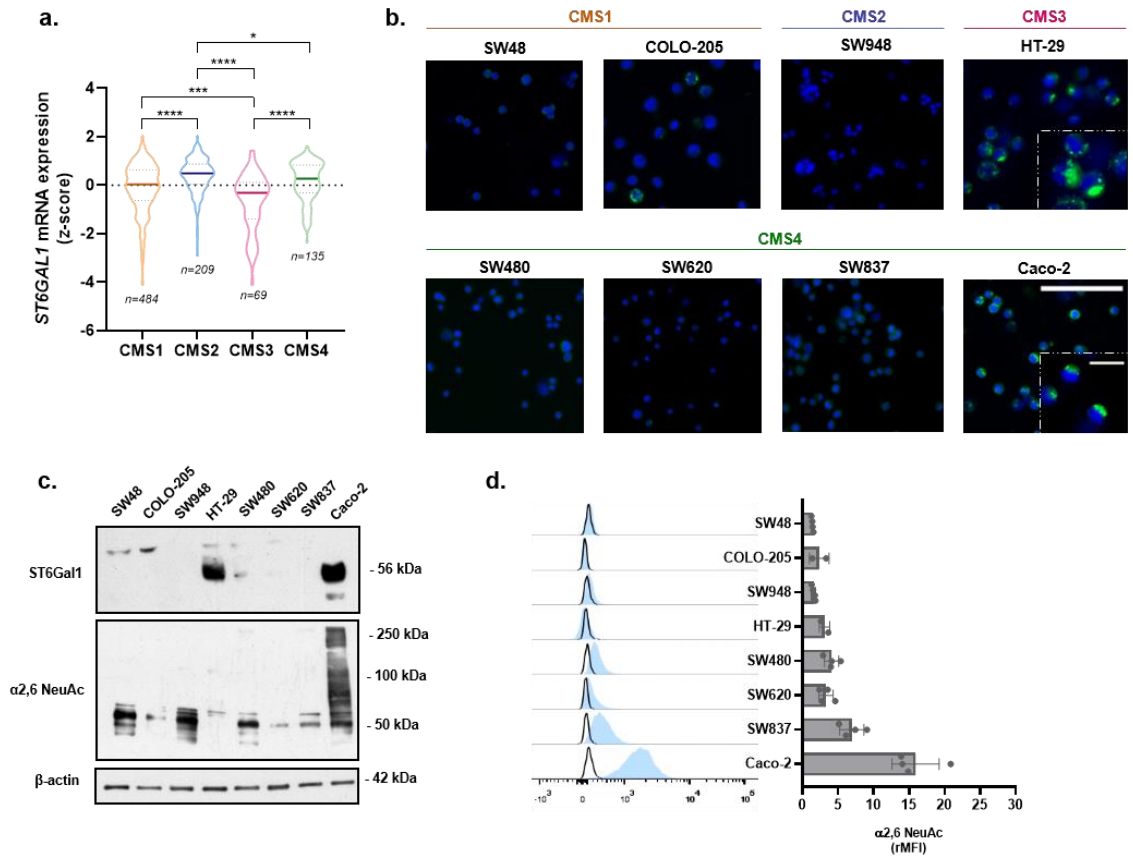

Figure 6. ST6Gal1 expression in colorectal cancer. **a.** *ST6GAL1* expression in tumor samples from CRC patients previously classified according to their consensus molecular subtype (CMS). Differences in gene expression were evaluated by Kruskall-Wallis test followed by Dunn´s multiple comparisons test. **b.** ST6Gal1 immunofluorescent labeling of CRC cells belonging to different CMS groups. Nuclei (blue) stained with DAPI. Scale bar and insert scale bar represent 100 and 25 μm, respectively. **c.** Western and lectin blots for the detection of ST6Gal1 protein levels and α2,6NeuAc motifs by reactivity with SiaFind™ α2,6-specific lectin in CRC cell lines, respectively. **d.** Flow cytometry analysis for detection of cell surface α2,6NeuAc motifs by reactivity with FITC-conjugated SNA on CRC cells. Data presented as representative flow cytometry histograms for each CRC cell line (black line represents the unstained control) and as median fluorescence intensity values relative to the respective unstained control (rMFI) (n ≥ 2, mean ± SD). **p*<0.05; ***p*<0.01; ****p*<0.001; *****p*<0.0001.

4.2. Impact of α2,6-sialylation on EV uptake by macrophages

Isolation and characterization of EVs produced by the Caco-2 *ST6GAL1* KO model (Fig. 7a) was previously performed in our group. EVs produced by Caco-2 WT cells were shown to carry ST6Gal1 and to be enriched in α2,6NeuAc glycans. In contrast, the EVs produced by the Caco-2 ST6Gal1 KO17 cells appear to mimic the phenotype of their parent cells, as ST6Gal1 and α2,6NeuAc motifs were not detected in the EVs produced by this cell line. Given the results from the EV characterization performed in our group, the immunomodulatory effect of EVs produced by the glycoengineered Caco-2 *ST6GAL1* KO model on macrophages was further evaluated.

The optimal amount of EVs for uptake assays, as well as the ideal timepoint, were determined by performing an uptake assay using human monocyte-derived macrophages isolated from healthy blood donors. Two distinct uptake assays were performed, either by an indirect co-culture system or by direct labelling of CRC cell-derived EVs.

