

DOUTORAMENTO
CIÊNCIAS BIOMÉDICAS

Glycans as novel immunomodulators in Colorectal cancer: an opportunity for innovative immunotherapeutic strategies

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D

2023







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TESE DE DOUTORAMENTO APRESENTADA AO INSTITUTO DE CIÊNCIAS
BIOMÉDICAS ABEL SALAZAR

DOUTORAMENTO EM CIÊNCIAS BIOMÉDICAS

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**GLYCANS AS NOVEL IMMUNOMODULATORS IN COLORECTAL
CANCER: AN OPPORTUNITY FOR INNOVATIVE
IMMUNOTHERAPEUTIC STRATEGIES**

Tese de Candidatura ao grau de Doutor em
Ciências Biomédicas;
Programa Doutoral da Universidade do Porto
(Instituto de Ciências Biomédicas de Abel
Salazar)

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Financiamento

Bolsa de doutoramento (SFRH/BD/136388/2018) da Fundação Portuguesa para a Ciência e Tecnologia (FCT) do Ministério da Ciência, Tecnologia e Ensino Superior.



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“The important thing is not to stop questioning. Curiosity has its own reason for existing.”

- Albert Einstein

Agradecimentos

O meu percurso e carreira como investigadora nasceram e cresceram neste grupo, neste instituto e com estas pessoas. O cancro e imunologia, com o toque doce da glicobiologia, foram a minha paixão ao longo destes últimos anos, e hoje sinto que não podia ter escolhido melhor tema para desenvolver o meu doutoramento. Foram anos felizes, de altos e baixos, que vou guardar comigo como uns dos melhores, certamente. Hoje concluo esta etapa com a sensação de missão cumprida, mas com um sentimento agri-doce de despedida. Quero por isso agradecer a todas as pessoas que tornaram isto possível:

À minha orientadora, Professora Doutora Salomé Pinho, que me recebeu em 2017 aquando da minha tese de mestrado e me “acolheu” até hoje. Obrigada por ter apostado em mim e me incentivar a crescer e a ser sempre melhor. Agradeço por ter acreditado, muitas vezes mais que eu própria, e me fazer acreditar no projeto e no meu trabalho. Vou guardar com carinho as nossas conversas e discussões científicas. Cresci muito consigo, mas também vi a Salomé crescer e levar o grupo a níveis de excelência que não imaginei no princípio. É para mim um grande orgulho ter feito parte desse crescimento e agradeço essa oportunidade.

Ao Professor Doutor Noel de Miranda, meu co-orientador, que me acolheu no seu grupo como se sempre fizesse parte dele. Que disponibilizou todos os recursos e o seu tempo para que o meu trabalho fosse feito. Obrigada pelos brainstormings e discussão de um projeto ambicioso e embrionário, e por toda a disponibilidade que demonstrou desde o início.

Ao Professor Doutor António Araújo, por aceitar ser meu co-orientador e toda a disponibilidade que sempre demonstrou.

Ao Professor Doutor Eduardo Rocha agradeço a disponibilidade e apoio enquanto diretor do Doutoramento em Ciências Biomédicas. À Helena Martins agradeço a disponibilidade em esclarecer todas as dúvidas e paciência em resolver todos os problemas, como membro dos serviços académicos do ICBAS.

À Ângela, o meu braço direito, a minha companheira. Foram tantas as experiências e as horas que passamos juntas! Sem o teu apoio, laboratorial e emocional, nada disto teria sido possível. Obrigada de coração por tudo que fizeste por mim e pelo que conquistamos juntas! À Inês, a pessoa que esteve sempre ao meu lado, literalmente, e que acompanhou este percurso desde o primeiro dia. Obrigada pela amizade que vai muito para além disto,

pelas conversas ao café, pelas discussões científicas e pela tua preocupação. À Edu, que foi a minha primeira aluna “não oficial”, e foi tão importante para o meu crescimento. Foram muitas noites juntas, horas intermináveis no biotério e no citómetro. Obrigada pelo carinho e amizade, obrigada por teres voltado! Ao Manu, pelos conselhos científicos, pela paciência que sempre tiveste comigo e pela amizade. À Márcia, por animar os meus dias, e por me mostrar que acima de tudo o importante é ser feliz e sentirmo-nos realizados. Ao Guilherme, o primeiro aluno “oficial”, pelo desafio e aprendizagem pessoal e profissional. À Bia, pela preocupação constante e por estar sempre pronta a ajudar. À Ana Dias, pelo exemplo de dedicação e coragem e por todos os ensinamentos. À Joana, pela disponibilidade e discussão científica. À Catarina, pela importante adição à “cancer team”. À Cláudia, por ser um exemplo de organização e foco. À Vanda, que foi e voltou, por todo o apoio de lab manager e paciência. À Carolina, pela dedicação à causa e a boa disposição. À Rafaela e ao Pedro e a todos os alunos que passaram pelo grupo, por me lembrarem a paixão pela ciência. À Ana Campar, Rita Amorim e Iago, por todo o input clínico. A todos os que por cá passaram e de alguma forma contribuíram para este trabalho.

Aos membros e ex-membros grupo *Glycobiology in Cancer*, por todos os bons momentos que passamos quando partilhávamos o laboratório. Em especial ao Henrique, Francisca, Joana, Rita e Juliana, pelos momentos de diversão para além do trabalho. Ao Doutor Celso Reis, por me ter recebido no grupo e pelos conhecimentos em glicobiologia.

To the Cancer Immunogenomics group, for the warm reception during my internship and for always integrating me in different activities. Especially to Joana, for her patience and help in solving all the problems, and to Dina, for all her help with my work.

A todos os colaboradores internos no i3S, Professor Doutor José Carlos Machado, Professor Doutor José Bessa, Professor Doutor Manuel Vilanova, Helena Xavier-Ferreira e em especial ao Doutor Carlos Resende por todo o trabalho e colaboração.

À Alexandra Correia, que mais que uma colaboração, orientou o meu percurso inicial e me ensinou tanto sobre imunologia.

A todos os colaboradores IPO do Porto, ao Professor Doutor Rui Henrique, Doutora Carina Pereira e Doutor Mário Dinis-Ribeiro. A todos os colaboradores do Centro Hospitalar Universitário de Santo António, em especial ao serviço de Gastroenterologia, ao Professor Doutor Ricardo Marcos-Pinto, Doutora Isabel Pedroto, à Doutora Paula Lago, e ao serviço de Hematologia, à Professora Doutora Margarida Lima e à Magda. Ainda um importante agradecimento à Dr. Joana Alves pela caracterização das amostras dos modelos animais.

À Professora Doutora Fátima Carneiro. Obrigada a todos pela vossa relevante colaboração e o vosso interesse e motivação por desenvolver investigação clínica.

To our external collaborators, Professor Bernd Lepenies, Professor Harry Sokol and Julio de Freitas-Junior. From *Hospital Clínico San Carlos*, Dr. Maria J. Fernandez-Aceñero and Professor Carlos Taxonera. Thank you all for your interest and collaboration.

Às minhas amigas mais próximas, Clara, Carla e Maguie, por todo o carinho e compreensão, por mesmo sem perceber bem o que isto é, me darem todo o apoio que precisei. Às amigas da faculdade que ficaram e ficarão, Celina, Alexandra, Mafalda e Inês, pelos jantares agora mais frequentes, pelas partilhas, conversas e desabafos. Ao Nuno Padrão, meu companheiro no estrangeiro, pela amizade genuína que perdura desde a faculdade, por toda a preocupação e disponibilidade, pelos fins de semana em Amesterdão e Leiden. À Diana, Jojo, Cláudio e Rita, o grupinho do costume, por todos os jantares, saídas e viagens, pela preocupação e apoio na minha luta. A todos aqueles amigos que de alguma forma fizeram parte e marcaram este percurso.

Ao Vitor, meu porto de abrigo. A tua paciência e preocupação foram incansáveis. Tiveste um papel tão, mas tão importante, que nem sei como expressar a minha gratidão. Foram períodos atribulados para os dois, e fomos o ombro um do outro em todos os momentos. A nossa vida “recomeça” agora, e estou muito grata por puder partilhar tudo isto contigo. Por todo o amor, compreensão, incentivo, força, e tantas outras coisas boas, obrigada!

À minha família, o meu pilar! Ao meu pai e à minha mãe, pelo apoio incondicional, pelo amor e preocupação. Pelo exemplo de vida, por terem sempre a palavra certa e me incentivarem a ser sempre melhor. Ao meu irmão, que sempre me quis bem e feliz. Ao meu avô, por todo o cuidado e preocupação pelo meu bem-estar e pela minha felicidade. Obrigada a toda a minha família por estarem sempre lá independentemente de tudo.

Resumo

O cancro colorretal (CRC) é um dos cancros mais frequentes e mortais em todo o mundo. O desenvolvimento do CRC é caracterizado por alterações na glicosilação. A glicosilação é um processo pós-traducional presente em praticamente todas as células, incluindo células tumorais e células imunitárias. Na verdade, os glicanos estão envolvidos em várias etapas fisiopatológicas da progressão tumoral, como proliferação celular, invasão, metastização e angiogénese. Além disso, a resposta imunitária também é mediada pela glicosilação, nomeadamente na ativação e função das células T, bem como o reconhecimento, captação e apresentação de antígenos. Por outro lado, as células tumorais desenvolvem vários mecanismos para escapar à resposta imunitária anti-tumoral, como a expressão de *checkpoints* imunitários (PD-L1), a diminuição da expressão do MCH classe I e a perda de antígenos imunogénicos. No entanto, a importância que a glicosilação anormal do tumor tem no escape à resposta imunitária ainda é pouco compreendida.

A presente tese teve como objetivo investigar o papel da glicosilação na resposta imunitária no CRC, a fim de melhorar a eficácia da resposta imunitária anti-tumoral e o design de imunoterapias contra o cancro mais eficientes.

Os resultados desta tese demonstraram que as células tumorais de CRC estão cobertas com *N*-glicanos β 1,6-GlcNAc ramificados, que parecem prejudicar a resposta imunitária anti-tumoral, inibindo o reconhecimento imunitário e a produção de interferão-gama (IFN- γ), promovendo assim a criação de redes imunossupressoras. Além disso, a remoção de *N*-glicanos ramificados complexos leva à exposição de glicanos de manose imunogénicos, que potenciam o reconhecimento imunitário e estimulam uma resposta imunitária anti-tumoral eficaz.

Posteriormente, explorámos ainda o potencial de antígenos glicosilados na modulação da resposta imunitária, especificamente em pacientes com CRC que não respondem a imunoterapias convencionais. Descobrimos que a perda de sítios de *N*-glicosilação em possíveis neoantígenos está associada ao estadió III do CRC e a amostras de CRC com deficiência em *mismatch repair* genes (MSI), destacando a relevância dos glico-antígenos na resposta imunitária anti-tumoral. Além disso, ensaios *in vitro* com células de pacientes com CRC, que apresentam deficiência na expressão de HLA, com células *natural killer* (NK) demonstraram que a remoção de *N*-glicanos complexos ramificados promove a morte tumoral mediada por células NK. Estes achados sugerem o importante papel dos glicanos como um *checkpoint* imunitário chave no CRC, destacando a necessidade de incluir os glicanos na imunoterapia do CRC.

Estudos anteriores do nosso grupo demonstraram que as alterações no perfil de glicosilação das células T modulam a sua atividade e função. Mostraram ainda um defeito na glicosilação das células T em pacientes com doença inflamatória do intestino (IBD). Nesta tese, exploramos como as alterações na glicosilação das células T estão associadas à progressão da IBD para cancro associado à colite (CAC). Descobrimos que ocorrem alterações em *N*-glicanos ramificados em células T durante o CAC, utilizando um modelo de ratinho com glicosilação modulada. Além disso, encontramos uma associação entre os níveis de glicanos complexos em células estromais/inflamatórias e o risco de desenvolver CAC. Estes achados destacam o potencial da glicosilação como um novo biomarcador e um alvo terapêutico para o CAC.

Em geral, estes estudos demonstraram o papel crítico da glicosilação na progressão do cancro e na evasão imunológica e sugeriram que a modulação da glicosilação poderia ser uma estratégia para a terapia do cancro. No entanto, são necessários mais estudos para elucidar os mecanismos de glicosilação na imunologia do cancro e para desenvolver terapias direcionadas à glicosilação eficazes para o tratamento do cancro.

Abstract

Colorectal cancer (CRC) is one of the most frequent and deadly cancers worldwide. CRC development is characterized by remarkable changes in glycosylation. The glycosylation is a post-translational mechanism, present in essentially all cells, including tumor cells and immune cells. Indeed, glycans are involved in several pathophysiological steps of tumor progression, such as cell proliferation, invasion, metastasis, and angiogenesis. Moreover, immune response is also mediated by glycosylation, for example T cell activation and function, as well as antigen recognition, uptake, and presentation. On the other hand, tumor cells develop several mechanisms to escape to anti-tumor immune response, by expressing of immune checkpoints (PD-L1), down expressing of MCH class I and losing of immunogenic antigens. However, the importance of abnormal glycosylation in immune escape is still poorly understood.

This thesis aimed to investigate the role of glycosylation in CRC immune response, in order to improve the efficacy of anti-tumor immune response and the design of more effective cancer immunotherapies.

The results from this thesis demonstrated that colorectal cancer cells are covered with a dense coat of β 1,6-GlcNAc-branched *N*-glycans, which appears to impair an effective anti-tumor immune response by inhibiting immune recognition and production of interferon-gamma (IFN- γ), thus promoting the creatin of immunosuppressive networks. Moreover, the removal of complex branched *N*-glycans was able to expose immunogenic mannose glycans, which potentiate immune recognition and stimulate an effective anti-tumor immune response.

Afterwards, we further explored the potential of glycosylated antigens in the modulation of immune response, specifically in CRC patients that do not respond to conventional immunotherapies. We found that the loss of glycosylation sites in putative neoantigens is associated with stage III CRC and microsatellite instable (MSI) CRC samples, highlighting the relevance of glycoantigens in anti-tumor immune response, a topic that should be further explored. In addition, *in vitro* assays with CRC cells, from HLA negative tumors, with NK cells demonstrated that the removal of complex branched *N*-glycans leads to a more effective NK cell-mediated tumor killing. These findings suggest the important role of glycans as key immune checkpoint in CRC, bringing to light the need to include glycans in CRC immunotherapy.

Previous studies from our group demonstrated that changes in the glycosylation profile of T cells module their activity and function. They also showed a defect in T cell glycosylation in inflammatory bowel disease (IBD) patients. In this thesis, we evaluated whether changes in T cell glycosylation associate with progression from IBD to colitis-associated cancer

(CAC). We found that changes in branched N-glycans on T cells occur along CAC, using a glycoengineered mouse model. Moreover, we found an association between levels of complex glycans in stromal/immune cells and the risk to develop CAC. These findings highlight the potential of glycosylation as a novel biomarker and a therapeutic target for CAC.

Overall, these studies demonstrated the critical role of glycosylation in cancer progression and immune evasion and suggested that targeting glycosylation could be a potential strategy for cancer therapy. Further research is needed to elucidate the mechanisms of glycosylation in cancer immunology and to develop effective glycosylation-targeted therapies for cancer treatment.

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Chapter 2:

Mariana C. Silva*, Ângela Fernandes*, Maria Oliveira, Carlos Resende, Alexandra Correia *et al*; ***Glycans as immune checkpoints: removal of branched N-glycans enhances immune recognition preventing cancer progression***. Cancer Immunology Research 2020. doi: 10.1158/2326-6066.CIR-20-0264. *These authors contributed equally to this article

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Charter 5:

Ângela Fernandes, Catarina M. Azevedo, Mariana C. Silva, Guilherme Faria, Carolina S. Dantas, Manuel M. Vicente, Salomé S. Pinho. ***Glycans as shapers of tumour microenvironment: A sweet driver of T-cell-mediated anti-tumour immune response***. Immunology 2022. doi: 10.1111/imm.13494

- This article is also included as an appendix in Manuel M. Vicente's PhD thesis 2023 untitled "*Dynamics and functions of N-glycans in T cell development*" submitted at ICBAS, University of Porto.

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Abbreviations

AOM	Azoxymethane
APCs	Antigen-presenting cells
CAC	Colitis-associated colorectal cancer
CAR-T	Chimeric antigen receptor T
CIMP	CpG island methylator phenotype
CIN	Chromosomal instability
CLRs	C-type lectin receptors
CMS	Consensus molecular subtypes
CRC	Colorectal Cancer
CTLA-4	Cytotoxic T lymphocyte antigen 4
CTLs	Cytotoxic T Lymphocytes
DC-SIGN	DC-specific intercellular adhesion molecule-3-grabbing non-integrin
DCs	Dendritic cells
DSS	Dextran sulfate sodium
ECM	Extracellular matrix
EMT	Epithelial-mesenchymal transition
EpCAM	Epithelial cell adhesion molecule
Gal-	Galectin
GBPs	Glycan-binding proteins
GlcNAc	N-acetylglucosamine
GNA	<i>Galanthus Nivalis Lectin</i>
GnT-	N-acetylglycosaminyltransferase
HLA	Human Leukocyte Antigen
IBD	Inflammatory bowel disease
ICIs	Immune checkpoint inhibitors
IFN-	Interferon
IL-	Interleukin
Le	Lewis
Man	Mannose
MGL	Macrophage galactose lectin
MHC	Major histocompatibility complex
MMR	Mismatch repair
MoDCs	Monocyte-derived dendritic cells
MR	Mannose Receptor
MSI	Microsatellite instable

MSS	Microsatellite stable
MUC-	Mucin
N-glyco-site	N-glycosylation site
NK	Natural killer
PD-1	Programmed cell death 1
PD-L1	Programmed cell death ligand 1
PHA	<i>Phaseolus vulgaris haemagglutinin</i>
Siglecs	Sialic acid-binding immunoglobulin-like lectins
SNA	<i>Sambucus Nigra Lectin</i>
TAA	Tumor -associated antigens
TAM	Tumor-associated macrophages
TCR	T cell receptor
TGF- β	Transforming growth factor β
Th	T helper
TIL	Tumor-infiltrating lymphocytes
TIM-3	T-cell immunoglobulin and mucin domain-3
TMB	Tumor mutation burden
TME	Tumor microenvironment
TMN	Tumor-node-metastasis
TNF-	Tumor necrosis factor
Treg	Regulatory T cell

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Chapter 1

General Introduction

Aims

General Introduction

1. Cancer

Cancer is a global problem with approximately 19.3 million new cases and almost 10 million cancer-related deaths worldwide in 2020 [1]. It is estimated that by 2040, the global cancer burden will increase to more than 28 million cases, representing a 47% rise [1]. These statistics highlight the importance of scientific research for improving cancer diagnosis and prognosis, as well as for the discovery of novel and more efficient treatments.

Cancer is a very complex disease, characterized by a coordinated process of several molecular and genetic alterations. In 2000, Hanahan and Weinberg propose the “hallmarks of cancer” for the first time, which represent 6 essential and common features for cancer development [2]. In 2011 a new update was published, adding 4 new hallmarks [3]. The most recent information dates from January 2022, including a total of 16 hallmarks for cancer development (**Figure 1**) [4]. This simple scheme describes the main processes involved in malignant transformation, such as evading growth suppressors, avoiding immune destruction, activating invasion and metastasis, genome instability and mutations, resisting cell death, sustaining proliferative signaling, among others.

This thesis is focused on colorectal cancer, one of the most prevalent cancers worldwide.

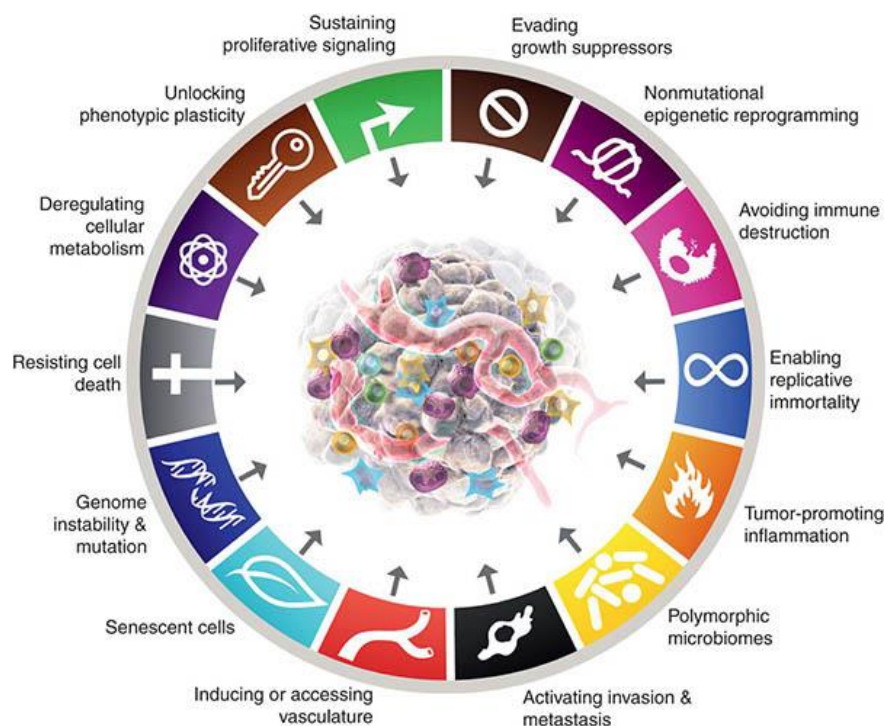


Figure 1 Hallmarks of Cancer: New Dimensions. Adapted from [4]

1.1. Colorectal Cancer

1.1.1. Epidemiology

Colorectal cancer (CRC) comprises, by definition, the colon, rectum and anus cancers. CRC is one of the most prevalent cancers, ranking third in incidence and second in mortality worldwide [1]. In 2020, there were more than 1.9 million new cases of CRC, with males being predominantly affected. Certain regions in Europe were found to have the highest incidence of CRC, according to the ranking. In fact, developed countries have a 4-fold higher incidence rate compared to developing countries [5].

Portugal is among the 10 countries with the highest incidence of CRC, with an age-standardized incidence rate incidence of 39.4 per 100 000. CRC is the most frequent type of cancer in both sexes in Portugal, with 10 501 new cases reported in 2020 [6].

1.1.2. Risk factors, screening and treatment

Countries with a high human development index have a higher incidence of CRC [5]. These geographic disparities can be explained by the fact that the risk of development CRC is influenced by environmental factors, such as smoking, alcohol, processed and red meat consumption, and obesity, which are associated with a higher socioeconomical level [5]. Moreover, patients with inflammatory bowel disease (IBD) and patients with type 2 diabetes *mellitus* present a higher risk of developing CRC [7, 8].

On the other hand, these countries have superior and well-established screening programs for the prevention and diagnosis of CRC. Colonoscopy and stool-based tests are the most commonly used methods to detect the presence of polyps or cancer. The guidelines for CRC screening recommend that individuals after the age of 50 with an intermediate risk and no symptoms should undergo fecal occult blood tests every year and colonoscopies every 5 years. During imaging screening colonoscopy, the polyps are removed, biopsies are collected, and histopathological analysis is performed to confirm the diagnosis. Definitely, appropriate screening is associated with a reduction in CRC incidence and mortality [9, 10].

Surgery is the most common treatment for CRC. In some cases, chemotherapy and/or radiotherapy need to be administered to completely eliminate of CRC. Colectomy is rare in CRC treatment, but it is more frequent in rectum cancer [11]. Moreover, immunotherapy is used to treat people with advanced disease, which will be described in detail in section 2.3.

1.1.3. Pathogenesis

Within CRC, only 30% of cases have a familial component and a genetic basis, with the majority of CRC cases occurring sporadically. [12]. Sporadic CRC develops through the progressive accumulation of several genetic and epigenetic alterations, which can be divided into three major genetic pathways: 1) Chromosomal instability (CIN), 2) Microsatellite instability (MSI), and 3) Epigenetic instability (or CpG island methylator phenotype; CIMP) [13]. The neoplastic transformation often develops through an adenoma-carcinoma cascade, driven by the CIN or/and the MSI pathway [14]. The cascade starts with the arising of a small polyp with an aberrant crypt that progresses into an early adenoma (<1cm in size). The tumor mass then grows to high grade dysplasia, called a large adenoma (>1cm in size), before becoming a carcinoma [11]. However, sporadic CRC could also be derived from the serrated polyp pathway, which mainly results from epigenetics alterations (CIMP-high) and *BRAF* mutations, with or without MSI [12].

Approximately 70% of sporadic CRC develop along the CIN pathway. This pathway is characterized by the accumulation of several mutations associated with the inactivation of tumor suppressor genes and activation of oncogenes [15]. Mutation in *Adenomatous polyposis coli* (*APC*) tumor suppressor gene are usually the inciting event of the cascade, leading to the activation of *Wnt* signaling pathway by degradation of β -catenin. In fact, this alteration is crucial to CRC tumorigenesis, and approximately 90% of patients have mutation in this gene [16]. Other key genes involved in CIN include *TP53*, *KRAS*, *PI3KCA* [17].

MSI tumors represent only 15% of sporadic CRC. The mismatch repair (MMR) mechanism plays a role in preventing errors during DNA replication. Nine different homologue genes have been identified as part of this mechanism [18, 19]. However, the majority of tumors with MSI have lost expression of the *MLH1* and *PMS2* genes. Additionally, hypermethylation of *MLH1* accounts for approximately 80% of the MMR alterations observed in sporadic CRC [20, 21]. The classification of MSI is based on altered size of various mono- and di-nucleotide repeat sequences (microsatellites), such as *BAT25*, *BAT26*, *D2S123*, *D5S346*, and *D17S250*, known as the Bethesda panel [22, 23]. An alteration in size of at least two of these markers is defined as MSI-high (MSI-H). Sporadic CRC with MSI-low (MSI-L) has an alteration in only one marker of the panel, and its clinical significance is controversial, being commonly grouped with microsatellite-stable (MSS) tumors. In MSI tumors, mutations in *APC* and *BRAF* are frequent, but *KRAS* mutations are rare [13, 24].

MSI-H tumors exhibit better outcomes and prognosis than MSS and MSI-L tumors, apparently due to the increased immune infiltration. Additionally, MSI-H tumors have a higher tumor mutation burden (TMB) and significant expression of tumor-specific antigens

(neoantigens) [24, 25]. This feature serves as a strong predictor of responsiveness to immunotherapies, including immune checkpoint blockade, due to the abundant tumor-infiltrating lymphocytes (TILs) (described in detail in section 2.3.2.) [26, 27].

CIMP-positive CRCs often are MSI-H due to methylation of the *MLH1* promoter, but more than 50% of CIMP tumors are MSS [28]. CIMP-positive tumors can be divided into two types, namely CIMP-high, with *BRAF* mutations and *MLH1* methylation, and CIMP-low, with *KRAS* mutations and MSS. CIMP-negative tumors are MSS with frequent *TP53* mutations [29]. The CIMP pathway is often associated with the development of serrated lesions.

Figure 2 provides a helpful summary of the genomic, epigenetic, and molecular features of different types of sporadic CRC, as well as examples of the immune microenvironments associated with each type. Moreover, **Figure 3** depicts the sporadic CRC cascade, showing the timing of some important mutations in the development of the disease.

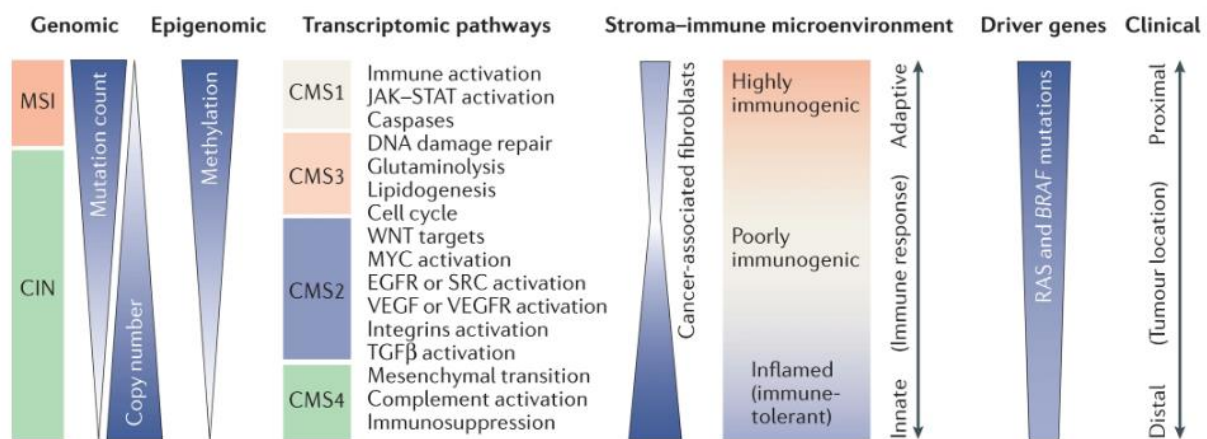


Figure 2 Microsatellite instability (MSI) is characterized by hypermutation, hypermethylation, high immune infiltration, activation of RAS, BRAF mutations, and locations in the proximal colon. Tumors with chromosomal instability (CIN) are more heterogeneous at the gene-expression level, showing a spectrum of pathway activation ranging from epithelial canonical (consensus molecular subtype 2 (CMS2)) to mesenchymal (CMS4). Tumors with CIN are mainly diagnosed in left colon or rectum, and their microenvironment is either poorly immunogenic or inflamed, with marked stromal infiltration. A subset of CRC tumors enriched for RAS mutations has strong metabolic adaptation (CMS3) and intermediate levels of mutation, methylation and copy number events. Adapted from [30].

1.1.4. Staging and Classification

The staging system that is most often used for CRC is the American Joint Committee on Cancer (AJCC) TNM system, based in 3 key characteristics: Tumor size, presence of lymph Nodes metastasis and distant Metastasis. Taking this into account, AJCC stages CRC from Stage 0 (very early cancer) to Stage IV (metastatic and very advanced cancer), each divided in several other subgroups [31].

The molecular characteristics of tumors have been recently considered with the emergence of the Consensus molecular subgroups (CMS) classification. This classification

distinguishes CRC into four different CMS: CMS1 (microsatellite instability immune, 14%), hypermutated, microsatellite unstable and strong immune activation; CMS2 (canonical, 37%), epithelial, marked WNT and MYC signaling activation; CMS3 (metabolic, 13%), epithelial and evident metabolic dysregulation; and CMS4 (mesenchymal, 23%), prominent transforming growth factor- β activation, stromal invasion and angiogenesis [32]. CMS1, which mainly consists of MSI tumors, is associated with a better prognosis due to its increased immunogenicity, higher tumor mutation burden and greater immune infiltration. On the other hand, CMS4, which is characterized by mesenchymal and inflammation-related type, is associated with a worse outcome [30, 32].

1.2. Inflammation-associated Colorectal Cancer

Inflammation is intrinsically associated to the development of cancer. Three types of inflammation can be distinguished in CRC based on the timing of their occurrence: inflammation as promotor of tumorigenesis, tumor-elicited inflammation, and therapy-induced inflammation. Chronic inflammation, triggered by infections, aberrant immune reactions (autoimmune diseases) or environmental factors, is known to be a driver of neoplastic progression and increases the risk of tumor development [33]. Inflammation generates oxidative stress-induced damage to DNA, which may activate tumor-promoting genes and inactivate tumor-suppressing genes [34]. Moreover, the activation of immune-mediated pathways and signaling, such as STAT3 signaling, and the NF- κ B and Wnt/ β -catenin pathways, among others, also provide tumor cells an important advantage in cancer development from inflammation [35]. On the other hand, the mutations present in tumor cells, as well as the hypoxic tumor environment can lead to the recruitment and polarization of immune cells and the establishment of an immunosuppressive environment [36].

1.2.1. Inflammatory bowel disease (IBD)-associated cancer

IBD, comprised by Ulcerative Colitis and Crohn's disease, is a chronic inflammation disorder of the gastrointestinal tract, associated with a 2-3-fold increased risk of CRC development [37]. In fact, while only 1-2% of the sporadic CRCs develop from IBD, patients with both IBD and CRC experience a 1.7-fold increased mortality rate when compared to CRC patients without IBD [38]. IBD patients who have been diagnosed for more than 8 years require close monitoring to detect early signs of tumor development. The degree of inflammation (in terms of severity and extent) and the persistence of the inflammation represent the main risk factors to the development of CRC, as well as concomitant primary sclerosing cholangitis and familiar history of CRC [39-42]. Considering these risk factors, IBD patients are stratified, and the type and frequency of monitoring are adjusted to screen

pre-malignant lesions as early as possible. However, the inflammation-dysplasia-carcinoma cascade, also known as colitis-associated CRC (CAC), is more challenging to detect macroscopically during conventional colonoscopy due to the flat characteristic of dysplastic lesions. This is one of the main reasons for late diagnosis of CAC and poor prognosis [43]. Therefore, there is an unmet need to discover novel biomarkers and prognostic markers for early detection and management of CAC.

Despite its inflammatory origin, CAC is derived from the same molecular pathways, CIN, MSI and CIMP, but the timing of the mutations across the tumorigenic cascade differs (**Figure 3**). *APC* gene mutation occurs less often and later in the carcinogenesis, while *TP53* mutation occurs frequently and earlier, even before dysplasia [44]. Indeed, it has been described that genomic and epigenomic alterations in some cell clones confer a survival advantage before histologic evidence of dysplasia [45]. In terms of CMS classification, CAC tumor development is characterized by a complete absence of CMS2 tumors and a skewing towards the CMS4-associated epithelial-mesenchymal transition pathway is described. This is accompanied by an increase in CD4+ T cells and monocytes when compared to sporadic CRC [46].

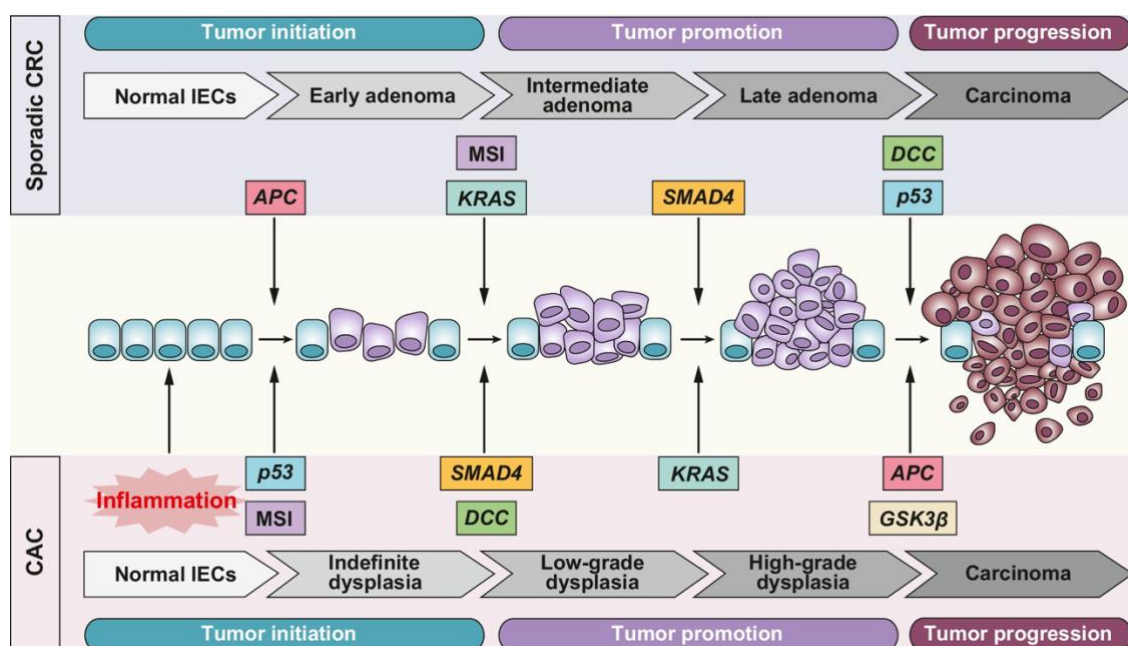


Figure 3 Tumor development in sporadic CRC and CAC. Differences in molecular pathogenesis of sporadic CRC (upper) and CAC (lower). From [35]. IECs: Intestinal epithelial cells.

The azoxymethane (AOM)/dextran sulfate sodium (DSS) mouse model of inflammation-associated tumorigenesis has been largely used for the study of inflammation-associated tumorigenesis. AOM is a potent carcinogenic agent that, in combination with DSS-induced inflammation and intestinal mucosal damage, leads to the appearance of tumor lesions [47].

However, this model lacks genetically defined mutations and rarely displays important gene mutations such as *APC*, *KRAS* and *TP53*, as well as MMR-associated genes [48]. It is worth noting that while the AOM/DSS model is the best and most commonly used mouse model to study CAC, it may not be well translated to human CAC.

2. Tumor immunology

The immune system is the primary system responsible for maintaining homeostasis of the organisms. In an ideal scenario, the immune system can identify non-self-molecules and pathogens, as well as abnormal tumor cells, and eliminate them. In the context of cancer, the sequence of events during interaction of immune system and tumor cells is commonly referred as the cancer-Immune cycle [49] (**Figure 4**). Tumor cells release tumor-associated antigens (TAAs), which are captured by antigen-presenting cells (APCs). In lymph nodes, APCs present the antigens through the major histocompatibility complex (MHC) class I or MHC class II to CD8⁺ or CD4⁺ T cells, respectively. T cells are then primed and activated, and traffic to tumor microenvironment (TME). Once in the tumor site, activated T cells recognize and kill tumor cells, which leads to the release of TAAs. This cycle repeats itself and amplifies the immune response [49]. However, tumor cells can adopt several mechanisms to evade immune response, causing a disfunction in the cycle and promoting tumor development.

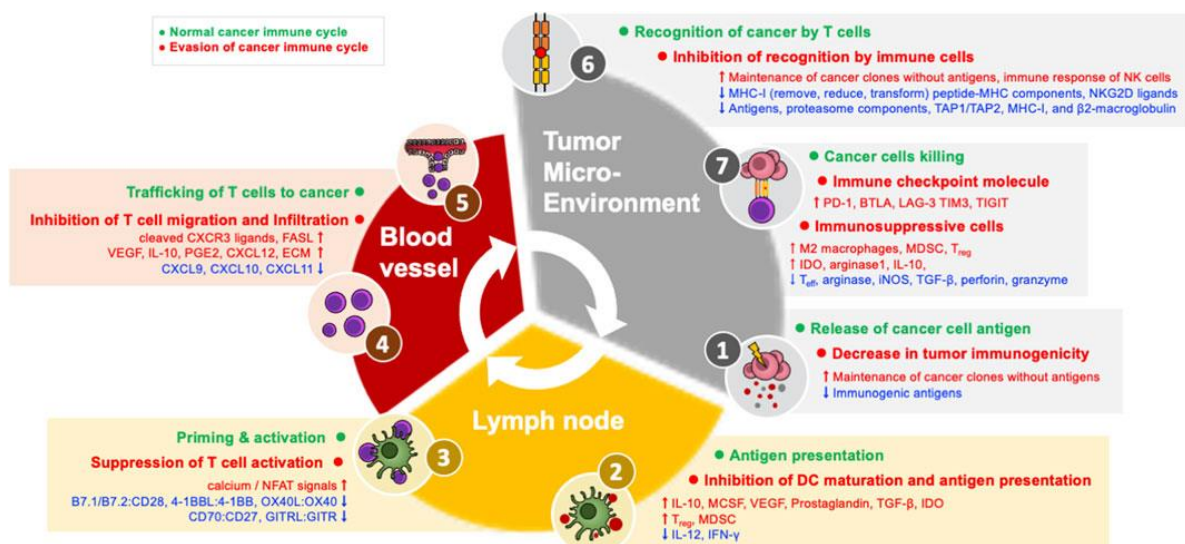


Figure 4 Evasion mechanisms in the cancer immune cycle. From [50].