The first assay consisted in directly labeling Caco-2 WT cells with the lipophilic fluorescent dye PKH67, for production of fluorescently labeled EVs secreted by the previously PKH67 labeled cells. An indirect co-culture transwell system was established, with different cell concentrations of PKH67-labeled Caco-2 WT cells in the upper compartment of the transwell and differentiated macrophages in the lower compartment (Fig. 7b.). Uptake of EVs by macrophages was assessed by flow cytometry as the percentage of $PKH67^{+}CD14^{+}$ macrophages (Fig. 7c.). No $PKH67^{+}CD14^{+}$ macrophages were detected after 24h of indirect co-culture (Fig. 7d.). Therefore, it was not possible to observe uptake of Caco-2 EVs by this method.

The second strategy consisted of labeling the EVs isolated from the conditioned medium of Caco-2 WT cells with PKH67. Two different amounts (2.5 µg and 5 µg) of Caco-2 WT EVs were labeled with PKH67 and macrophages were treated for 30 min, 1h or 2h. After 30 min of treatment, 30% of $PKH67^{+}CD14^{+}$ macrophages treated with 2.5 µg of labeled EVs were found, while 50% of $PKH67^{+}CD14^{+}$ macrophages treated with 5 µg of labeled EVs were identified, indicating that Caco-2 WT-derived EVs were being uptake by macrophages. Moreover, the uptake of labeled EVs increased over time, showed by the increase in the percentage of $PKH67^{+}CD14^{+}$ macrophages over the three tested timepoints. A plateau was reached after 1h of treatment, with 70% and 80% of EVs uptake being found in macrophages treated with 2.5 µg and 5 µg of labeled EVs, respectively. The percentage of $PKH67^{+}CD14^{+}$ cells was higher in macrophages treated with 5 µg of EVs in all timepoints, indicating that the uptake of Caco-2 WT-derived EVs by macrophages is time- and concentration-dependent (Fig. 7e.). To evaluate the effect of sialylation on EVs uptake by recipient cells, macrophages were treated with 2.5 µg of EVs isolated from Caco-2 WT and Caco-2 ST6Gal1 KO17 cells, during 30 min and 1h. Similar to the previous results, the percentage of $PKH67^{+}CD14^{+}$ macrophages treated with EVs isolated from both cell lines increased over time, with a higher percentage of $PKH67^{+}CD14^{+}$ events being found when macrophages were treated with Caco-2 ST6Gal1 KO17 EVs compared with macrophages treated with Caco-2 WT EVs (Fig. 7f.). These findings suggest that ST6Gal1-mediated α2,6-sialylation of EVs impairs their uptake by macrophages.

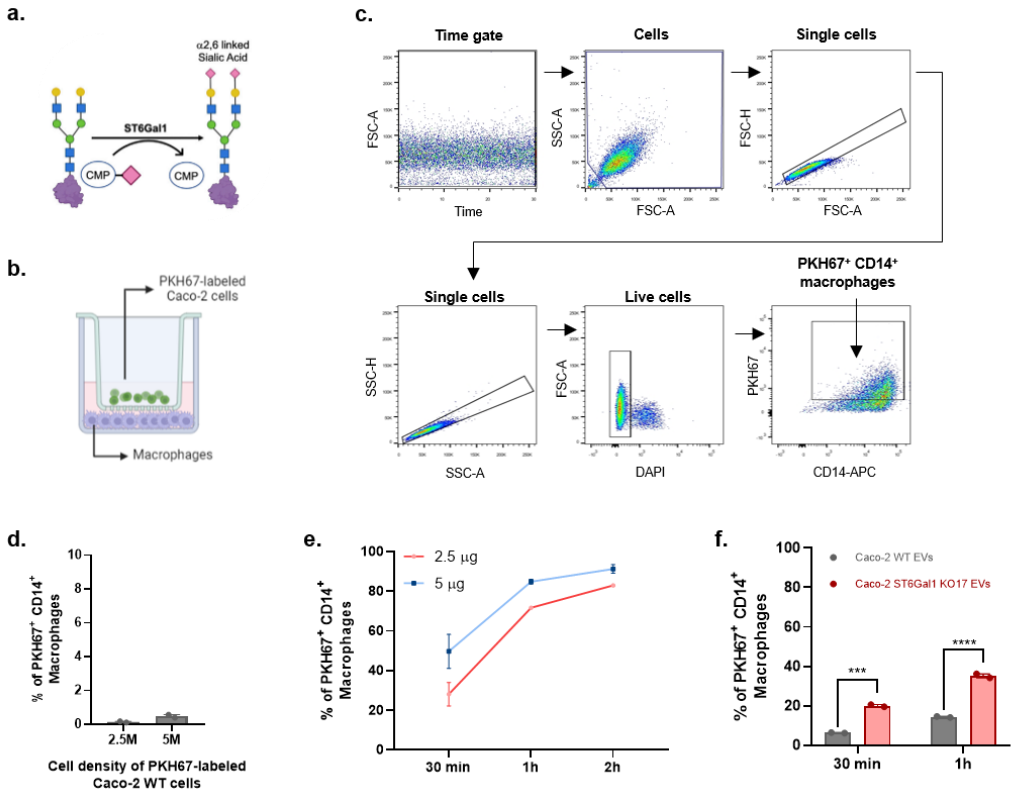

Figure 7. Caco-2 WT- and Caco-2 ST6Gal1 KO17-derived EVs from CRC cell models uptake by human monocyte-derived macrophages. **a**. Schematic representation of *N*-glycan terminal α2,6-sialylation by ST6Gal1. **b**. Schematic representation of the transwell system used for indirect co-culture. Monocyte-derived macrophages were seed in the lower compartment and PKH67-labeled Caco-2 WT cells in the upper compartment of the transwell. **c.** Flow cytometry gating strategy for uptake analysis of Caco-2 PKH67-labeled EVs by macrophages (PKH67$^+$ CD14$^+$). **d.** Percentage of PKH67$^+$ CD14$^+$ macrophages after indirect co-culture with 2.5 million (2.5M) or 5 million (5M) PKH67-labeled Caco-2 WT cells. **e.** Percentage of PKH67$^+$ CD14$^+$ macrophages treated with 2.5 or 5 µg of Caco-2 WT-derived EVs labeled with PKH67, in three timepoints (30 min, 1h and 2h). **f**. Percentage of PKH67$^+$ CD14$^+$ macrophages treated with 2.5 µg of Caco-2 WT and Caco-2 ST6Gal1 KO17 EVs labeled with PKH67 for 30 min and 1h. Data shown as mean ± SEM (*n* = 2 donors). Comparisons with multiple groups were made with two-way ANOVA, with macrophages treated with Caco-2 WT-derived EVs as control. ***p<0.001; ****p<0.0001.

4.3. Interplay between ST6Gal1-carrying EVs and macrophages

To unravel potential immunomodulatory roles of ST6Gal1 carried by CRC EVs, human monocyte-derived macrophages were treated with EVs produced by Caco-2 WT and Caco-2 ST6Gal1 KO17 CRC cell models to assess the expression of classic co-stimulatory and anti-inflammatory markers associated with macrophage polarization (Fig. 8a.). To determine the ideal timepoint to treat macrophages with Caco-2-derived EVs, monocyte-derived macrophages were incubated with 5 µg of Caco-2 WT EVs for 24h and 48h. After each timepoint, macrophages were collected and the expression of CD40, CD86, CD163, CD206, HLA-ABC and HLA-DR surface markers was assessed through flow cytometry (Fig. 8b.). For each timepoint, macrophages

stimulated with pro- or anti-inflammatory cytokines served as polarization controls (Fig. 8c., 8d.). In both timepoints, LPS and IFN-γ increased the classic co-stimulatory markers HLA-DR (MHC class II) and CD40 typically expressed by pro-inflammatory (M1-like) macrophages. Additionally, MHC class I (HLA-ABC) expression was elevated in M1 macrophages, in line with the reported upregulation of MHC class I by IFN-γ stimulation [173]. IL-4 treatment upregulated the expression of the classic anti-inflammatory CD206 marker. After 24h of treatment with Caco-2 WT EVs, no significant alterations were found in the expression of the majority of cell surface markers in treated macrophages when compared to the untreated M0 macrophages (Fig. 8e.). However, after 48h, macrophages appeared to have responded to EV treatment, indicated by the increase in CD163 and CD206 expression when compared to M0 macrophages. An increase in MHC class I (HLA-ABC) expression was also observed in one donor (Fig. 8f.). Considering these results, the 48h treatment timepoint was chosen to evaluate the effect of EVs produced by the glycoengineered Caco-2 cell line model on the expression of markers associated with macrophage polarization.

Differentiated macrophages were treated with 5 μg of EVs isolated from the Caco-2 WT and Caco-2 ST6Gal1 KO17 cells for 48h and flow cytometry analysis was performed with at least four independent donors. The same polarization controls were applied (Fig. 9a., 9b.). Stimulation of macrophages with LPS and IFN-γ induced an increase in the expression of MHC class I (HLA-ABC) and MHC class II (HLA-DR) molecules. An increase in CD40 expression was also observed. IL-4 treatment induced a significant increase in the expression of the CD206 anti-inflammatory marker compared to M0 and M1 macrophages, together with an upregulation of CD163 compared to M1 macrophages. Interestingly, IL-4 also increased CD86 expression in comparison to M0 macrophages. Despite being a co-stimulatory marker typically associated with pro-inflammatory macrophages, reports have demonstrated that IL-4 may upregulate CD86 expression in anti-inflammatory subsets of macrophages [174, 175] (Fig. 9b.). The overall results regarding the expression of co-stimulatory and anti-inflammatory markers by macrophages treated with CRC cell-derived EVs showed that ST6Gal1 does not have a relevant impact on macrophage polarization (Fig. 9c., 9d.), as no significant difference in the expression of CD40, CD86, CD163, HLA-ABC and HLA-DR markers were observed between macrophages treated with Caco-2 WT or Caco-2 ST6Gal1 KO17 EVs. Macrophage treatment with the Caco-2 cell line-derived EVs increased CD206 expression compared to untreated M0 macrophages, but no difference was observed between macrophages treated with Caco-2 WT EVs and Caco-2 ST6Gal1 KO17 EVs (Fig. 9d.). Therefore, these results suggest that there is no clear effect of ST6Gal1-carrying EVs produced by Caco-2 cells on macrophage polarization. However, macrophages treated with Caco-2 WT-derived EVs showed a lower viability than Caco-2 ST6Gal1 KO17 EV-treated macrophages, hinting that ST6Gal1 carried by CRC EVs has an impact on macrophage survival (Fig. 9e.).