2.1. Cancer immunosurveillance and immunoediting

The immune system has a dual function in the development of cancer. In most cases, the immune system is capable of identifying and irradiating newly transformed cells that

arise continuously in the body. However, the tumor itself is heterogeneous, and some cells are able to evade the anti-tumor immune response. This creates selective pressure, where resistant variants are chosen and proliferate, known as the tumor immunoediting process [51]. This process is commonly divided into three phases (3 E's): Elimination, Equilibrium and Escape. The elimination phase, corresponding to immunosurveillance, involves the recognition of transformed cells by the innate and adaptive immune system, through mechanisms that directly kill tumor cells. If some tumor cells persist in the elimination phase, the process can progress to the equilibrium phase, in which tumor does not expand due to immune pressure. In the escape phase, tumor cells become less immunogenic, evading the anti-tumor immune response. This escape phase occurs concomitantly with the appearance of clinically detectable progressively growing tumors [51-54].

Tumor cells employ various mechanisms to evade the host immune system (**Figure 4**), including: (1) reduced immune recognition, resulting from the downregulation of MHC class I expression, loss of TAAs and decreased priming and activation of T cells; (2) selection of less immunogenic clones; (3) establishment of an immunosuppressive TME through the expression of inhibitory immune checkpoint molecules (e.g. PD-L1/PD-1) by tumor cells or T cells, and recruitment of regulatory T cells (Tregs), M2-like macrophages and myeloid-derived suppressor cells (MDSCs), which promotes the production of anti-inflammatory cytokines, such as Transforming Growth Factor β (TGF- β) and Interleukin 10 (IL-10), and reduces the capacity to produce pro-inflammatory cytokines, like granzyme, Interferon- γ (IFN- γ) and Tumor necrosis factor α (TNF- α) [50, 52, 55].

2.2. Tumor microenvironment (TME)

TME includes the tumor and its surrounding environment, which is modulated to support tumor development. In addition to tumor cells, the TME is composed of immune cells, cancer-associated fibroblasts (CAFs), mesenchymal stromal cells (MSCs), endothelial cells, and extracellular matrix (ECM) [56].

The immune cells in TME comprise lymphocytes, macrophages, neutrophils and dendritic cells (DCs). TILs are a group of immune cells composed by T cells, such as CD4⁺ and CD8⁺ cells, B cells and Natural Killer (NK) cells.

Particularly, CD8⁺ cytotoxic T lymphocytes (CTLs) are a key component of the adaptive immune response and play a crucial role in the anti-tumor immune response. CTLs directly recognize tumor antigens through MHC class I molecules, and, in response, secrete pro-inflammatory cytokines such as IFN- γ , TNF- α , IL-12, and IL-2 to induce tumor cell death and recruit other effector cells such as NK cells and macrophages. CTLs exhibit important cytotoxic activity by secreting perforin, granzymes, and granulysin, and by expressing Fas

ligand, leading to tumor cell apoptosis [57]. The presence of CTLs significantly impacts the survival of cancer patients [58-61]. In fact, in CRC, tumor-infiltrated CTLs have been strongly associated with an effector anti-tumor immune response and a good prognosis [62-65].

CD4⁺ T cells are categorized into T helper (Th)1, Th2 and Th17, and Treg, based on their cytokine production and effector function. Th1 cells are involved in cell-mediated immunity and activation of other immune cells, such as CTLs, macrophages, and B cells, by releasing pro-inflammatory cytokines IFN- γ and TNF- α . IFN- γ regulates various biological programs that could participate in tumor rejection, including the capacity to inhibit cellular proliferation, induction of apoptosis, and inhibition of angiogenesis [66, 67]. Moreover, IFN- γ can substantially increase the levels of IL-12 production by stimulated DCs, which in turn polarize naïve T cells into the Th1 phenotype [68]. By interacting with the IL-12 receptor, IFN- γ stimulates downstream signaling routes and increases STAT1 activity, which subsequently induces T-bet expression [69]. Additionally, this cytokine also upregulates the MHC-class I expression by APCs and tumor cells, facilitating the recognition of antigens by CTLs [70, 71]. However, IFN- γ secretion can also lead to suppression of immune response indirectly through the upregulation of IFN- γ -inducible genes. Indoleamine 2,3-dioxygenase (IDO) is an IFN- γ -inducible enzyme that catalyzes tryptophan catabolism, an essential amino acid to T cells, causing proliferation arrest of T lymphocytes [72]. In addition to this, Th1 cells and their cytokines have been associated with favorable clinical outcomes in several cancers, including CRC, due to their significance in anti-tumor immune responses [59, 60, 73, 74].

Regarding the other CD4⁺ cell subtypes, the results are controversial. Th2 cells are frequently associated with aggressive tumors and a worse prognosis [75-78]. However, other studies suggest an association with the presence of Th2 cells and a good prognosis [79, 80]. Th2 cells are characterized by GATA3 expression and IL-4, IL-5, IL-9 and IL-13 production [81]. Th17 cells, which are characterized by IL-17 production and ROR γ T expression, have been associated with poor prognosis in different cancer types [82, 83]. In fact, IL-17 is known to increase vascular growth, which can lead to increased tumor growth and metastasis in some models [84]. In contrast, Th17 cells also recruit CTLs and DCs to the TME to promote tumor clearance. Moreover, they have the ability to convert into a Th1 phenotype that secretes IFN- γ under specific environmental factors [85]. Therefore, Th17 cells are associated with an improved survival in various cancers [86-89]. However, a dual role of IL-17-producing cells has been described in CRC, showing that the presence of Th17 cells also promotes the tumor infiltration by neutrophils and CTLs, due to the secretion of IL-8 and chemokines. Additionally, the intraepithelial Th17 cells, but not stromal, have been

shown to positively correlate with improved survival in patients with CRC [90]. In contrast, some studies suggest that IL-17 promotes intestinal tumorigenesis [84, 91-95].

Treg (CD4⁺CD25^{high}Foxp3⁺) cells are overexpressed in the TME, despite their low frequency in the peripheral circulation of patients with cancer. These cells are capable of suppressing the proliferation of other T cells in the microenvironment through contact-dependent mechanisms or by secreting anti-inflammatory cytokines, such as IL-10 and TGF- β [96, 97]. IL-10 has potent immunosuppressive effects on APCs and effector T cells, by reducing expression of type 1 cytokines, inhibiting antigen-specific T cell proliferation and preventing the production of pro-inflammatory cytokines by macrophages and APCs [98, 99]. TGF- β is commonly overexpressed in many tumors and it has significant immunosuppressive effects, including the inhibition of T cell proliferation and their differentiation into CTLs and T-helper cells [100].

Macrophages present within tumor, also known as tumor-associated macrophages (TAMs), are classified as M1 or M2-macrophages. M1-macrophages are associated with an anti-tumor immune phenotype, characterized by the expression of IFN- γ and the release of nitric oxide synthase (iNOS) and reactive oxygen species (ROS). Conversely, the majority of TAMs are M2-macrophages, which promote tumor progression by producing high levels of IL-10, TGF- β , epithelial growth factor (EGF), and vascular endothelial growth factor (VEGF). This ultimately lead to immune suppression of CTLs and NK cells, recruitment of Treg, tumor cell proliferation and angiogenesis [101].

NK cells possess significant anti-tumor potential, but their expression in the solid TME is low. NK cells present killer-cell immunoglobulin-like receptors (KIRs), which recognize MHC I and exert an inhibitory signal to protect healthy cells from an NK cell attack. Tumor cells frequently decrease or lose MHC I expression in cell surface, impairing KIR activation and enhancing anti-tumor immune response [102]. Moreover, NK cells also express the activation receptor, lectin-like receptor natural killer group 2D (NKG2D), which binds to MHC class-I-chain related proteins A and B (MICA and MICB), on tumor cells. This binding promotes NK cytotoxicity and cytokine release and represents the most well-characterized mechanism for NK cell activation in tumor surveillance [102]. Additionally, the presence of NK cells in the TME has been linked to improved survival, while the lack of NK cells is associated with an increased risk for cancer and worse clinical outcomes in CRC [103, 104]. However, similar to T cell evasion, tumor cells have some mechanisms to avoid NK cell function, by releasing anti-inflammatory cytokine TGF- β and expressing immune checkpoints, or by promoting a hostile microenvironment with an increase in ROS and hypoxia [105]. Moreover, shedding of MICA/B from the tumor cell surface is one of the most described mechanisms responsible for the impaired NKG2D recognition and subsequent tumor immune evasion [106].

By taking advantage of all these features of tumor immunity, several immunotherapies have been developed to promote anti-tumor immune response.

2.3. Anti-tumor Immunotherapies in CRC patients

The field of cancer treatment has been recently transformed by the advent of immunotherapies, which have introduced a revolutionary approach by leveraging the host's immune system to fight cancer. Immunotherapies have been shown to be highly efficient in treating several types of cancer, including hematopoietic cancer, melanoma and lung cancer. For CRC, significant progress has been made in the development of immunotherapies. However, the application of this type of therapy is limited to advanced and/or metastatic stages of CRC.

Several types of immunotherapies are being explored in ongoing clinical trials, including targeted monoclonal antibodies, immune checkpoint inhibitors (ICIs), chimeric antigen receptor T (CAR-T) cells and cancer vaccines, having demonstrated promising results (**Figure 5**). However, in clinical practice, only targeted antibodies and immune checkpoint inhibitors have received approval from the U.S. Food and Drug Administration (FDA) for the treatment of metastatic CRC.

2.3.1. Targeted monoclonal antibodies

There are two types of targets for monoclonal antibodies in immunotherapy: tumor antigens or immune cell specific molecules. Cetuximab and Panitumumab (anti-EGF receptor), and Bevacizumab and Ramucirumab (anti-VEGF), targeting tumor growth and angiogenesis, respectively, have already been approved by FDA and used for the treatment of CRC. Antibodies against Human epidermal growth factor receptor-2 (HER2) and Hepatocyte growth factor (HGF) are currently undergoing clinical trials [107].

Bi-specific antibodies are a new generation of monoclonal antibodies for cancer treatment. These antibodies are characterized by combining the antigen-binding domains of two different monoclonal antibodies. The advantage of bi-specific antibodies is that they can bind simultaneously two different antigens from tumor cells and T cells, improving the T cell anti-tumor immune response. These antibodies have been approved for the treatment of leukemia, lung cancer and melanoma, although in CRC, some bi-specific antibodies are still under investigation in phase I clinical trials [108]. MGD007, RO6958688 and MT110 are examples of bi-specific antibodies tested in CRC, which binds to CD3 receptor and to anti-glycoprotein A33, Carcinoembryonic antigen (CEA) and Epithelial cell adhesion molecule (EpCAM), respectively [108].

2.3.2. Immune checkpoint inhibitors

Immune checkpoint molecules, such as programmed cell death 1 (PD-1), cytotoxic T lymphocyte antigen 4 (CTLA-4), and T cell immunoglobulin and mucin domain-3 (TIM-3), are expressed in T cells upon activation. These co-inhibitory molecules bind to their respective receptors, programmed cell death ligand 1 (PD-L1), B7 and Galectin (Gal)-9 (among others), respectively, on immune cells or tumor cells. This interaction leads to T cell exhaustion and apoptosis, thereby regulating the immune response [109]. ICIs are monoclonal antibodies, which target these molecules and prevent their binding, thereby enhancing T cells activation and cytotoxic killing of tumor cells. However, this specific type of immunotherapy is restricted to patients with advanced MSI CRC.

MMR deficient tumors present a hypermutated phenotype, with a considerable high number of somatic mutations, the so-called TMB, and an increased expression of neoantigens compared to MSS tumors. TMB is closely related to tumor immune infiltration and associated with a good prognosis. In fact, several studies have demonstrated that MSI tumors present a higher density of TILs intratumorally, mainly CTLs [57]. Furthermore, TMB has been considered a predictor of response to ICIs therapy in MSI-high metastatic CRC [110]. Controversially, a significant percentage of MSI tumors do not present MCH class I in tumor cell surface, which conceals their neoantigens to CTLs and hinders anti-tumor immune response. This is one among other mechanisms of acquired resistance to immunotherapies [111, 112].

Dostarlimab (PD-1/PD-L1), Ipilimumab (CTLA-4), Nivolumab (PD-1/PD-L1) and Pembrolizumab (PD-1/PD-L1) are ICIs already approved by FDA in metastatic CRC, and many others have been tested in ongoing clinical trials [113].

2.3.3. Chimeric antigen receptor T cells and Adoptive T cell transfer

Adoptive T cell transfer of CAR-T cells is a novel genetically engineered, cell-based immunotherapy. T cells from cancer patients are modified with a receptor that recognizes specific tumor antigens, enabling them to identify and destroy cancer cells. CAR-T cell therapy has revolutionized the treatment of hematopoietic tumors. However, in the context of solid tumor, the efficacy of this target-specific immunotherapy needs to be improved and further investigated, as several problems have been identified: (1) Immunosuppression in the tumor microenvironment, (2) inefficient chemotaxis to tumor tissue, (3) incapacity to proliferate and survival in the blood or tumor area, (4) low levels of CAR-T cells invasion in tumor tissues [114].

In CRC, several clinical trials are ongoing, targeting EpCAM, CEA, Epidermal growth factor receptor (EGFR), NKG2D, Human epidermal growth factor receptor-2 (HER2), among others, all expressed in CRC [115].

2.3.4. Cancer Vaccines

Cancer vaccines are based on tumor-associated antigens (TAAs) and tumor-specific antigens and can be delivered via various approaches including (1) DNA/mRNA, proteins or peptides, (2) cell lysates, (3) antigen-primed DCs or (4) vector-based vaccines. The main goal of this approach is to enhance the anti-tumor immune response by targeting APCs. Although some cancer vaccines have been approved, in the case of CRC, these therapies are still in their early stages, with ongoing clinical trials [114].

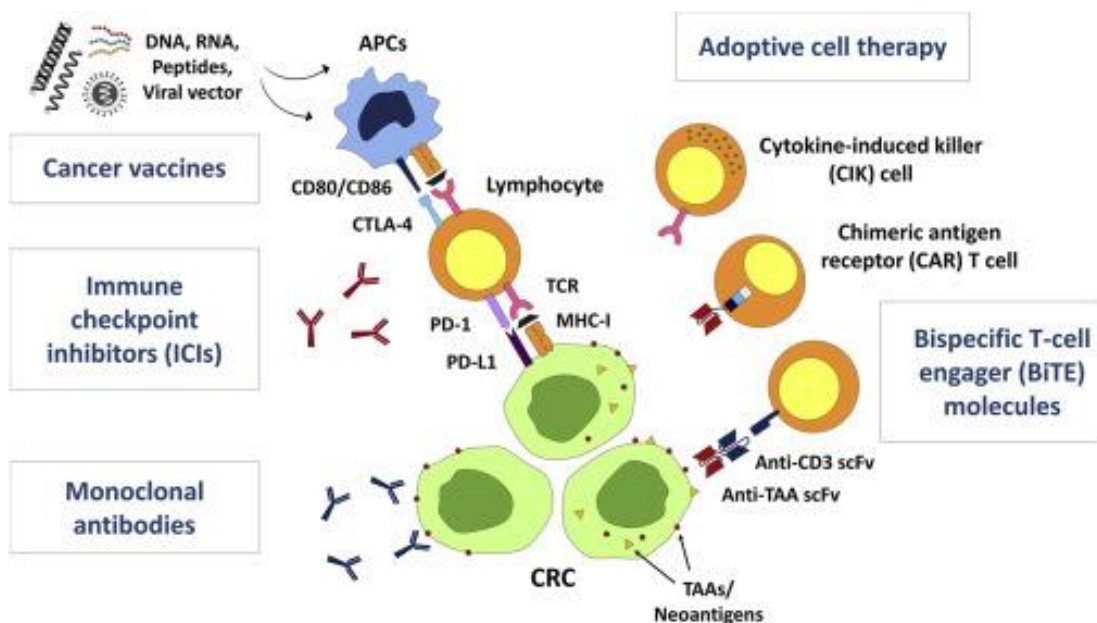


Figure 5 Different strategies for the development of immunotherapies in CRC. From [116].

3. Glycosylation

Glycosylation is defined as the enzymatic process that produces glycosidic linkages of saccharides to other saccharides, lipids, proteins and other organic molecules, producing different families of glycoconjugates: glycoproteins, glycolipids and glycosaminoglycans [117, 118].

Glycans play key roles in several physiopathological processes, including inter- and intracellular signaling, immune defense, inflammation, cell adhesion, molecular recognition and pathogen invasion [119]. In fact, nearly all cells are covered by glycans, giving rise to the so-called glycocalyx. The mammalian glycome is estimated to contain hundreds to thousands of glycan structures which can be larger than the proteome and the genome [118].

Protein glycosylation occurs in the secretory pathway, which includes the endoplasmic reticulum (ER) and the Golgi apparatus. This process involves the action of different glycosyltransferases and glycosidases enzymes, which, together with moieties availability, sugar nucleotides donors, and other biological factors, determine the type of glycoconjugates and glycan structures [118, 120]. The two main types of glycans known to be attached to proteins are O-glycans and N-glycans. O-linked glycans are attached to Serine (Ser) or Threonine (Thr) residues of a protein and are particularly found on secreted or membrane-bound mucins. N-glycosylation is a highly frequent post-translational process that occurs in Asparagine (Asn) residues of proteins in the consensus peptide sequence Asn-X-Ser/Thr, where X is any amino acid except proline [121].

3.1. N-Glycosylation

More than 90% of all mammalian glycoproteins are known to carry N-linked glycans, with an average of 2 N-linked glycans per polypeptide chain [122]. The process initiates in extramembrane of the ER, where N-acetylglucosamine (GlcNAc) residue is attached to dolichol phosphate, a lipid carrier in ER membrane. The glycan structure is built upon this residue, adding one more GlcNAc and 5 mannose residues (Man). This structure is then translocated to ER luminal side, where is decorated with more 4 Man and 3 Glucose residues (Glc). This specific 14-residue oligosaccharide, consisting of $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$, is *en bloc* transferred to the amine of Asn in the consensus sequence Asn-X-Ser/Thr, during the protein translation (co-translational) by an oligosaccharyltransferase (OST) [123].

Before leaving ER, the glycoprotein is submitted to a quality control system managed by the calnexin-calreticulin cycle. These two homologous lectins chaperones, calnexin (membrane-bound) and calreticulin (soluble), bind to glycans and control protein folding through a deglycosylation-reglycosylation system. When the protein is correctly folded, the last Glc is trimmed, and the glycoprotein makes its way to the Golgi apparatus, where the glycans are further elongated and extended. On the other hand, if the protein is misfolded, it is maintained in the calnexin-calreticulin cycle and it will be targeted to ER-associated degradation (ERAD) pathway [124].

Once in the Golgi, the glycans are trimmed by glycosidases and further elongated by a sequential activity of different glycosyltransferases that add a specific monosaccharide through a specific glycosidic bond in a stepwise manner. The resulting N-glycans can be classified in three main categories according to the diversity of sugar residues: High-Mannose, constituted mainly by mannose residues; Complex, where one or more “antennae” are yielded by the N-acetylglucosaminyltransferases; and Hybrid, in which

mannose residues are attached to the Man α 1-6 glycosidic bond and one or two antennae are on the Man α 1-3 arm [121].

N-acetylglucosaminyltransferase I (GnT-I, encoded by *MGAT1* gene) catalyzes the addition of the first GlcNAc residue to core mannose, and it is a crucial step for the conversion of high-mannose to hybrid or complex-type *N*-glycans. Afterward, two outer mannose residues are removed by α -Mannosidase II and a second GlcNAc residue is added to mannose α 1-6 in the core by the action of GnT-II, converting the hybrid glycans in complex *N*-glycans. By the action of GnT-III, hybrid and complex *N*-glycans may carry a “bisecting” GlcNAc residue linked on the internal β 4Man residue. The presence of a bisecting GlcNAc impedes a possible elongation and branching reactions. Otherwise, the process goes further with the addition of a GlcNAc residue to the Man α 1-3 arm via β 1,4 linkage by GnT-IV, and the addition of a β 1,6-GlcNAc branch to the Man α 1-6 arm by GnT-V, to form tri- and tetra-antennary *N*-glycans respectively [125].

During subsequent terminal glycosylation, in trans-Golgi, further sugar additions may occur, including 1) fucose (Fuc) residue addition in first GlcNAc residue adjacent to Asn (core fucosylation), 2) elongation of the branch GlcNAc structures, with, for example, poly-*N*-acetylglucosamine (polyGlcNAc), and/or “capping” and “decoration” of elongated branches with the addition of Fuc, *N*-Acetylneuraminic acid (Neu5Ac), Galactose (Gal), *N*-acetylgalactosamine (GalNAc) and Sialic Acid [121].

The *N*-glycosylation pathway is summarized in **Figure 6**.

3.2. Altered glycosylation in cancer

The glycosylation profile of malignant cells undergoes significant changes when compared to their healthy counterparts. [117]. The expression of aberrant glycans has been implicated in different steps of tumor progression, such as proliferation, invasion, metastasis, and angiogenesis [117, 126]. The altered protein glycosylation can occur due to under- or overexpression of glycosyltransferases, their mislocalization in the ER/Golgi compartments, dysregulation of chaperone function and availability of the sugar nucleotide donors and cofactors [117].

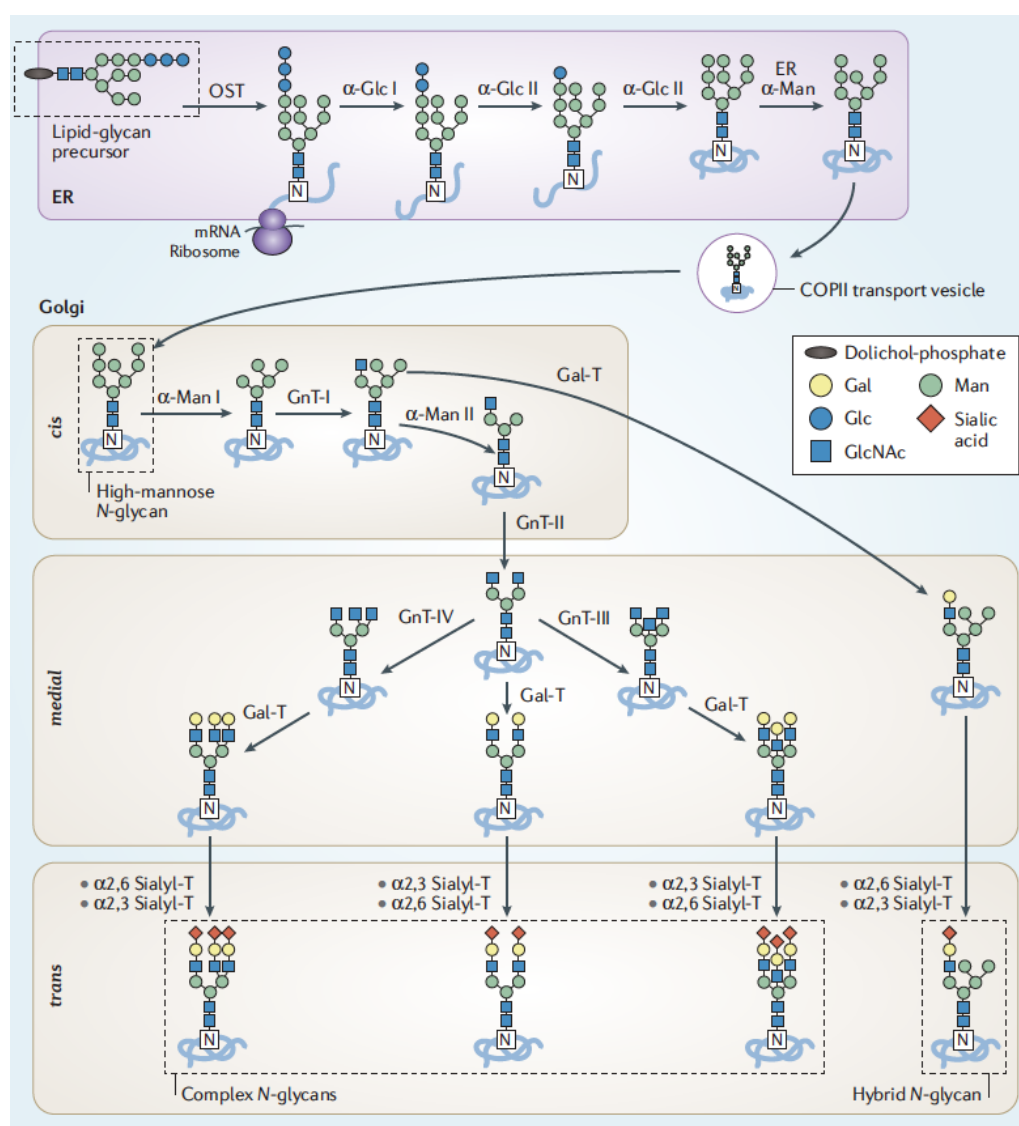


Figure 6 N-glycosylation pathway across endoplasmic reticulum (ER) and Golgi apparatus. The formation of N-glycans is mediated by the sequential action of several enzymes, glycosyltransferases, and glycosidases. During the pathway, high-mannose, hybrid and complex N-glycans can be synthesized. From [127].

The most-widely occurring changes in glycosylation in cancer include an overall increase in sialylation, branched N-glycans and core fucosylation. Specifically, an increase in sialyl Lewis X (SLe^x), SLe^a and O-linked truncated glycans, such as sialyl Tn (STn), Tn and T structures, are commonly found in cancer. Furthermore, an overexpression of branching N-glycans often occurs in N-linked glycans, caused by increased activity of GnT-V. There is also an increase in terminal sialylation, α 2,8-linked polysialic acid, and an increase of core fucosylation by fucosyltransferase VIII (Fuc-VIII, encoded by *FUT8* gene) [117].

Particularly, the abnormal expression of the complex β 1,6-GlcNAc branched N-linked glycans results from the GnT-V activity, encoded by *MGAT5* gene, which is upregulated by the activation of the RAS-RAF-MAPK pathway in cancer [128]. This alteration is associated

with several mechanisms of tumor progression, such as enhanced invasion and metastasis in mouse mammary carcinoma, glioma and colon cancer cells [129-131]. Indeed, *Mgat5*-deficient mice display significant suppression of mammary tumor growth and metastasis [132]. In gastric cancer, aberrant expression of complex *N*-glycans mediates the cell-cell adhesion, by controlling E-Cadherin function, a transmembrane glycoprotein and a major epithelial cell-cell adhesion molecule in cancer [133, 134]. GnT-V overexpression has been shown to induce E-cadherin cellular mislocalization in the cytoplasm, resulting in loss of function and non-functional adherent junctions [135, 136]. Additionally, the capping of complex *N*-glycans with α 2,6-sialic acids has been shown to have implications in E-cadherin regulation [135, 137, 138]. Moreover, the *O*-GlcNAcylation of E-cadherin has also been associated with its retention in the ER complex, which interferes with E-cadherin binding to p120-catenin and type I γ -phosphatidylinositol phosphate kinase (PIPK γ), which is required for the recruitment of E-cadherin to the adhesion sites [139].

The presence of β 1,6-GlcNAc branched *N*-glycans in integrins controls the interaction between cell and ECM, thereby increasing migration and invasion [140-142]. Moreover, α 2,6-sialylation of β 1-integrin, which is a result of the activity of the β -galactoside α -2,6-sialyltransferase 1 (ST6Gal1), enhances the adhesion of CRC cells to ECM. Consequently, it has been shown to promote tumor cell survival, adhesion, migration and invasion [143-148].

Besides the well described implications of aberrant glycosylation in tumor development, invasion, and metastasis, these altered glycans have a key role in tumor immune evasion (addressed below and in Chapter 2).

4. Glycans at the interface of immune responses and cancer

Glycans play a crucial role in mediating immune responses. Nearly all immune cell receptors are glycosylated, and their function is tightly controlled by glycans. Moreover, immune cells are equipped with glycan-binding proteins (GBPs), which sense and bind glycans in cell surface, mediating the immune response.

This section will describe the role of glycans in immune response and how they influence the tumor immunity. Specifically, the role of glycans on T cell mediated immune response in cancer was recently reviewed by us and published in *Immunology* journal, in 2022 (Chapter 5 of this PhD thesis).

4.1. Glycan-binding proteins as mediators of tumor immunosuppression

Immune cells express a variety of GBPs, also known as lectins, which are involved in the recognition of glycans. These proteins can be secreted or located on the cell surface of immune cells. They can be classified into several families and types, including galectins, C-type lectins receptors (CLRs) and sialic acid-binding immunoglobulin-like lectins (Siglecs). All these GBPs carry one or more carbohydrate recognition domains that coordinate their interaction with a specific glycan (**Figure 7**) [149-152].

Galectins are a family of soluble lectins that recognize glycans containing disaccharide *N*-acetylglucosamine (Gal- β 1,3-GlcNAc or Gal- β 1,4-GlcNAc) [153]. In the immune system, galectins are expressed by activated T cells, Treg cells, B cells and activated macrophages, but also by tumor cells. Galectins play distinct roles in immune response. For example, galectin (Gal)-3 has a dual role depending on its location on T cells. Intracellular Gal-3 blocks apoptosis, while extracellular Gal-3 binds to glycosylated receptors on the T cell surface, leading to the inhibition of T cell activation [143, 154-156]. In fact, Gal-3, produced by activated T cells, interacts with T cell receptor (TCR), negatively regulating T cell activation through the destabilization of the immunological synapse, which precludes TCR clustering [157-159]. In cancer, Gal-3 establishes glycan-dependent lattices with the TCR and CD8, impairing cytotoxic T cell function and IFN- γ secretion [160]. Additionally, Gal-1 and Gal-9 have also been shown to negatively affect T cell function, controlling survival and cytokine production, with particular importance in cancer. The overexpression of Gal-1 inversely correlates with the number of TILs [161]. In fact, Gal-1 can specifically control the function of Th1 and Th17 cells, but not Th2, due to their differential glycosylation profile enriched in α 2,3-sialic acids, which promotes the binding of Gal-1 and induces apoptosis [162]. In breast cancer, it has been demonstrated that Gal-1 leads to the expansion of Treg in the TME [163]. Regarding Gal-9, it is known to specifically bind Tim-3 and induce apoptosis of TILs [164, 165].

Siglecs can convey regulatory signals that positively or negatively control immune responses. However, most Siglecs are known to be negative regulators of cell signaling due to the presence of immune receptor tyrosine-based inhibitory motifs (ITIMs). In the context of cancer, Siglecs bind to abnormally expressed sialic acids on tumor cells, mediating an immunosuppression role in TME. Specifically, Siglec-9 has been demonstrated to be expressed on tumor-infiltrating CD8⁺ T cells in several cancers and is associated with poor survival [166, 167]. Siglec-9 of TAMs binds to Mucin-1 (MUC-1), which is expressed by several types of cancer, through ST truncated glycans, inhibiting the immune signal by activating the MAPK/ERK pathway [168]

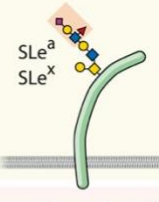
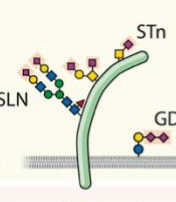
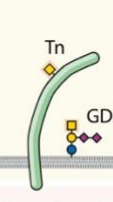
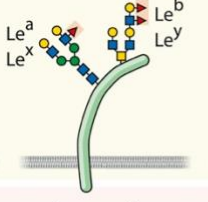
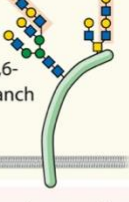
Lectin	Selectins	Siglecs	MGL	DC-SIGN	Galectins
Cell	Endothelial cells Platelets 	Monocytes, Macrophages, DCs, NK cells, CD8 ⁺ T Cells 	DCs and macrophages 	DCs and macrophages 	Various cells 
Ligand					
Acquired glycan alteration	Expression of sialyl Lewis antigens	Increased sialylation	Truncation of O-glycans and altered ganglioside expression	Increased fucosylation	Increased branching

Figure 7 Glycan-binding proteins (GBPs) recognition of glycosylation changes in cancer. Representation of expressing cells and main ligands in cancer context. From [169].

CLRs are a superfamily of calcium-dependent carbohydrate-binding proteins [170]. They are mainly expressed by APCs, and can bind to different types of glycans, including mannose, fucose and galactose. These glycans are essential for recognizing not only pathogens but also other cells, such as tumor cells. For instance, DC-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN; CD209), receptor of mannose and fucose expressed mainly by DCs and Macrophages, is known to recognize CEA and Mac-2 binding protein, which are well-known tumor-associated antigens overexpressed in CRC. The binding of DC-SIGN to non-sialylated Lewis (Le) glycans in those proteins inhibits the maturation of monocytes-derived DCs (MoDCs) and induces the production of anti-inflammatory cytokines [171, 172]. Additionally, the interaction between Le structures and DC-SIGN also enhances IL-10 production and impairs production of pro-inflammatory cytokines in TAMs, leading to decreased capacity to elicit anti-tumor T cell responses [173]. On the other hand, Tn or STn modified tumor-associated MUC-1 binds to macrophage galactose lectin (MGL) on immature DCs [174]. This interaction promotes the up-regulation of maturation markers of DCs, decreases phagocytosis, enhances motility, and also increases antigen-specific CD8⁺ T cell activation. However, MGL binding also increases the expression of Toll-like receptor 2 on APCs. Upon interaction with its ligand, this promotes the secretion of IL-10 and TNF- α [175]. Additionally, the interaction of MGL with terminal GlcNAc on CD45 negatively regulates TCR signaling, resulting in decreased T cell proliferation and increased T cell death [176]. Mannose receptor (MR; CD206) is another CLR that has implications in tumor immunity. M2-macrophages, which are highly present in

TME, express high levels of MR in cell surface. However, the activation of MR repolarizes M2-macrophages to M1-type anti-inflammatory TAMs, thereby improving innate and adaptative anti-tumor immune responses [177, 178].

All these data highlight the potential of the glycans and these GBRs in the development of anti-tumor immune therapies.

4.2. Alteration in immune cell glycosylation in cancer

The glycosylation of immune cells itself also plays an important role in cancer immunity. Almost all receptors in T cell and APCs surface are glycosylated, and different types of glycosylation can have different effects on immune cell function.

The immune response of T lymphocytes depends on MHC-I/II antigen presentation by APCs or tumor cells. *N*-glycans play a critical role in protein folding, traffic to the cell surface and peptide loading on MHC-I [179, 180]. In fact, the blocking of MHC-Ia *N*-glycosylation leads to a significant increase in intracellular misfolded protein, along with a decrease in cell surface expression [181]. Moreover, the knockout of the *Mgat2* gene, which is involved in the synthesis of complex branched *N*-glycans, decreases antigen presentation by MHC-II and leads to a loss of T cell stimulatory activity [182]. On the other hand, TCR *N*-glycosylation has been described to have a critical function in T cell activity. For instance, *N*-glycans on TCR are important in the stabilization of co-receptors at the immunological synapse, protection against the action of proteases and restriction of non-specific protein-protein interactions [183]. We and others have been demonstrated that decoration of TCR with complex β 1,6-GlcNAc branched *N*-glycans is particular important for T cell activity, by modulating the threshold of T cell activation and signaling [159, 184]. Furthermore, these complex *N*-glycans can be extended with poly-*N*-acetylglucosamine structures, allowing the binding of Gal-3, which establishes a molecular lattice with TCR and CD45 receptor, prevents TCR clustering and controls T cell activation and signaling [159]. This is in line with the function of Gal-3 in cancer described in section 4.2. Additionally, it has been described that the presence of β 1,6-GlcNAc branched *N*-glycans on the TCR inhibits Th1 cell differentiation and promotes Th2 cells polarization [185]. Moreover, a decrease in UDP-GlcNAc, which is necessary for the synthesis of complex branched *N*-glycans, promotes Th17 cells over the anti-inflammatory-induced Treg differentiation [186]. In fact, the removing of glycosylation on Treg cell surface impairs their suppressing function [187].

N-glycans also mediate the activation of the co-stimulatory receptor CD28. In fact, the binding of CD28 with its ligands CD80/86 is negatively regulated by its glycosylation. Mutation of all potential *N*-glycosylation sites of CD28 leads to an enhanced binding to CD80

expressed APCs [188]. However, the absence of branched *N*-glycans does not affect CD28 surface levels [189].

The immune checkpoint molecules are glycoproteins, and *N*-glycans are essential for their inhibitory activity. For example, PD-1 requires *N*-glycans to its stabilization on T cell surfaces, specifically those containing core fucose and poly-LacNAc [190]. Similarly, PD-L1 also depends on *N*-glycans to maintain its stability at the cell membrane [191]. In line with this, a recent study has shown that supplementation of tumor cells with D-mannose impairs the normal glycosylation of PD-L1, leading to its degradation and decreasing PD-L1 levels at the cell surface, thereby enhancing anti-tumor immune response [192]. Moreover, the immunosuppressive activity of PD-1/PD-L1 binding is dependent on *N*-glycans in both receptors [191, 193]. Additionally, CTLA-4 glycosylation is also crucial for its retention in T cell surface [194]. Likewise, the core fucosylation of TIM-3 is essential for Gal-9 binding, and its removal impairs Gal-9 interaction and the regulatory function of TIM-3 [165].

4.3. Glycan-based anti-tumor therapeutic strategies

As described above, the glycosylation of tumor cells, as well as immune cells, has potential to be good targets for immunotherapies. Actually, in the past decade, several advances have been made in this field, and some therapies have already been approved for use in cancer.

Immunotherapies targeting galectins are one of the strategies to achieve anti-tumor immunity. Gal-3 inhibitors GM-CT-01 and GR-MD-02 have shown promising results in improving anti-tumor CD8⁺ T cell response by binding and inhibiting Gal-3 function [195, 196]. Clinical trials testing these inhibitors are currently ongoing but are still in phase 1 and 2 [196]. Regarding Gal-1, no effective therapeutic has been developed, although there are promising results that support Gal-1 as a possible target. For instance, inhibition of LacNAc biosynthesis in T cells, using 4-fluoro-GlcNAc or thiodigalactoside, impairs Gal-1 binding and increases tumor-infiltration of CD8⁺ T cells and IFN- γ production [143, 197, 198]. Additionally, the overexpression of ST6Gal1 sialyltransferase on T cells, and consequently the increase of α -2-6 sialylated *N*-glycans expression on the CD45 receptor, has also been demonstrated to be efficient in impairing Gal-1 binding, preventing T cell death, which can represent an effective anti-tumoral strategy [199]. Moreover, knockout or knockdown of *Lgals1* gene, which encodes Gal-1 protein, promotes T cell infiltration and increases mice survival in cancer models [200, 201].

Within Siglecs, some advances have been made. Antibodies targeting Siglec-7 and -9 have also been demonstrated to promote an efficient anti-tumor immune response [202, 203]. Interestingly, modulation of sialylation in tumor cells with sialic acid analogs reduces

cell surface Siglec recognition and improves the activation of intratumoral T cells [204]. On the other hand, CAR-T cell composed of the exodomain (recognition without function) of Siglec-7 and -9 have demonstrated efficiency in recognizing sialylated glycans on tumor cells, both *in vitro* and *in vivo*, consequently improving anti-tumor CAR-T cell target function [205].

In line, CAR-T cells specifically targeting glycosylated antigens have shown good results in immunotherapy. For instance, CAR-T cell targeting Tn-MUC-1 have been demonstrated to be efficient in controlling tumor growth, being currently clinical trials ongoing [206]. Additionally, CAR-T cells anti-LewisY antigens have also shown efficacy and are now under clinical trials [207].

One of the major problems of CAR-T cells therapy is the inhibition of its function by the expression of PD-1. In this line, removing PD-1 N-glycosylation, by editing the coding sequence of the N74 residue of *PDCD1* gene, has been shown to reduce PD-1 expression at the cell surface, improving overall survival and increasing anti-tumor activity *in vivo* [208]. Similarly, abolishing core fucose in T cells decreases surface PD-1 expression and enhances T cell activation, leading to more efficient tumor eradication [190]. Furthermore, ICIs that specifically target the N-glycosylated form of PD-1 or PD-L1 have been shown to improve their capacity to promote anti-tumor immune responses [193, 209]. Additionally, antibodies against glycosylated-CTLA-4 are also being considered to increase its therapeutic efficacy against tumors [210].