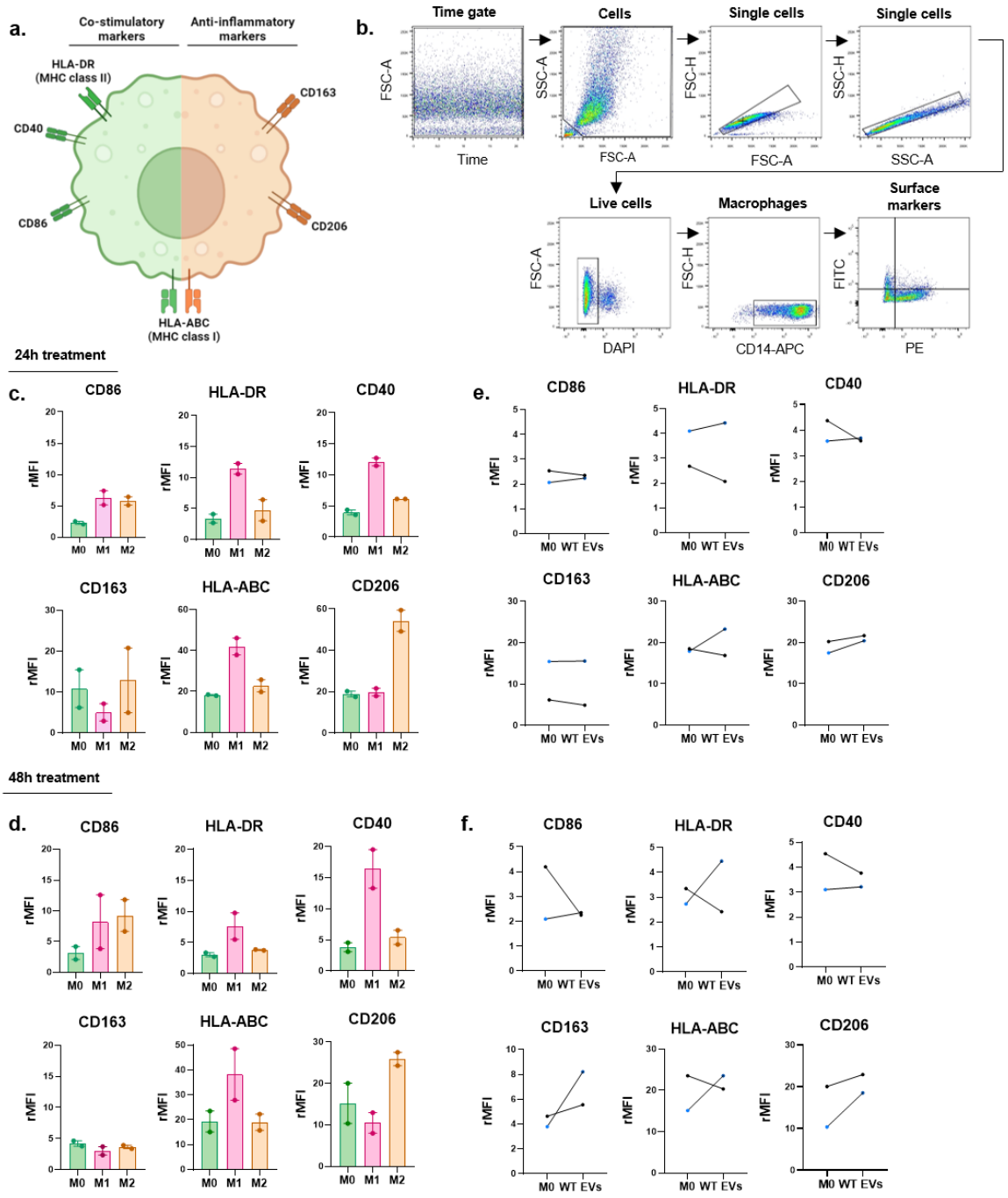

Figure 8. Macrophage polarization analysis in different timepoints after treatment with Caco-2 WT-derived EVs. **a**. Schematic representation of co-stimulatory and anti-inflammatory markers expressed by pro-inflammatory (M1) and anti-inflammatory (M2) macrophages, respectively. **b.** Flow cytometry gating strategy for the analysis of surface markers expressed by macrophages treated with 5 ug of Caco-2 WT-derived EVs for 24h or 48h. Monocyte-derived macrophages were identified as $CD14^+$ cells within the live cell population. **c.-d.** Expression of surface markers by cytokine-stimulated macrophages in the (**c.**) 24h and (**d.**) 48h treatment timepoints. Macrophages were stimulated with LPS and IFN-γ for M1 polarization or IL-4 for M2 polarization. Macrophages stimulated only with M-CSF served as control (M0). Data presented as MFI values relative to live $CD14^+$ macrophages (rMFI) (mean ± SEM; *n* = 2 donors). **e.-f.** Individual rMFI values for each surface marker expressed by macrophages after (**e.**) 24h and (**f.**) 48h treatment with 5 µg of Caco-2 WT-derived EVs compared to M0 macrophages (*n* = 2 donors; connecting lines indicate matched donors).

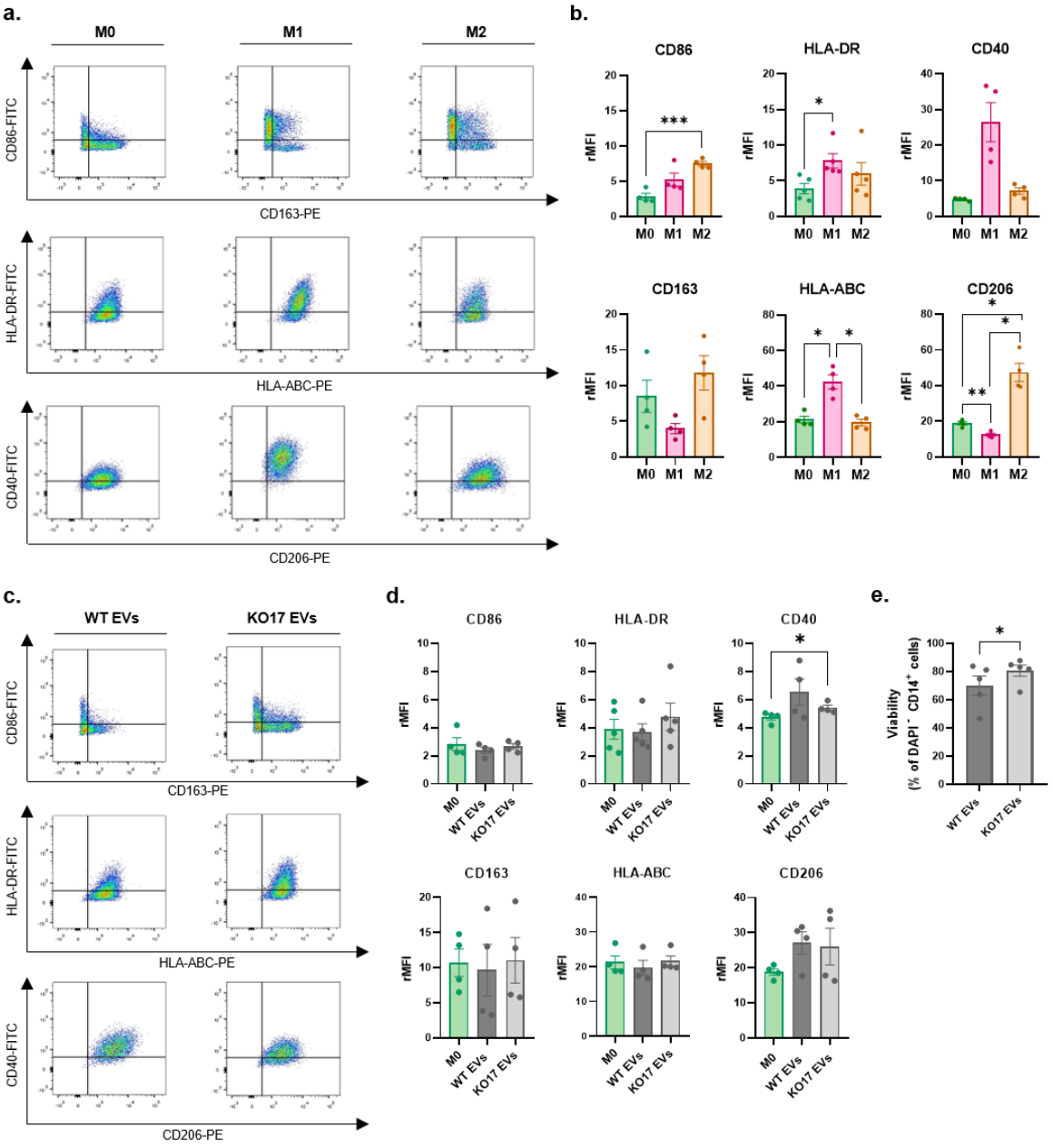

Figure 9. ST6Gal1-carrying EVs effect on macrophage polarization and survival. **a.-b.** Expression of co-stimulatory and anti-inflammatory markers by cytokine-stimulated macrophages. Macrophages were stimulated with LPS and IFN-γ for M1 polarization or IL-4 for M2 polarization. Macrophages stimulated only with M-CSF served as control (M0). Data presented as (**a.**) representative flow cytometry blots for each marker and as (**b.**) MFI values relative to live $CD14^+$ macrophages (rMFI). **c.-d.** Expression of co-stimulatory and anti-inflammatory markers by monocyte-derived macrophages after 48h treatment with 5 µg of Caco-2 WT- and Caco-2 ST6Gal1 KO17-derived EVs. M0 macrophages were used as control. Data presented as (**c.**) representative flow cytometry blots for each marker and as (**d.**) rMFI. **e.** Percentage of viable macrophages ($DAPI^-$ $CD14^+$ cells) after treatment with 5 µg of Caco-2 WT-derived EVs or Caco-2 ST6Gal1 KO17-derived EVs for 48h. Data shown as mean ± SEM of at least 4 donors. Multiple comparisons were made with one-way ANOVA for polarization assays and paired t-test for viability assessment. *p < 0.05; **p<0.01; ***p<0.01.