Regarding APCs, the use of glycosylated antigens to stimulate APCs and to lead anti-tumor immune response has been explored. Taking advantage of CLRs present in these cells, mainly DC-SIGN and MR, APCs are stimulated through glycosylated peptides or molecules. For instance, priming of DCs with synthetic long peptides conjugated with mannose specifically promotes antigen cross-presentation and CD8⁺ T cell activation [211]. Interestingly, nanoparticles or lysosomes decorated with mannose interact with MR present in TAMs and polarize M2-macrophages into M1-type anti-tumor TAMs, improving anti-tumor immune response [177, 212]. Moreover, a dendrimer carrying DC-SIGN ligand Lewis Y glycans conjugated to gp100 synthetic long peptide has been shown to stimulate gp-100-specific CD8⁺ T cell responses [213].

Altogether, the evidence presented highlights the important role of glycans as potential targets for immunotherapy in cancer. The modulation of glycosylation in tumor cells, immune cells, and APCs can improve anti-tumor immune response through various mechanisms, such as enhancing T cell activation, inhibiting immune checkpoint molecules, and modulating TAM polarization. Therefore, targeting glycans represents a promising strategy for developing effective immunotherapies in cancer.

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Aims

CRC is still in top 3 both in terms of incidence and mortality worldwide. Cancer cells and immune response are tightly interconnected by glycans. For instance, tumor cells are covered by glycans, which constitute the first barrier between tumor cells and immune cells. Additionally, immune cell function is strongly mediated by glycosylation.

The main goal of this thesis is to explore how tumor-specific complex branched *N*-glycans have a role in the regulation of the immunological landscape in CRC. This new knowledge is expected to foster the development of innovative glycan-based immunotherapeutic strategies to improve CRC clinical management.

To achieve this goal, this thesis is divided into 3 main specific aims:

1. **To characterize the CRC glycosylation profile, addressing its impact in the regulation of the tumor immune response along CRC development.** Does the aberrant *N*-glycosylation on tumor cells contribute to tumor immune evasion? Can we improve anti-tumor immune response by removing the complex glycan layer of tumor cell surface? ([Chapter 2](#), Silva and Fernandes, *et al. Cancer Immunology Research* 2020)
2. **To investigate the role of *N*-glycans in (A) neoantigens-mediated T cell immune response and (B) antigen-independent innate immune response.** What is the role of *N*-glycosylation in tumor neoantigens in the regulation of T-cell mediated tumor immune response? Can glycosylation profile of HLA negative tumors influence antigen-independent recognition by NK cells? ([Chapter 3](#))
3. **To evaluate the impact of T cell *N*-glycosylation in inflammation-associated cancer.** How does the T cell *N*-glycosylation profile change from colitis to cancer and how does this associate with tumor immune response? Can we modulate T cell glycosylation to control tumor development? ([Chapter 4](#) and [Chapter 5](#), Fernandes *et al. Immunology* 2022)

Chapter 2

Glycans as Immune Checkpoints: Removal of Branched
N-glycans Enhances Immune Recognition Preventing
Cancer Progression



Glycans as Immune Checkpoints: Removal of Branched N-glycans Enhances Immune Recognition Preventing Cancer Progression

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ABSTRACT

Tumor growth is accompanied with dramatic changes in the cellular glycome, such as the aberrant expression of complex branched N-glycans. However, the role of this protumoral N-glycan in immune evasion and whether its removal contributes to enhancement of immune recognition and to unleashing an antitumor immune response remain elusive. We demonstrated that branched N-glycans are used by colorectal cancer cells to escape immune recognition, instructing the creation of immu-

nosuppressive networks through inhibition of IFN γ . The removal of this “glycan-mask” exposed immunogenic mannose glycans that potentiated immune recognition by DC-SIGN-expressing immune cells, resulting in an effective antitumor immune response. We revealed a glycoimmune checkpoint in colorectal cancer, highlighting the therapeutic efficacy of its deglycosylation to potentiate immune recognition and, thus, improving cancer immunotherapy.

Introduction

Colorectal cancer is a global and lethal disease, remaining the third most commonly diagnosed cancer in males and the second in females worldwide (1). Despite the clinical success of cancer immunotherapies, the majority of patients with metastatic colorectal cancer fail to respond or develop resistance to therapy, which constitutes a major clinical concern (2). In fact, only 5% of the patients with metastatic colorectal cancer diagnosed with mismatch repair deficiency (MMR-d)/microsatellite instability-high (MSI-H) respond to immunotherapies (3, 4), indicating a critical gap in our understanding of how these tumors regulate immune responses (5). In this era of cancer immunotherapy, there is an urgent clinical need to understand the dynamic cross-talk between tumor and immune cells, aiming to unlock new targets for the development of more effective cancer immunotherapeutic strategies.

Cancer progression is guided by the selective pressure of the immune system on malignant cells, resulting in the selection of tumor cell variants that are more prone to survive/escape in an immunocompetent host. Some mechanisms have been described in past studies showing the mechanisms used by tumor cells to elude immunosurveillance, including the expression of immune inhibitory checkpoints. This has boosted the development of immunotherapies for CTLA-4 and PD-1/PD-L1 blockade, revolutionizing cancer treatment (6–9). Nevertheless, only a small proportion of patients with cancer ultimately benefit from immunotherapy, which supports the hypothesis that cancer cells evade immune recognition through other immune escape strategies.

All cells are covered with a dense and complex coat of glycans (the glycocalyx), which constitutes a major biological interface between cells and their environment. Glycosylation is the enzymatic process responsible for the attachment of glycans to proteins or lipids in essentially all cells. The cellular glycosylation signature is altered in cancer cells, and the aberrant expression of glycans by tumor cells regulates each pathophysiologic step of cancer development and progression, including tumor cell dissociation and invasion, signaling,

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Note: Supplementary data for this article are available at Cancer Immunology Research Online (<http://cancerimmunolres.aacrjournals.org/>).

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Cancer Immunol Res 2020;8:1407–25

doi: 10.1158/2326-6066.CIR-20-0264

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angiogenesis, and metastasis (10, 11). Particularly, one of the most widely occurred tumor-associated glycan structure is the complex β 1,6-GlcNAc-branched N-glycans (12). The aberrant overexpression of this structure, catalyzed by the N-acetylglucosaminyltransferase V (GnT-V) enzyme, has been consistently shown to induce promalignant, proinvasive, and prometastatic cancer phenotypes through mechanistic interference with tumor cell adhesion, and is associated with poor survival rates of patients with gastrointestinal cancer (13–17). The cellular stabilization of PD-L1 is also known to be modulated by complex N-glycosylation (18, 19). The importance of glycans' modifications of cancer proteins and cancer (neo)antigens on the regulation and instruction of the surrounding immune response is now starting to be explored (20). However, whether and how the abnormal expression of the tumor-associated complex branched N-glycans alters the way that the immune system recognizes malignant transformation and cancer progression remains unknown.

Immune cells express a variety of specific glycan-binding receptors called C-type lectins [such as dendritic cell (DC)-specific intercellular adhesion molecular-3-grabbing nonintegrin (DC-SIGN); mannose receptor (MR)], galectins, and Siglecs, which are able to recognize alterations in the glycan signature of tumor cells, instructing either immunostimulatory or immunoinhibitory pathways (21, 22). In this study, we hypothesized that the abnormal cancer glycoprofile characterized by the overexpression of complex branched N-glycans was able to impact cancer immunoediting, contributing to the creation of immunosuppressive networks associated with disease progression. From a clinical point of view, we also postulated that the removal of branching N-glycosylation on colorectal cancer cells may expose relevant immunogenic (glyco)epitopes, constituting a glycoengineered strategy to enhance immune recognition and immunosurveillance, promoting antitumor immunity. Whether and how this altered host glycoprofile perturbed the gut microbiome composition associated with the modulation of tumor immune responses in colorectal cancer were also investigated.

Materials and Methods

Mice

All mouse procedures were approved by the i3S ethics committee for animal experimentation under Portuguese regulations. Mice were housed at the animal facility of the Institute for Research and Innovation in Health, University of Porto (i3S, Porto, Portugal). C57BL/6 wild-type (WT; acquired at The Jackson Laboratory), *Mgat5*^{−/−} (kindly provided by Michael Pierce, University of Georgia, Athens, GA), and *Rag2*^{−/−}*Il2rg*^{−/−} (kindly provided by Prof. James Di Santo, Institut Pasteur, Paris, France) mice were used for tumorigenicity assays. The *Apc*^{Min/+} (acquired at The Jackson Laboratory) and *VCMsh2*^{LoxP/LoxP} (kindly provided by Prof. Winfried Edelmann, Albert Einstein College of Medicine, New York, NY) mice were crossed (first generation) with *Mgat5*^{−/−} mice (*Apc*^{Min/+} *Mgat5*^{−/−} and *VCMsh2*^{LoxP/LoxP} *Mgat5*^{−/−}) to evaluate spontaneous tumor growth and tumor immune infiltrate, comparing with *Apc*^{Min/+} and *VCMsh2*^{LoxP/LoxP} *Mgat5* WT background mice.

Cell culture

The MKN45 gastric cancer cell line transfected with an empty vector (MKN45 Mock) or with GnT-V cDNA vector (MKN45 T5) were used in coculture assays (23) (kindly provided by Naoyuki Taniguchi, Osaka University Medical School/Graduate School of Medicine, B1, 2–2 Yamadaoka, Suita, Osaka, Japan). These cells were cultured in RPMI1640 GlutaMAX culture medium (Gibco), supplemented with

10% FBS (Biowest) and penicillin/streptomycin (100 U/mL; Gibco), under selection pressure of G418 (500 μ g/mL; InvivoGen) at 37°C in 5% CO₂. All the cell lines used were tested for the presence of *Mycoplasma* and maintained in culture during approximately 3 passages before using in the experiments. Authentication of cell lines (genotyping) was performed before the experiments.

In *in vivo* assays, the murine colon adenocarcinoma cell line, MC38 (kindly provided by our collaborator and coauthor Julio C. de-Freitas, junior), was manipulated using CRISPR/Cas9 technique to knockout the *Mgat5* gene (MC38 T5KO), as described below. The MC38 WT and MC38 T5KO cells were maintained in DMEM culture medium (Gibco), supplemented with 10% FBS and penicillin/streptomycin (100 U/mL) at 37°C in 5% CO₂.

CRISPR/Cas9, insertion-deletion validation, and functional assays

To obtain the cell line MC38 KO to *Mgat5*, 3 different single-guide RNA (sgRNA) were used: AAGTTGTCCTCTCAGAAAGCTGGG; TCAGAAGCTGGGCTTTTCCTGG and CTAGCAATGTAC-CATTCCTTGG (3'–5') (all from IDT). Briefly, these sgRNA were cloned in pSpCas9(BB)-2A-GFP (#48138, AddGene) and transfected to cell line, using Lipofectamine 2000 (Invitrogen) according to manufacturer's recommended protocol. The clonal cell lines were isolated by flow cytometry sorting (BD FACSaria II, BD Biosciences) 24 hours after transfection (around 10% of the cells were positive to GFP). One positive cell per well was plated in 96-well plates, and the clonal cell line was expanded for 2–3 weeks in DMEM supplemented with 10% FBS and penicillin/streptomycin (100 U/mL) at 37°C in 5% CO₂. The genomic DNA from the clonal cell lines was extracted with Quick Extract reagent (Epicentre) and the *Mgat5* gene was amplified by PCR using the primers forward: CAACCTGCACCATGAACCTTGC and reverse: ATGTAGTTCCGTGACCGTCT. The PCR products were sequenced by Sanger method and INDELs presence was inspected by *DSDecodeM* online software (developed in ref. 24).

Three different clones transfected from the same sgRNA and with proved indel, were inspected for the glycoprofile to validate the functional KO of the enzyme. The cells were labeled with different lectins (1:1,000; Vector Laboratories)—Fluorescein-conjugated *Phaseolus vulgaris* leucoagglutinin (a lectin that recognizes the β 1,6-GlcNAc arm of branched complex-type N-glycans; L-PHA-Fluorescein), *Galanthus nivalis* agglutinin (a lectin that recognizes the terminal α -1,3 mannose residues; GNA-Fluorescein), and biotinylated *Concanavalin A* (a lectin that recognizes α -mannose residues; ConA-biotin)—for 15 minutes at 4°C, after the staining with Fixable Viability Dye (FVD)–APC-eFluor780. For the staining with biotinylated lectins, an additional incubation was performed with streptavidin-PE (BioLegend). Samples were measured on a FACSCanto II machine and the data was analyzed using FlowJo software v10.

The absence of β 1,6-GlcNAc branched N-glycans was also confirmed by immunofluorescence. Samples were fixed with 4% formaldehyde and blocked with 5% BSA in PBS-Tween 0.1% for 1 hour. After incubation for 1 hour with L-PHA-Fluorescein (1:250; Vector Laboratories) or GNA-Fluorescein (1:500; Vector Laboratories) diluted in the blocking solution, nuclei were stained with DAPI and cell fluorescence was visualized using fluorescence microscopy Zeiss Axio Imager Z1 (Carl Zeiss, Germany). Analysis was performed in Axiovision 4.9 software (Carl Zeiss, Germany).

For the proliferation assay, 5×10^4 MC38 WT and T5KO clonal cells were labeled with 5-(and-6)-carboxyfluorescein diacetate succinimidyl ester (CFSE), using CellTrace CFSE Cell Proliferation Kit

(Invitrogen). Cells were washed and maintained in culture at 37°C in 5% CO₂. After 60 and 96 hours of culture, the fluorescence of cells was measured on a FACSCanto II machine and data analyzed using FlowJo v10 software.

Human samples

Formalin-fixed, paraffin-embedded (FFPE) tissue samples were collected in routinely medical exams or as part of patients' treatment. The cohort of clinical samples from colon carcinogenesis cascade were characterized by biopsies of normal colon ($n = 5$), colonic low-grade dysplasia (LGD; $n = 13$), and colonic high-grade dysplasia (HGD; $n = 7$) from Hospital Center of Porto (CHP, Porto, Portugal), and colonic dysplasia ($n = 57$) and colon adenocarcinoma ($n = 55$; stage I–III) from Portuguese Oncology Institute of Porto (IPO Porto, Porto, Portugal). The samples representing the gastric carcinogenesis cascade: normal gastric mucosa ($n = 5$), gastritis ($n = 5$), and atrophy and/or intestinal metaplasia ($n = 5$), were assigned by CHP, whereas gastric adenocarcinomas ($n = 5$) were assigned by Hospital Center of São João (CHSJ, Porto, Portugal). Whole blood from healthy donors, firstly used for peripheral blood mononuclear cells (PBMC) isolation, were kindly donated by healthy volunteers from a bank of blood donors. Buffy coats from healthy donors, used in CD14⁺ cells and T-cell isolation (described below), were obtained from Blood Bank of CHSJ (Porto, Portugal).

All samples were collected after written informed consents, according to directives 2004/23/EC and 2001/83/EC, which sets the standards of quality and safety for the donation, procurement, testing, processing, preservation, storage, and distribution of human samples in agreement with the World Medical Association Declaration of Helsinki for the ethical Principles for Medical Research Involving Human Subjects. The study was performed after approval by the institutional review board (IRB) of CHP and CHSJ.

Human PBMC isolation, monocyte-derived DC differentiation, naïve CD4⁺ T-cell isolation, and mouse bone marrow-derived DC isolation

To isolate PBMCs, 1 volume of Lymphoprep (StemCell Technologies) was used for 2 volumes of diluted whole blood (diluted 1:2 in PBS) and centrifuged for 30 minutes at 800 × *g* with the brake off. The interphase between the plasma/PBS and Lymphoprep, that contain PBMCs was then isolated. The cells were resuspended in RPMI supplemented with 10% FBS and penicillin/streptomycin (100 U/mL).

To isolate CD14⁺ monocytes, the PBMCs isolation was performed as before, but it used buffy coats (diluted 1:6 in PBS) from blood donors. From 1×10^8 PBMCs, CD14⁺ cells were purified using Human CD14 MACS Microbeads (Miltenyi Biotec) by magnetic separation with MS columns according to manufacturer's instructions (purity ≥ 95%). The enriched CD14⁺ cells were cultured in 6-well flat plates in RPMI supplemented with 10% FBS, penicillin/streptomycin (100 U/mL), and human recombinant proteins IL4 and GM-CSF (50 ng/mL; *E. coli*, PeproTech) at a concentration of 1×10^6 cells/mL to drive differentiation into monocyte-derived DCs (moDC). On the third day of culture, the medium was replaced, and the cells were in cultured for 3 more days, for a total of 6 days. At that time, monocytes were differentiated into immature moDCs and in half of the wells, lipopolysaccharide (LPS, 100 ng/mL; Sigma-Aldrich) was added to mature moDCs. Forty-eight hours later, the matured moDCs were resuspended in X-VIVO 15 serum-free medium (Lonza) for posterior cocultures with cancer cells. The remaining PBMCs were frozen in freezing medium [90% FBS and 10% dimethyl sulfoxide (DMSO, PanReac AppliChem)] for later isolation of naïve CD4⁺ T cells.

Naïve CD4⁺ T cells were isolated from same donors' PBMCs used for moDCs. PBMCs were thawed in RPMI plus 10% FBS and penicillin/streptomycin (100 U/mL) and kept in culture overnight. After this period, naïve CD4⁺ T cells were enriched using the EasySep Human naïve CD4⁺ T Cell Isolation Kit II (StemCell Technologies) according to manufacturer's recommended protocol (purity ≥ 90%).

The bone marrow was extracted from the femur and tibiae of C57BL/6 WT mice, and the isolated cells were maintained in culture in RPMI plus 10% FBS, penicillin/streptomycin (100 U/mL), and mouse recombinant proteins IL4 and GM-CSF (50 ng/mL; *E. coli*, PeproTech). On the third day of culture, the medium was replaced, and the cells were in cultured for 3 more days. The bone marrow-derived DCs (BMDC) were matured with LPS (100 ng/mL) and used in cocultures with MC38 cells as described below.

IHC and lectin histochemistry

For lectin histochemistry, 3-μm sections of FFPE tissues from distinct stages of colon or gastric carcinogenesis were used. To block endogenous peroxidase, 3% H₂O₂ (Thermo Fisher Scientific) in methanol (Thermo Fisher Scientific) solution was used. Before staining, unspecific proteins were blocked with 10% BSA (Sigma-Aldrich). The sections were incubated for 1 hour with biotinylated lectin [13.3 μg/mL; L-PHA, GNA; Vector Laboratories]. To provide the detection, the sections were incubated with ABC HRP solution from the Vectastain ABC Kit (Vector Laboratories) followed by 3,3'-Diaminobenzidine tetrahydrochloride (0.67 mg/mL; DAB, Sigma-Aldrich) substrate.

Foxp3 and Tbet IHC was performed in sequential sections from the same samples. The slices were deparaffinized and hydrated, and antigen retrieval was performed using vapor heat during 40 minutes (in 1 mol/L citrate buffer, pH = 6). At this time, all steps before and after the staining were performed using solutions provided by the UltraVision Quanto Detection System HRP DAB kit (Thermo Fisher Scientific) according to the manufacturer's recommended protocol. The sections were incubated with primary anti-human anti-Foxp3 (6.7 μg/mL, 236A/E7, eBioscience) or anti-Tbet (5 μg/mL, eBio4b10, eBioscience) diluted in 5% BSA overnight at 4°C for colon samples or 2 hours at room temperature for gastric samples. The sections were contrasted with hematoxylin, dehydrated, and preserved with appropriate mounting medium (Entellan new, Merck Millipore) and coverslips. The results were analyzed by three independent observers, to establish an observational classification of the slides (Zeiss Axioskop 2 Microscope was used). The characterization of lectin histochemistry followed a semiquantitative classification (<25%, 25–50%, 50–75%, and >75%), whereas the IHC for Tbet and Foxp3 followed a qualitative classification (±, +, ++, +++) accordingly with the staining extension and intensity.

Real-time PCR

For all FFPE tissues of colon and gastric carcinogenesis, RNA was extracted from 10-μm sections using the RecoverAll Total Nucleic Acid Isolation Kit (Invitrogen) according to the manufacturer's recommended protocol. For colon adenocarcinomas and dysplasias, only the selected and identified lesion tissue (accordingly with the histopathologic classification performed by a pathologist) was processed. For cell lines, RNA of MKN45 or MC38 cell pellets were extracted with TRI reagent (Sigma-Aldrich) according to the manufacturer's instructions. Total RNA was quantified using the Nanodrop system, and cDNA synthesis was performed using SuperScript IV Reverse Transcriptase (Invitrogen) according to the manufacturer's recommended protocol. Real-time PCR was performed in 96-well reaction plates, and cDNA was amplified using the respective TaqMan assays: *MGAT5*

(Hs00159136), *FOXP3* (Hs01085834), *Mgat5* (Mm00455036), *Mgat5b* (Mm01252571), *POMT1* (Hs01059558), *POMT2* (Hs00203575), *MGAT3* (Hs02379589), *MAN2A1* (Hs01123597; all from Applied Biosystems) and *18S* (Hs.PT.39a.22214856.g; IDT). Amplification data were acquired with 7500 Fast Real-time PCR system (Applied Biosystems). The mRNA expression of the genes of interest was normalized using the internal control 18S mRNA expression (ΔC_t). For the MC38 cell line, RQ values were calculated by the equation $2^{-(\Delta C_t)}$, whereas for each carcinogenesis and MKN45 cell line, RQ values were calculated by the equation $2^{-(\Delta\Delta C_t)}$, where $\Delta\Delta C_t$ represents the relative expression of ΔC_t of the gene in comparison with mean of normal clinical samples or Mock cell line.

Distal small intestine sections from *Apc^{Min/+}Mgat5^{+/+}* and *Apc^{Min/+}Mgat5^{-/-}* mice with or without lesions were digested and RNA was extracted using RNeasy-Micro Total RNA Isolation Kit (Invitrogen) according to the manufacturer's recommended protocol. The total RNA was quantified in Nanodrop system and the cDNA synthesis was performed using SuperScript IV Reverse Transcriptase (Invitrogen) protocol.

The real-time PCR were performed in 96-well reaction plates, and cDNA was amplified using LightCycler 480 SYBR Green I Master (Roche) and respective primers (IDT): *Ifng_Fw*: TGGCTGTTTCTGGCTGTACT, *Ifng_Rv*: GTTGCTGATGGCCTGATTGTC; *Il17a_Fw*: TACCTCAACCGTTCCACGTC, *Il17a_Rv*: TTCCCTCCGCATTGACACAG; *Il10_Fw*: TAACTGCACCCACTTCCCAG, *Il10_Rv*: AGGCTTGCAACCCAAGTAA; *Cd45/Ptprc_Fw*: GGGTTGTTCTGTGCCTTGTT, *Cd45/Ptprc_Rv*: CTGGACGGACACAGTTAGCA; *Gapdh_Fw*: GAAGGTCGGTGTGAACGGAT, *Gapdh_Rv*: CTCGCTCCTGGAAGATGGTG. Amplification data were acquired with 7500 Fast Real-Time PCR System (Applied Biosystems). The mRNA expression of the genes was normalized using the internal control *Gapdh* mRNA levels (ΔC_t). RQ values were calculated by the equation $2^{-(\Delta\Delta C_t)}$, where $\Delta\Delta C_t$ represents the relative expression of ΔC_t of the gene in comparison with mean of the controls *Apc^{Min/+}Mgat5^{+/+}* samples. A ratio of gene of cytokines and *Cd45/Ptprc* gene expression was performed to normalized to immune infiltrate.

Colorectal cancer and gastric carcinogenesis: Bioinformatics analysis

Differentially expressed genes in colorectal ($n = 275$) and gastric cancer ($n = 408$) versus adjacent normal tissue ($n = 349$ and $n = 211$, respectively) were evaluated using GEPIA2 (information and analysis details are included in <http://gepia2.cancer-pku.cn/#about>), a web server for large-scale expression profiling and interactive analysis that extracts data from The Cancer Genome Atlas (TCGA; <https://www.cancer.gov/about-nci/organization/ccg/research/structural-genomics/tcga>) and The Genotype-Tissue Expression (GTEx; <https://gtexportal.org/home/index.html>). Pairwise gene expression correlation analysis was performed with the TCGA data, using the GEPIA2 web server.

Cocultures

Initially, PBMCs were cocultured with MKN45 Mock or MKN45 T5 cells (1:1) during different time points, from 18–96 hours, to select the best time point (18 hours) to use in the subsequent experiments. Cocultures with PBMCs were done in 96-well U plates coated with anti-CD3 (0.1 μ g/well, OKT3, eBioscience). The wells were washed, and 1×10^5 PBMCs were directly cocultured with 1×10^5 MKN45 Mock or MKN45 T5 cells in RPMI supplemented with 10% FBS, penicillin/streptomycin (100 U/mL) and anti-CD28 (0.1 μ g/well,

CD28.2, eBioscience) to induce T-cell activation. After 18 hours, supernatants were collected for cytokine analysis, and the cells were then labeled for CD4⁺ T-lymphocyte transcription factor analysis by flow cytometry, as described below. Three hours before, Brefeldin A (10 ng/mL, Sigma-Aldrich), Phorbol 12-myristate 13-acetate (PMA; 20 ng/mL, Sigma-Aldrich), and ionomycin (200 ng/mL, Sigma-Aldrich) were added to the wells designated for intracellular cytokine expression analysis by flow cytometry. Single cultures of PBMCs, MKN45 Mock, or MKN45 T5 cells were used as controls.

Similar to PBMC cocultures, immature or mature moDCs were cocultured with MKN45 Mock or MKN45 T5 (1:1) in X-VIVO 15 medium. After 18 hours, the supernatants were collected for cytokine analysis, and the moDCs were labeled to analyze their differentiation and activation by flow cytometry. The moDCs were preincubated with inhibitory anti-DC-SIGN (50 ng/mL, 120507, Invitrogen), a C-type lectin that recognizes high-mannose N-glycans and fucose residues, and anti-MR (50 ng/mL, 15–2, Invitrogen), a C-type lectin that recognizes high mannose N-linked glycans, for 1 hour, followed by the addition of MKN45 Mock or MKN45 T5 cells. In addition, transwell cocultures were also performed to determine contact dependency. The moDCs (1×10^5) were cultured in bottom of the plate, while tumor cells (1×10^5) were culture in the transwell (HTS transwell-96-well plate, 0.4- μ m polycarbonate membrane, Corning). Supernatants were collected for cytokine analysis. Single cultures of moDCs, MKN45 Mock, or MKN45 T5 cells were used as controls.

For triple cocultures with tumor cells, moDCs and naïve CD4⁺ T cells, MKN45 Mock or MKN45 T5 (1:1:1) were cultured for 18 hours, and then of CD4⁺ T cells and anti-CD3 (0.1 μ g/well, OKT3, eBioscience) were added in X-VIVO 15 medium. After 96 hours, the supernatants were collected, and the cells were stained against transcription factors and intracytoplasmic cytokines, as done before in PBMCs.

The BMDCs (1×10^5 cells) and MC38 WT or MC38 T5KO (1×10^5 cells) were also cocultured in X-VIVO 15 medium. After 18 hours, the supernatants were collected, and the released cytokines were assessed by ELISA as described below.

In vivo tumorigenicity assays

MC38 cells were treated with kifunensine (KF; 20 μ mol/L; Sigma-Aldrich) for 48 hours and 2×10^5 MC38 WT or treated (MC38 KF) cells were subcutaneously inoculated into the right flank of 6- to 8-week-old C57BL/6 WT mice. After tumor becomes visible, KF (100 μ L, 20 μ mol/L) was injected directly in the tumor every 2 days to maintain KF efficiency. Similarly, MC38 WT and T5KO cells (2×10^5 cells) were subcutaneously inoculated into the right flank of WT and *Rag2^{-/-}Il2rg^{-/-}* C57BL/6 mice. Beginning day 7 postinoculation, tumor size was measured with a caliper every two days, and tumor volume (mm^3) was monitored using the formula $(\text{length} \times \text{width}^2)/2$. Mice were euthanized when one of the tumors from one mouse reached a volume greater than 2,000 mm^3 (human endpoint established). The collected tumors were divided in 2 pieces for explant *ex vivo* assays and to isolate and analyze immune cell infiltrates.

The tumors were manually minced, digested in a dissociation medium (RPMI1640 GlutaMAX medium (Gibco) with collagenase IV (1 mg/mL; Sigma-Aldrich) supplemented with 10% FBS, penicillin/streptomycin (100 U/mL), 1 mmol/L CaCl₂ and 1 mmol/L MgCl₂) for 45 minutes with agitation at 37°C, and subjected to two semiautomated dissociation steps, before and after enzymatic digestion, using the gentleMACS Dissociator (Miltenyi Biotec), program 02.01 and 03.01, respectively, of tumor dissociation, using gentleMACS M Tubes (Miltenyi Biotec). Then, cells were filtered through 70- μ m cell

strainers. The mononuclear cells were enriched by Lymphoprep gradient density centrifugation at $800 \times g$ with no break for 30 minutes. The isolated cells were after incubated with ammonium chloride potassium (ACK) erythrocyte lysing buffer [from in-house made $10\times$ buffer: 1.5 mol/L ammonium chloride (NH_4Cl), 100 mmol/L potassium bicarbonate (KHCO_3), 10 mmol/L EDTA; all from Sigma-Aldrich] for 3 minutes. The cells were then stained for flow cytometry as described below.

The other smaller piece of the tumor was placed in 300 μL of RPMI medium for 24 hours in a 48-well plate. The weight of tumor pieces was determined, and supernatants were stored at -80°C for cytokine analysis by ELISA.

Colorectal cancer spontaneous mouse models— $\text{Apc}^{\text{Min}/+}$ and $\text{VCMsh2}^{\text{LoxP/LoxP}}$

The $\text{Apc}^{\text{Min}/+}$ mice crossed with $\text{Mgat5}^{-/-}$ mice ($\text{Apc}^{\text{Min}/+} \text{Mgat5}^{-/-}$) or WT to Mgat5 were euthanized at 140 days (based on ref. 25). The number and size of lesions per zone of small intestine (proximal, medial, and distal) and colon were evaluated using a caliper. To evaluate the tumor immune infiltrates, a portion of 3 cm of distal small intestine, region with more incidence of lesions, with several lesions (WT), and without or with small lesions ($\text{Mgat5}^{-/-}$) were collected, dissociated as described for the tumorigenicity assays, and CD45^+ cells were purified using Mouse CD45 MACS Microbeads (Miltenyi Biotec) by magnetic separation with MS columns according to manufacturer's instructions. The survival rate of mice was carried out until the same human endpoint established in-house appeared and when animals were euthanized. Kaplan–Meier survival plots were used to evaluate the survival differences between $\text{Apc}^{\text{Min}/+} \text{Mgat5}^{-/-}$ and $\text{Apc}^{\text{Min}/+} \text{Mgat5}^{+/+}$ mice.

$\text{VCMsh2}^{\text{LoxP/LoxP}}$ mice are described to have a median survival around 12 months and develop up to four lesions in small intestine and colon (26), as opposed to the $\text{Apc}^{\text{Min}/+}$ model that develops several lesions. After 330 days, $\text{VCMsh2}^{\text{LoxP/LoxP}} \text{Mgat5}^{-/-}$ or WT background mice were euthanized, and tumor burden was evaluated as described previously. The region with lesion (WT) and a portion of proximal small intestine (region with more incidence of lesions) without lesion ($\text{Mgat5}^{-/-}$) were collected, dissociated, and CD45^+ cells were isolated, as described above, for flow cytometry analysis.

Another portion was placed in 300 μL of RPMI medium for 24 hours in 48-well plates. The weight of tumor pieces was determined, and supernatants were stored at -80°C for later cytokine analysis by ELISA.

Flow cytometry

For lectin staining, performed in tumor cell lines and isolated tumor cells (1×10^6), the cells were incubated with conjugated lectins (Vector Laboratories): L-PHA-fluorescein, GNA-fluorescein, and ConA-biotin for 15 minutes after incubation with FVD–APC–eFluor780 (eBioscience). The biotinylated lectin was then incubated with streptavidin-PE (BioLegend) for 30 minutes.

PBMCs and triple cocultures were stained for transcription factors and intracellular cytokines. For transcription factors, dead cells were excluded by staining with FVD–APC–eFluor780 (eBioscience), followed by extracellular staining with anti- CD45 -BV510 (HI30, BioLegend) and anti- CD4 -eFluor450 (RPA-T4, eBioscience). For intracellular staining of transcription factors, fixation and permeabilization were performed using Foxp3/Transcription Factor Staining Buffer Set (eBioscience) according to manufacturer's instructions. After blocking with 2% mouse serum (obtained in-house from C57BL/6 mouse), anti-Tbet-PerCP-Cy5.5 (eBio4B10, eBioscience) was added.

Intracellular cytokine analysis was performed using Golgi blockade with Brefeldin A. Cells were collected, washed, and stained with FVD-eFluor780, followed by anti- CD45 -BV510 (HI30, BioLegend) and anti- CD4 -PerCP-Cy5.5 (RPA-T4, BioLegend). Cells were washed and fixed with 2% formaldehyde (PanReac ApplieChem). Permeabilization was done with 0.5% saponin (Sigma-Aldrich), and staining with anti-IFN γ -APC (4S.B3) and anti-TNF α -AF488 (MAB11; all from eBioscience) was performed.

For cell surface markers analysis on moDCs, cells from cocultures were stained with anti- CD45 -BV510 (HI30, BioLegend), anti- CD11c -FITC (BU15, Immunotools) or anti- CD11c -APC (BU15, eBioscience), anti- CD14 -PE (MEM-18, Immunotools) or anti- CD14 -PE (61D3, eBioscience), anti-HLA-DR-PerCPy7 (G46-6, BD Pharmingen) or anti-HLA-DR-PerCPy7 (L243, eBioscience), and anti- CD86 -PE-Cy5 (IT2.2, eBioscience) after FVD staining and Fc γ R blocking (Fc Receptor binding inhibitor polyclonal antibody, eBioscience).

For tumorigenicity assays, single-cell suspensions enriched in mononuclear cells were stained with anti- CD45 -FITC (30-F11), anti- CD3 -eFluor 506 (17A2), anti- CD4 -eFluor 450 (RM4-5), anti- CD8a -PE-Cy7 (53-6.7), anti- CD279 -PE (J43), and anti-TCR $\gamma\delta$ -APC (eBioGL3) to assess adaptive immune responses, or with anti-MHCII-Per-Cy5 (M5/114.15.2), anti- CD24 -PE (M1/69), anti- CD64 -APC (X54-5/7.1), anti- CD11c -eFluor 450 (N418), anti- CD11b -eFluor 506 (M1/70), and anti- CD206 -PE-Cy7 (MR6F3; all from eBioscience) to assess innate immune responses after staining with FVD and blocking with 2% rat serum (StemCell Technologies).

For tumors from spontaneous models, isolated CD45^+ cells were stained with FVD-eFluor780 before the staining with anti- CD3 -FITC (17A2, Invitrogen), anti- CD279 -PE (J43, eBioscience), anti- CD4 -PerCP-eFluor 710 (GK1.5, eBioscience), anti- CD8a -V500 (53-6.7, BD Horizon), and anti- CD25 -PE-Cy7 (PC61.5, eBioscience). After fixating and permeabilizing the cells using the Foxp3/Transcription Factor Staining Buffer Set (eBioscience) according to manufacturer's instructions, cells were blocked with 2% rat serum and stained with anti-Foxp3-APC (FJK-16s, Invitrogen) and granzyme B (GZMB)-eFluor450 (NGZB, eBioscience). To determine the innate immune profile, the same protocol used in tumorigenicity assays was performed. Samples were acquired on a FACSCanto II machine (BD Biosciences) and the data were analyzed using FlowJo software v10. The gating strategy to assess to all cell populations is presented in the Supplementary Data.

ELISA and Cytometric Bead Array

Supernatants from tumor explants were collected and the concentrations of TNF α , IFN γ , IL1 β , IL17A (mouse ELISA Ready-SET-Go! Kits from eBioscience) and IL10 (mouse DuoSet ELISA Kit from R&D systems) were measured by ELISA, according to the manufacturer's protocol and using as substrate 3,3',5,5'-tetramethylbenzidine (TMB, eBioscience) and stop solution 2 N H_2SO_4 . The absorbance was detected using a microplate reader (Biotek Instruments) at 450 nm and 570 nm, the concentration was determined using a standard curve, and the results were posteriorly normalized to the tumor explant dry weight (g).

For cocultures, cytokine concentrations were analyzed by flow cytometry using two different Cytometric Bead Arrays (CBA): the BD Cytometric Bead Array Human Th1/Th2/Th17 Kit and the BD Cytometric Bead Array Human Inflammatory Cytokine Kit (BD Biosciences), following manufacturer's instructions. The samples were measured on BD Accuri C6 Machine (BD Biosciences) using a specific template provide by BD Biosciences. The human TGF β 1 DuoSet

ELISA (R&D Systems) was also used to assess the concentration of TGFβ in mDCs cocultures.

C-type lectin receptor-binding glycan assay

The production of the C-type lectin receptor (CLR)-Fc fusion proteins used in this experiment was performed as described previously (27, 28). SDS-PAGE with subsequent Coomassie stain, as well as Western blot analysis, were performed to check the identity and purity of the respective CLR-Fc fusion proteins. CLR-Fc concentrations were determined using the Micro BCA Protein Assay Kit (Thermo Fisher Scientific). The glycan domains were assessed by flow cytometry, where MKN45 Mock and MKN45 T5 cells were first stained with FVD-APC-eFluor780; eBioscience), followed by incubation with DC-SIGN-Fc (2 μg/mL) and MGL1-Fc (2 μg/mL) fusion proteins in lectin-binding buffer (50 mmol/L HEPES, 5 mmol/L MgCl₂, and 5 mmol/L CaCl₂) for 1 hour at 4°C. MGL-1, which recognizes galactose residues, was used because that the glycan motifs recognized in humans are similar to those recognized in mice, as described previously (29). After washing with lectin-binding buffer, the cells were stained with a PE-conjugated goat anti-human Fc (Dianova) for 25 minutes at 4°C. Samples were analyzed on a BD FACSCanto II machine (BD Biosciences), and the data were analyzed using FlowJo v10 software (FlowJo LLC).

Immunofluorescence

FFPE sections from small intestine of *Apc^{Min/+} Mgat5^{+/+}* and *Apc^{Min/+} Mgat5^{-/-}* mice ($n = 4/\text{group}$) were deparaffinized and hydrated as before, and antigen retrieval was performed using vapor heat for 40 minutes (1 mol/L citrate buffer, pH = 6). The slices are blocked with 10% BSA and 20% rabbit normal serum (Vector Laboratories) for 1 hour and incubated with primary anti-IFNγ-APC (5 μg/mL, XMG1.2) or anti-IL17A-PE (5 μg/mL, eBio17B7) diluted in 5% BSA overnight. Nuclei were stained with DAPI, and cell fluorescence was visualized using the fluorescence microscope Zeiss Axio Imager Z1 (Carl Zeiss, Germany). Analysis was performed in Fiji software (ImageJ, NIH, Bethesda, MD), and qualitative classification (±, +, ++, +++) was used to score three different areas from the same tissue. The mean of staining for each was considered to classify the samples.

Microbiome and 16S bioinformatic analysis

Before euthanasia, the feces from *Apc^{Min/+} Mgat5^{+/+}* and *Apc^{Min/+} Mgat5^{-/-}* were directly collected from mice into Eppendorf tubes and frozen immediately in liquid nitrogen. Bacterial amplicon sequence analysis targeted a 16S DNA gene fragment comprising the V3/V4 hypervariable regions (16S sense 5'-TACGGRAGGCAGCAG-3' and antisense 5'-CTACCGGGTATCTAAT-3'). 16S amplicon library preparation was performed according to an optimized and standardized protocol (Metabiot, GenoScreen). In brief, PCR of the 16S DNA was performed using 5 ng of genomic DNA according to the manufacturer's protocol (Metabiot), utilizing bar-coded primers (Metabiot MiSeq Primers) at 0.2 μmol/L final concentration. An annealing temperature of 50°C for 30 cycles was used. The Agencourt AMPure XP-PCR purification system (Beckman Coulter) was used to purify PCR products and following quantification according to the manufacturer's instructions, they were multiplexed at equal concentrations. 250 bp paired-end sequencing was performed on an Illumina MiSeq platform (Illumina) at GenoScreen (accession number PRJNA660736). Raw paired-end reads were subjected to the following processes: (i) trimming of low-quality bases from the 3' end (quality <30, based on the Phred algorithm); (ii) paired-end read assembly

using fast length adjustment of short reads to improve genome assemblies (30), requiring a minimum overlap of 30 bases and a 97% overlap identity; and (iii) removal of forward and reverse primers using CutAdapt (31), with no mismatches allowed in the primer sequences. Sequences without perfectly matching forward and reverse primers were eliminated.

The merged sequences were analyzed using Deblur in the Qiime 2 environment (version 2019.7), which allows for theoretical single-nucleotide resolution [although we continued to use the conventional operational taxonomic unit (OTU) nomenclature]. Taxonomic classification was performed using a classifier trained specifically on the primers (available through the Qiime2 pipeline (version 2019.7) using the Silva reference database (version 132; www.arb-silva.de/silva-license-information). Bacterial OTUs that could not be assigned at the Phylum-level were excluded. Alpha diversity was estimated by the Shannon diversity index or the number of observed OTUs. Differences in diversity between *Apc^{Min/+} Mgat5^{+/+}* and *Apc^{Min/+} Mgat5^{-/-}* were tested using the Wilcoxon rank-sum test. Diversity values are expressed as median and range. Comparisons in taxonomic composition were made using the linear discriminant analysis with effect size (LEfSe) algorithm using recommended parameters. Statistical analysis and plotting were performed in R (version 3.6.1) using the phyloseq (version 1.28.0), ggplot2 (version 3.2.1), and ggpvr (version 0.2.3) packages. The single OTU assigned to the genus *Enterococcus* had the sequence (TAGGGAATCTTCGGCAATGGACGAAAGTCTGACC-GAGCAACGCCGCGTGAGTGAAGAAGTTTTCCGATCGTAA-AACTCTGTTGTAGAGAAGAACAAGGACGTTAGTAAGTGA-ACGTCCTCCCTGACGGTATCTAACAGAAAGCCACGGCTAAC-TACGTGCCAGCAGCCGCGTAATACGTAGGTGGCAAGCGT-TGTCCGGATTATTGGGCGTAAAGCGAGCGCAGGCGGTTT-CTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGG-GTCATTGGAAACTGGGAGACTTGAGTGCAGAAGAGGAGAG-TGGAATTCCATGTGTAGCGGTGAAATGCGTAGATATATGG-AGGAACACCAGTGGCGAAGGCGGCTCTCTGGTCTGTAAC-TGACGCT). This was submitted to nucleotide blast (megablast) against the 16S ribosomal RNA sequence database using standard settings. Raw sequence data are accessible in the Sequence Read Archive (accession number PRJNA660736).

Quantification and statistical analysis

Data were processed using GraphPad Prism 7.0 (GraphPad Software) or SPSS v26 Software (IBM). Flow cytometry analysis was collected with BD FACSDiva v8.0.3 Software (BD Biosciences) and analyzed with FlowJo v10 Software (FlowJo LLC). Means and SEM or SD are presented as averages and error bars as indicated in the figure legends. For *in vitro* studies, points represent biological replicates, from mean of technical duplicates/triplicates, and the bars represent the mean of different experiments ± SD. When described, the mean fluorescence intensity (MFI) or % of cells were normalized (fold change) to the mean of MKN45 Mock cocultures of each experiment. Unpaired *t* test was performed to compare two different groups. Ordinary one-way or two-way ANOVA was performed to compare more than two experimental groups. Tumor measurements of *in vivo* experiments are represented as tumor volume (mm³) and represented as mean tumor volumes of a group of mice ± SEM and a two-way ANOVA was performed. Survival differences between groups were analyzed by Kaplan-Meier estimator, performed by SPSS v26 Software (IBM). Multiple *t* tests were used to compare different immune populations, determined by flow cytometry, between the groups of mice. Statistical significance was considered at $P \leq 0.05$, with (*), (#), (§), or (\$) symbols.

representing the following scheme: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; and ****, $P < 0.0001$.

Results

Complex branched N-glycans are upregulated during colorectal cancer: Correlation with Tregs

Using a well-characterized cohort of human clinical samples from colorectal carcinogenesis, we evaluated the correlation between the *in situ* expression of β 1,6-GlcNAc-branched N-glycans and the local tumor immune response. We showed a clear increased expression of branched N-glycans, predominantly at the tumor cell surface, that were positive for L-PHA lectin, along colorectal carcinogenesis (Fig. 1A). This gradual increase of β 1,6-GlcNAc-branched N-glycan expression occurred early, reaching the highest expression in high-grade dysplasia and colon adenocarcinoma (Fig. 1A). In colon adenocarcinoma stage, low expression of mannosylated N-glycans (positive to GNA lectin) were detected (Fig. 1A). This *in situ* glycoprofile was accompanied with an increase in Foxp3-expressing regulatory T cells (Tregs) and decreased expression of Tbet-expressing cells in the tumor microenvironment (Fig. 1A; Supplementary Fig. S1A). This colon cancer-associated glycoimmune phenotype suggests a potential relationship between branching N-glycans and the local tumor immune response.

This correlation was further validated at the transcriptional level (Fig. 1B–D), where a significant increase of *MGAT5* mRNA expression (gene encoding GnT-V, mediate β 1,6-GlcNAc-branched N-glycan) was observed from normal to dysplasia stages, with a slight decrease in colon adenocarcinomas (Fig. 1B). Concomitantly with the upregulation of *MGAT5* expression, the *FOXP3* expression also increased gradually from normal to dysplasia to colon adenocarcinoma (Fig. 1C). This further supported a correlation between *MGAT5* and *FOXP3* expression, both at protein/glycan and mRNA levels. The Pearson correlation coefficient, performed at normal, dysplasia, and colon adenocarcinoma stages, revealed a significant positive association between *MGAT5* and *FOXP3* expression (Fig. 1D). To further validate these results, we used data from TCGA and GTEx databases, and we observed a significant increase in *MGAT5* and *FOXP3* mRNA expression in this larger cohort of patients with colorectal cancer compared with the normal context (Fig. 1E). A significant positive correlation between *MGAT5* mRNA expression and *FOXP3* mRNA expression or a Treg signature (composed of *ENTPD1*, *FOXP3*, and *CTLA4* genes) were also observed in this large colorectal cancer cohort (Fig. 1F).

The analysis of other glycoenzymes that participate in N-glycan branching pathway showed a trend increase of *MAN2A1* (encoding α -mannosidase II) expression and a trend decrease in *MGAT3* (encoding GnT-III glycosyltransferase) expression in colon cancer compared with normal colon (Supplementary Fig. S1B and S1C), which is in line with the upregulation of *MGAT5*-mediated branched N-glycans. Although without statistical significance, a decreased expression of *POMT1/2* (encoding O-mannosyltransferase) was also observed in colon cancer compared with normal controls (Supplementary Fig. S1D), in accordance with a decreased expression of mannose glycans observed in colorectal cancer (Fig. 1A). This transcriptional profile further supported the observed overexpression of branched N-glycans and the downregulation of mannose-enriched glycans along colorectal carcinogenesis.

To validate these observations, we analyzed a gastric carcinogenesis cascade. We observed that during gastric carcinogenesis, branched N-glycan expression also gradually increased, early in premalignancy

(gastric atrophy/metaplasia stage; Supplementary Fig. S1E). At the gastric adenocarcinoma stage, overexpression of branched N-glycans was also accompanied by an increased frequency of Foxp3⁺ cells and low frequency of Tbet-expressing cells compared with normal gastric mucosa (Supplementary Fig. S1E). Using data from TCGA, we also observed a significant increase of *MGAT5* and *FOXP3* mRNA expression in gastric cancer compared with normal stomach (data from GTEx), further supporting our data (Supplementary Fig. S1F). A significant positive correlation between *MGAT5* mRNA expression and the Treg signature were also observed in the same gastric cancer cohort (Supplementary Fig. S1G). Taken together, these results suggest a positive correlation between the expression of β 1,6-GlcNAc-branched N-glycans at the tumor cell surface (concomitantly with low mannose-enriched glycans) and an increased differentiation and/or recruitment of Foxp3⁺ Tregs associated with immunosuppression in the tumor microenvironment.

Branched N-glycans expression hampers immune recognition of tumor cells

To gain further insights on the impact of the overexpression of β 1,6-GlcNAc-branched N-glycans by tumor cells in the modulation of the surrounding immune response, we performed short-term cocultures using the gastrointestinal cancer cell line MKN45, stably transfected with GnT-V enzyme (MKN45 T5) and overexpressing β 1,6-GlcNAc-branched N-glycans (with reduced levels of mannosylated glycans; Supplementary Fig. S2A–B). Short-term cocultures of MKN45 T5 or Mock cells and PBMCs from healthy donors were performed for 18, 24, 48, and 72 hours. Immunophenotyping (gating strategy in Supplementary Fig. S2C) and cytokine release were analyzed. The most pronounced differences were observed at 18 hours, and therefore this time point was selected for further experiments (Supplementary Fig. S2D). We observed a significant decrease in the MFI of Tbet and IFN γ in CD4⁺ T cells when cancer cells overexpressed branched glycans, compared with Mock cells (Fig. 2A and B). We also detected decreased IFN γ in the supernatants of PBMCs and MKN45 T5 cell cocultures (Fig. 2C). We further observed that overexpression of branched N-glycans by MKN45 T5 cells is associated with decreased expression of HLA-ABC (MHC-I) in tumor cells, when compared with Mock cells, further supporting a role of branched N-glycans on immune escape (Fig. 2D). Higher expression of MHC-I was observed intracellularly in MKN45 T5 cells when compared with cell surface expression, suggesting an internalization of MHC-I when branched N-glycans were overexpressed in tumor cells (Fig. 2D).

Considering antigen-presenting cells (APC), such as DCs, express glycan-recognizing receptors, namely DC-SIGN and MR, we cocultured tumor cells with moDCs from healthy donors to further explore the cross-talk between tumor cells overexpressing branched N-glycans and immune cells. No differences in the expression of surface/activation markers CD86 and HLA-DR was observed in different cocultures (Supplementary Fig. S2E). However, analysis of the supernatants from MKN45 T5 cocultures showed a significantly decreased concentration of proinflammatory cytokines IL6 and IL8 compared with Mock cell cocultures (Fig. 2E). A trending increase of the inhibitory cytokine TGF β was also observed when moDCs were cocultured with MKN45 T5 cells (Fig. 2E).

To test whether this immunosuppressive phenotype was mediated by specific glycan-binding proteins (GBP) expressed on moDCs, we analyzed the expression of the glycan-binding domains on MKN45 cells that are recognized by DC-SIGN and macrophage galactose type lectin-1 (MGL-1). We found that MKN45 Mock cells (with increased

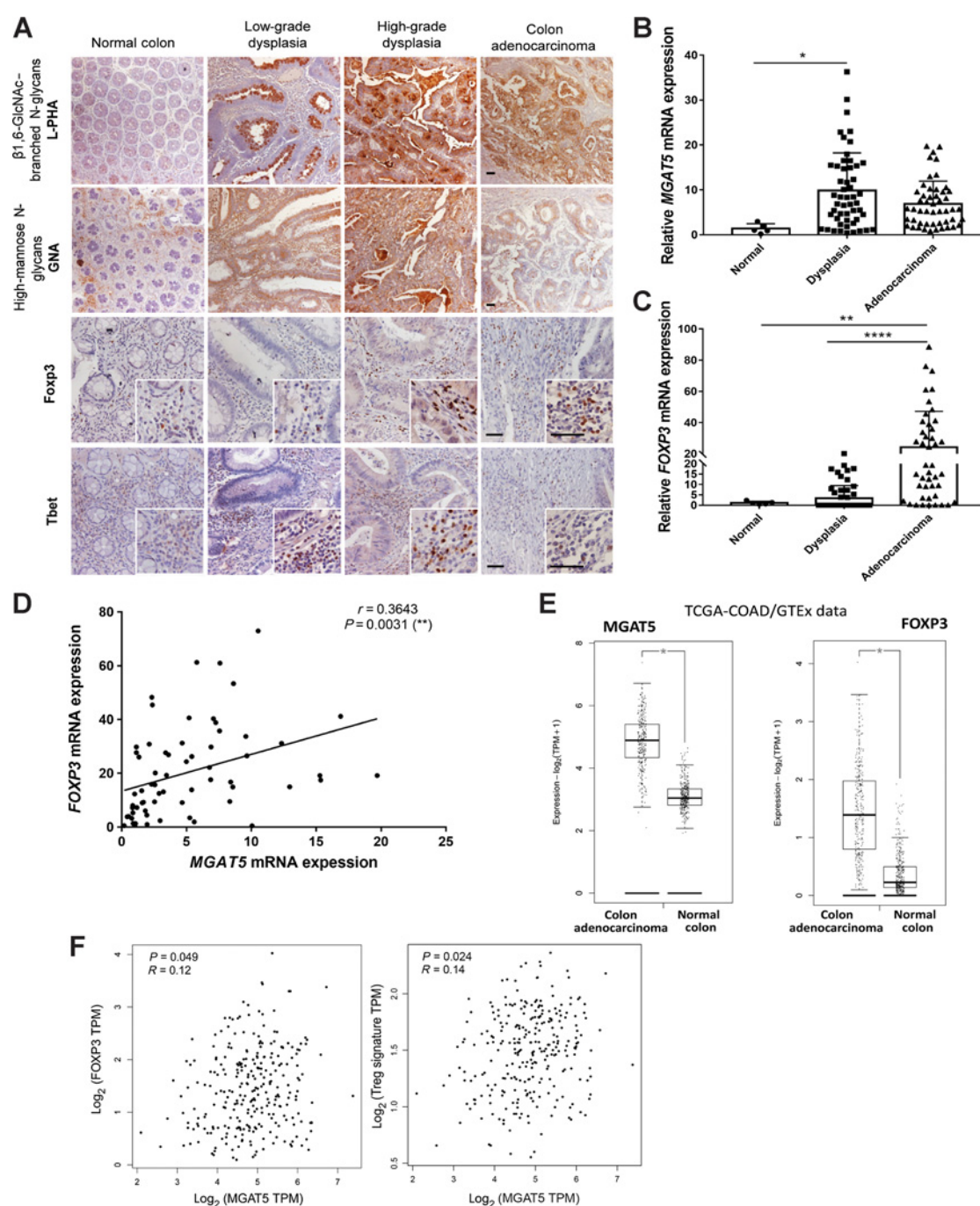
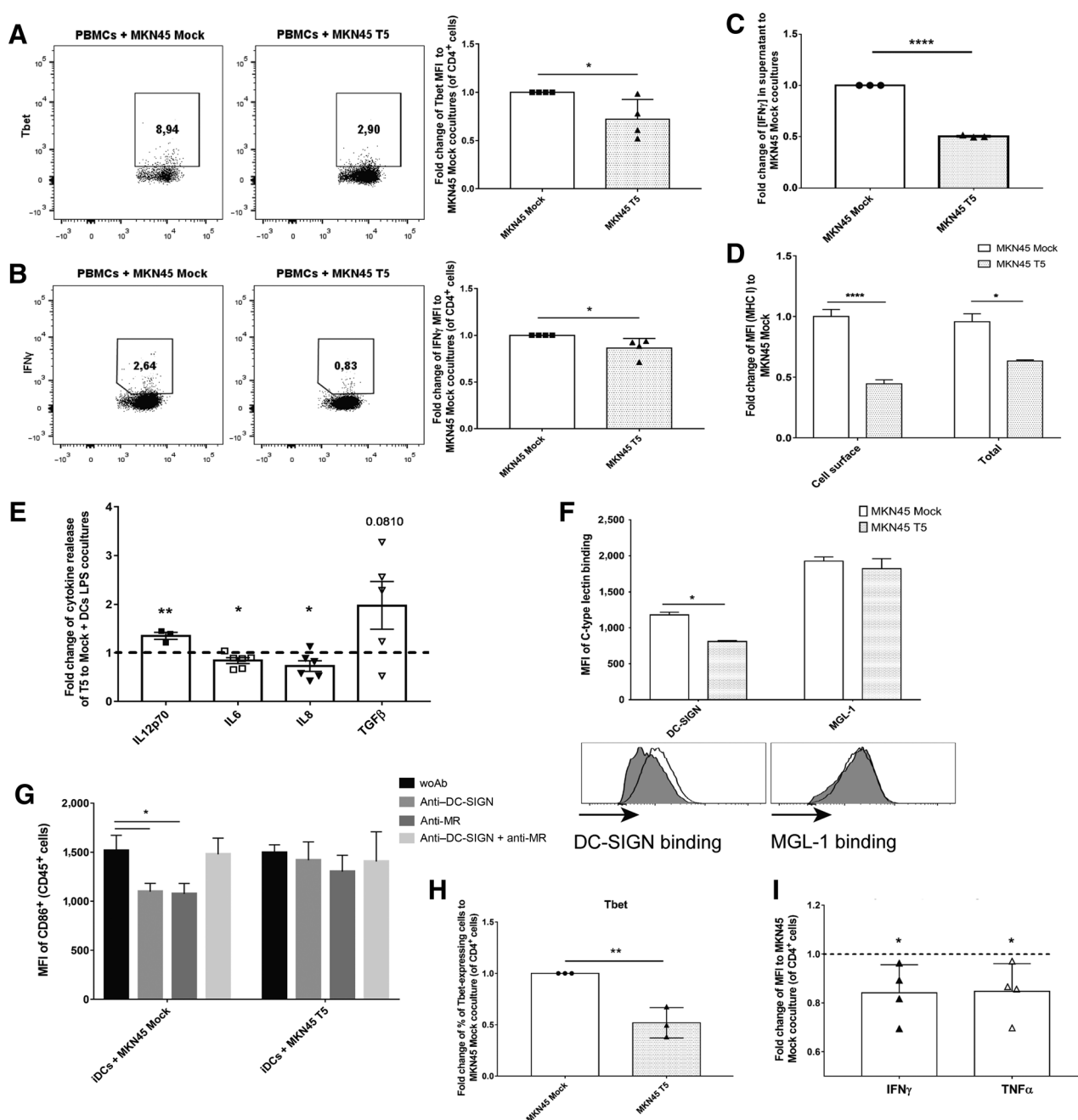


Figure 1.

Overexpression of complex branched N-glycans and downregulation of mannose-enriched glycans during colorectal carcinogenesis. Correlation with regulatory T cells and tumor immunosuppression. **A**, Histochemistry on normal colon, low-grade dysplasia, high-grade dysplasia, and colon adenocarcinoma samples using lectins (L-PHA and GNA) that recognized, respectively, branched N-glycans and mannose glycans, as well as the transcription factors Foxp3 and Tbet. For L-PHA staining: $n = 5$ normal colon, $n = 13$ low-grade dysplasia, $n = 7$ high-grade dysplasia, and $n = 55$ colon adenocarcinoma. For GNA, Foxp3, and Tbet staining: $n = 5$ tissue samples for all stages. Image magnification for L-PHA/GNA: 100 $\times$; image magnification for Foxp3/Tbet: 200 $\times$ and 400 $\times$ (inserts). Scale bar, 50 μ m. Relative expression of *MGAT5* (**B**) and *FOXP3* mRNA (**C**) from FFPE samples of normal colon ($n = 5$), colon dysplasia (low + high-grade dysplasias, $n = 65$), and colon adenocarcinoma ($n = 52$). All samples were normalized to 18S housekeeping expression. *, $P < 0.05$; **, $P < 0.01$; and ****, $P < 0.0001$; ordinary one-way ANOVA; error bars represent SEM of different biological samples. **D**, Pearson correlation of *MGAT5* mRNA versus *FOXP3* mRNA expression considering all samples of colorectal carcinogenesis, normal colon, dysplasia, and colon adenocarcinoma samples. **E**, The expression profiling of *MGAT5* and *FOXP3* in colorectal cancer samples ($n = 275$) versus normal colon samples ($n = 349$), using GEPIA2. TCGA-COAD dataset for colon adenocarcinoma samples. One-way ANOVA; *, $P = 0.049$; line represents median. **F**, Pearson correlation analysis of *MGAT5* versus *FOXP3* and *MGAT5* versus the Treg signature (composed of *ENTPD1*, *FOXP3*, and *CTLA4*) in colorectal cancer samples ($n = 275$), using GEPIA2. $P = 0.049$, $R = 0.12$; $P = 0.024$, $R = 0.14$. See also Supplementary Fig. S1. TPM, transcripts per million.

**Figure 2.**

Aberrant overexpression of β 1,6-GlcNAc-branched N-glycans in tumor cells hinders its recognition by DC-SIGN-expressing immune cells, leading to tumor immunosuppression. **A–C**, Cocultures of MKN45 Mock and MKN45 T5 cells with PBMCs activated with anti-CD3 and anti-CD28 for 18 hours. **A**, Tbet MFI gated on CD4⁺ cells in MKN45 T5 versus MKN45 Mock cocultures ($n = 4$). Representative flow cytometry scatter plots with a gate on CD4⁺Tbet⁺ cells are shown (percentage represented). **B**, IFN γ -producing CD4⁺ cells ($n = 4$). Representative flow cytometry scatter plots with a gate on CD4⁺IFN γ ⁺ cells are shown (percentage represented). **C**, IFN γ concentration in coculture supernatants ($n = 3$). **D**, Cell surface and total MFI of HLA-ABC (MHC-I) in MKN45 Mock and MKN45 T5 cells from intra- and extracellular staining ($n = 3$). **E**, moDCs, stimulated with LPS (100 ng/mL) for 48 hours, cocultured with MKN45 cells for 18 hours. Cytokines in the supernatants of cocultures were measured with CBA Human Inflammatory Cytokines Kit or TGF β ELISA ($n = 6$; undetected cytokines were not considered). **F**, Flow cytometry of DC-SIGN-Fc and MGL-1-Fc (MFI) binding to MKN45 Mock and MKN45 T5 cells. Detection used a PE-conjugated goat anti-human Fc. Representative histograms shown; MKN45 Mock (unshaded) and MKN45 T5 (gray). **G**, MFI of CD86 in moDCs, preincubated with anti-DC-SIGN and/or anti-MR (50 ng/mL), in coculture with MKN45 Mock or T5 cells for 18 hours ($n = 3$). iDC, immature moDC. **H** and **I**, Naïve CD4⁺ T cells cocultured with moDCs and MKN45 cells under stimulation with anti-CD3. Frequency of Tbet⁺CD4⁺ T cells ($n = 3$; **H**) and IFN γ - and TNF α -producing CD4⁺ T cells ($n = 4$; **I**) after 96 hours. Fold-change results were normalized to the mean of cocultures with MKN45 Mock cells, represented by the dotted line. *, $P < 0.05$; **, $P < 0.01$; and ****, $P < 0.0001$; Student t test or ordinary one-way ANOVA. Error bars represent SEM of different biological replicates and/or technical replicates. See also Supplementary Fig. S2.

surface expression of mannose-enriched glycans and decreased expression of branched N-glycans; Supplementary Fig. S2B) displayed higher DC-SIGN-recognized glycan domains that significantly decreased with branched glycans overexpression (Fig. 2F). These results suggested that the specific recognition of mannose glycans by DC-SIGN exposed on Mock cells was significantly hampered when branched glycans were overexpressed in MKN45 T5 cells (Fig. 2F; Supplementary Fig. S2B).

To further validate this interplay, we inhibited the glycan-binding recognition of DC-SIGN and MR on MKN45 Mock and T5 cells (Fig. 2G). When Mock cells (display higher mannose-enriched glycans than T5 cells) were cocultured with moDCs, CD86 expression significantly decreased after treatment with inhibitory anti-MR or anti-DC-SIGN (Fig. 2G). This observation supports the role of these GBPs in the immunomodulation, whereby overexpression of branched N-glycans in T5 cells appeared to mask and hamper the recognition of mannose-enriched glycans by DC-SIGN (Fig. 2F and G), thus impairing immune activation. This contact-dependent mechanism between glycans expressed by tumor cells and GBP present on the DC surface was confirmed using transwell assays, which showed that in the absence of cell-cell contact, a decreased production of IL8 was observed (Supplementary Fig. S2F).

To further assess the consequence of DC glycans recognition on T-cell function, triple cocultures with tumor cells, moDCs, and autologous naïve CD4⁺ T cells were performed. CD4⁺ T cells exhibited decreased expression of Tbet, IFN γ , and TNF α in the cocultures with cells overexpressing complex branched N-glycans compared with Mock cells (Fig. 2H and I). Altogether, these evidences support the functional effects of branched N-glycans on tumor cells in the modulation of immunosuppressive networks through masking and hampering immune recognition by specific GBPs, together with an apparent internalization of MHC-I molecules, revealing a “sweet” escape strategy and immune checkpoint in cancer cells.

Removing branched N-glycans exposes immunogenic glycans on colorectal cancer cells

Considering the immunosuppressive profile of branched N-glycans expressed at tumor cell surface, we inhibited the synthesis of branching N-glycosylation using KF, a potent inhibitor of the α -mannosidase I enzyme that blocks the biosynthesis of branching N-glycosylation, generating high mannose glycoproteins. Treatment of MC38 murine colon adenocarcinoma cells with KF (MC38 KF) showed a significant overexpression of high-mannose N-glycans and decreased expression of β 1,6-GlcNAc-branched N-glycans, revealed by the increased reactivity to GNA/ConA lectins and decreased L-PHA binding, respectively (Fig. 3A). The subcutaneous inoculation of MC38 KF cells in C57BL/6 WT immunocompetent mice resulted in a significant delay in tumor growth compared with vehicle-treated MC38 cells (Fig. 3B and C). These results demonstrated that the removal of branched N-glycans promoted the exposure of mannose residues associated with suppression of tumor growth.

To further investigate the role of branched versus nonbranched/mannose-enriched N-glycans in colorectal cancer immunoediting, the *Mgat5* glycogene was silenced using CRISPR-Cas9 in MC38 cells. Among all clones obtained, three different MC38 clones were selected, namely clones 1, 2, and 3, and validated for the presence of insertion-deletions (INDEL) in *Mgat5* gene. The three clones showed the presence of different INDELs on *Mgat5* gene associated with a decrease in *Mgat5* mRNA transcription (Supplementary Fig. S3A and S3B). The three clones also showed lower β 1,6-GlcNAc-branched N-glycans at their cell surfaces, together with higher expression of high-mannose

glycans compared with MC38 WT (Fig. 4A; Supplementary Fig. S3C and S3D). MC38 T5KO clone 2 was chosen to proceed on the basis of the lowest expression of L-PHA-recognizing β 1,6-GlcNAc-branched N-glycans and the highest GNA-recognizing mannose glycans compared with the other clones (Supplementary Fig. S3C). Clone 2 showed no impact on *Mgat5b* mRNA transcription level, which could be associated with a compensatory mechanism for complex branched N-glycans biosynthesis, and no impact on cell proliferation was observed using CFSE MFI versus MC38 WT cells (Supplementary Fig. S3E and S3F).

Tumorigenicity assays were conducted using the selected MC38 T5KO clone 2. We found that this clone into immunocompetent WT mice resulted in a significant suppression of tumor growth compared with controls (MC38 WT; Fig. 4B and C), which was more evident compared with KF-treated cells (Fig. 3B and C) due to the stable and effective removal of the branched N-glycans upon *Mgat5* gene KO. Only 1 of 11 mice in this group developed a very small tumor (Fig. 4C), demonstrating the effects of exposing the mannose structures (Fig. 4A) in the suppression of colorectal cancer development and progression.

To address the effects of this on cancer immunoediting and to determine how this MC38 T5KO glycoprofile modulated the surrounding immune responses, tumors were collected for immunophenotyping of immune infiltrates and evaluation of cytokines. We observed a slight increase in the percentage of infiltrating monocytes, macrophages, and DCs after removal of complex branched N-glycans (MC38 T5KO) compared with MC38 WT tumors (Fig. 4D). The innate immune response analysis is adapted from ref. 32 and described in Supplementary Fig. S4A. For adaptive immune responses (Supplementary Fig. S4B), MC38 T5KO tumors exhibited an increase in the number of CD3⁺ T cells, most likely due to the increase in $\gamma\delta$ T cells, compared with MC38 WT tumors (Fig. 4E). Cytokine analysis of the tumor explants was also in line with an immunostimulatory phenotype, showing higher concentrations of TNF α released by MC38 T5KO tumors compared with controls (Fig. 4F). No major differences were detected in the concentrations of IFN γ and IL1 β between the two conditions (Fig. 4F). Together, these observations, although not significant due to the limitation of tumor number availability for analysis, indicated that removal of β 1,6-GlcNAc-branched N-glycans exposed mannose glycans that contributed to tipping the balance toward immunostimulation and antitumor immune attack. To overcome the aforementioned technical limitation and to further validate our results, MC38 WT and T5KO cells were cocultured with BMDCs, and cytokines were evaluated by ELISA. We observed that MC38 T5KO cells cocultured with BMDCs induced an increased production of the proinflammatory cytokine IL23 compared with controls (Fig. 4G). No major differences were detected in the concentrations of other cytokines (including TNF α , IL1 β , IL12p70, and IL6) at different time points. Together, these results corroborated the proinflammatory and antitumor immune effects of branched N-glycans removal through exposure of immunogenic mannose glycan epitopes.

Branched N-glycans are direct modulators of the immune microenvironment

To further demonstrate the existence of a dynamic cross-talk between tumor-expressing branched N-glycans and immune cells in the microenvironment, MC38 WT and T5KO cells were subcutaneously inoculated in syngeneic immunocompetent C57BL/6 WT mice or immunodeficient *Rag2*^{-/-}IL2 γ ^{-/-} mice (deficient in functional T, B, and natural killer (NK) cells and reduced myeloid cells). Our results showed that the removal of complex branched N-glycans only resulted

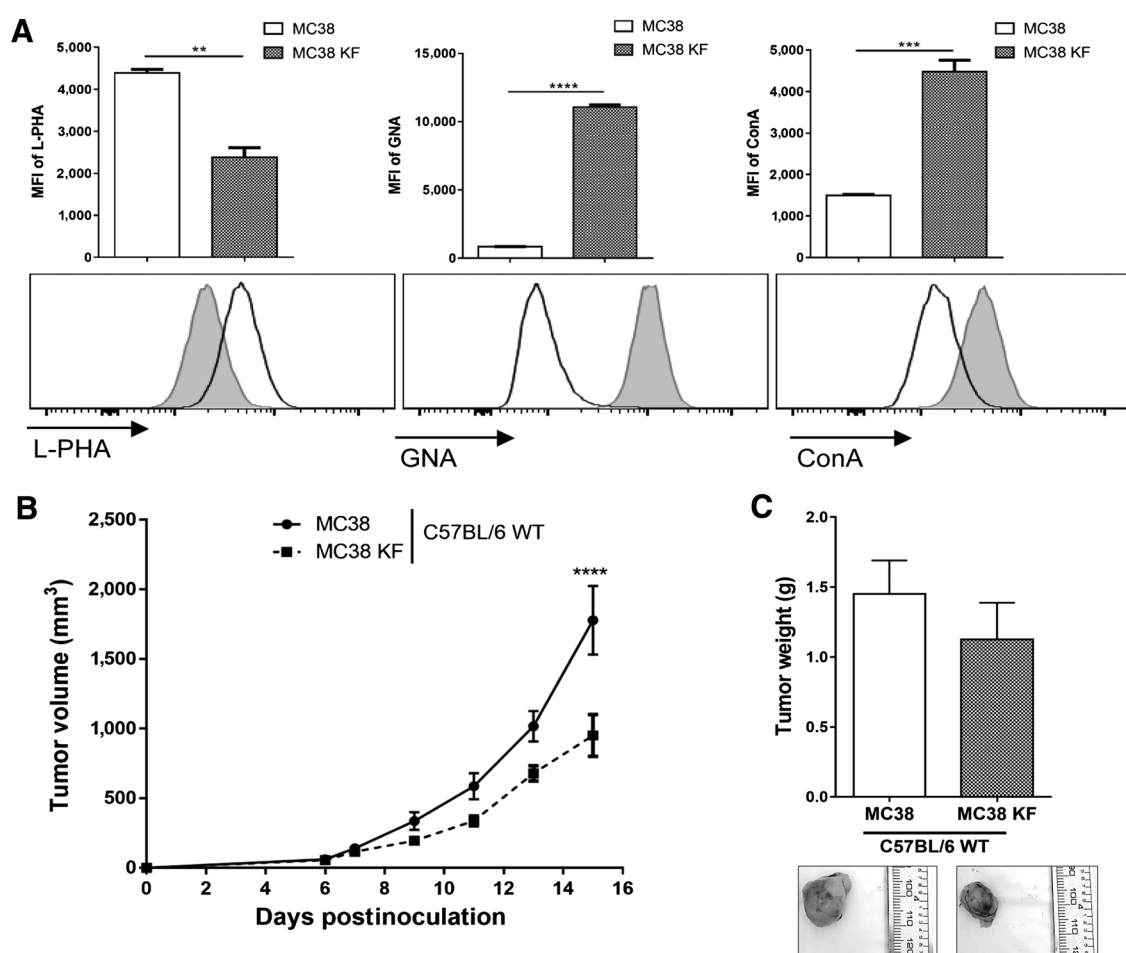


Figure 3.

Treatment with the branched glycan inhibitor KF exposes mannose glycans associated with suppression of tumor growth. **A**, Cell-surface N-glycan profile of MC38 cells untreated or treated with KF analyzed by flow cytometry ($n = 3$). Staining performed with L-PHA-fluorescein, GNA-fluorescein, or ConA-biotin lectins. Lectin binding expressed in MFI. Representative histograms shown; MC38 (unshaded) and MC38 KF (gray). **B**, Tumor growth kinetics ($n = 4$ /group) of MC38 cells, untreated or treated with KF, in C57BL/6 mice until the day of euthanasia, when one of the tumors reached 2,000 mm³. Tumor length and width (mm) were measured every 2 days beginning day 7 postinoculation. **C**, Tumor weight (g) upon euthanasia and representative images of the tumors ($n = 4$ /group). $P = 0.5714$. **, $P < 0.01$; ***, $P < 0.001$; and ****, $P < 0.0001$; Student t test or two-way ANOVA. Error bars represent SEM of different cell line/mouse groups.

in nontumor growth in immunocompetent mice, which demonstrated the dependency on immune recognition of mannose glycans to control tumor growth (Fig. 4H and I). MC38 T5KO cells exposing mannose glycans (through removal of branched N-glycans) in immunodeficient mice exhibited an increase in tumor growth, whereas immunocompetent mice showed regression of tumors (Fig. 4H and I). The same was observed in MC38 WT cells expressing branched N-glycans (Supplementary Fig. S5A–S5C). These results demonstrated the interplay and functional cross-talk between glycans expressed on cancer cells and the immune cells in the tumor microenvironment.

Deletion of branched glycosylation results in spontaneous suppression of colorectal cancer

To translate the observations into a preclinical model of spontaneous colorectal cancer, we crossed the $Apc^{Min/+}$ mice (widely used to study intestinal tumorigenesis; ref. 33) with $Mgat5^{-/-}$ mice and after 140 days (based on ref. 25) the tumor burden was evaluated, as well as the tumor-infiltrating immune cells. The $Apc^{Min/+}Mgat5^{-/-}$ mice developed a lower number of tumor lesions with smaller size in the

different parts of small intestine compared with $Apc^{Min/+}Mgat5^{+/+}$ mice (Fig. 5A–C). Analysis of overall survival showed that $Apc^{Min/+}Mgat5^{-/-}$ mice survived longer compared with $Apc^{Min/+}Mgat5^{+/+}$ mice (Fig. 5D). These findings highlight that β 1,6-GlcNAc-branched N-glycan removal, and the consequent exposure of mannose glycans (Fig. 5E), can inhibit colorectal cancer development and progression.

Given the *in vivo* antitumor effects of the $Mgat5$ deletion, we next examined its impact in the modulation of antitumor responses. A segment of the distal part of the small intestine was collected from $Apc^{Min/+}Mgat5^{+/+}$ and $Apc^{Min/+}Mgat5^{-/-}$ mice and the immunoprofile was analyzed. Our results showed that inhibition of tumor growth in $Apc^{Min/+}Mgat5^{-/-}$ mice was accompanied with an increased infiltration of DCs and lymphocytes, together with a significant decreased expression of MR⁺ M2 protumoral macrophages (Fig. 5F and G). We further showed that colorectal cancer development in $Mgat5$ -null mice was accompanied by a significant decrease of Foxp3⁺ Tregs in the tumor microenvironment and a significant decrease of the immune checkpoint molecule PD-1, predominantly in CD4⁺ T cells (Fig. 5H). Intestinal explant supernatants collected

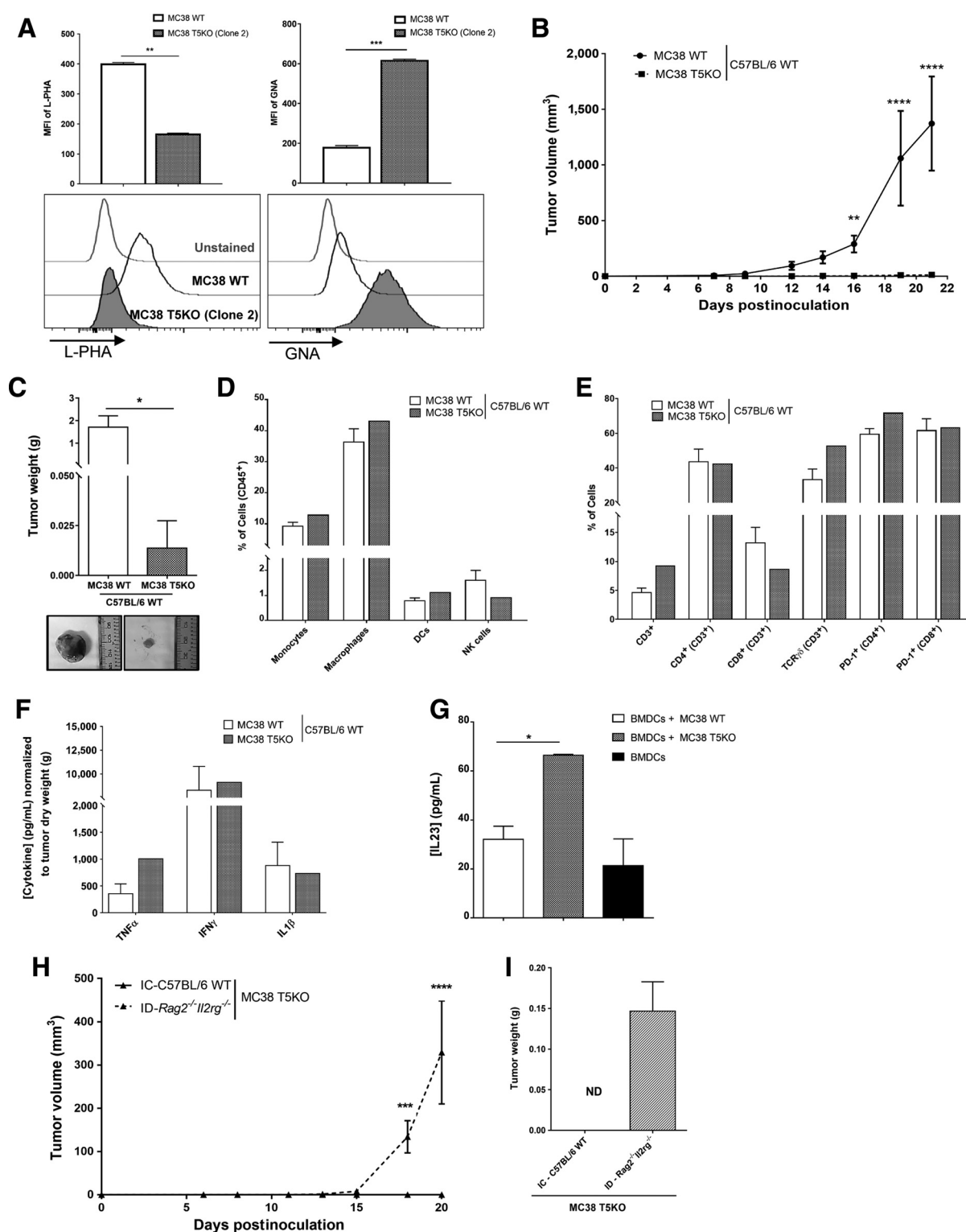


Figure 4.