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5. DISCUSSION

The aberrant glycosylation patterns that arise in cancer can alter immune recognition and induce immunosuppressive signaling through glycan-binding protein receptors. These glycan signatures, or glyco-code, expressed by tumor cells can act as immune checkpoints that modulate T cell differentiation, macrophage polarization, and antigen presentation. Overexpression of sialyltransferases leads to hypersialylation of tumor cells, which dampens immune responses and favors the establishment of a tumor-supportive microenvironment [176]. In this work, we evaluated the role of ST6Gal1 carried by CRC cell-derived EVs in the modulation of human macrophages. As previously mentioned, CRC heterogeneity gives rise to unique tumors with specific gene signatures and activated signaling pathways, as well as unique intratumoral immune populations [2]. Transcriptomic-based analysis allowed the stratification of colorectal tumors into four consensus molecular subtypes (CMS) that account for the genetic and molecular features of colorectal tumors, as well as their distinct immune profiles. In our study, we analyzed the *ST6GAL1* gene expression in tumor samples from CRC patients from the TCGA public database and previously classified according to their CMS, showing that *ST6GAL1* is mostly expressed in CMS2 tumors, followed by CMS4 tumors. The “canonical” CMS2 subtype is the most frequently identified in CRC, accounting for 37% of all CRC cases. The CMS4 "mesenchymal" subtype follows the CMS2 subtype, accounting for 23% of CRC cases [2]. Our results, therefore, indicate that *ST6GAL1* is mainly expressed in the most frequent CMS of CRC, which seems to be in accordance with the reported *ST6GAL1* upregulation frequently observed in CRC samples [74, 177]. Furthermore, we found high levels of ST6Gal1 and its product, α2,6NeuAc motifs, in the CRC cell line Caco-2, which belongs to the CMS4 group. The "mesenchymal" CMS4 subtype is frequently associated with metastasis, possibly explained by the immunosuppressive TME and activation of EMT programs that, together with TGFβ secretion, favors tumor growth and dissemination [2, 178]. The immunosuppressive TME of CMS4 tumors can be a result of the infiltration of immune cells with immunosuppressive features. For example, macrophages found in CMS4 tumors have a predominantly anti-inflammatory phenotype, in contrast to the reduced frequency of pro-inflammatory macrophages found in these tumors [2]. This suggests an inherent effect associated to the TME of this subtype in educating macrophages to adopt a protumor phenotype. Interestingly, activation of EMT programs and TGFβ secretion has also been linked to ST6Gal1 overexpression in cancer, which appears to favor immune evasion and metastasis [73]. Therefore, considering the upregulation of *ST6GAL1* in CMS4 colorectal tumors, as indicated by our transcriptomic analysis and CRC cell line characterization, we hypothesized that there may be a functional role for ST6Gal1 in mediating tumor immune evasion in this particular subtype of CRC.

EVs produced by cancer cells actively shape intercellular dynamics in the TME by promoting cell growth, survival, invasion and metastasis. By secreting these mediators, cancer cells can reprogram recipient cells, such as immune cells, and promote the establishment of pre-metastatic niches [129, 143, 156]. Considering the expression of ST6Gal1 in the CMS4-type Caco-2 cell line,

we chose to study the immunomodulatory effect of EVs produced by the glycoengineered Caco-2 *ST6GAL1* KO model previously established in our group. Our results show that ST6Gal1-mediated α2,6-sialylation of EVs impairs EV uptake by macrophages, following previous studies that report an increase in the uptake efficiency of desialylated EVs by recipient cells [162]. Indeed, removal of terminal sialic acids from neuraminidase-treated EVs appeared to increase their uptake by ovarian cancer cells [179]. Since sialic acid residues are negatively charged, removal of these residues may decrease electrostatic repulsions and facilitate the uptake of EVs by recipient cells. Moreover, sialic acids present in EVs may interact with lectin receptors present on macrophages, which may delay their uptake and activate downstream signaling pathways that may affect macrophage activation and function. Recognition of linkage-specific sialic acid motifs by Siglecs expressed on immune cells activates signaling pathways that suppress immune responses [180]. For example, the sialylated CD24 glycoprotein was reported to engage with the Siglec-10 receptor expressed by macrophages and inhibit phagocytosis of breast and ovarian cancer cells [113]. EVs can be uptaken by recipient cells through phagocytosis and a link between EV internalization and Siglecs has already been reported, as Siglec-1 (CD169) expressed by macrophages was shown to mediate the capture of B cell-derived EVs in the spleen and lymph nodes in murine models [116, 181]. Considering the anti-phagocytic roles of Siglec receptors expressed by macrophages, the uptake of Caco-2 WT-derived EVs carrying α2,6NeuAc epitopes could be delayed by interactions with Siglec-expressing macrophages. The interplay of sialylated glycans with Siglecs has long been reported to modulate myeloid regulatory cells in the TME [63]. Rodríguez et al. previously reported the involvement of α2,3-sialyltransferases ST3Gal1 and ST3Gal4 in the synthesis of ligands for Siglec-7 and Siglec-9 in pancreatic ductal adenocarcinoma (PDAC) cancer. These Siglecs were found to be expressed by monocyte-derived macrophages present in the myeloid compartment of PDAC and to dictate macrophage differentiation after binding to their respective sialylated partners. Interestingly, triggering of Siglec-9 induced anti-inflammatory programs in macrophages, evidenced by upregulation of PD-L1 and the anti-inflammatory marker CD206, as well as immunosuppressive cytokines [109]. This demonstrates a possible role of sialic acids and Siglecs in modulating macrophage differentiation in the TME. However, the role of ST6Gal1 and α2,6-sialylation in macrophage polarization is yet to be disclosed. Our current results suggest that EVs carrying ST6Gal1 and α2,6NeuAc glycans do not affect macrophage polarization. However, the expression of co-stimulatory and anti-inflammatory markers seemed to be affected in few specific donors, as well as showing a great variability between donors, which is expected when handling human primary cells. Further assays are needed to ensure that ST6Gal1 does not play a role in modulating macrophage polarization.