Mgat5 CRISPR/Cas9 gene knockout in MC38 colorectal cancer cells exposes immunogenic mannose glycans associated with suppression of tumor growth via immunostimulation. **A**, Cell-surface N-glycan profile of MC38 WT and T5KO (clone 2) cells analyzed by flow cytometry after staining with L-PHA-fluorescein or GNA-fluorescein lectins ($n = 3$). Lectin binding expressed as MFI. Representative histograms shown. **B**, Tumor growth kinetics ($n = 11$ /group) of MC38 WT and T5KO cells in C57BL/6 mice until the day of euthanasia, when one of the tumors reached 2,000 mm³. Tumor length and width (mm) were measured every 2 days beginning on day 7 postinoculation. **C**, Tumor weight (g) upon euthanasia and representative images of the tumors ($n = 11$ /group). (Continued on the following page.)

from $Apc^{Min/+}Mgat5^{-/-}$ mice released higher concentrations of proinflammatory cytokines, predominantly IL17A, compared with $Apc^{Min/+}Mgat5^{+/+}$ lesions. The concentration of the anti-inflammatory cytokine IL10 was slightly lower in $Apc^{Min/+}Mgat5^{-/-}$ samples compared with $Apc^{Min/+}Mgat5^{+/+}$ samples (Fig. 5I). In accordance, a higher *Il17a* mRNA expression and a slight decrease of *Il10* expression was detected in $Apc^{Min/+}Mgat5^{-/-}$ mice tissues compared with the controls (Supplementary Fig. S6A). No major differences were observed in *Ifng* mRNA expression (Supplementary Fig. S6A). The data obtained by immunofluorescence also showed a higher expression of IFN γ and IL17A in $Apc^{Min/+}Mgat5^{-/-}$ mice intestinal samples compared with $Apc^{Min/+}Mgat5^{+/+}$ mice (Fig. 5J). We also observed these immunostimulatory effects upon increased exposure of mannose-enriched glycans in *Mgat5* KO mice (Fig. 5E).

As a proof-of-concept of the *in vivo* effects of *Mgat5* deletion in the suppression of colorectal cancer progression, via immunostimulation, we used a different colorectal cancer mouse model based on conditional knockout of *Msh2* in villin-expressing intestinal tissue, $VCMsh2^{LoxP/LoxP}$ mice. These mice develop intestinal adenomas and carcinomas similar to human Lynch syndrome and are microsatellite instability-high (MSI-high). $VCMsh2^{LoxP/LoxP}Mgat5^{-/-}$ mice developed a lower number of intestinal lesions with smaller size than control mice ($VCMsh2^{LoxP/LoxP}Mgat5^{+/+}$; Fig. 6A–C). As was observed in the $Apc^{Min/+}$ model, the immunoprofiling showed that the removal of complex branched glycans also skewed the balance towards immunostimulation, with decreased frequency of inhibitory Foxp3⁺ Tregs, associated with control of tumor growth (Fig. 6D–F). Together, our data demonstrated that the abnormal branched N-glycan expression in colorectal cancer constituted an immune checkpoint mechanism in this cancer type. We demonstrated that glycoengineering through *Mgat5* deletion results in a significant exposure of relevant immunogenic glycan epitopes, such as mannose glycans, leading to suppression of tumor growth associated with immune stimulation and effective antitumor responses.

The gut microbiome composition in the absence of branched N-glycans: Impact on antitumor immune responses

The gut microbiota has been described as a major player in shaping immune response and inflammation (34). Changes in microbiota composition have been associated with major intestinal inflammatory disorders, such as inflammatory bowel diseases (IBD) and cancer (reviewed in refs. 35–37). In the cancer context, it is known that intestinal microbiota contribute to the immune system activation against tumors and response to chemotherapy (38, 39). However, the contribution of glycans to the composition and function of the gut microbiota, and their potential implications in the immunomodulation associated with cancer progression, remains completely unknown. To establish a correlation between changes in N-glycan expression, upon *Mgat5* deletion, and the modulation of gut microbiota composition associated with antitumor immune responses

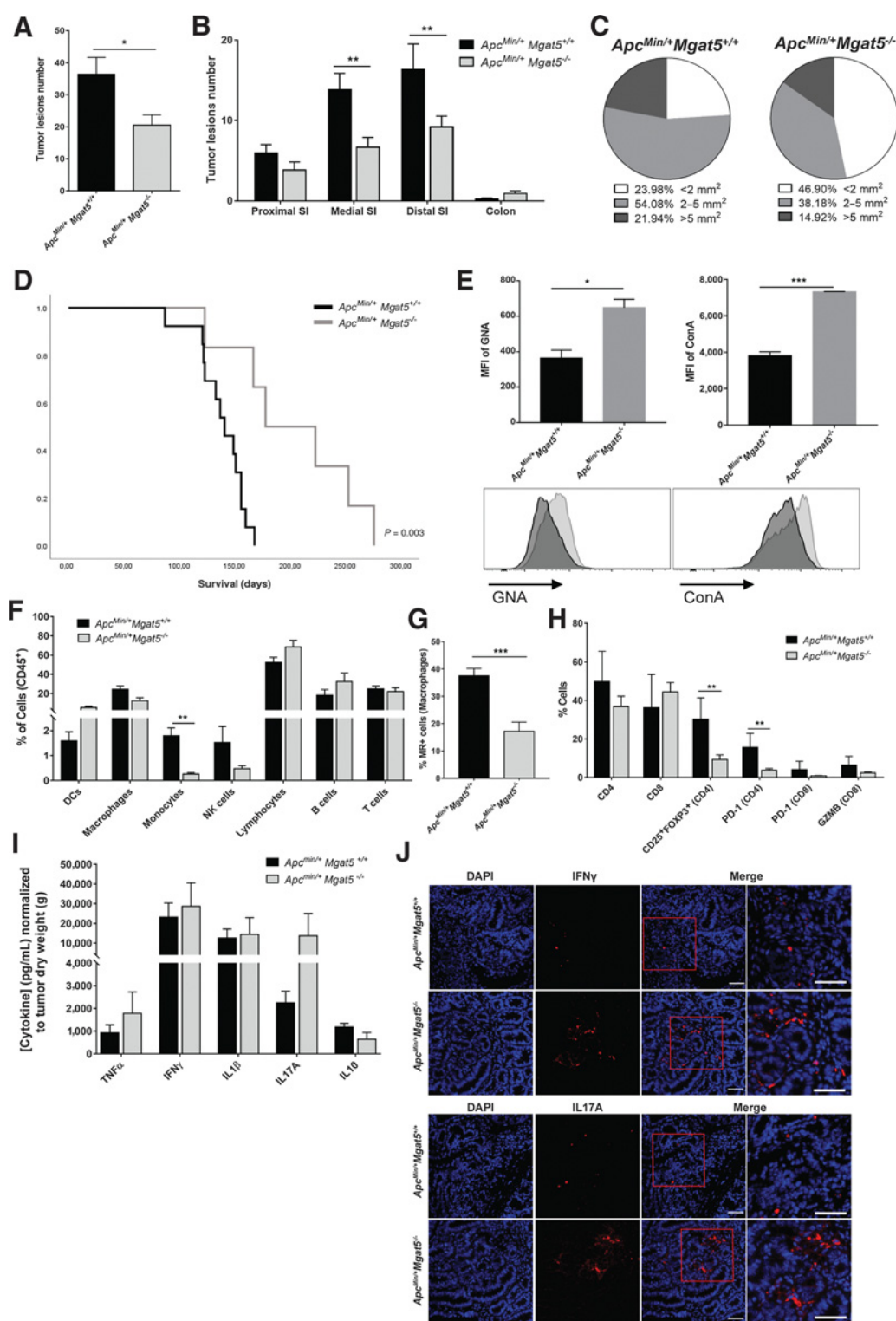
in colorectal cancer, stools from $Apc^{Min/+}Mgat5^{+/+}$ and $Apc^{Min/+}Mgat5^{-/-}$ mice were collected, and the microbiome composition was analyzed by 16S rDNA gene sequencing. We observed a reduction in alpha diversity in $Apc^{Min/+}Mgat5^{-/-}$ mice compared with $Apc^{Min/+}Mgat5^{+/+}$ mice (Fig. 7A). Alterations in composition were also detected between $Apc^{Min/+}Mgat5^{+/+}$ and $Apc^{Min/+}Mgat5^{-/-}$ mice (Fig. 7B and C). Our results suggest that the absence of complex branched N-glycans modulated the gut microbiota composition, with an increase in *Enterococcus* in $Apc^{Min/+}Mgat5^{-/-}$ mice compared with $Apc^{Min/+}Mgat5^{+/+}$ mice (Fig. 7C). The single strain of *Enterococcus* detected, although not assigned to a species by the classifier, had a 100% 16S sequence identity match to *Enterococcus faecalis* NBRC 100480 and other *Enterococcus faecalis* strains by blast. In accordance, it was reported that different strains from *Enterococcus* bacteria exhibited an immunostimulatory effect, being able to promote DC activation and IFN γ production and associated with reduction of polyps progression in $Apc^{Min/+}$ mice, which supports the hypothesis that these microorganisms have a role in the modulation of the tumor microenvironment (40–42). Altogether, our data suggest that the absence of branched N-glycan expression potentiates an effective antitumor immune response associated with the modulation of intestinal microbiota composition and function.

Discussion

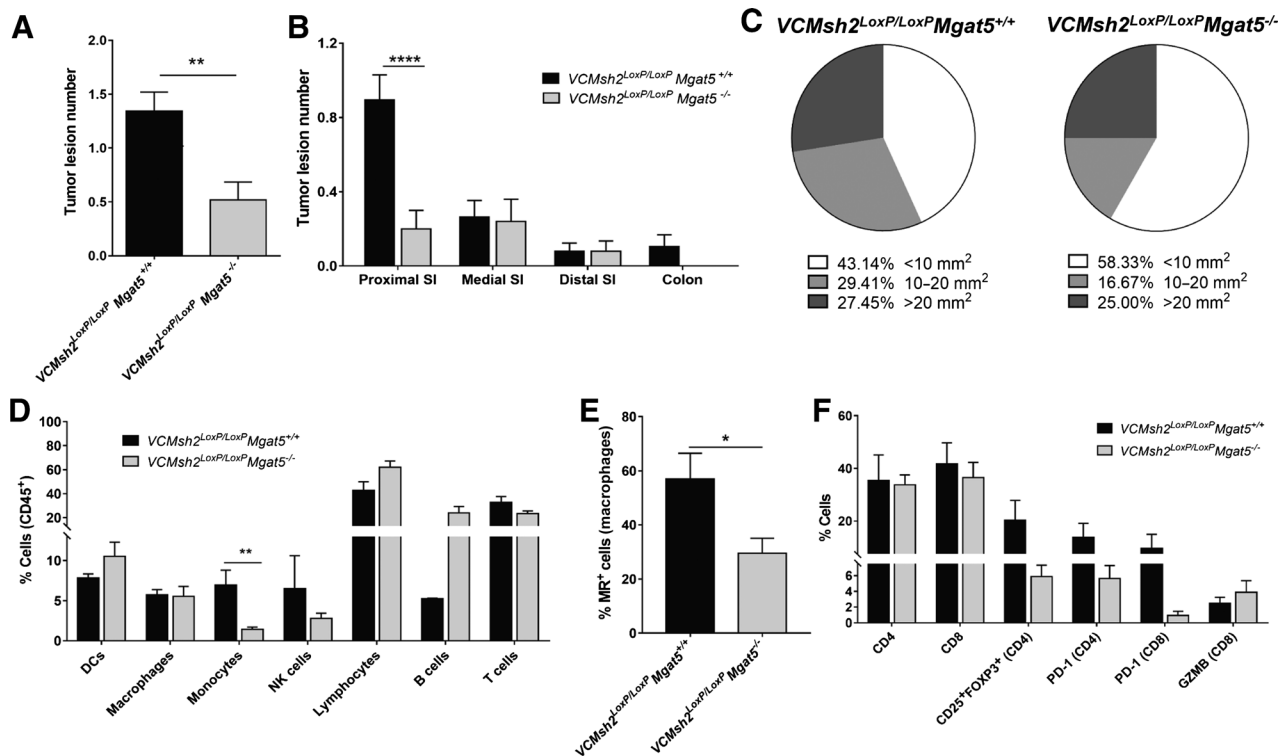
Changes in protein glycosylation is a hallmark of cancer development and progression, and particularly, the overexpression of branched N-glycans by cancer cells is one of the most widely occurring cancer-associated glycan modifications (13–15). In gastrointestinal cancer, overexpression of GnT-V-mediated branched N-glycans by cancer epithelial cells exert a proinvasive and prometastatic behavior in cancer cells through mechanistic interference with tumor cell adhesion (13–15). The detection of this aberrant glycoform in patients with gastric cancer also correlates with poor survival (16). The role of GnT-V-mediated branched N-glycans in chronic inflammatory gastrointestinal disorders, such as IBD, has been demonstrated and shows that branched N-glycans regulate T-cell-mediated immune responses (22, 35, 43, 44). However, whether this abnormal expression of cancer-associated branched N-glycans is associated with immune modulation and immune evasion correlated with cancer progression remains unknown.

The immune system is primed with a variety of different glycan-binding receptors (collectively designated as lectins) that have the ability to sense changes in cellular glycosylation and instruct either immunosuppressive or immunostimulatory responses. GBPs involved in glycan immune recognition and signaling include the C-type lectins, galectins, and siglecs and can be expressed or secreted by DCs, macrophages, B cells, T cells, and NK cells (21, 45). Changes in both O- and N-linked glycans occur in cancer (11) and both types of glycosylation can be sensed and recognized by specific immune cell glycan-binding receptors (46, 47). We previously reported a

(Continued.) **D**, Percentage of innate immune cells within MC38 WT ($n = 6$) and MC38 T5KO ($n = 1$, the only case that developed a full tumor mass) tumors in C57BL/6 mice. Gating strategy to assess different immune populations represented in Supplementary Fig. S4A. NK, natural killer. **E**, Percentage of adaptive immune cells within MC38 WT ($n = 6$) and MC38 T5KO ($n = 1$) tumors in C57BL/6 mice. Gating strategy to assess different immune populations represented in Supplementary Fig. S4B. **F**, Concentrations of TNF α , IFN γ , and IL1 β (pg/mL) after 24 hours in the supernatants of tumor explants of MC38 WT ($n = 6$) and MC38 T5KO ($n = 1$) tumors developed in C57BL/6 mice, evaluated by ELISA. Cytokine concentrations were normalized to tumor dry weight (g). **G**, Concentration of IL23 (pg/mL) released by BMDCs in coculture with MC38 WT ($n = 3$) and MC38 T5KO ($n = 3$) for 18 hours, evaluated by ELISA. **H**, Tumor growth kinetics ($n = 7$ /group) of MC38 T5KO cells in immunocompetent (IC) C57BL/6 mice or immunodeficient (ID) $Rag2^{-/-}Il2rg^{-/-}$ mice until the day of euthanasia, when one of the tumors reached 2,000 mm³. Tumor length and width (mm) were measured every 2 days beginning day 7 postinoculation. **I**, Tumor weight (g) upon euthanasia ($n = 7$ /group). ND, not determined. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$; Student *t* test or two-way ANOVA. Error bars represent SEM of different mouse groups. See also Supplementary Figs. S3 and S4.

**Figure 5.**

In vivo glycoengineering of the *Apc^{Min/+}* mouse model by MGAT5 KO results in spontaneous suppression of colorectal cancer associated with an effective antitumor immune response. **A**, Total number of tumor lesions per mouse in *Apc^{Min/+} Mgat5^{+/+}* ($n = 23$) and *Apc^{Min/+} Mgat5^{-/-}* ($n = 25$) mice. Mice were euthanized at 140 days. **B**, Tumor number lesions per intestinal area (proximal small intestine, medial small intestine, distal small intestine, and colon) in *Apc^{Min/+} Mgat5^{+/+}* ($n = 23$) and *Apc^{Min/+} Mgat5^{-/-}* ($n = 25$) mice. **C**, Percentage of lesions per tumor size (mm²): <2 mm², 2 to 5 mm², and >5 mm² in both genotypes of *Apc^{Min/+}*. **D**, Kaplan-Meier survival curve for *Apc^{Min/+} Mgat5^{+/+}* ($n = 13$) and *Apc^{Min/+} Mgat5^{-/-}* ($n = 6$) mice. (Continued on the following page.)

**Figure 6.**

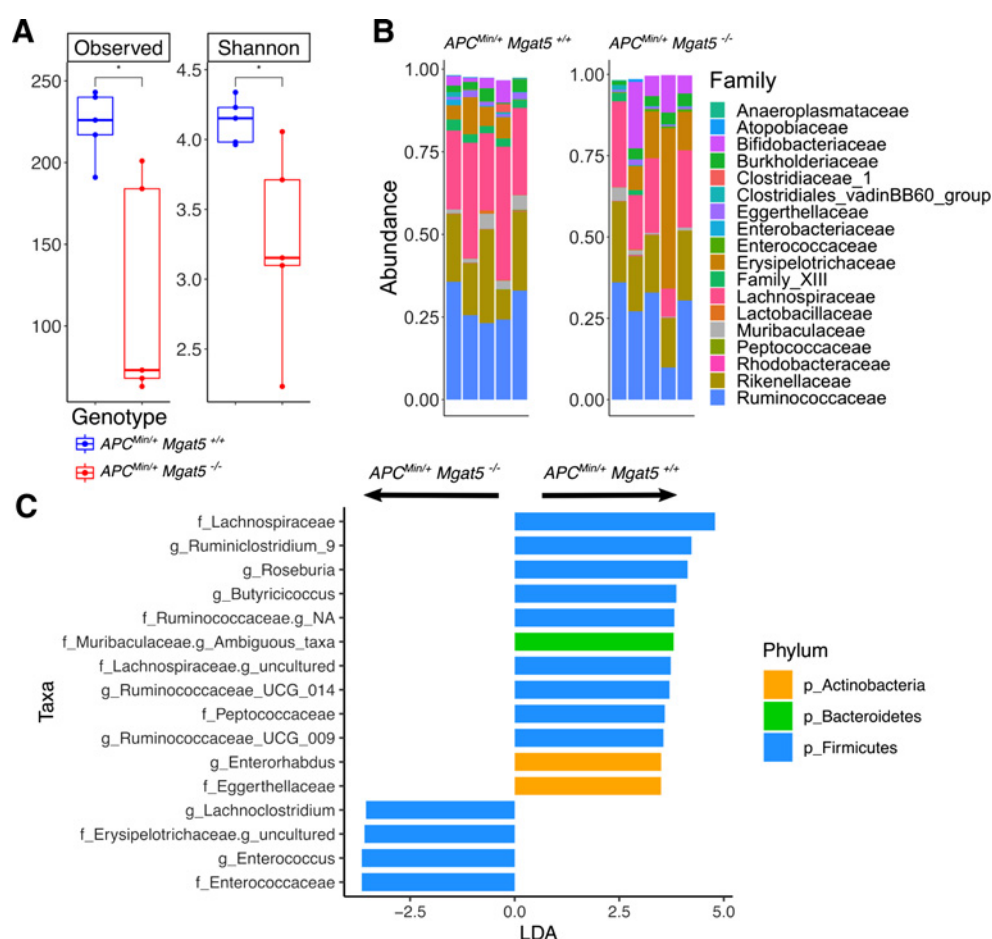
In vivo gene engineering of the *VCMsh2^{LoxP/LoxP}* mouse model. **A**, Total number of tumor lesions per mouse in *VCMsh2^{LoxP/LoxP}Mgat5^{+/+}* ($n = 38$) and *VCMsh2^{LoxP/LoxP}Mgat5^{-/-}* ($n = 25$) mice. **B**, Tumor number of lesions per intestinal area (proximal small intestine part, medial small intestine part, distal small intestine part, and colon) in *VCMsh2^{LoxP/LoxP}Mgat5^{+/+}* ($n = 38$) and *VCMsh2^{LoxP/LoxP}Mgat5^{-/-}* ($n = 25$) mice. **C**, Percentage of lesions per tumor size (mm²): <10 mm², 10 to 20 mm², and >20 mm² in both genotypes of *VCMsh2^{LoxP/LoxP}* ($n = 38$ and 25, respectively). **D**, Percentage of innate and adaptive immune cells within segments of the small intestine proximal part collected from *VCMsh2^{LoxP/LoxP}Mgat5^{+/+}* ($n = 2$) and *VCMsh2^{LoxP/LoxP}Mgat5^{-/-}* ($n = 9$) mice. Gating strategy to assess different immune populations represented in Supplementary Fig. S4A. **E**, Percentage of MR⁺ macrophages within segments of the small intestine proximal part collected from *VCMsh2^{LoxP/LoxP}Mgat5^{+/+}* ($n = 6$) and *VCMsh2^{LoxP/LoxP}Mgat5^{-/-}* ($n = 9$) mice. **F**, Percentage of adaptive immune cells within segments of the small intestine proximal part collected from *VCMsh2^{LoxP/LoxP}Mgat5^{+/+}* ($n = 6$) and *VCMsh2^{LoxP/LoxP}Mgat5^{-/-}* ($n = 9$) mice. Gating strategy to assess different immune populations represented in Supplementary Fig. S4B. *, $P < 0.05$; **, $P < 0.01$; ****, $P < 0.0001$; Student t test, multiple t test, or ordinary one-way ANOVA; error bars represent SEM of different mouse groups.

correlation between *N*- and *O*-glycosylation and cancer cell behavior (15); however, the interplay in immune evasion remains to be defined and is worth exploring further.

Here, we investigated how the aberrant expression of complex branched *N*-glycans by gastrointestinal cancer cells affects immune responses within the tumor microenvironment by addressing the impact of removing branched *N*-glycosylation on the exposure of immunogenic (glycan) epitopes (like mannose-enriched glycans) and how this affects antitumor immune recognition and surveillance, along

with exploring the relationship with the gut microbiome. We demonstrated that *MGAT5*-mediated branched *N*-glycans are an immune checkpoint in colorectal cancer and are overexpressed during colorectal cancer carcinogenesis. This process displaying a positive correlation with *Foxp3* expression. *In vitro* studies further showed that cancer cell branched *N*-glycans prevented immune recognition and contributed to tumor immune escape. The overexpression of complex branched *N*-glycans by gastrointestinal tumor cells imposed an immunosuppressive response within the microenvironment by inhibiting

(Continued.) **E**, Cell-surface *N*-glycan profile of epithelial cells (CD45⁻) isolated from tissue segments of the small intestine distal part of *Apc^{Min/+}Mgat5^{+/+}* ($n = 4$) and *Apc^{Min/+}Mgat5^{-/-}* ($n = 3$) mice analyzed by flow cytometry. Staining with GNA-fluorescein and ConA-biotin lectins. Lectin binding expressed as MFI. Representative histograms shown; *Apc^{Min/+}Mgat5^{+/+}* (dark gray) and *Apc^{Min/+}Mgat5^{-/-}* (light gray). **F**, Percentage of innate and adaptive immune cells within segments of the small intestine distal part collected from *Apc^{Min/+}Mgat5^{+/+}* ($n = 6$) and *Apc^{Min/+}Mgat5^{-/-}* ($n = 7$) mice. Gating strategy to assess different immune populations represented in Supplementary Fig. S4A. **G**, Percentage of MR⁺ macrophages within segments of the small intestine distal part collected from *Apc^{Min/+}Mgat5^{+/+}* ($n = 6$) and *Apc^{Min/+}Mgat5^{-/-}* ($n = 7$) mice. **H**, Percentage of adaptive immune cells within segments of the small intestine distal part collected from *Apc^{Min/+}Mgat5^{+/+}* ($n = 10$) and *Apc^{Min/+}Mgat5^{-/-}* ($n = 7$) mice. Gating strategy to assess different immune populations represented in Supplementary Fig. S4B. **I**, Concentrations of TNF α , IFN γ , IL1 β , IL17A, and IL10 (pg/mL) released by tissue explants from the small intestine distal part of *Apc^{Min/+}Mgat5^{+/+}* ($n = 8$ –13) and *Apc^{Min/+}Mgat5^{-/-}* ($n = 3$ –7) mice after 24 hours, evaluated by ELISA. Cytokine concentrations were normalized to tumor dry weight (g). **J**, Immunofluorescence for IFN γ and IL17A (red) using FFPE sections from intestinal parts of *Apc^{Min/+}Mgat5^{+/+}* ($n = 4$) and *Apc^{Min/+}Mgat5^{-/-}* ($n = 4$) mice. Unstained cells were used as a control, and DAPI was used to stain cell nuclei (blue). Scale bar, 50 μ m. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; Student t test, multiple t test, or ordinary one-way ANOVA; error bars represent SEM of different mouse groups. See also Supplementary Figs. S4 and S6.

**Figure 7.**

Removal of branched N-glycans impacts the microbiome composition associated with antitumor immune responses. **A**, Alpha diversity of microorganisms present in the gut microbiome of $APC^{Min/+} Mgat5^{+/+}$ ($n = 5$) and $APC^{Min/+} Mgat5^{-/-}$ ($n = 5$) mice [observed OTUs: median 226 (range 191–243) vs. 73 (63–201), $P = 0.016$; Shannon diversity: 4.15 (3.96–4.34) vs. 3.15 (2.23–4.06), $P = 0.032$]. Boxplots use the median line, the 25% and 75% percentiles for the upper and lower box hinges, respectively, and the highest whisker extends to the largest value no further than 1.5 times the interquartile range (IQR) from the upper hinge, while the lower whisker extends to the smallest value no further than 1.5 times the IQR from the lower hinge. **B** and **C**, Composition of microorganisms present in the gut microbiome of $APC^{Min/+} Mgat5^{+/+}$ ($n = 5$) and $APC^{Min/+} Mgat5^{-/-}$ ($n = 5$) mice. Microbiome composition was analyzed by 16S rDNA gene sequencing. LDA, linear discriminant analysis.

IFN γ production. The role of branched N-glycans in immune escape was further demonstrated by removing this glycan by KF or *Mgat5* CRISPR-Cas9 editing. The elimination of *Mgat5*-mediated branched N-glycans in colon epithelial cancer cells revealed and exposed mannose-enriched glycans associated with suppression of tumor growth *in vivo*, along with an increased inflammatory response. Accordingly, in colorectal cancer clinical samples, mannose-enriched glycans were downregulated during cancer progression. These evidences support that branched glycans were covering/masking immunogenic glycans, such as mannose, that upon exposure, could unleash effective antitumor immune responses.

In fact, high-mannose glycans are typically found in lower organisms and can be recognized as “non-self” by specific C-type lectins, such as MR and DC-SIGN expressed by macrophages and DCs, eliciting a proinflammatory immune response (48). We showed that treatment of gastrointestinal cancer cells with inhibitory anti-MR/DC-SIGN resulted in downregulation of the activation marker CD86, which supports the role of mannose-recognizing C-type lectins in the recognition of glycans and tumor immunomodulation. Accordingly,

treatment with mannose is shown to cause tumor growth retardation via enhancement of tumor cell death (49). The immunogenic role of mannose glycans in autoimmunity shows that deficiency of α -mannosidase-II results in the expression of mannosylated glycans and induction of an autoimmune-like disease in mice (50). Moreover, the lack of branched glycans (by blocking galectin-1 binding) reduces angiogenesis and can increase immune cell infiltration (51). On the other hand and in line with the immunosuppressive functions of the β 1,6-GlcNAc-branched glycans, the presence of sialic acid, commonly found in the branched N-glycans terminal part, associates with inhibition of effector T cells and induction of *de novo* Tregs through recognition by Siglecs (52).

The dynamic cross-talk between branched/mannose glycans expressed at the tumor cell surface and on immune cells in the tumor microenvironment was demonstrated when glycoengineered colon cancer cells were inoculated either in immunocompetent or immunodeficient mice. We demonstrated that mannose glycan exposure unleashed an antitumor immune attack in immunocompetent mice, suggesting a direct interaction between glycans and immune cells

needs to occur to modify antitumor immunity and tumor control. These results provide a strategy whereby glyco-engineering can unmask immunogenic glycan epitopes, such as mannose, resulting in enhanced antitumor immune responses.

The proof-of-concept of these findings was revealed in our pre-clinical models of colorectal cancer. When *Mgat5*-null mice were crossed with *Apc* or *Msh2*-mutated mice, we observed suppression of tumor growth that associated with an increased survival rate. The absence of branched N-glycans, and the consequent exposure of immunogenic mannose-enriched glycans, was found to trigger a proinflammatory immune response characterized by a significant decrease of Tregs and decreased expression of classical checkpoint molecules, such as PD-1. These immunomodulatory effects imposed by the absence of branched N-glycans and the revealing of mannose glycans led to an effective antitumor immune attack associated with colorectal cancer prevention. The future identification of relevant protein carriers of these branched/mannose-enriched N-glycans will be important for developing future targeted immunotherapies.

Given that the colon mucosa is covered with a dense and complex coat of glycans, called the glycocalyx, and that the gut microbiota use glycans as a source of energy, we evaluated the impact of gut mucosa glycosylation in cancer immunoediting, immunosurveillance, and its complementary/synergistic effects on the microbiota composition. We also asked whether and how an altered host glycoprofile (via *Mgat5*-glyco-engineering and the consequent remodeling of mucosa branched N-glycosylation) affected the microbiome composition that associated with immunomodulation in colorectal cancer. We found that in *Apc^{Min/+}* mice, the *Mgat5* deletion significantly altered the microbiome diversity and composition whereby the absence of branched N-glycans led to an increased abundance of *Enterococcus* bacteria. Accumulating evidences support an important role of gut microbiome in antitumor immunity (38, 53, 54). Changes in intestinal microbiome composition associate with resistance to anti-PD-1-based immunotherapy (55). Here, the identification of *Enterococcus* bacteria that associated with suppression of colorectal cancer growth and antitumor immune responses (triggered by the removal of branched N-glycans) supports the effects of altered host glycosylation in tumor immunomodulation and tumor growth. These evidences are in line with a study showing that *Enterococcus faecalis* CECT7121 induces immune activation, characterized by the activation of DCs and IFN γ production (40, 42). Another study describes that heat-killed *Enterococcus faecalis* EC-12 is able to reduce polyp progression in *Apc^{Min/+}* mice by suppressing β -catenin signaling (41). Our evidence reveals the effects of the remodeling of mucosa glycosylation in the selection of specific microorganisms that together converge to regulate the antitumor immune response and tumor growth.

Altogether, this study described a glycan-associated immune checkpoint in colorectal cancer, in which the expression of abnormal branched N-glycans induced immunosuppressive networks in the tumor microenvironment through hidden immunosurveillance. This tumor-specific glycosignature could be a tool to identify high-risk individuals of colorectal cancer progression that can be potentially

eligible for early immunopreventive interventions. From the clinical point of view, we also demonstrated that glycoengineering to remove tolerogenic branched N-glycan is a strategy to expose relevant immunogenic glycan epitopes that not only impose changes in microbiome composition, but also triggers an effective antitumor immune response (Supplementary Fig. S7, summary of data).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

M.C. Silva: Conceptualization, software, formal analysis, validation, investigation, methodology, writing—original draft, writing—review and editing. **Á. Fernandes:** Conceptualization, software, formal analysis, validation, investigation, visualization, methodology, writing—original draft, writing—review and editing. **M. Oliveira:** Investigation, methodology. **C. Resende:** Resources, investigation, methodology. **A. Correia:** Resources, investigation, methodology. **J.C. de-Freitas-Junior:** Conceptualization, formal analysis, investigation, methodology. **A. Lavelle:** Software, formal analysis, methodology. **J. Andrade-da-Costa:** Investigation, methodology. **M. Leander:** Methodology. **H. Xavier-Ferreira:** Methodology. **J. Bessa:** Resources. **C. Pereira:** Resources. **R.M. Henrique:** Resources. **F. Carneiro:** Resources. **M. Dinis-Ribeiro:** Resources. **R. Marcos-Pinto:** Resources. **M. Lima:** Resources. **B. Lepenies:** Resources. **H. Sokol:** Resources. **J.C. Machado:** Resources. **M. Vilanova:** Resources. **S.S. Pinho:** Conceptualization, resources, supervision, funding acquisition, investigation, writing—original draft, project administration, writing—review and editing.

Acknowledgments

This article is a result of the project NORTE-01-0145-FEDER-000029, supported by the Norte Portugal Regional Programme (NORTE 2020) under the PORTUGAL 2020 Partnership Agreement through the European Regional Development Fund. This work was also funded by Fundo Europeu de Desenvolvimento Regional (FEDER) funds through the COMPETE 2020—Operacional Programme for Competitiveness and Internationalization (POCI), Portugal 2020, and by Portuguese funds through the Portuguese Foundation for Science and Technology in the framework of the projects (POCI-01/0145-FEDER-016601/PTDC/DTP-PIC/0560/2014 and POCI-01-0145-FEDER-028772). M.C. Silva acknowledges Fundação para a Ciência e a Tecnologia (FCT; SFRH/BD/136388/2018) for a fellowship. J. Bessa acknowledges FCT, for an FCT Investigator position (Grant IF/00654/2013) and support from European Research Council (ERC) under the European Union's Horizon 2020 research and innovation programme (grant agreement no. ERC-2015-StG-680156-ZPR). J.C. Machado acknowledges FCT for funding (PTDC/MED-PAT/32462/2017; PTDC/BIM-MEC/2834/2014). The authors thank Prof. Naoyuki Taniguchi for kindly providing the MKN45 Mock and T5 cell lines. They thank Prof. Michael Pierce for kindly providing the *Mgat5*-knockout mice. The authors thank Prof. Winfried Edelmann, from Albert Einstein College of Medicine, New York, NY, for kindly providing the VCMsh2^{loxP/loxP} mice. They also thank Prof. Adriane Todeschini for kindly providing the MC38 cell line. The authors thank Renata Carriço, from i3S, for the technical support in the CRISPR/Cas9 approach. They acknowledge the support of the i3S Scientific Platforms: Animal Facility and Translational Cytometry (TraCy). The Institute of Molecular Pathology and Immunology of the University of Porto integrates the i3S research unit, which is partially supported by the Portuguese Foundation for Science and Technology.

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Received April 9, 2020; revised July 3, 2020; accepted September 8, 2020; published first September 15, 2020.

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Supplementary Data

Figure S1.

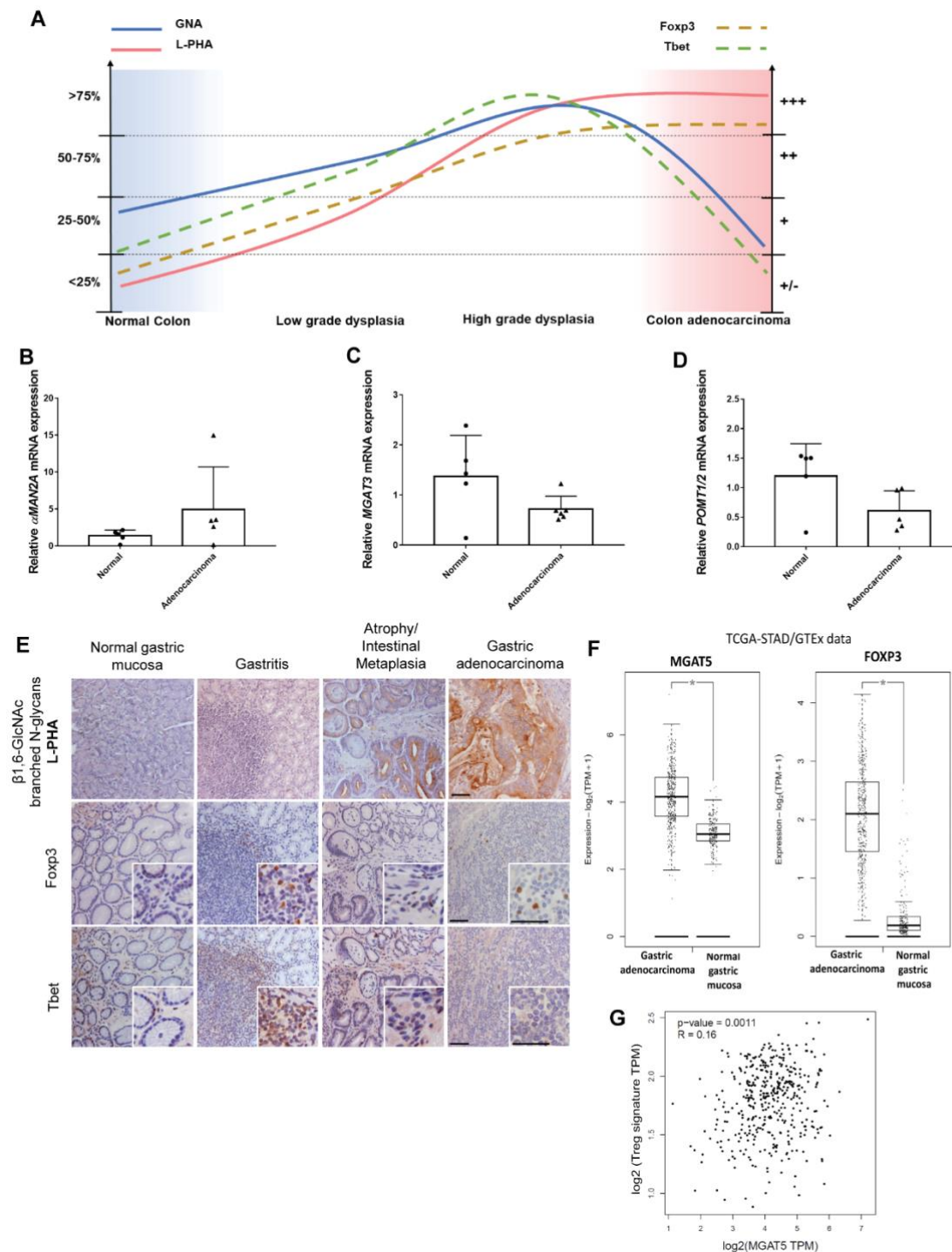


Figure S1, related to Figure 1

(A) Schematic representation of histochemistry staining, translating the overall profile of glycans and T cells expression along colon carcinogenesis, Figure 1A. The characterization of lectin

histochemistry follows the Y axis on the left, with a semi-quantitative classification, and immunohistochemistry for Tbet and Foxp3 follows a qualitative classification represented in Y axis on the right (representing the mean of 3 independent observers). **(B-D)** Relative expression of *αMAN2A* **(A)**, *MGAT3* **(B)** and *POMP1/2* **(C)** mRNA of FFPE samples of normal colon (n=5) and colon adenocarcinoma (n=5), normalized to 18S housekeeping expression; Student's T-test; Error bars represent SEM of different biological samples. **(E)** Glycoimmuneprofiling along gastric carcinogenesis: Normal gastric mucosa (n=5), gastritis (n=5), atrophy and/or intestinal metaplasia (n=5) and gastric adenocarcinoma (n=5). Image magnification for lectin staining (L-PHA): 100x; Image magnification for antibodies staining (Foxp3; Tbet): 200x and 400x (inserts). Scale bars length=50 μm. **(F)** The expression profiling of *MGAT5* and *FOXP3* in gastric cancer samples (n=408) vs normal gastric mucosa (n=211), using GEPIA2. One-way ANOVA; line represent median **(G)** Pearson correlation analysis of *MGAT5* versus "Treg signature" (composed by *ENTPD1*, *FOXP3* and *CTLA4* genes) in gastric cancer samples (n=408), using GEPIA2. P value=0.0011.

Figure S2.

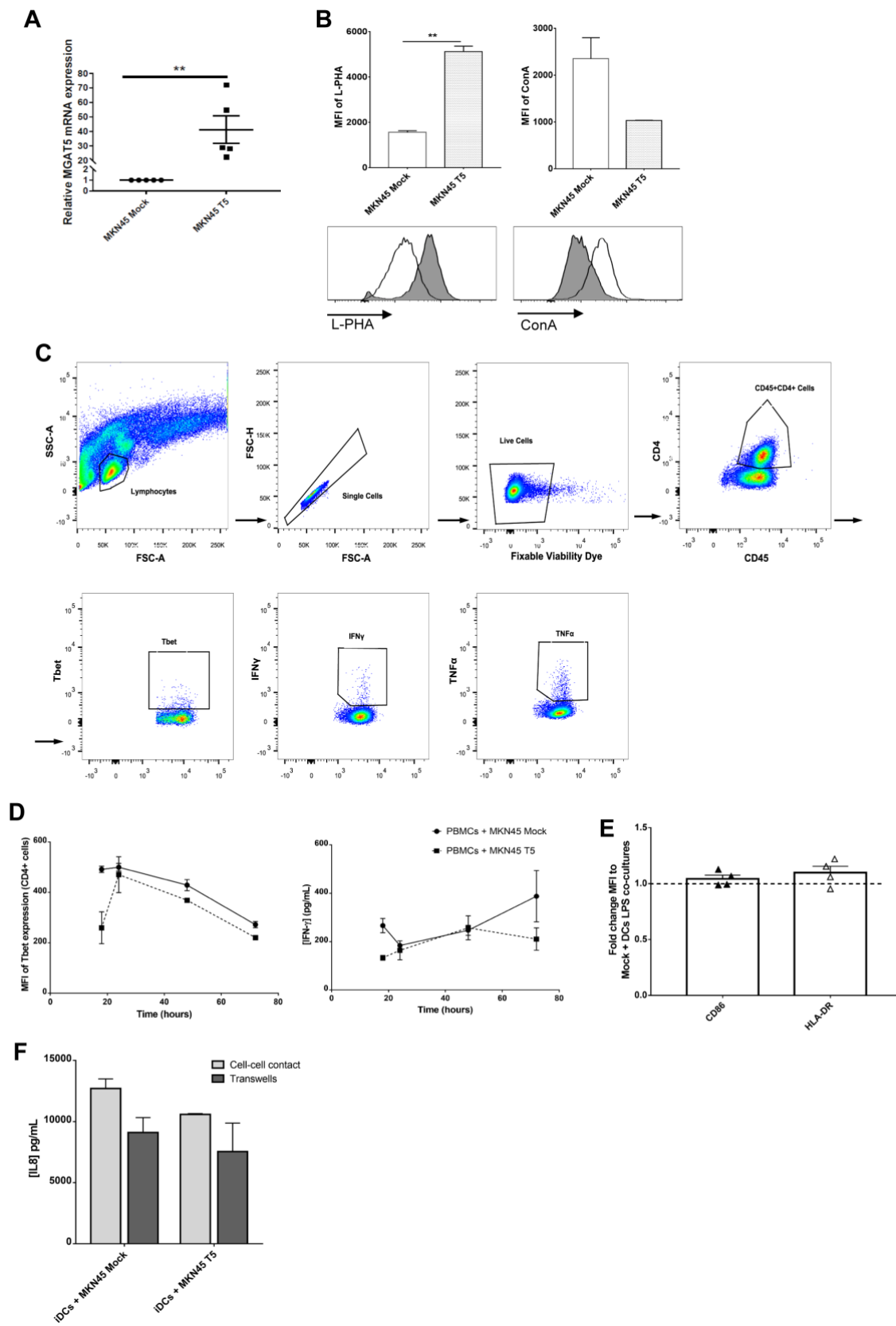


Figure S2, related to Figure 2

(A) Relative *MGAT5* mRNA expression of MKN45 T5 vs Mock cells, normalized to 18S housekeeping expression. (n=5, represented different time points of cell line culture). **(B)** Cell-surface N-glycan profile of MKN45 Mock and T5 cell line analyzed by FACS staining with L-PHA-fluorescein and ConA-biotin lectins. The levels of lectin binding are expressed in MFI. Representative histograms shown; MKN45 Mock (unshaded) and MKN45 T5 (gray) **(C)** Gating strategy used to distinguish Tbet⁺, IFN γ ⁺ and TNF α ⁺ populations within CD4⁺ population in FACS analysis, related to figure 2. **(D)** Co-cultures of MKN45 Mock and MKN45 T5 with PBMC, activated with anti-CD3 and anti-CD28 antibodies during 18 h, 24 h, 48 h and 72 h. MFI of Tbet expression in CD4⁺ cells and [IFN γ] (pg/mL) along the time. **(E)** The co-stimulating receptors CD86 and HLA-DR analyzed by FACS (n=4) in moDCs, activated with LPS, in co-culture with MKN45 Mock or T5 cell lines after 18 h. **(F)** Release of [IL8] (pg/mL) in transwell co-cultures of moDCs (cultured in the bottom) with MKN45 Mock and MKN45 T5 after 18 h. Cytokine concentration assessed by CBA Human Inflammatory Cytokines Kit.

**p < 0.01; Student's t test; Error bars represent SEM of different biological replicates and/or technical replicates.

Figure S3.

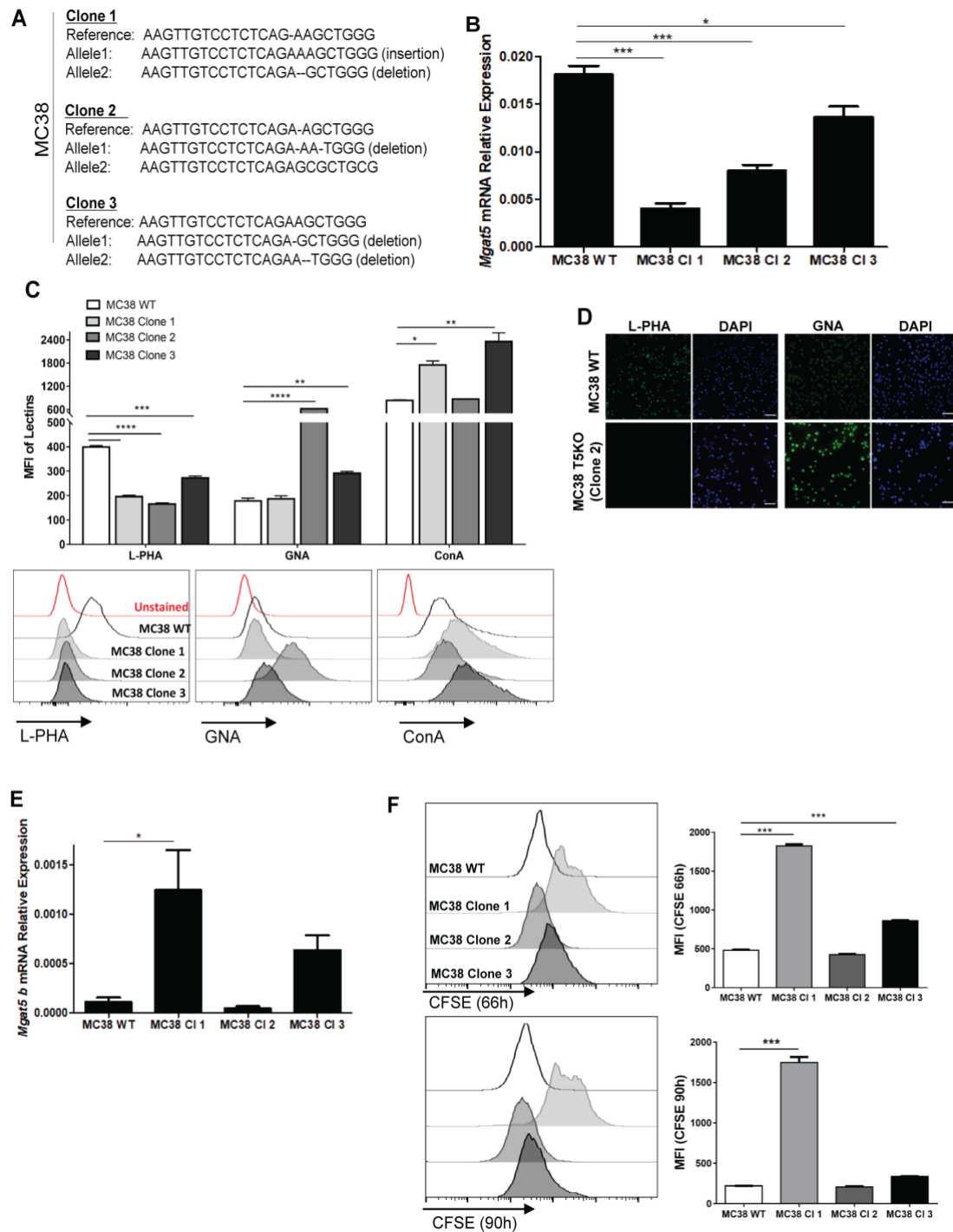


Figure S3, related to Figure 4

(A) DNA sequencing of the three MC38 T5KO clones, in comparison to the DNA sequence of the MC38 WT cells. Different insertions or deletions (indels) are detected in both alleles, indicating a biallelic disruption of the *Mgat5* gene. (B) Relative *Mgat5* mRNA expression levels in MC38 WT and MC38 T5KO cells, evaluated by RT-PCR and normalized to the mRNA levels of the housekeeping gene *18S*. (C) Cell-surface N-glycan profile of MC38 WT cells and MC38 T5KO

clones analyzed by flow cytometry after staining with L-PHA-fluorescein, GNA- fluorescein and ConA-biotin lectins. The levels of lectin binding are expressed in MFI. **(D)** Immunofluorescence results for terminal α 1-3-linked mannose glycans after staining with GNA conjugated with fluorescein (green color). Unstained cells were used as a control and DAPI was used to stain cell nuclei (blue color). Scale bars length=50 μ m. **(E)** Relative *Mgat5b* mRNA expression levels in MC38 WT and MC38 T5KO cells, evaluated by RT-PCR and normalized to the mRNA levels of the housekeeping gene *18S*. **(F)** Detection of MC38 WT and MC38 KO cells fluorescence after 66h and 90h of CFSE-labelling, to evaluate the proliferation rates of these samples.

*p < 0.05; **p < 0.01; ***p < 0.001 and ****p < 0.0001; Ordinary One-way ANOVA. Error bars represent SEM of different technical replicates.

Figure S4.

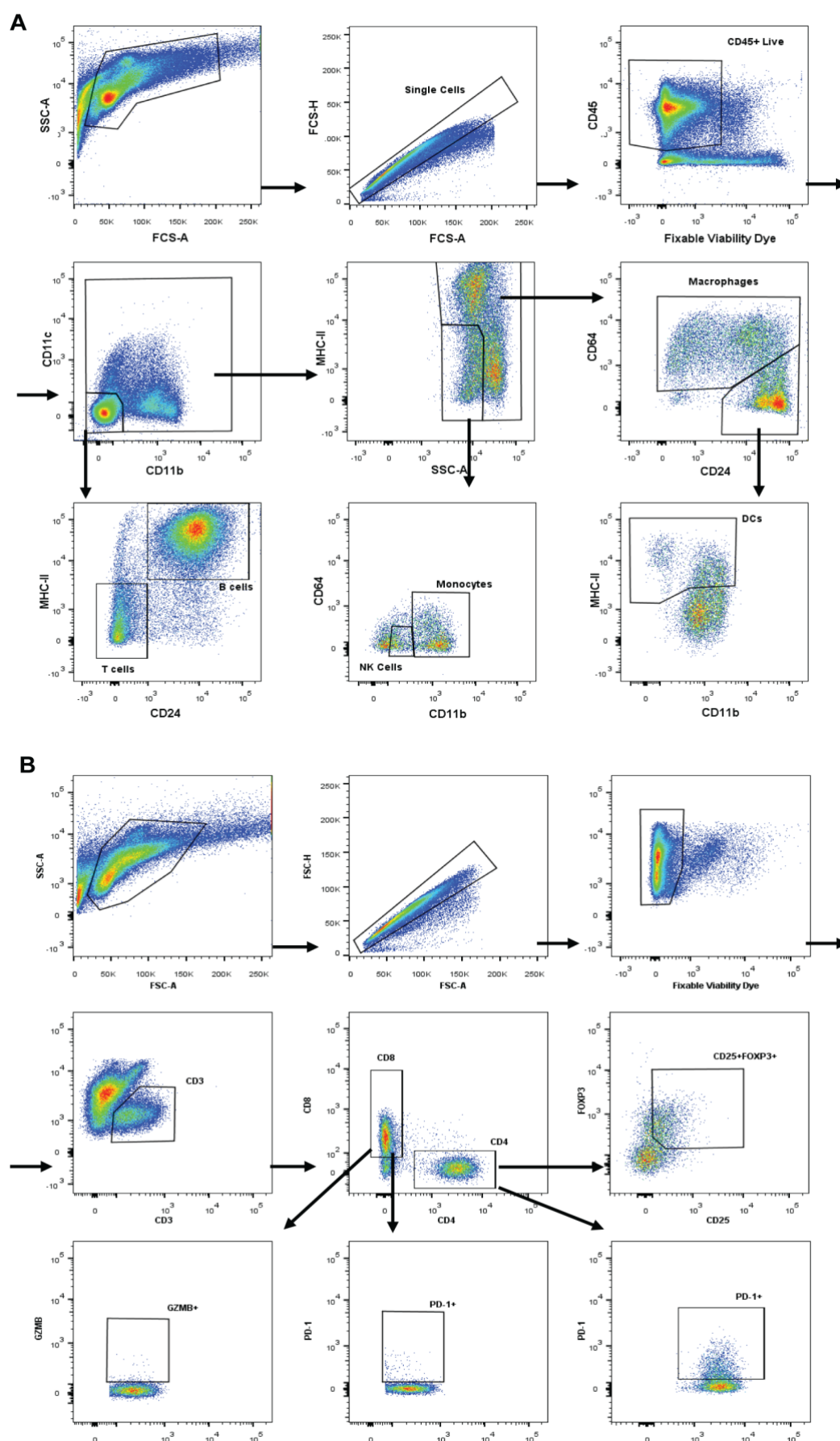


Figure S4. Gating strategy used to distinguish the different populations of immune cells in FACS analysis, related to figure 4, 5 and 6.

(A) Gating to exclude dead cells and define CD45⁺ cells, which is the population to obtain innate population of cells (CD11c⁺ and/or CD11b⁺) and lymphocyte population (CD11c⁻CD11b⁻). With this strategy, we could distinguish, on innate gate, monocytes (CD11b^{hi}CD64^{int}) and NK cells (CD11b^{int}CD64⁺), inside SSC^{low}MHCII⁻ gate. On the other hand, macrophages (CD64⁺CD24⁺ and CD24⁻) and DCs (CD24⁺CD64⁻MHCII⁺CD11b⁺ and CD11b⁻) were identified in the remaining cells. The B cells (MHCII⁺CD24⁺) and T cells (MHCII⁻CD24⁻) were obtain inside lymphocyte gate. Analysis adapted from (1) and obtain using FlowJo V10 Software. **(B)** PD-1⁺ and GZMB⁺ within CD8⁺ population and PD-1⁺ and FOXP3⁺ CD25⁺ within CD4⁺ population, identified inside CD3⁺ gating.

Figure S5.

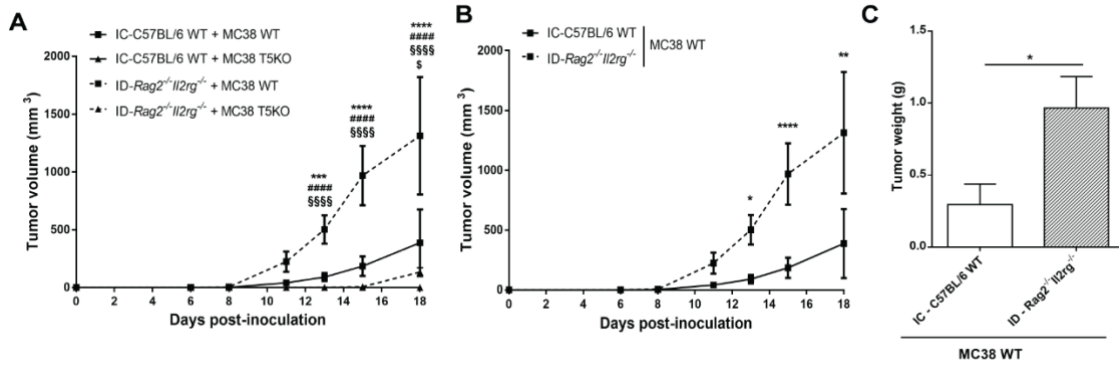


Figure S5, related to Figure 4

(A) Tumor growth kinetics (n=7 for each condition) upon the inoculation of MC38 WT cells and MC38 T5KO in IC (immunocompetent) - C57BL/6 WT mice or ID (immunodeficient) - *Rag2*^{-/-}*Il2rg*^{-/-} mice until the day of euthanasia, when one of the tumors reached 2000mm³. Tumor length and width (mm) were measured with a caliper every two days since day 7 post-inoculation and tumor volume (mm³) was calculated according to the formula [(length x width²)/2]. ***p < 0.001 and ****p < 0.0001 represents comparison between ID - *Rag2*^{-/-}*Il2rg*^{-/-} + MC38 WT and IC - C57BL/6 WT + MC38 WT; ##### p < 0.0001 represents comparison between ID - *Rag2*^{-/-}*Il2rg*^{-/-} + MC38 WT and ID - *Rag2*^{-/-}*Il2rg*^{-/-} + MC38 T5KO; §§§§ p < 0.0001 represents comparison between ID - *Rag2*^{-/-}*Il2rg*^{-/-} + MC38 WT and IC - C57BL/6 WT + MC38 T5KO. \$ p ≤ 0.05 represents comparison between IC - C57BL/6 WT + MC38 WT and IC - C57BL/6 WT + MC38 T5KO. **(B)** Tumor growth kinetics (n=7 for each condition) upon the inoculation of MC38 WT cells in IC (immunocompetent) - C57BL/6 WT mice or ID (immunodeficient) - *Rag2*^{-/-}*Il2rg*^{-/-} mice until the day of euthanasia, when one of the tumors reached 2000mm³. Tumor length and width (mm) were measured with a caliper every two days since day 7 post-inoculation and tumor volume (mm³) was calculated according to the formula [(length x width²)/2]. * p ≤ 0.05; **p < 0.01 and ****p < 0.0001. **(C)** Tumor weight (g) upon euthanasia. Two-way ANOVA. Error bars represent SEM of different mice groups.

Figure S6.

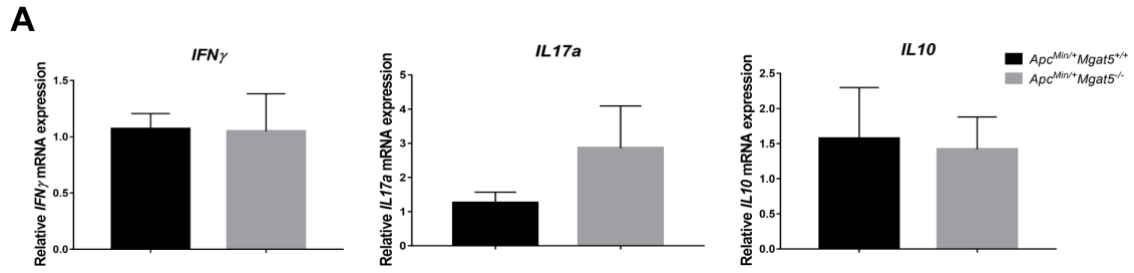


Figure S6, related to Figure 5.

(A) Relative *IFN γ* , *IL17a* and *IL10* mRNA expression levels in small intestine distal part of *Apc^{Min/+} Mgat5^{+/+}* (n=5 with 2 biological replicates) and *Apc^{Min/+} Mgat5^{-/-}* (n=4 with 2 biological replicates) mice, evaluated by RT-PCR and normalized to the mRNA levels of the housekeeping *GAPDH* gene and *CD45* gene expression.

Figure S7.

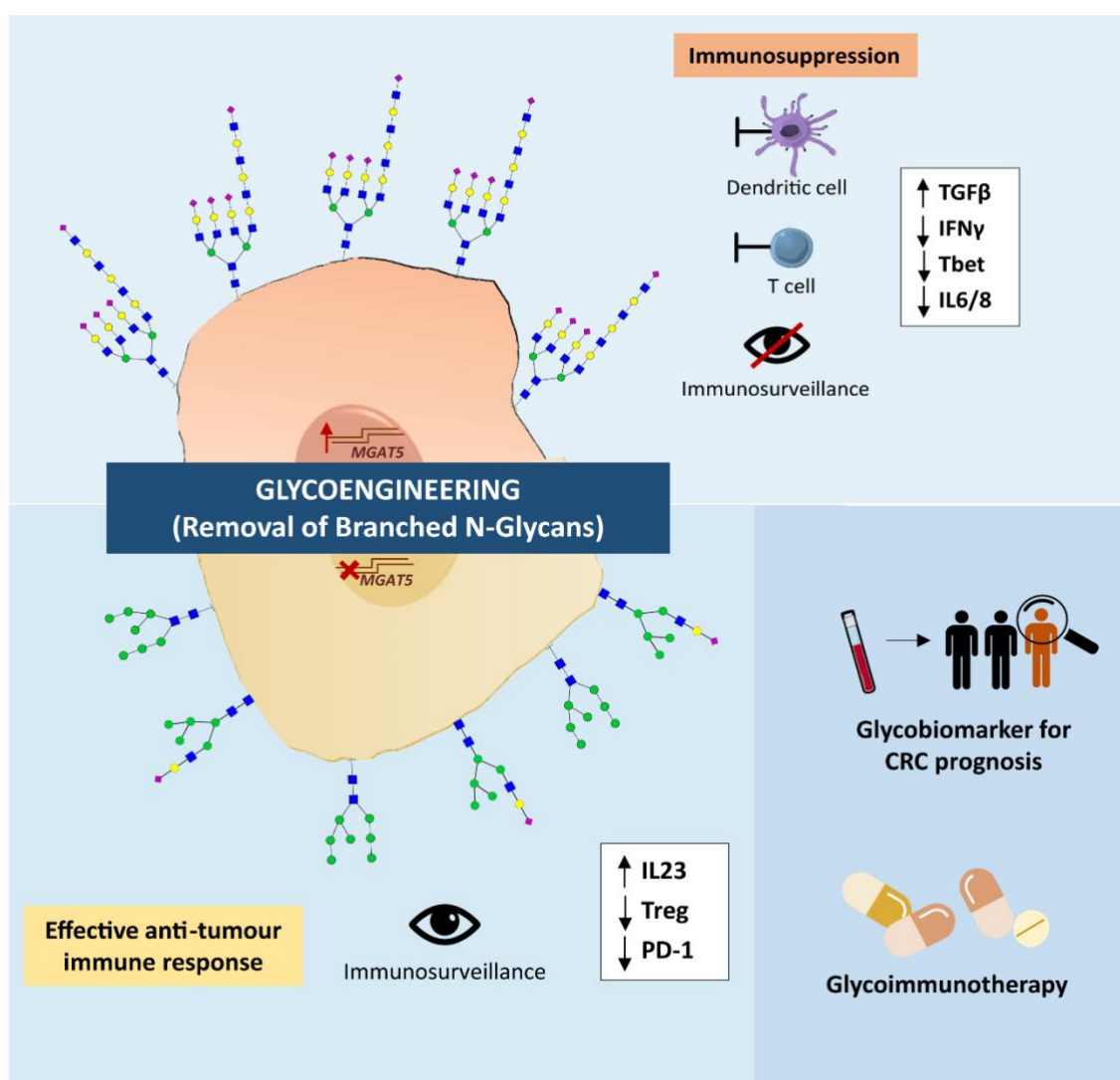


Figure S7. Graphical abstract.

MGAT5 gene is overexpressed in tumorigenesis, leading to the aberrant expression of β 1,6-GlcNAc branched N-glycans on tumour cells and to an enhanced anti-inflammatory response – increased $TGF\beta$ levels; reduction of $IFN\gamma$, Tbet and IL6/8 levels. These features contribute to cancer immunoediting and enhanced tumour growth. However, when *MGAT5* gene expression is silenced by glycoengineering, tumour cells do not express branched N-glycans resulting in an increased anti-tumour immune response and tumour suppression – IL23 production; reduction of Treg cells presence; decrease of PD-1 levels. This study highlights how tumor-specific glycosignature may constitute a biomarker to identify high risk individuals of CRC progression and paves the way for novel glycoimmunotherapeutic strategies in CRC.

Supplemental References

1. Yu Y-RA, O’Koren EG, Hotten DF, Kan MJ, Kopin D, Nelson ER, et al. A Protocol for the Comprehensive Flow Cytometric Analysis of Immune Cells in Normal and Inflamed Murine Non-Lymphoid Tissues. PloS one. 2016;11(3):e0150606.

Chapter 3

Exploring the role of *N*-Glycosylation of Neoantigens in the modulation of tumor immune response: an opportunity to improve cancer immunotherapy

Exploring the role of N-Glycosylation of Neoantigens in the modulation of tumor immune response: an opportunity to improve cancer immunotherapy

(In preparation)

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Abstract

Colorectal cancer (CRC) is the second leading cause of cancer-related death worldwide, highlighting the importance to identify relevant etiological factors and new therapeutic targets. Additionally, the vast majority of patients with CRC are not eligible or do not respond to immune-targeted therapies, which are restricted to CRC patients with metastatic disease and microsatellite instability (MSI). In fact, approximately 15% of CRC cases have a defect in DNA mismatch repair (MMR) genes, resulting in MSI, with a high tumor mutation burden (TMB) and high neoantigen expression. However, MSI tumors frequently present deficient Human Leukocyte Antigen class I (HLA-class I) molecule expression. In these cases, CRC patients do not respond to conventional immunotherapies. Therefore, there is an urgent need to find new strategies to improve eligibility and response to immunotherapy, as well as to increase the effectiveness of antitumor immune response.

Glycosylation is a post-translational modification of proteins and lipids with impact in cancer and immune response. In fact, the glycosylation profile in cancer cells significantly changes, being associated with tumor progression and worse prognosis. Particularly, the abnormal expression of complex β 1,6-branched N-glycans in cancer is considered an immune checkpoint in tumor evasion, masking the recognition of neoantigens, and promoting an anti-inflammatory response.

Cellular immunotherapy, such as adoptive T cell transfer, has been proposed as an alternative strategy to conventional immune checkpoint inhibitor immunotherapy. Using glycosylated antigens to train immune cells could be a complementary approach to increase cellular immunotherapy efficacy. In the case of HLA-negative patients, other immune cells such as natural killer (NK) cells or $\gamma\delta$ T cells should be considered.

This study aims to develop a novel strategy to increase the effectiveness of cellular immunotherapy, mainly for MSS and in MSI HLA-negative CRC tumors. We found that the loss of N-glycosylation sites in putative neoantigens is associated with stage III of CRC, and these peptides are more related to MSI CRC samples. However, MSI tumors are also negative for HLA molecules, and neoantigen recognition by T cells is compromised. In this case, we performed *in vitro* assays with NK cells, which demonstrated that the removal of complex branched N-glycans leads to more effective NK-mediated tumor killing.

These preliminary results highlight the potential of glycans to revolutionize the way we understand and use immunotherapy by filling the gap of non-responders to immunotherapies in CRC.

Background

Colorectal cancer (CRC) is the third most commonly diagnosed cancer and the second deadliest cancer worldwide [1]. The great majority of CRC develop sporadically, which approximately 30% derived from familial component and a genetic basis [2]. Sporadic CRC develops through the progressive accumulation of several genetic and epigenetic alterations, which can be divided in three major genetic pathways: 1) Chromosomal instability (CIN), 2) Microsatellite instability (MSI), and 3) Epigenetic instability (or CpG island methylator phenotype; CIMP) [3]. Only 10-15% of sporadic CRC have defects in DNA mismatch repair (MMR) proteins, which are known as MSI tumors [4]. These tumors are characterized by high tumor mutation burden (TMB) and significantly frequent expression of tumor specific antigens (neoantigens) [5]. MSI is a good predictor of response to immunotherapies, such as immune checkpoints inhibitors (anti-PD-1/PD-L1), due to the abundant tumor-infiltrating lymphocytes (TILs) [6, 7]. On the other hand, the majority of CRC (approximately 85%) has a low TMB with microsatellite stable (MSS). Additionally, in MSS tumors, a very small number of antigens is capable to induce effective T cell anti-tumor immune response [8]. Enhancing the ability of immune system to recognize neoantigens is a critical unmet need to improve the eligibility and efficacy of immunotherapy.

Post-translational modifications, such as glycosylation, should be considered for the recognition of neoantigens. Protein glycosylation is characterized by the addition of carbohydrate chains to proteins, which mediate a wide variety of functions in physiological and pathophysiological states [9]. Essentially all cells are covered with glycans, which constitutes a major biological interface between cells and their environment. However, the glycosylation profile dramatically changes in cancer cells [10]. The abnormal expression of complex β 1,6-branched *N*-glycans, catalyzed by the *N*-acetylglucosaminyltransferase V (GnT-V) enzyme, has been shown to induce pro-malignant, pro-invasive, and pro-metastatic cancer phenotypes through mechanistic interference in tumor cell adhesion. Additionally, these complex glycans are associated with poor survival rates of patients with gastrointestinal cancer [11-14]. Moreover, we have also found that increased expression of these complex *N*-glycans in CRC blocks recognition of neoplastic cells by the immune system, which has been described as a mechanism of tumor immune escape. In line, when this glycan mask is removed from tumor cells, the anti-tumor immune response is improved, impairing CRC development [15].

Hereupon, the real impact of glycans in neoantigen recognition by T cells needs to be explored, in order to develop new strategies for target/personalized immunotherapy. Indeed, it is known that T cells can recognize directly glycosylated antigens [16, 17]. Moreover, antigen-presenting cells (APCs) recognize, via C-type Lectins, and process

glycosylated tumor antigens, presenting them through MHC class II or MHC class I to CD4⁺ or CD8⁺ T cells, respectively. During processing, the peptides can maintain glycans, which could be recognized by specific T cell receptor (TCR), whereas other T cell clones only recognize naked antigens [16, 18]. These findings highlight the relevance of glycoantigens for T cell recognition and function. Moreover, innate immune cells have glycan binding receptors that are able to recognize different types of glycans. For instance, dendritic cells, macrophages, and NK cells have specialized receptors, such as C-type lectins and sialic acid-binding immunoglobulin-type lectins (Siglecs), that mediate immune responses [19, 20]. In cancer, aberrant glycosylation of tumor cells is recognized by the immune cells through these receptors and impairs anti-tumor immune response [21]. Recently, our group have described that $\gamma\delta$ T cells are also able to recognize and respond to mannosylated epitopes, which is mediated by C-type lectin DC-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) [22].

The main aim of this study is to explore the presence of *N*-glycans in neoantigens and how it could impact in tumor immune evasion and immune response. To address that, we defined two different approaches: (A) explore the role of *N*-glycans in the antigen-mediated T cell immune response and (B) investigate the impact of glycosylation profile in antigen-independent innate cell recognition and response. We started by generating a data base of neoantigens for a total of 24 CRC patients (20 MSS and 4 MSI). A survey of *N*-glycosylation sites (Asparagine-X-Serine/Threonine, where X is any amino acid (aa) except Proline) was performed. We found that the presence of *N*-glycosylation sites is more prevalent in missense-mutated neoantigens and peptides with frameshift mutations led more frequently to alterations in *N*-glycosylation sites. MSI-derived peptides are also more related with gain or loss of *N*-glycosylation sites. In a second part, an *in vitro* experiment was performed, using primary HLA negative cell lines and NK cells. This has shown that the removal of complex *N*-glycans improves NK cell reactivity and anti-tumor immune response.

All together this preliminary study demonstrates the regulatory power of glycosylated neoantigens in mediating immune responses in cancer, pinpointing glycopeptide antigens as a new strategy for the design of novel cancer immunotherapies. However, the influence of glycans in HLA-presented neoantigens in T cell recognition and reactivity, as well as the role of glycans in NK cell recognition need to be further explored.

Materials and methods

Collection of patient material

This study was approved by the Medical Ethical Committee of the Leiden University Medical Centre (protocol P15.282), and all patients provided informed consent. It was collected a total of 24 CRC samples from different patients. MMR status was determined initially through diagnostic procedures by making use of PMS2 and MSH6 immunodetection and was further confirmed by the observation of numerous nucleotide insertions and deletions by exome sequencing in the samples classified as MSI. Patient samples were anonymized and handled according to the medical ethical guidelines described in the Code of Conduct for Proper Secondary Use of Human Tissue of the Dutch Federation of Biomedical Scientific Societies. This research was conducted according to the recommendations outlined in the Helsinki Declaration.

Whole-exome and RNA sequencing of tumor and corresponding normal tissue

The sequencing libraries for CRC samples were obtain as described before in [8]. With exception of synonymous substitutions, all other coding variants were further investigated if at least one read displaying a mutation was present in the RNA sequencing data. To this purpose, RNA sequencing reads were first mapped against the same hg38 genome build using gsnap [23], followed by read count at variant positions using the samtools mpileup tool. Allele frequencies at DNA level were extracted from the .vcf files and an mpileup file was generated for all mutated sites to inform on the number of variant-supporting reads at RNA level. Purity estimates of the tumor content were determined using Sequenza [24].

51-mer peptides were generated for all the identified variants. In case of frameshifts and stop loss mutations, several peptides were generated which overlapped for at least half of the sequence. A file for each sample was prepared with information of Identified variant, genomic position, gene identification, wildtype (WT) transcript, 51-mer WT peptide, 51-mer Altered (ALT) peptide, WT protein sequence and ALT protein sequence, among other variables.

Identification of *N*-glycosylation sites in 51-mer peptides

The files generated previously were processed using Python language and Jupyter Notebook version 6.0.3 software with a focus on WT and ALT peptide sequences. The *N*-glycosylation sites (*N*-glyco sites) Asparagine-X-Serine/Threonine, where X is any amino acid except Proline ($N-X-S/T$, $X \neq P$), were searched in peptide sequences, and the position of the motif was annotated. ALT peptides with *N*-glycosylation motifs that were not identified

in WT peptides are considered a gain of N-glycosylation site. Conversely, N-glyco sites identified in WT peptides but not in ALT peptides were lost due to the mutation. Mutations that eliminate N, S, or T or generate a P aa on the N-glyco consensus sequence led to a loss of N-glyco sites, and the opposite occurs in the case of the generation of an N-glyco site. In frameshift variants, some cases lead to the complete abolish or generation of an N-glyco motif.

The data obtained from this analysis were processed using SPSS Statistics software (IBM), and associations between different variables and the presence, gain, or loss of N-glyco sites were analyzed.

HLA binding prediction

HLA binding prediction test was performed for a sample of peptides from different patients. An 8-mer peptide, which includes the mutation in the middle, were tested for the HLA binding using the method developed in [25]. The number of binders per peptide was annotated, as well as the strong and weak binders, for further analysis.

Cell culture

Cell lines from CRC patients (called NIC cell lines) were established and kindly provided by Noel de Miranda, Leiden University Medical Center (LUMC), Leiden, The Netherlands. NIC12 and NIC13 cell lines were cultured in 1:1 mixture of DMEM/F12:RPMI1640 GlutaMAX (Gibco) supplemented with 10% FBS (Biowest) and penicillin/streptomycin (100 U/mL; Gibco) at 37°C in 5% CO₂. NIC12 cell line was treated with kifunensine (KF; 20 mM; Sigma-Aldrich) for 24 hours before used in co-culture.

NK cell co-culture

NK cells were isolated from healthy donors' buffy coats from *Centro Hospitalar de São João* (CHSJ), Porto, Portugal. Peripheral blood mononuclear cells (PBMCs) were first obtained using LymphoPrep™ (StemCell technologies) and then NK cells were isolated using EasySep™ Human NK Cell Isolation Kit (StemCell technologies) accordingly manufacture instructions. NK cells were maintained in culture overnight before being used in co-culture, in RPMI1640 GlutaMAX supplemented with 10% FBS and penicillin/streptomycin at 37°C in 5% CO₂. NIC cell lines and NK cells are culture 1 x 10⁵ cells 1:1 in 96-well plate with RPMI1640 GlutaMAX supplemented with 10% FBS and penicillin/streptomycin (100 U/mL). Recombinant human IL-2 (15 ng/mL; Peprotech) and anti-CD107a-FITC (H4A3, BioLegend) was added for 1 hours and then Brefeldin A (10 ng/mL; Sigma-Aldrich) and GolgiStop-Monensin (BD Bioscience) for more 5 hours. After 6 hours of culture, cells were stained for flow cytometry to evaluate NK activation and function.

Flow Cytometry

Co-cultured NK and NIC cells were stained with fixable viability dye (FVD-eFluor780), anti-CD56-PE (HCD56, BioLegend), anti-CD16-Brilliant Violet 421 (3G8, BioLegend), anti-GranzymeB-APC (GB12, Invitrogen) and anti-CD3-eFluor506 (OKT3, Invitrogen). In parallel, NIC cells were stained with FVD-eFluor780, *Phaseolus Vulgaris* *Leucoagglutinin* (L-PHA)-FITC (Vector Laboratories), *Galanthus nivalis lectin* (GNA)-biotin (Vector Laboratories), anti-PD-L1-APC (MIH1; eBioscience), anti-HLA-ABC-PE-Cy7 (W6/32; Invitrogen) and Streptavidin-PE (BioLegend).

Data acquisition was performed in the FACS Canto v.2 flow cytometer (BD Bioscience), using the FACSDiva software (BD Bioscience). The files were then analyzed with FlowJo software version 10.

Statistical analysis

One-way ANOVA or Chi-square test were performed to compare % number of glyco sites between samples or each peptide individually, respectively. This statistical analysis was performed using SPSS software (IBM). For in vitro assays, unpaired student t-test and One-way ANOVA were accomplished, using Prism version 9 software. Results were considered statistically significant when $p \leq 0.05$.

Results

A total of 24 samples from CRC patients were analyzed and the putative neoantigens were identified across different types of mutation.

To identify putative neoantigens, we followed the strategy outlined in *Figure 1A*. We used data from RNA sequencing and whole exome sequencing to identify expressed variants. Mutated variants were identified if at least one read at RNA sequencing was found. Based on this, we generated 51-mer peptide sequences, which were defined as potential neoantigens. We analyzed a total of 24 CRC patients, including 20 MSS patients and 4 MSI patients. MSI tumors presented a higher number of identified mutations than MSS tumors, as shown in *Figure 1B*. Clinical parameters and the distribution of mutations per mutation type are presented in Table 1. The majority of mutations were missense mutations, accounting 59.76% of the total 3743 mutations analyzed.