Our results hint that α2,6-sialylated EVs affect macrophage viability, as macrophages treated with ST6Gal1-carrying EVs showed a lower viability compared to macrophages treated with EVs lacking ST6Gal1. While the α2,6NeuAc motifs carried by EVs may interact with receptors expressed on the cell surface of macrophages, EVs can also be internalized and release ST6Gal1 to sialylate intracellular targets. Zhang et al. reported that ST6Gal1 carried in exomeres, a subfraction of EVs, is transferred to recipient cells and it is able to efficiently sialylate β1-integrins

[167]. The α2,6-sialylation of β1-integrins has been reported to enhance migration and invasion of cancer cells, contributing to metastasis [88, 182]. As such, ST6Gal1 transferred to recipient cells via EVs seems to induce phenotypic alterations via hypersialylation of cell surface receptors. Nonetheless, our results seem to be in agreement with the reported role of ST6Gal1 in modulating survival and apoptotic pathways [73]. For example, ST6Gal1-mediated α2,6-sialylation of TNFR1 has been shown to prevent receptor internalization and activation of apoptosis in cancer cells [81]. The α2,6-sialylation of TNFR1 has also been reported to regulate apoptosis and inflammatory signaling in macrophages [82, 183]. Additionally, it has been shown that extracellular ST6Gal1 is capable of sialylate the M-CSF receptor of macrophages, resulting in its activation and regulation of macrophage differentiation and survival [184]. Most results reported so far seem to indicate that ST6Gal1 has a protective role, either by preventing apoptosis through sialylation of apoptotic receptors or by regulating mediators involved in survival pathways. However, Wang et al. demonstrated that $CD8^+$ T cells co-cultured with ST6Gal1-overexpressing hepatocarcinoma cells exhibited higher apoptotic rates [105]. This seems to indicate a complex modulatory role for ST6Gal1 in survival and apoptotic pathways that, depending on the context, may promote survival or induce cell death. While we hypothesize that ST6Gal1 carried in EVs is released and sialylates intracellular targets involved in apoptotic pathways, the interaction between sialic acids present in EVs and receptors expressed by macrophages, such as Siglecs, cannot be overlooked. Activation of Siglecs by interaction with its ligands can also induce apoptotic signaling [180]. For example, during the resolution phase of inflammation, Siglec-9 has been reported to induce neutrophil apoptosis [185]. Also, CD24-mediated signaling through Siglec-10 expressed on B cells is thought to promote B cell apoptosis by recruiting signal transduction mediators to glycolipid-enriched microdomains (GEMs), which results in the upregulation of caspases and other pro-apoptotic proteins [186]. Siglec-2 (CD22) has also been shown to induce B cell apoptosis [187, 188]. Interestingly, CD22 binds α2,6NeuAc motifs, while ST6Gal1 has been shown to negatively regulate B cell activation by modulating the colocalization of CD22 with BCR in lipid rafts [111]. As such, we believe that macrophage viability after treatment with Caco-2 WT-derived EVs is being modulated by a dynamic network that involves ST6Gal1-mediated sialylation of signaling mediators and the interaction of sialic acids present in EVs with receptors expressed on the cell surface of macrophages. Therefore, the induction of macrophage cell death could be possible a mechanism of immune evasion mediated by the secretion of ST6Gal1-carrying extracellular vesicles by CRC cells.

Although we are considering a functional interaction between ST6Gal1 carried in cancer EVs with intracellular targets in macrophages, the role of glycosylation in the biological roles of EVs must not be overlooked. Cells release heterogeneous populations of EVs with distinct biological and biophysical roles. In cancer, heterogeneous EV populations are thought to have unique biological roles that support tumor development and progression [156, 189]. Zhang et al. demonstrated that both membrane-bound and soluble forms of ST6Gal1 are only found in a subset of CRC cell-derived exosomes enriched in CD81 and the EGFR ligand, amphiregulin (AREG) [167]. Besides, ST6Gal1-mediated α2,6-sialylation of EGFR has been shown to activate

its tyrosine kinase activity and promote resistance to the cytotoxic effect of Cetuximab mAb [83, 167]. Additionally, Jung et al. also demonstrated that ST6Gal1 appears to have a role in controlling EV protein sorting in CRC cells [166]. Therefore, these findings seem to indicate that ST6Gal1 may be enriched in specific subpopulations of EVs and regulates protein sorting mechanisms. In our study, Caco-2-derived EVs were previously characterized to assess the presence of ST6Gal1 and α2,6NeuAc motifs. However, the overall cargo present in these EVs and the impact of ST6Gal1 abrogation on EV protein sorting in these cells, which may affect macrophage response to EV treatment, remains to be elucidated. Additionally, Rodrigues et al. reported that ST6Gal1 abrogation shifts cell surface glycosylation patterns by altering *N*-glycan terminal motifs, as seen by the increased α1,2-linked fucoses in Caco-2 ST6Gal1 KO cell clones [83]. This shift in the glycosylation profile of Caco-2 ST6Gal1 KO cells may also be reflected in the EVs produced by these cells and consequently alter their recognition by GBP receptors, possibly inducing a different response on macrophages.