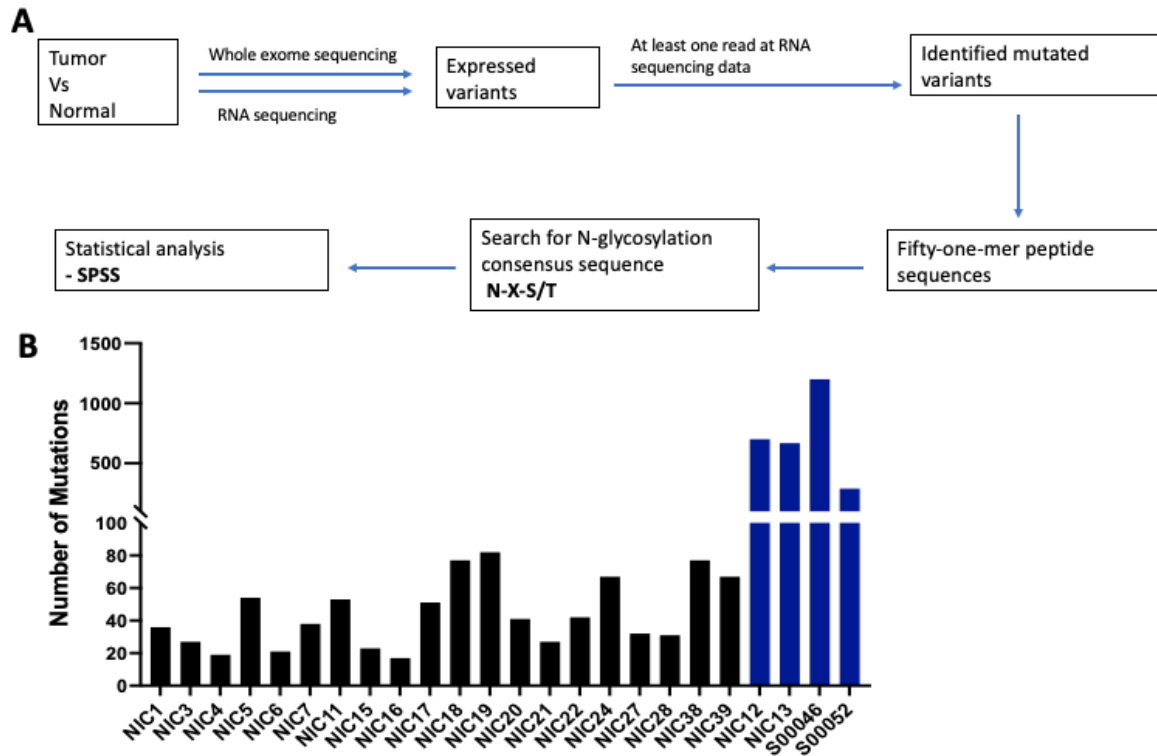


Figure 1 | (A) Workflow of data process and analysis, described in detail on methods. (B) Number of mutations identified per sample (n=24). Blue bars represent MSI tumors.

Alterations in N-glycosylation sites are associated with Stage III colorectal cancer

Screening for the presence of N-glycosylation sites (N-glyco-sites; Asn-X-Ser/Thr) was performed for all putative neoantigens (Table 2). Alterations in N-glyco sites due to the mutation was also considered, and the majority of MSS samples presented no gain or loss of glyco-sites due to the mutation (Table 2). Associations between the % of peptides with N-glyco sites and clinical features were performed, and no statistical differences was found within MMR status, consensus molecular subtypes (CMS) classification and Tumor-Node-Metastasis (TNM) classification (Figure 2). Regarding alterations of N-glyco site, gain or loss, Stage III colorectal cancer samples presented significantly more incidence of lost glyco sites (2.18%) compared to stage I CRC (0.31%) (Figure 2C), but no differences were found in CMS classification or MMR status (Figure 2 A-B).

Table 1 | Characterization of the cohort. Distribution of mutations across mutation type

Sample ID	TNM	MMR status	HLA Staining	CMS	Number of mutated peptides	MUTATION TYPE							
						Synonymous		Missense		Frameshift		Inframe deletion /insertion	
NIC1	Stage IV	MSS	weak	-	36	9	25.0%	25	69.4%	0	0.0%	2	5.6%
NIC3	Stage I	MSS	positive	CMS4	27	7	25.9%	19	70.4%	1	3.7%	0	0.0%
NIC4	Stage II	MSS	positive	CMS4	19	7	36.8%	12	63.2%	0	0.0%	0	0.0%
NIC5	Stage III	MSS	positive	CMS2	54	9	16.7%	43	79.6%	2	3.7%	0	0.0%
NIC6	Stage III	MSS	positive	CMS3	21	2	9.5%	16	76.2%	0	0.0%	3	14.3%
NIC7	Stage III	MSS	weak	CMS2	38	9	23.7%	27	71.1%	2	5.3%	0	0.0%
NIC11	Stage III	MSS	positive	CMS4	53	19	35.8%	33	62.3%	1	1.9%	0	0.0%
NIC12	Stage II	MSI	negative	CMS1	701	174	24.8%	389	55.5%	127	18.1%	11	1.6%
NIC13	Stage III	MSI	negative	CMS1	668	159	23.8%	338	50.6%	159	23.8%	12	1.8%
NIC15	Stage I	MSS	negative	CMS4	23	11	47.8%	12	52.2%	0	0.0%	0	0.0%
NIC16	Stage I	MSS	positive	CMS2	19	2	10.5%	16	84.2%	1	5.3%	0	0.0%
NIC17	Stage III	MSS	positive	CMS2	51	10	19.6%	40	78.4%	1	2.0%	0	0.0%
NIC18	Stage I	MSS	positive	CMS3	77	22	28.6%	53	68.8%	2	2.6%	0	0.0%
NIC19	Stage I	MSS	positive	CMS3	82	20	24.4%	59	72.0%	1	1.2%	2	2.4%
NIC20	Stage II	MSS	positive	CMS2	41	12	29.3%	27	65.9%	2	4.9%	0	0.0%
NIC21	Stage I	MSS	positive	CMS2	27	8	29.6%	19	70.4%	0	0.0%	0	0.0%
NIC22	Stage I	MSS	positive	CMS2	42	15	35.7%	26	61.9%	1	2.4%	0	0.0%
NIC24	Stage I	MSS	positive	CMS4	67	14	20.9%	51	76.1%	2	3.0%	0	0.0%
NIC27	Stage I	MSS	weak	CMS2	32	13	40.6%	17	53.1%	2	6.3%	0	0.0%
NIC28	Stage III	MSS	-	CMS4	31	9	29.0%	22	71.0%	0	0.0%	0	0.0%
NIC38	Stage II	MSS	positive	CMS3	77	28	36.4%	47	61.0%	2	2.6%	0	0.0%
NIC39	Stage IV	MSS	positive	CMS3	67	16	23.9%	47	70.1%	3	4.5%	1	1.5%
S00046	-	MSI	negative	CMS1	1200	299	24.9%	731	60.9%	152	12.7%	18	1.5%
S00052	-	MSI	weak	CMS1	290	76	26.2%	168	57.9%	42	14.5%	4	1.4%
Total	-	-	-	-	3743	950	25.38%	2237	59.76%	503	13.44%	53	1.42%

Table 2 | Absence, Presence or Alteration, gain or loss, of N-glycosylation sites, per sample.

N-GLYCOSYLATION SITES IN PEPTIDES								
	ABSENCE		PRESENCE		GAIN		LOSS	
NIC1	28	77.8%	7	19.4%	1	2.8%	0	0.0%
NIC3	20	74.1%	7	25.9%	0	0.0%	0	0.0%
NIC4	13	68.4%	6	31.6%	0	0.0%	0	0.0%
NIC5	40	74.1%	12	22.2%	1	1.9%	1	1.9%
NIC6	15	71.4%	5	23.8%	0	0.0%	1	4.8%
NIC7	32	84.2%	5	13.2%	0	0.0%	1	2.6%
NIC11	43	81.1%	8	15.1%	0	0.0%	2	3.8%
NIC12	565	80.6%	109	15.5%	14	2.0%	13	1.9%
NIC13	536	80.2%	110	16.5%	7	1.0%	15	2.2%
NIC15	19	82.6%	4	17.4%	0	0.0%	0	0.0%
NIC16	17	89.5%	2	10.5%	0	0.0%	0	0.0%
NIC17	47	92.2%	4	7.8%	0	0.0%	0	0.0%
NIC18	57	74.0%	19	24.7%	0	0.0%	1	1.3%
NIC19	69	84.1%	12	14.6%	1	1.2%	0	0.0%
NIC20	31	75.6%	9	22.0%	0	0.0%	1	2.4%
NIC21	21	77.8%	6	22.2%	0	0.0%	0	0.0%
NIC22	35	83.3%	7	16.7%	0	0.0%	0	0.0%
NIC24	46	68.7%	19	28.4%	1	1.5%	1	1.5%
NIC27	23	71.9%	9	28.1%	0	0.0%	0	0.0%
NIC28	26	83.9%	4	12.9%	1	3.2%	0	0.0%
NIC38	58	75.3%	17	22.1%	1	1.3%	1	1.3%
NIC39	57	85.1%	9	13.4%	0	0.0%	1	1.5%
S00046	962	80.2%	193	16.1%	23	1.9%	22	1.8%
S00052	243	83.8%	43	14.8%	1	0.3%	3	1.0%
TOTAL	3003	80.23%	626	16.72%	51	1.36%	63	1.68%

The type of mutation influences the probability to gain or loss N-glycosylation sites

In order to increase the statistical power and achieve different associations, each mutated peptide was treated individually. We found that frameshift mutations give rise to more alterations in glyco-sites, both gain and loss ($p = 0.000$) (Table 3). In fact, frameshift mutations lead to alterations in several amino acids, while in missense mutations only one amino acid is altered, increasing the probability to gain or loss glyco-sites. In line, the presence of glyco-sites was more frequent in peptides with missense mutation (17.9%). Moreover, the presence or alteration in glyco-sites did not influence the prediction of HLA binding of the peptide, being the great majority identified as binders of HLA (Table 3).

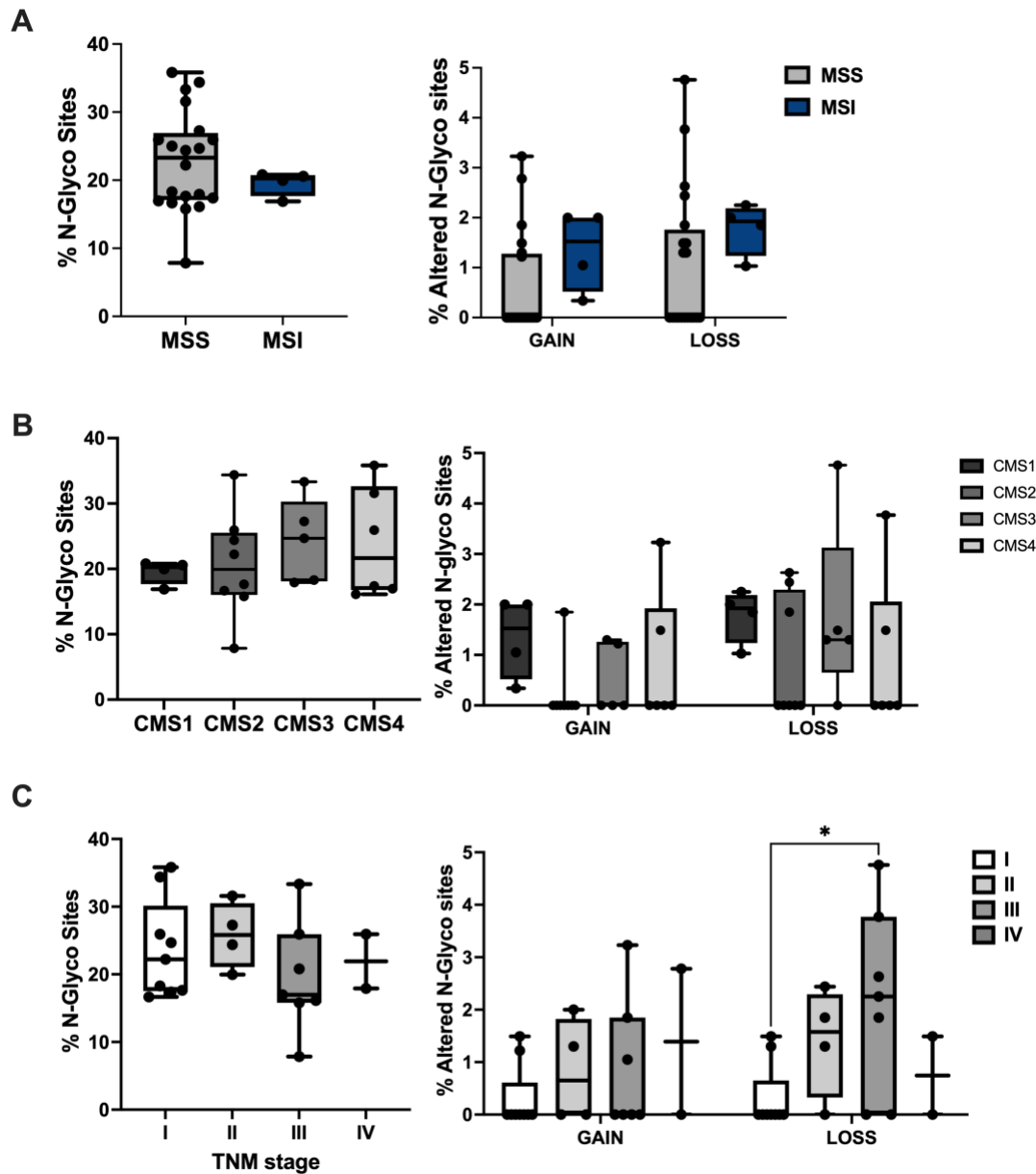


Figure 2 | Percentage of N-glycosylation sites present in peptides and percentage of alteration in N-glycosylation sites to the mutations, according to clinical features: (A) MMR status, (B) CMS classification and (C) TNM stage. Points represent different samples. Unpaired t-test or One-way ANOVA test with multiple comparisons were performed. *, $P \leq 0.05$

Table 3 | Peptides according to type of mutation and HLA binding prediction. Chi-square test.

	N-GLYCOSYLATION SITE IN MUTATED PEPTIDES				p-value
	ABSENCE	PRESENCE	GAIN	LOSS	
SYNONYMOUS (n=950)	785 (82.6%)	165 (17.4%)	0	0	0.000
MISSENSE (n=2237)	1795 (80.2%)	400 (17.9%)	28 (1.3%)	14 (0.6%)	
FRAMESHIFT (n=503)	382 (75.9 %)	50 (9.9%)	23 (4.6%)	48 (9.5%)	
NO HLA BINDER (n=254)	198 (78.0%)	45 (17.7%)	5 (2.0%)	6 (2.4%)	0.873
HLA BINDER (n=683)	546 (79.9%)	114 (16.7%)	11 (1.6%)	12 (1.8%)	

Neoantigens with alterations in N-glycosylation sites (loss or gain) are related to MSI samples

The presence of glyco-sites was also associated with the different clinical features. For instance, MSI-associated neoantigens had more probability to gain or loss glyco-sites than MSS-associated peptides ($p = 0.011$) (Table 4). In accordance, HLA negative-associated peptides also presented more probability to loss or gain glyco-sites ($p=0.004$), which are tightly related with MSI status, since all MSI samples present defects in HLA expression. CMS and TNM classification-associated peptides had found no difference in the presence of glyco-sites.

All together, these results suggest that peptides from MSI samples and with frameshift mutations have more probability to be altered, gain or loss, glyco-sites due to the mutation.

Table 4 | Peptides according to MMR status, CMS classification, HLA presence and TNM stage. Chi-square test.

	N-GLYCOSYLATION SITE IN MUTATED PEPTIDES				p-value
	ABSENCE	PRESENCE	GAIN	LOSS	
MSS (n=884)	697 (78.8%)	171 (19.3%)	0.7 (0.7%)	10 (1.1%)	0.011
MSI (n=2859)	2306 (80.7%)	455 (15.9%)	45 (1.6%)	53 (1.9%)	
CMS1 (n=2859)	2306 (80.6%)	455 (15.9%)	45 (1.6%)	53 (1.9%)	0.145
CMS2 (n=304)	246 (80.9%)	54 (17.8%)	1 (0.3%)	3 (1.0%)	
CMS3 (n=324)	256 (79%)	62 (19.1%)	2 (0.6%)	4 (1.2%)	
CMS4 (n=220)	167 (75.9%)	48 (21.8%)	2 (0.9%)	3 (1.4%)	
HLA NEGATIVE (n=2592)	2082 (80.3%)	416 (16.0%)	44 (1.7%)	50 (1.9%)	0.004
HLA POSITIVE (n=1120)	895 (79.9%)	206 (18.4%)	6 (0.5%)	13 (1.2%)	
TNM stage I (n=396)	307 (77.5%)	85 (21.5%)	2 (0.5%)	2 (0.5%)	0.096
TNM stage II (n=838)	667 (79.6%)	141 (16.8%)	15 (1.8%)	15 (1.8%)	
TNM stage III (n=916)	739 (80.7%)	148 (16.2%)	9 (1.0%)	20 (2.2%)	
TNM stage IV (n=103)	85 (82.5%)	16 (15.5%)	1 (1.0%)	1 (1.0%)	

Glycosylation of MSI HLA negative samples impairs antigen-independent reactivity

As HLA negative samples are not capable to present neoantigens directly to T cells, we tested reactivity of these samples in an antigen independent way, using NK cells. NIC12 and NIC13A established primary cell lines were used in this experiment. The glycosylation profile of these cells showed that NIC13A cells are less glycosylated overall, compared to NIC12, except for α 1,6-sialic acids (SNA-binding motifs) (Figure 3A). In accordance, genes

related to complexity of glycans were also decreased in this cell line (*Figure 3B*). Curiously, in contrast with lectin staining, *ST6GAL1* gene, responsible for α 1,6-sialic acids, was significantly less expressed in NIC13A, as well as *MGAT3*, *FUT8* and *MGAT5* genes (*Figure 3B*). The cells with higher expression of glycans (NIC12) were treated with Kifunensine (KF), in order to abolish complex N-glycans and increase high-mannose glycans (*Figure 3C-D*). Moreover, we observed that the treatment with KF led to a decrease of PD-L1 expression in cell surface and NIC13A had a complete absence of PD-L1 expression (*Figure 3E*). We found that NIC12 cell line without complex N-glycans (NIC12 KF) induced a more efficient NK cell anti-tumor response, when compared to cells without treatment, by the increasing of the percentage of Lysosomal-associated membrane protein 1 (LAMP1)⁺ Granzyme B (GzmB)⁺ NK cells (*Figure 3F*). LAMP1 is a protein present in lysosomes that appears in cell surface upon degranulation, and as anti-LAMP1-FITC was added to co-culture media, cells positive to LAMP1 are those that suffered degranulation in contact with tumor cells. Interestingly, NIC13A cell line, which seems to present a defective of overall glycosylation, induced less degranulation of NK cells than the other cell lines, supporting the idea of a glycan-mediated response (*Figure 3F*). However, this experiment should be repeated in order to increase the number of independent and biological replicates and better understand the anti-tumor immune response mechanism.

These results demonstrated that modulation of glycosylation of tumor cells could be used to promote the NK cell anti-tumor immune response, as a potential cellular immunotherapeutic strategy. Moreover, as NK cell effector immune response is antigen-independent, they could be an important target to use in anti-tumor immunotherapies in HLA negative tumors.

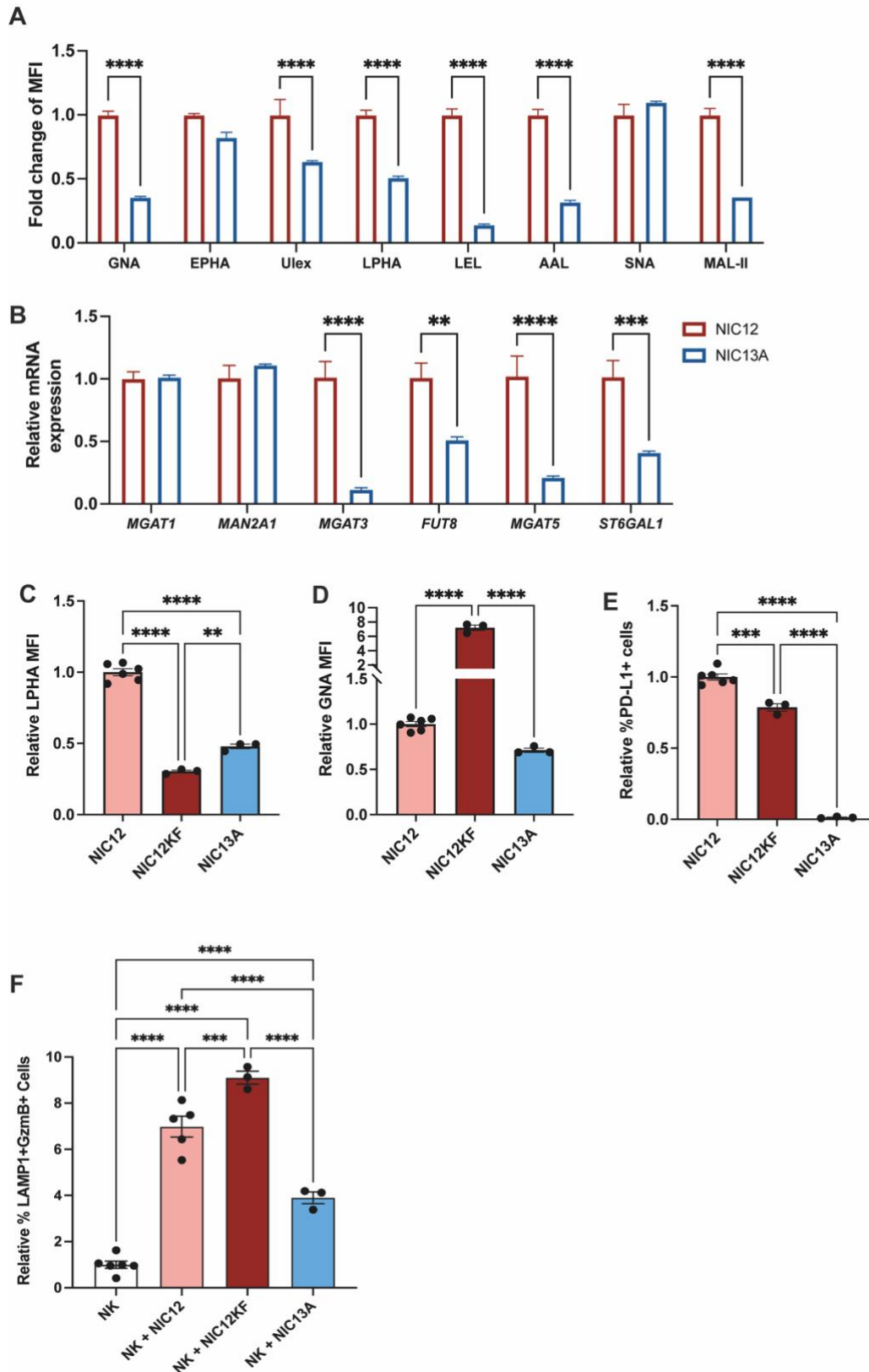


Figure 3 | Characterization and co-cultures of NIC cell lines with NK cells. (A) Lectin binding quantification in NIC12 and NIC13A cell lines, by flow cytometry. Fold change of MFI for each lectin. (B) Relative mRNA expression for several complex N-glycosylation-related genes. (C-E) Characterization of NIC12, NIC12 treated with kifunensine (20 mM) and NIC13A of LPHA and GNA staining, as well as % of PD-L1 positive cells. (F) % of LAMP1+GzmB+ NK cells in co-culture with different tumor cells, NIC12, NIC12 treated with kifunensine (20 mM) and NIC13A. Error bars represent SEM of different technical replicates.

Discussion

The results of this study highlight the relevance of glycans both in innate and adaptative immune response. We reveal that the type of mutation and the clinical and molecular characteristics of the samples are predominately associated with presence, or alterations (gain or loss) of *N*-glycosylation sites. In fact, mutated proteins and neoantigens could be also glycosylated, which might have a relevant impact in immune recognition, both through HLA presentation and cellular surface antigens. Glycans are the first barrier in cell recognition, and it were described as important immune checkpoints in tumor immunology [15].

Presence of putative *N*-glycosylation sites in neoantigens could be a predictor for the presence of *N*-glycans, although not all Asn-X-Ser/Thr motifs carry glycans. Regarding this, further work needs to be done in order to clarify the presence of glycans in neoantigens, especially in HLA presented peptides and how it impacts immune response. Glycoproteomics and Mass spectrometry analysis of eluted peptides from HLA will be the strategy to identify glycopeptide antigens. Moreover, bioinformatic analysis of *N*-glycosylation prediction and identification of already described and annotated variants is also in our pipeline of this study. Nevertheless, we found some interestingly associations of clinical variables with the presence, gain, or loss of *N*-glycosylation sites. We described that the presence of *N*-glycosylation motifs nearby a somatic mutation presents the same distribution within MSS status, CMS classification or TMN stage, although MSS (25.8%), CMS3 (24.3%) and TMN stage II (25.8%) tend to have more % of neoantigens with *N*-glyco sites. Indeed, stage II CRC have been described to display a pick of glycan expression in tumor associated antigens, such as Carcinoembryonic antigen (CEA), decreasing in subsequent stages [26]. In line with this, we observed a significant increase of percentage of peptides with loss of *N*-glyco sites due to a mutation in stage III CRC compared to early stages CRC. In accordance, stage II and stage III exhibited a significantly distinct glycoprofile [27]. These preliminary results were the first step in the study of neoantigens glycosylation and they underlined that glycans could be a player in the antigen-dependent T cell anti-tumor immune response in CRC. However, more experiments need to be done in order to identify the presence of glycosylated neoantigens *in vivo* and understand the real impact of glycans in neoantigen immune recognition and response.

Frequently, MSI CRC tumors have mutations in HLA class I molecule, stopping to present antigens to CD8⁺ T cells through HLA [28]. In fact, in our samples, all MSI tumors presented defects in HLA expression. These patients do not benefit with conventional immunotherapies, nether neoantigen-specific immunotherapies [29]. Therefore, alternative immunotherapies have been considered, using innate antigen-independent immune cells,

such $\gamma\delta$ T cells and NK cells [30, 31]. For instance, HLA-deficient tumors are associated with increased of $\gamma\delta$ T cells, which contribute to a positive response to immune checkpoint blockade in these patients [30]. In line, we used primary cell lines from NIC12 and NIC13, MSI and HLA-negative samples, and co-cultured with NK cells in an antigen-independent manner. The co-cultures have shown that NK cells have a better response, exhibiting increased % of LAMP⁺GzmB⁺ cells in culture with NIC12, which increases with the treatment with KF, by inhibiting complex *N*-glycosylation. These results are in line with our previous results that have shown an increase in anti-tumor immune response when complex branched *N*-glycans are removed [15]. Compared to NIC12, NIC13 cell line is hypoglycosylated, with down regulation in almost all tested complex glycosylation-related genes and with reduced staining of lectins specific to high-mannose, β 1,6-GlcNAc branched, sialylated and fucosylated glycans. It could be related with mutations in glycosidases, for example, that affect its function, or due to the mutations that affects glycosylation sites. For instance, NIC13 is a stage III tumor, and we observed an increasing of peptides with loss of *N*-glyco sites in this stage, which also could be related to less glycosylation. These data support the hypothesis of a distinct immune response against different tumors depending of their glycosylation, and how glycans could be critical for the design of immunotherapies.

However, the specificity of NK cells to glycans and which receptors mediate their activation/inhibition will need to be further investigated. NK cells present several sialic acids binding receptors, such as Siglec-7 and -9 and Lectin-like receptors natural killer group 2D (NKG2D) [32, 33]. Siglec-7 and -9, when bind to sialic acids on tumor cells, have a strong inhibitory effect on NK cell-mediated killing [34-36]. In line, removal of sialic acids on tumor cells reduces the binding of Siglecs and simultaneously enhanced the binding of NKG2D to its ligands, MHC class-I-chain related proteins A and B (MICA and MICB), leading to a better NK cell-mediated tumor cell lysis [37]. On the other hand, the binding of NKG2D to sialyl-Lewis X (sLeX) activates the NK cells and promotes tumor killing, therefore tumors that overexpressed sLeX are more susceptible to NK cell-mediated lysis [38]. In our results, the presence of sialic acids in tumor cells need to be clarified. For instance, the treatment of NIC12 with KF will probably inhibits sialylated glycans, since glycosylation blocks MAN1A1 enzyme earlier in the pathway, preventing the sialic acids expression of *N*-glycans. Nevertheless, KF also decreases the immune checkpoint molecule PD-L1 in tumor cells, supporting the idea of a less inhibitory signaling and a more efficient anti-tumor immune response. Additionally, NK cells also expressed the killer immunoglobulin-like receptors (KIRs), inhibitory receptors which identify self and non-self MHC molecules, preventing the attack against host cells [39]. In line, as samples are HLA negative samples, the inhibitory signal promoted by its binding was suppressed, improving NK cell activation and function

[40]. However, we used NK cells from healthy donors instead of NK cells from tumor cells' patient, which could influence the results. Nonetheless, as the cell lines lack HLA, we believe that the influence of KIRs would be minimal in *in vitro* assays, although it is something that need to have in consideration in further experiments.

These are preliminary results that need to be further clarified by exploring how glycosylated neoantigens act as mediators in both adaptive and innate immune response. We open the windows for a new era of glycoengineered immunotherapies, targeting glyco-antigen-specific T cells and NK cells anti-tumor immune response.

Authors contributions:

M.C. Silva: conceptualization, software, formal analysis, validation, investigation, visualization, methodology, writing—original draft, writing—review and editing. **D. Ruano:** software, visualization, methodology. **M.M. Vicente:** software, validation, methodology. **J.van den Bulk:** methodology. **N.F.C.C. de Miranda:** conceptualization, resources, supervision, funding acquisition, investigation, project administration. **S.S. Pinho:** conceptualization, resources, supervision, funding acquisition, investigation, writing—original draft, project administration, writing—review and editing.

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Chapter 4

The role of T cell glycosylation in colitis-associated
colorectal cancer progression

The role of T cell glycosylation in colitis-associated colorectal cancer progression

(In preparation)

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Abstract

Inflammatory bowel disease (IBD), which comprises ulcerative colitis and Crohn's disease, is a chronic inflammatory disorder of the gastrointestinal tract. Patients with IBD have an increased risk to develop colorectal cancer (CRC), driven by long-lasting and poorly controlled inflammatory activity, extension of the disease, among others. It is known that inflammation plays a critical role in driving malignant transformation, activating several immune-mediated pathways. CD4⁺ T cells are the major players in the pathology of IBD.

Glycosylation, a post-translational modification that occurs in essential all cells, mediates the immune response, including CD4⁺ T cell function. Previous results shown that glycosylation of colonic CD4⁺ T cells in IBD patients present a defect in β 1,6-N-acetylglucosamine (GlcNAc) branched N-linked glycans in TCR, and it is associated with severity of the disease. Additionally, mice with absence of these branched N-glycans (*Mgat5*^{-/-} mice) are more susceptible to develop DSS-induced colitis. On the other hand, in CRC, these glycans are important to sustain an immunosuppressive microenvironment. β 1,6-GlcNAc branched N-glycans are overexpressed in tumor cells, which is related with the inhibition of pro-inflammatory response, promoting tumor growth. In line, removal of these complex glycans enhances anti-tumor immune response and impairs tumor development. However, how hyperactivation of T cells gives rise to the immunosuppressive microenvironment necessary for the development of colitis-associated cancer (CAC) and how glycans are implicated in this switch remain completely unknown, and it is the main goal of this study.

In IBD patients, we found that the absence of branched N-glycans in the stromal compartment was associated with increased risk to develop CAC, presenting higher levels in carcinoma stage. Moreover, in a mouse model of CAC, we shown that the absence of branched N-glycans by the deletion of *Mgat5* gene delayed tumor development and enhanced anti-tumor immune response. In fact, an augment in CD3⁺ T cells and M1-macrophages was observed in the absence of these structures. Additionally, wildtype (WT) mice presented an increase of *Mgat5*-mediated glycans along the cascade, that was accompanied by a decrease of CD8⁺ T cells and MHC class I expression, which was not found in *Mgat5*^{-/-} mice.

These data support the hypothesis of an alteration in glycosylation profile in T cells in CAC, following the shift from inflammation to cancer. Moreover, the modulation of glycosylation in CAC model seems to be a promisor approach to precludes CAC development.

Background

Inflammatory Bowel Disease (IBD) is a chronic inflammatory disease of the gastrointestinal tract, that comprises Ulcerative Colitis (UC) and Crohn's Disease (CD). IBD patients have a 2-3-fold increased risk to develop Colorectal Cancer (CRC), related to family history of CRC, long-lasting and poorly controlled inflammatory activity, extension of the disease, presence of colonic strictures, presence of inflammatory pseudo polyps and concomitant primary sclerosing cholangitis [1-5]. Colitis-associated CRC (CAC) is driven by inflammation, which lead to activation of several immune-mediated pathways and signaling, giving tumor cells an important advantage in cancer development [6]. Moreover, the continuous activation of NF- κ B pathway induces oxidative stress-induced damage to DNA, that may activate tumor-promoting genes and inactivate tumor-suppressing genes [7, 8].

As an inflammatory disease, immune response and immune cells have a crucial role in the development of CAC. It has been described that IBD patients have increased levels of Interleukin (IL)-17-producing cells, T helper (Th)-17, which triggers a severe inflammation and promotes tissue damage and cancer progression [9, 10]. Besides the Th17 cells, other CD4⁺ T cells are also involved in CAC development. In fact, it has been shown that Th2 immune response promotes tumor growth in a model of CAC [11].

Glycans have been largely described as mediators in IBD, CRC and inflammation-driven CRC [12-15]. Glycosylation is a post-translational modification present in essential all cells, as colonic or immune cells. Glycans play key roles in several physiopathological processes, such as inter- and intracellular signaling, immune defense, inflammation, cell adhesion, molecular recognition and pathogen invasion [16]. The gut mucosa is covered by a dense and complex coat of different glycans structures, giving rise to the so-called mucosa glycocalyx. The glycocalyx is the first physical and biological barrier that protects the epithelium against the harsh gut environment.

Changes in the profile of glycans structures have been associated with many pathological processes, including inflammation and cancer. For instance, in CRC, increase in overall sialylation, branched N-glycans and core fucosylation are the major changes. Specifically, in N-linked glycans, it is frequently observed an overexpression of β 1,6-N-acetylglucosamine (GlcNAc) branched N-linked glycans, caused by an increased activity of GnT-V, encoded by *MGAT5* gene [15]. On the other hand, in IBD, these glycans have a key role on the regulation of T cell activity. IBD patients have shown a decrease of these complex N-glycans specifically on the TCR of CD4⁺ colonic T cells, being associated with disease severity [17]. This major alteration has implications in the T cell activation threshold, by affecting the TCR clustering, and promoting a hyperactivation of T cells in IBD [18, 19]. In line, the metabolic supplementation of colonic T cells *in vitro* and DSS *Mgat5*^{-/-} mouse

model with *N*-acetylglucosamine (GlcNAc) have been described to repair the deficiency in complex branched *N*-glycans, which are related to a controlled T cell-mediated immune response and disease control [19]. In the opposite, in cancer context, it has shown that Galectins, such as galectin (Gal)-1 and Gal-3, which are released by tumor cells or immune cells, are able to bind to galactose of complex branched glycans present in the TCR, inducing apoptosis and impairing anti-tumor immune responses [20, 21]. However, how the hyperactivation transforms into immunosuppression in CAC, and how glycans are implicated in this switch is something that remain unknown.

The aim of this study is to uncover the role of *N*-glycosylation, with focus on T cells, in the transition of IBD to CRC, and to identify a potential new risk biomarker for tumor development in IBD. In IBD patients, we found an association between decreased levels of branched *N*-glycans and increased risk to develop dysplasia. On the other hand, this deficiency in branching has been restored in carcinoma. Moreover, the gold standard *in vivo* model of CAC, the azoxymethane (AOM)/dextran sulfate sodium (DSS) mouse model, demonstrated a tendency to increase β 1,6-GlcNAc branched *N*-glycans in T cells from inflammation to dysplasia, that was accompanied with a decrease of CD8⁺ T cell levels. In contrast, the abolishment of branched *N*-glycans precluded tumor development and enhanced anti-tumor immune response, with increased infiltration of T lymphocytes and M1-macrophages.

These data support the hypothesis that glycosylation switching, particularly in T cells, in CAC appears to accompany the transition from inflammation to cancer. Moreover, we highlight the importance of T cell glycosylation, as biomarkers of prognosis and monitoring in CAC progression and a potential target to therapy.

Materials and Methods

Mice

All mouse procedures were approved by the Institute for Research and Innovation in Health (i3S) ethics committee for animal experimentation under Portuguese regulations. Mice were housed at the animal facility of i3S, University of Porto, Porto, Portugal (i3S, Porto, Portugal). C57BL/6 wild-type and *Mgat5*-knockout (*Mgat5*^{-/-}) mice (established and provided by Prof. Michael Pierce, CCRC, Georgia University, Athens, GA, USA) with 8-10 weeks old were used for the induction of AOM/DSS CAC model.

Human samples

Formalin-fixed paraffin-embedded sections (FFPE) of colonic mucosa were obtained from a cohort of patients from *Centro Hospitalar Universitário de Santo António*

(CHUdSA), Porto, Portugal and from *Hospital Clínico San Carlos* (HCSC), Madrid, Spain. From CHUdSA, 42 patients were included in the cohort based on the European Crohn's and Colitis Organisation (ECCO) guidelines for the surveillance of CRC in IBD patients [22]. From these patients, 21 were classified as low risk to develop CAC, 11 were classified with an intermediate risk and 10 were considered high risk to develop CAC. From these 10 patients, 9 of them prograde to dysplasia (either low-grade or high-grade dysplasia). 190 FFPE sections of colonic tissue were provided from all these 42 patients in different stages of CAC development. From HCSC, 38 FFPE sections of colonic tissue were provided from 18 patients diagnosed with UC, with or without dysplasia or carcinoma (UC n=24; Dysplasia n=5 or IBD-associated carcinoma n=9). Provided material was used to carry out lectin histochemistry and RNA extraction for analysis of gene expression through Real Time PCR (RT-PCR).

AOM/DSS mouse model

CAC development was explored using a chemical induced mouse model. The model consists in the repeated dextran sulfate sodium (DSS) administration combined with the treatment with the genotoxic agent azoxymethane (AOM) [23]. Two independent experiments were performed from 2019 to 2021, using WT and *Mgat5*^{-/-} mice. The AOM/DSS model was initiated by an intraperitoneal injection of 8 mg/kg AOM (Sigma-Aldrich), and DSS treatment started up to seven days after AOM injection. Oral DSS (MP Biomedicals) was carried in three cycles of seven days (first cycle) or five days (last two cycles), separated by a recuperation period of at least two weeks. DSS was dissolved in the drinking water at 2% for the first cycle and 1.5% for the last two cycles. Throughout the experiment, mice were monitored 3-5 times a week, and rectal bleeding, blood in stools, stool consistency, as well as mice weight were assessed, and Disease Activity index (DAI) was calculated. To reach different stages of colitis-associated carcinogenesis (inflammation, dysplasia, and carcinoma), four different timepoints for euthanasia were consider across the course of the disease for each experiment. The experiment was concluded when animals present signals of CAC, such as big lesions identified in colonoscopies, or after 20 weeks from AOM injection.

Mice were euthanized with lethal doses of isoflurane or CO₂ and the colon was collected. Upon opening the colon, the entire luminal surface was observed, and the lesion size was measured in the last time point. A small portion of the distal and proximal colon was bisected to perform *ex vivo* culture (explant) of the tissue to further analyze the cytokine production. Distal colon from AOM/DSS group was bisected longitudinally in three equal portions. One portion was stored in *RNA later* stabilization solution (Sigma-Aldrich) for further transcriptome analysis and other for flow cytometry analysis. The third portion of the

colon was fixed in 4% formaldehyde (Enzymatic) for further paraffin embedding, processing, and slicing for hematoxylin and eosin (HE) staining and lectin histochemistry.

Tissue digestion

The tumors were manually minced, digested in a dissociation medium (RPMI1640 GlutaMAX medium (Gibco) with collagenase IV (0.9 mg/mL; Sigma-Aldrich) supplemented with 10% FBS, 100 U/mL penicillin/streptomycin, 1 mmol/L CaCl₂ and 1 mmol/L MgCl₂ for 45 minutes with agitation at 37°C. Immune cells compartment were isolated with LymphoPrep™ solution, according to the manufacturer's protocol. Finally, the immune mononuclear cell layer was collected, and cells were centrifuged to follow for flow cytometry staining. Epithelial cells, deposited on the bottom, were also collect and analyzed.