The notion of EV-mediated intercellular communication mechanisms offers new insights for novel designs of therapeutic strategies to treat metastatic cancers. The ability of EVs to modulate the immune system can also be exploited for immunotherapies as anti-cancer vaccines. In the case of macrophages, such strategy could be employed to reprogram macrophage polarization in the TME and induce a pro-inflammatory and anti-tumor phenotype. Glycosylated EVs are also being explored as therapeutic agents for cancer treatment and efforts are being conducted to isolate and manipulate the heavily glycosylated surface of EVs [156]. For example, modification of EVs with high mannose-type glycans increased the uptake of EVs by dendritic cells through DC-SIGN and enhanced $CD8^+$ T cell responses against melanoma cells [190]. The desialylation and insertion of palmitoyl-LeY in EVs produced by glioblastoma cells enhanced their uptake by dendritic cells and upregulated $CD4^+$ and $CD8^+$ T cells responses [191]. Considering our results, a possible therapeutic strategy could involve EV treatment with neuraminidase to remove sialylated structures and block signaling mediated by inhibitory receptors, thus modulating the activation status of macrophages in a therapeutic context. However, it is necessary to consider that manipulation of EV glycosylation may affect their *in vivo* biodistribution. For example, Royo et al. demonstrated that removal of sialic acids in EVs by treatment with neuraminidase altered the biodistribution of EVs in mice, observing a greater accumulation of vesicles in lung tissues and axillary lymph nodes [162]. Furthermore, while considering the interaction of sialic acids with receptors on macrophages, EVs can also transfer ST6Gal1 to recipient cells. Therefore, it is also necessary to delineate a strategy to prevent ST6Gal1-mediated sialylation in cancer. Although sialyltransferase inhibitors have been synthetized, it cannot be ignored that ST6Gal1 is also found in stem cell niches and may have a physiological role that, if altered, could generate undesirable off target effects [76, 79, 192].

In conclusion, our results suggest that the uptake and survival of macrophages might be affected by the glycosylation profile of CRC cell-derived EVs, possible underlying a mechanism for tumor immune evasion and cancer progression mediated by EVs. Thus, the modification of

cancer EVs glycosylation could be a possible therapeutic strategy to modulate patient's immune system and elicit antitumor responses to prevent cancer progression and metastasis.

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6. CONCLUSION AND FUTURE PERSPECTIVES

The main goal of the present work was to evaluate the functional effect of ST6Gal1 carried by CRC-derived EVs in the modulation of macrophage function. For this purpose, transcriptomic and molecular analysis were performed to characterize the expression of ST6Gal1 in CRC and its impact in immunosuppression and metastasis. Primary human monocyte-derived macrophages were treated with EVs isolated from the Caco-2 WT cell line and from the glycoengineered Caco-2 *ST6GAL1* KO cell model. While no relevant effect was observed on macrophage polarization, we report that ST6Gal1-mediated α2,6-sialylation has an impact on the uptake of EVs by macrophages. Additionally, we found that ST6Gal1-carrying EVs negatively affect macrophage viability. Although it remains unclear the mechanism by which ST6Gal1-carrying EVs induce macrophage cell death, we hypothesize the involvement of a complex and dynamic signaling network regulated by ST6Gal1-mediated α2,6-sialylation, but also by interactions between α2,6NeuAc residues present at the surface of EVs and immunomodulatory receptors expressed by macrophages. Therefore, the secretion of ST6Gal1- and α2,6NeuAc-carrying EVs may be a possible mechanism employed by CRC cells to evade macrophage recognition and promote tumor immune evasion. Nonetheless, to further confirm and validate these results, more detailed cell death assays should be performed to measure apoptosis on macrophages treated with CRC cell-derived EVs. Additionally, the main targets of ST6Gal1-mediated sialylation remain unknown. Therefore, it would be interesting to analyze the sialylation profile of apoptotic receptors, such as FasR and TNFR1, in macrophages treated with CRC cell-derived EVs. The involvement of immunosuppressive sialic acid receptors in the uptake of EVs and macrophage survival should also be addressed in the future. Altogether, our findings show an impact of ST6Gal1-carrying EVs in the biological functions of macrophages and further support the crucial role of glycans in the development of cancer and antitumor immunity. Moreover, modification of EV glycosylation could be a future approach for the development of effective anti-cancer vaccines. In fact, considering the role of glycosylation in the biological properties of EVs, the whole cargo of CRC cell-derived EVs needs to be assessed in order to comprehend whether ST6Gal1 controls EV protein sorting mechanisms. Glycoproteomic analysis should be performed to characterize the EV glycome and assess differences in the glycan repertoire of EVs produced by glycoengineered CRC cell lines. Finally, while this study was carried out with EVs isolated from a specific cell model, it would also be interesting to perform ST6Gal1 knockout or overexpression in other CRC cell lines belonging to different CMS to further address the role of the oncogenic ST6Gal1 in CRC malignancy. Additionally, ST6Gal1 is not only involved in CRC carcinogenesis, as its oncogenic role has already been reported in other malignancies, such as gastric, lung and breast cancers. In this way, we could extend this work and address the immunomodulatory properties of ST6Gal1-carrying EVs produced by cell lines from different cancer models.

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