Histological analysis (HE and Lectin histochemistry)

Glycan profile from 3 µm FFPE tissue sections of colonic biopsies from IBD patients and from AOM/DSS mice model were performed. It was accomplished by lectin histochemistry using biotinylated lectins *Phaseolus Vulgaris Leucoagglutinin (L-PHA)* that recognize β1,6-GlcNAc branched. To block endogenous peroxidase, 3% H₂O₂ (Thermo Fisher Scientific) in methanol (Thermo Fisher Scientific) solution was used. Before staining, unspecific proteins were blocked with 10% BSA (Sigma-Aldrich). The sections were incubated for 1 hour with biotinylated lectin (13.3 mg/mL; L-PHA; Vector Laboratories). To provide the detection, the sections were incubated with ABC-HRP solution from the Vectastain ABC Kit (Vector Laboratories) followed by 3,30-Diaminobenzidine tetrahydrochloride (0.67 mg/mL; DAB, Sigma-Aldrich) substrate.

Sections from colonic tissue of AOM/DSS mice model were stained with HE, in order to analyze histologically the morphology and stage of lesions. Slides were stained with hematoxylin (Richard-Allan Scientific™ Modified Mayer's Hematoxylin, Thermo Scientific, DE, USA), washed with water and embedded in ammoniacal water 1% to differentiate the nuclei. The slides were washed again with water and ethanol 95% and then embedded in eosin (Eosin-Y, Thermo Scientific, DE, USA).

Histological evaluation of the mice samples was performed by the pathologist Dr. Joana Alves, MD, and samples were regrouped accordingly the histologic classification, either in colitis, dysplasia or carcinoma.

Flow Cytometry

Single cells from AOM/DSS mice colon were stained by flow cytometry, in order to distinguish different immune populations. Dead cells were stained with Fixable Viability Dye

(FVD), and then stained with different lectins L-PHA, *Galanthus Nivalis Lectin* (GNA), *Sambucus Nigra Lectin* (SNA).

Table 1 | Monoclonal anti-mouse antibodies and Lectins used in Flow Cytometry

Anti-mouse mAb / Lectin	Conjugation	Clone	Supplier
L-PHA	FITC	-	Vector Laboratories
GNA	Biotin	-	Vector Laboratories
SNA	Cy5	-	Vector Laboratories
FVD	APC-eFluor780	-	Invitrogen
CD45	FITC	30-F11	Invitrogen
CD3E	Brilliant Violet 510	145-2C11	BioLegend
CD4	eFluor450	RM4-5	Invitrogen
CD8a	PE-Cy7	53-6.7	Invitrogen
$\gamma\delta$ TCR	APC	eBioGL3	Invitrogen
PD-1	PE	J43	Invitrogen
CD24	PE	M1/69	Invitrogen
CD64	APC	X54-5/7.1	Invitrogen
CD11b	eFluor506	M1/70	Invitrogen
CD11c	eFluor450	N418	Invitrogen
MHC-II	PE-Cy5	M5/114.15.2	Invitrogen
CD206 (MR)	PE-Cy7	MR6F3	Invitrogen
EpCam	Brilliant Violet 421	G8.8	BioLegend
MCHI	eFluor450	AF6-88.5.5.3	Invitrogen
PD-L1	PerCP/Cy-5.5.	10F.9G2	BioLegend
Streptavidin	PE	-	BioLegend

FITC: fluorescein isothiocyanate; **PE**: phycoerythrin; **APC**: allophycocyanin; **PerCP**: peridinin chlorophyll; **Cy**: cyanine

After that, extracellular markers were stained: anti-CD45, anti-CD3E, anti-CD4, anti-CD8a, anti- $\gamma\delta$ TCR, anti-PD-1, anti-CD24, anti-CD64, anti-CD11b, anti-CD11c, anti-MHC-II, anti-CD206, anti- EpCam, anti-MCHI, anti-PD-L1. Antibody and lectin details are described in *Table 1*.

Data acquisition was performed in the FACS Canto v.2 flow cytometer (BDBioscience), using the FACSDiva software (BDBioscience). The files were then analyzed with FlowJo software version 10.

RT-PCR

Real-time PCR was performed to analyze the glycogene and immune gene expression in samples from FFPE samples of the human IBD-associated cancer cohort, as well as from experiments of AOM/DSS model.

Prior to conversion into complementary DNA (cDNA), RNA concentration was quantified with a NanoDrop 1000 UV-Vis spectrophotometer (Thermo Scientific). cDNA conversion was prepared by using SuperScript IV Reverse transcriptase (Invitrogen) according to the manufacturer's instructions. Quantification of amplified transcripts relied either on TaqMan probes or through SYBR Green fluorescence with specified primers.

For TaqMan-based quantification, cDNA samples were added to TaqMan probes conjugated to either FAM or VIC fluorophores and TaqMan™ Universal PCR Master Mix (1:2; Thermo Fisher). The probes used were: *MGAT5* (Hs00159136_m1), *MAN2A1* (Hs01123597_m1), *18S* (Custom human and mouse), *Mgat5* (Mm00455036_m1), *Ifng* (Mm011g8134_m1); all from Taqman, except *18S* (IDT).

For SYBR Green-based quantification, cDNA was combined with reverse and forward primers (250 ng/mL; IDT, IA, USA) and NZYSupreme qPCR Green Master Mix (1:2; NZYTech). The primers used in this samples were: *Cd3* Fw: 5'-CGTCCGCCATCTTGGTAGAG-3', Rv: 5'-ATTCAATGTTCTCGGCATCGT-3'; *Tnfa* Fw: 5'-GTGACAAGCCTGTAGCCAC-3', Rv: 5'-GCAGCCTTGTCCTTGAAGA-3'; *Foxp3* Fw: 5'-CCTATGGCTCCTTCCTTGGC-3', Rv: 5'-GATCATGGCTGGGTTTGCCA-3'; *Gapdh* Fw: 5'-GAAGGTCGGTGTGAACGGAT-3', Rv: 5'-CTCGCTCCTGGAAGATGGTG-3'; all from IDT.

Ct values were normalized with *18S* or *Gapdh* or as the internal controls for TaqMan probes or SYBR Green, respectively, and relative expression values were calculated with the $2^{-\Delta\Delta Ct}$ formula. In both instances, reaction mixtures were in MicroAmp™ Optical 96 well reaction plates and amplification data were acquired in a 7500 Fast Real-Time PCR system (Thermo Fisher).

In silico data

Microarray data was obtained from the ArrayExpress repository (accession number E-GEOD-37283) and analyzed in RStudio. The accessed dataset consisted of raw microarray fluorescence measurements belonging to a 2013 study [24] obtained with the Affymetrix platform. Samples of colonic mucosa were obtained from a total of 20 patients: 11 had neoplasia deriving from ulcerative colitis, 4 only quiescent ulcerative colitis and the remaining 5 consisted of normal controls. Raw data was pre-processed in accordance with

the protocol established by Klaus & Reisenhauer [25]. Briefly, this involved the removal of probes for which measure fluorescence was below the measured non-specific background followed by calibration by across-array normalization. This normalized dataset was then refined by filtering for genes with low expression and low variation across all samples. Finally, all probes for which multiple mapping detected were eliminated to rule out redundant transcript identification. The final processed dataset consisted of 27 800 gene expression values (expressed as $\log_2(\text{normalized fluorescence})$) across 19 samples, with one sample removed owing to substantial variation in gene expression when compared to other samples of the same type and to all other samples.

A list of 19 genes related to glycan biosynthesis was selected to perform principal component analysis with *prcomp* package. This same list was also utilized for calculating Spearman correlation coefficients between glycogenes and genetic markers of T cell populations and function. The packages *Hmisc* and *knitr* were utilized to determine correlation coefficients and their significance. All plots were produced with the *ggplot* package.

In addition, microarray data from a 2012 study exploring the AOM/DSS model of colitis-associated inflammation was also analyzed [26]. Pre-processing was performed as detailed above yielding $\log_2(\text{normalized fluorescence})$ values for a total of 20 838 genes across 18 samples, with 3 replicates for each of the following stages: normal, inflamed, low grade dysplasia, high grade dysplasia at 6 weeks, high grade dysplasia at 8 weeks and carcinoma. For evaluating differences between the relative expression of glycogenes, normalization was performed by dividing each value of gene expression for each replicate by the average of the same gene across samples from the normal condition.

Statistical analysis

Outliers test was performed for all experiments prior to analysis, and outliers identified were excluded. One-way ANOVA followed by Dunn's multiple comparison test was employed for analysis of gene expression in RT-PCR from human samples. Correlations were tested with Spearman's correlation coefficient. For all remaining data, two-way ANOVA with mixed effects was performed. Statistical significance was established as any p-value below 0.05.

Results

***In silico* analysis indicates associations between T cell function and N-glycosylation-related genes in IBD-associated carcinogenesis**

The first evidence of the influence of glycosylation in T cell function during CAC development was shown by an *in silico* analysis of a public dataset of transcriptional data (E-GEOD-37283; [24]). This microarray data includes a total of 19 samples from healthy, UC patients and UC with neoplasia patients. Data was accessed and processed, thus producing a calibrated and normalized dataset of gene expression values (calculated as $\log_2(\text{relative expression})$). Specifically, a glycogene module containing genes for key enzymes in N-glycan branching was selected to test whether differences within this module could differentiate between normal and unhealthy mucosal tissue (UC and neoplasia). Principal component analysis was carried out for the selected glycogene module and shown a differential segregation between healthy and diseased states (*Figure 1A*).

In order to reveal possible associations between the expression of N-glycan branching-related glycogenes and T cell markers, this same glycogene module was employed and correlated with different T cell-associated genes (*Figure 1B*). Notable, it was found pronounced positive associations of *CD4* with *MGAT1*, *MAN2B1* and *ST3GAL3* expression, overall. In addition, *ST6GAL1* and *FUT8* expression was broadly correlated with genetic markers for T cells, such as a marker of T cell activation *CD69* and the signaling molecule *STAT4*, which induces expression of the T-bet transcription factor for Th1 differentiation. Of note, we have also observed that *MGAT5* glycogene expression presented negative correlations across nearly all T cell markers, mainly to *CD3E* and *IL2RA*, which suggests that high levels of branching N-glycans may be related to decreased T cell abundance and activation.

Although insufficient to infer the T cell-specific glycosylation and how this shapes its phenotype and function, this approach revealed important associations of T cell infiltration and glycosylation partners.

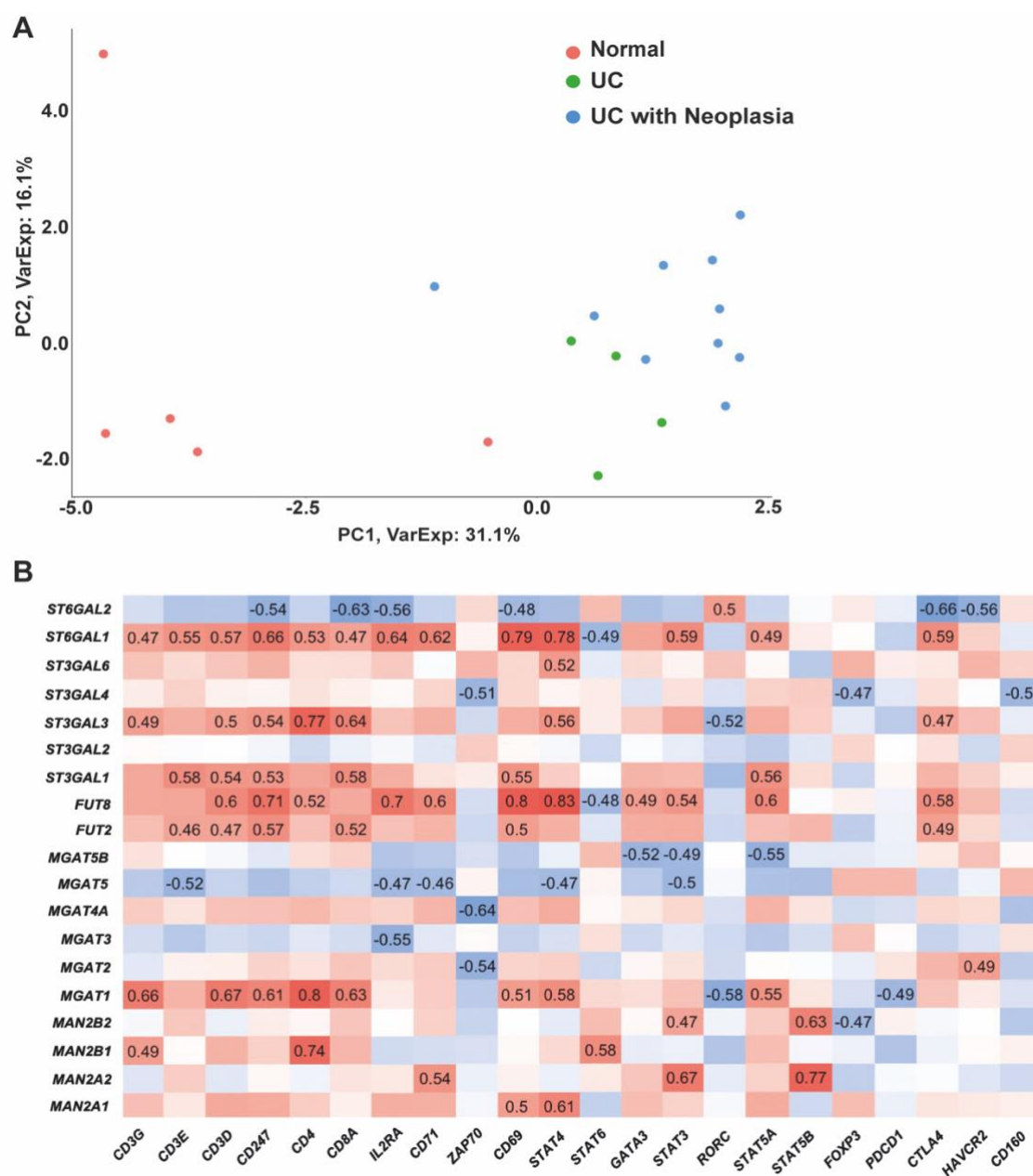


Figure 1 | Analysis of complex N-glycosylation-related genes and immune genes. (A) Principal component analysis of N-glycan branching gene expression distinguishes non-dysplastic mucosa of UC patients with neoplasia and normal controls. Data are plotted as scatterplots of samples into the low-dimensional space defined by the first and second principal components (PC1 and PC2). **(B)** Correlation analysis between various glycogenes and genetic T cell markers. Correlations were tested between genetic markers for T cell populations (including markers for T cell activation, T helper cell differentiation and T cell exhaustion) and N-glycan branching-related genes through the Spearman's method. Red color represents positive correlations, while blue color represents negative correlations. Values for obtained correlation coefficients are only shown for significant correlations ($p \leq 0.05$).

A decrease of branched N-glycans expression in stroma is associated with the risk to develop dysplasia

The risk of IBD patients to progress to neoplastic transformation is associated with prolonged and extension of inflammation, presence of inflammatory pseudo polyps, among

others. In this line, we stratify the samples accordingly ECCO guidelines [22]. Lectin histochemistry for L-PHA, which binds to β 1,6-GlcNAc branched *N*-glycans, of FFPE biopsies from IBD patients revealed an increased expression of branched *N*-glycans in epithelial cells across the cascade, with a peak of expression in carcinoma (*Figure 2A-B*). On the other hand, looking at the stromal compartment, the expression of this complex glycans seems to decrease with the increased risk to neoplasia (*Figure 2A and C*). This observation corroborates with the hypothesis that less branched *N*-glycans on T cells leads to a more severe inflammation, increasing the risk to develop carcinoma. Curiously, there is a number of samples in IBD patients without neoplasia that presents increased levels of L-PHA staining in stromal compartment, which could be interesting to explore the difference in prognosis between these patients and the ones with low straining levels. Regarding overall gene expression in IBD patient samples, *MGAT5* gene showed no significant differences between the groups, but it was interesting to note a peak of expression in low risk IBD patients and in dysplasia, whereas higher risk samples have decreased levels of *MGAT5* expression (*Figure 2D*). Furthermore, overall expression of *MAN2A1* gene, responsible for the transition from high-mannose *N*-glycans to complex *N*-glycans, had a peak of expression in intermediate risk, significantly higher than normal, dysplasia and carcinoma samples (*Figure 2E*). This interesting result points towards a potential risk biomarker for tumor development, since the higher levels of *MAN2A1* gene precedes the appearance of increased levels of branched *N*-glycans in high risk IBD and dysplasia of epithelial compartment, which was observed in L-PHA histochemistry. However, the analysis of RT-PCR needs to be refined in order to clarify these results, including analysis of specific cells (epithelial vs immune cells) from fresh biopsies, and perhaps a longitudinal study of some patients over time could help to elucidate the biomarker potential of this glycogene.

Altogether, the analysis of the glycosylation of IBD patient samples demonstrates an important prognostic glyco-biomarker for the risk to develop dysplasia, associated with low levels of complex *N*-glycans in stroma, which includes T cells, and high levels of *MAN2A1* gene expression in intermediate risk IBD patients.

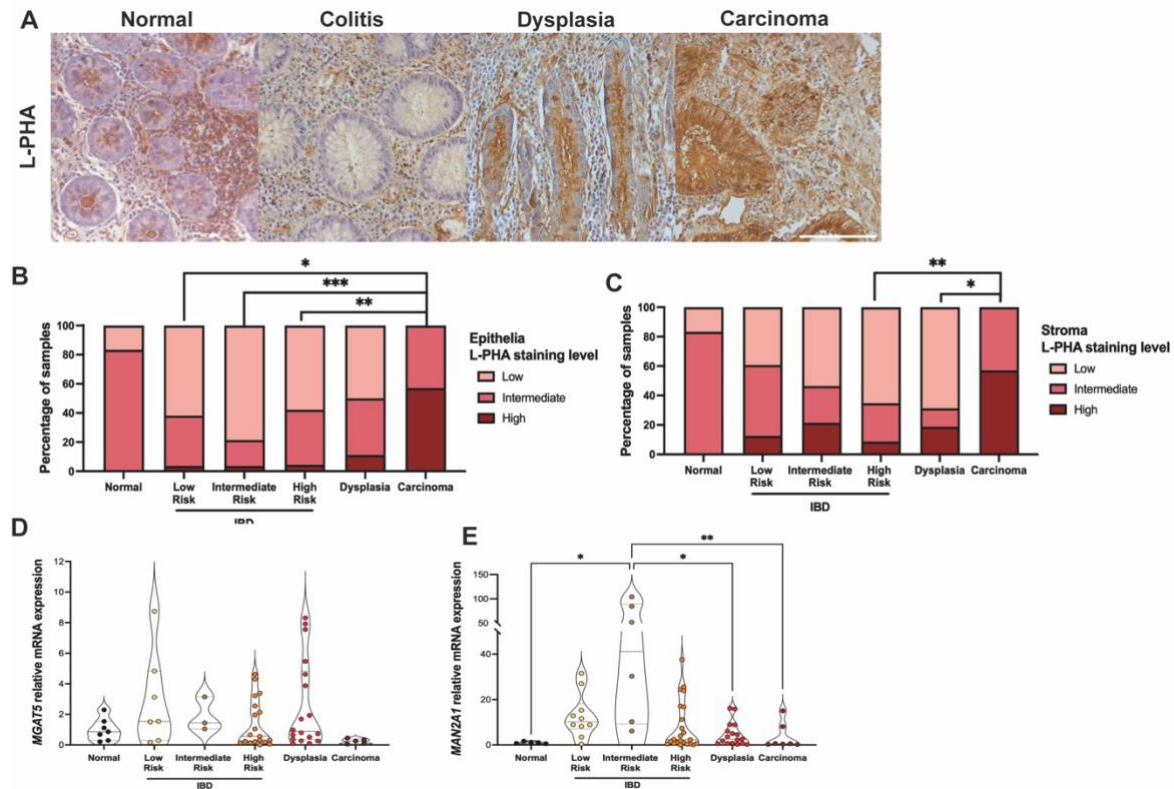


Figure 2 | Increased expression of complex N-glycans change in CAC cascade. (A) Representation of L-PHA histochemistry from Normal tissue to Carcinoma. Scale bar, 100 μ m. **(B-C)** Qualitative classification of CAC cascade samples of Normal (n=6), Low Risk colitis (n=56), Intermediate Risk colitis (n=28), High risk colitis (n=46), Dysplasia (n=16) and Carcinoma (n=7), for epithelial compartment (B) and stromal compartment (C). **(D-E)** Relative expression of *MGAT5* (D) and *MAN2A1* (E) gene, for Normal (n=5), Low Risk colitis (n=10), Intermediate Risk colitis (n=6), High risk colitis (n=25), Dysplasia (n=19) and Carcinoma (n=8). Outliers and non-expressed samples are excluded. Non-parametric test Kruskal-Wallis test with multiple comparisons was performed for all graphs; *, $P \leq 0.05$; **, $P < 0.01$; and ***, $P < 0.001$.

Complex N-glycans in the regulation of T cell-mediated anti-tumor response

In order to further explore the impact of glycosylation in CAC development, we used a mouse model of CAC, the AOM/DSS mouse model. A list of glycogenes was accessed from a data base of AOM/DSS mouse model (GSE31106) [26]. In line with what we observed in human samples, *Mgat5* gene expression increased from inflamed to carcinoma, while *Man2b2* gene, homolog of *Man2a1* gene, presented a decrease from inflamed tissue to dysplasia and carcinoma (Figure 3A). Interestingly, *St6gal1* gene expression decreased in inflamed tissue, which could be associated with a pro-inflammatory feature in T cells (Figure 3A), since α 2-6 acid-sialic in T cells precludes Gal-1 binding and apoptosis.

In order to disclose the role of *Mgat5*-mediated complex N-glycans in CAC development, AOM/DSS was performed in mice with no complex β 1,6-GlcNAc branched N-glycans (*Mgat5*^{-/-}), comparing with wildtype mice (*Mgat5*^{+/+}) with a normal N-glycosylation pathway.

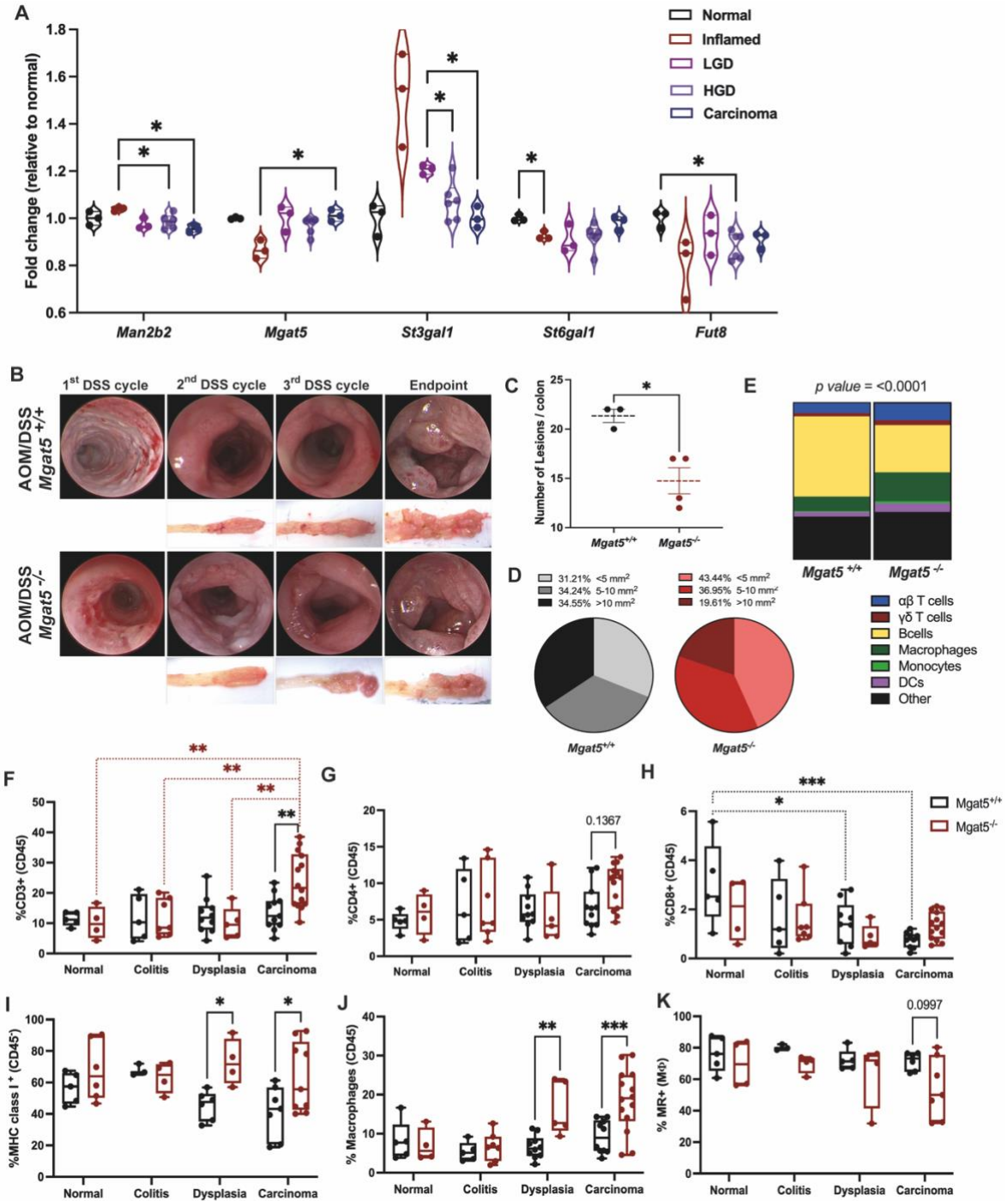


Figure 3 | Tumor burden and immune infiltration associated with complex N-glycan expression in mouse model of CAC AOM/DSS. (A) Relative expression of complex N-glycosylation-relates genes in AOM/DSS mouse model of normal (n=3), inflamed (n=3), low grade dysplasia (LGD, n=3), high grade dysplasia (HGD, n=6) and Carcinoma (n=3). (B) Colonoscopies and image of colon of animals after 1st, 2nd and 3rd DSS cycle and endpoint of experiment (20 weeks), comparing *Mgat5* WT mice with *Mgat5*^{-/-}. (C-D) Number of lesions per animal (C) and segregation of lesions per size (D) in *Mgat5*^{+/+} and *Mgat5*^{-/-} mice at last time point of carcinoma. Unpaired t-test. (E) Tumor immune infiltration of different types of immune cells (CD45⁺ cells) at last timepoint of carcinoma. (F-H) Percentage of CD3⁺ cells (F), specifically CD4 (G) and CD8 (H), across tumor development. (I) MHC class I⁺ tumor cells across tumor development. (J-K) Tumor infiltrated Macrophages (J) and specifically MR⁺ Macrophages (M2-Macrophages). Histological classification of samples by a pathologist, *Mgat5*^{+/+} mice: Normal (n= 5), Colitis (n=5), Dysplasia (n=10) and Carcinoma (n=11) and *Mgat5*^{-/-}: Normal (n= 5), Colitis (n=7), Dysplasia (n=5) and Carcinoma (n=16). Identified outliers were excluded. 2way ANOVA with multiple comparisons. *, P < 0.05; **, P < 0.01; and ***, P < 0.001.

The mice were monitored by colonoscopies, and it was defined several timepoints of euthanasia in order to achieve different stages of disease progression: Colitis, Dysplasia and Carcinoma. Colonoscopies images and macroscopic images showed the progression of tumorigenesis, and it was possible to see the differences in tumor development in *Mgat5*^{-/-} mice (Figure 3B). In fact, the results in the last timepoint revealed a decrease of tumor burden in *Mgat5*^{-/-} mice, with development of significant less lesions with a smaller size (Figure 3C-D). Additionally, anti-tumor immune response of these mice appeared to be more efficient, since KO tumors presented more infiltration of T cells and macrophages (Figure 3E). For instance, in the absence of complex branched N-glycans, we observed a significant increase in CD3⁺ T cells levels, mostly due to the increase of CD4⁺ T cells, compared to WT tumors (Figure 3F-G). Moreover, in WT mice it was observed a decrease in CD8⁺ T cell infiltration from normal to dysplasia and carcinoma, which was not found in *Mgat5*^{-/-} mice (Figure 3H). These results could be related to the stability of MHC class I molecules at the tumor cells surface in *Mgat5*^{-/-} mice, comparing with WT mice that showed a decrease in MHC-I over time (Figure 3I). Regarding macrophages, besides the significant increase of its frequency in dysplasia and carcinoma, the M2-type pro-tumor macrophages, which are characterized by the expression of Mannose Receptor (MR), showed a tendency to be fewer in KO than in WT mice (Figure 3J-K), which pinpoint for an increase of anti-tumor M1-macrophages.

These data support the idea that the absence of branched N-glycans in tumor cells is capable to modulate the microenvironment and promote a more effective anti-tumor immune response in a mouse model of CAC.

Glycosylation of T cells affects their anti-tumor immune function in CAC

In accordance, several glycosylation features related to a proficient anti-tumor immune response was observed in AOM/DSS model. For instance, the absence of β 1,6-GlcNAc branched N-glycans in T cells was confirmed, which was balanced with an increase of mannosylated less complex N-glycans in advanced stages of disease (Figure 4A-B). Moreover, these mice appeared to present increased levels of α 2,6-sialic acids in homeostatic T cells that was drastically decreased in colitis but was restored in dysplasia (Figure 4C). As previous mentioned, expression of α 2,6-sialic acids in T cells is associated with a protection against galectins-mediated apoptosis, which supports the idea of an improved anti-tumor immune response.

In line with the previous results *in silico* and human samples, it was demonstrated a tendency to increase *Mgat5*-mediated complex N-glycans in WT mice T cells during the carcinogenic cascade (Figure 4A, right panel up). Curiously, this trend occurs also in *Mgat5*^{-/-} mice, although in a very low levels compared with WT, which could be due to an increased

activity of homolog *Mgat5b* gene and perhaps have a role in T cell suppression (Figure 4A, right panel down).

All these results support the point that glycosylation of T cells could in fact mediate their function in the context of CAC. However, the T cell function, such as T cell polarization, cytokine production and tumor killing activity, need to be further explored.

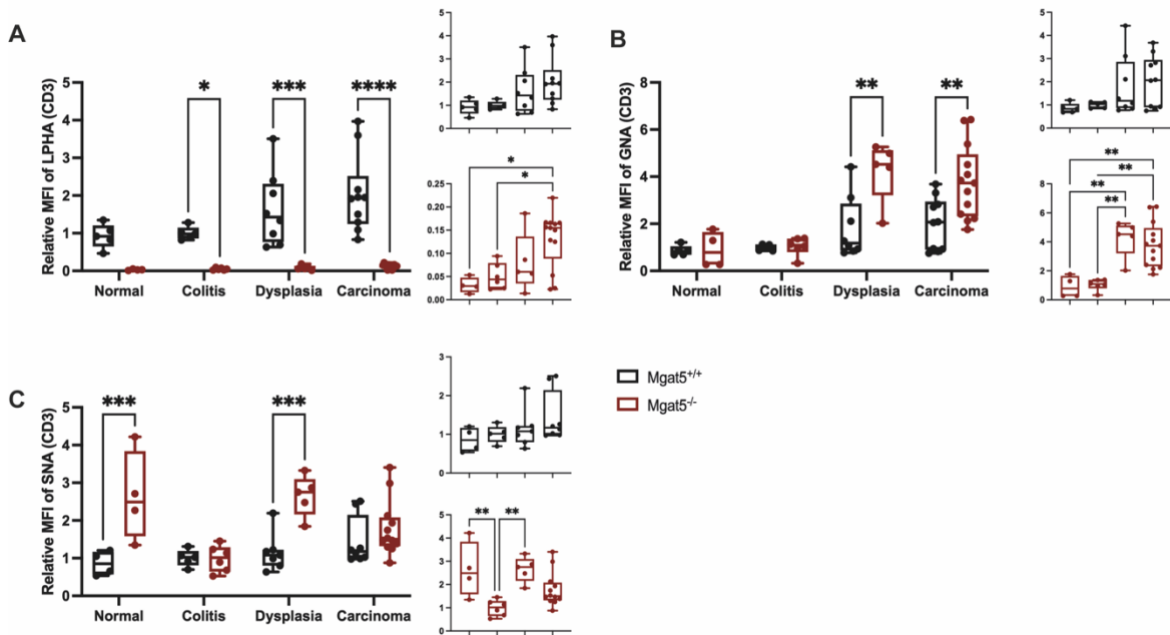


Figure 4 | T cell glycosylation in AOM/DSS model. (A-C) Relative MFI of L-PHA (A), GNA (B) and SNA (C) staining in CD3⁺ T cells across tumor development. Black bars, WT; Red bars, *Mgat5*^{-/-} mice. Histological classification of samples by a pathologist, *Mgat5*^{+/+} mice: Normal (n= 5), Colitis (n=5), Dysplasia (n=10) and Carcinoma (n=11) and *Mgat5*^{-/-} mice: Normal (n= 5), Colitis (n=7), Dysplasia (n=5) and Carcinoma (n=16). Identified outliers were excluded. 2way ANOVA or Ordinary one-way ANOVA with multiple comparisons. *, P < 0.05; **, P < 0.01; ***, P < 0.001 and ****, P < 0.0001.

Discussion

The role of glycosylation in the context of IBD has been extensively investigated, with focus on T cell glycosylation. The T cell glycosylation mediates its function, activation and differentiation, and its glycosylation profile can be very different in inflammation or cancer, highlighting the potential of glycans to predict tumor progression in CAC model. In this line, this study reveals the N-glycosylation profile of immune infiltrated cells from IBD to carcinoma and uncovers the potential of T cell glycosylation as a target to therapy and a biomarker of prognosis. Our group has been previously described that IBD patients display a defect of β 1,6-GlcNAc branched N-glycans on T cells, specifically on TCR, which are associated with a more severe disease [17]. In line, *Mgat5*^{-/-} mice, with no β 1,6-GlcNAc branched N-glycans, developed a more severe DSS-induced colitis [19]. Moreover, we have also been demonstrated that overexpression of β 1,6-GlcNAc branched N-glycans modulates an inhibition of pro-inflammatory response, and the removal of *Mgat5*-mediated N-glycans improves anti-tumor immune response and impairs tumor growth [27]. Understanding how this glyco-mediated hyperreactive immune response in IBD shifts into an immune suppressive microenvironment in cancer was the main aim of this study.

In silico data demonstrated that the signature of glycogenes involved in the process of branching is different from disease to normal tissues, supporting the idea of a glycosylation switch from IBD to CAC. Associations of these glycogenes with the expression of immune genes showed the significance of glycosylation in immune modulation. In this line, the negative association of *MGAT5* gene with different T cell infiltration and activation genes suggests that increased expression of *MGAT5*, with consequent increase in β 1,6-GlcNAc branched N-glycans, impairs T cell infiltration and function. In accordance, this relationship was also demonstrated in mice model of CAC, which showed that the KO of *Mgat5* in mice lead to an increased T cell infiltration in carcinoma.

We believe that N-glycosylation of T cells has a crucial role in IBD-cancer transition, which presents a decreased expression in branched N-glycans, switching their profile to increased levels of complex glycans to suppress T cell activity. In fact, the stromal compartment of CAC histological samples presents higher levels of β 1,6-GlcNAc branched N-glycans compared with the inflammation stage. Interestingly, the decrease of branched N-glycans in stromal cells appears to be related with the risk to develop dysplasia. The hyperactivation of T cells caused by their deficiency in the *MGAT5* gene might be involved in both increased severity of disease and risk to develop cancer. Moreover, it has been already described that *MGAT5* defect in T cells from IBD patients is associated with disease severity [17]. However, how the severity of IBD and risk to develop cancer are related need

to be further explore. For instance, the severe colitis implicates an advanced and extended inflammation, which are features that are related also to risk to neoplasia. However, IBD patients with increased risk to develop cancer are distinguished from severe IBD patients by presenting more than 8 years of diagnosis and a long-term colitis. Therefore, we should have special attention to these patients, as changes in T cell glycosylation may be related to a poor prognosis and an increased risk to develop CRC.

Regarding epithelial compartment, our results have been shown that levels of branched *N*-glycans increase early in high risk IBD patients, even before dysplasia, until carcinoma. In line, an increase in epithelial branching *N*-glycosylation before carcinoma has been already described in CRC by our group, where high grade dysplasia samples present higher levels of β 1,6-GlcNAc branched *N*-glycans [27]. Furthermore, it was interesting to observe a peak of *MAN2A1* gene expression in colonic biopsies of intermediate risk patients. *MAN2A1* gene mediates the transition from high-mannose and hybrid glycans to complex branched *N*-glycans, preceding the coordinated action of *MGAT2*, *MGAT4* and *MGAT5*-encoded enzymes that give rise to β 1,6-GlcNAc branched *N*-glycans [28]. Like *MGAT5* gene, *MAN2A1* have been associated with dysfunctional T cells in cancer context. In fact, the *MAN2A1* defeat renders cancer cells more susceptible to T cell-mediated killing and increases the efficacy of anti-PD-L1 therapy [29]. Therefore, the increase expression of *MAN2A1* in IBD risk patients seems to be the trigger for the develop of cancer, which is followed by increasing of β 1,6-GlcNAc branched *N*-glycans in high risk samples and neoplastic tissue. Altogether, these results highlight the potential of *MAN2A1* gene as a marker of prognosis that could help monitoring the cancer risk in IBD patients. Nevertheless, these results need to be better explored and a longitudinal study in IBD patients should be done in order to understand its predictive role in colitis to carcinoma transition.

Of note, the increased expression of *MAN2A1* gene seems to appear as an attempt to reestablish *N*-glycosylation pathway in IBD patients. For instance, IBD patients present a truncated *N*-glycosylation pathway, showing high levels of mannose in serum and a deficiency in branched *N*-glycans in T cells [19, 30, 31]. Moreover, *Mgat5*^{-/-} T cells have been previous described as hyperactivated cells, which are associated with a more severe inflammation and autoimmune disorders [19, 32]. It has been demonstrated that supplementation with mannose reposes the gut homeostasis and attenuates experimental colitis by protecting intestinal barrier integrity [31]. More recently, defects in *N*-glycosylation pathway have been related with endoplasmic reticulum stress, which causes tissue damage and inflammation. The mannose supplementation reduces endoplasmic reticulum stress and blocks TNF- α -mediated pro-inflammatory circuit [30]. In accordance, the supplementation GlcNAc, which promotes the synthesis of complex glycans, have been

shown to control inflammation by restoring branched *N*-glycans in T cells [19]. These results highlight that a correct *N*-glycosylation pathway is essential to control the disease, reinforcing the importance of *N*-glycans as mediators in inflammation and cancer development.

In accordance, changes of *N*-glycosylation profile also occur in mice model of CAC. For instance, from inflammation to cancer it was observed a progressive increase of *Mgat5* and a decrease of *Man2b2* gene expression, as we found in human samples. The modulation of this profile by removing the β 1,6-GlcNAc branched *N*-glycans (*Mgat5*^{-/-} mice) induces protection against cancer development, mainly due to a more effective anti-tumor immune response. In fact, these mice showed an enhanced infiltration of CD3⁺ T cells, both CD4⁺ and CD8⁺ T cells, and M1-like macrophages in cancer stage in contrast with WT mice. Furthermore, MHC class I in epithelial cells presented constant expression over time, counteracting the tumor immune evasion that occurs during the cancer development [33]. Together with stable levels of CD8⁺ T cells along carcinogenesis, *Mgat5*^{-/-} mice seem to present a more efficient immunosurveillance, being able to impair tumor development. In mice model of CAC, we also identified an increase in high-mannose and sialylated glycans in T cells in dysplasia stage. In fact, the presence of α 2,6-sialic acids in T cells has been described to play a role against Gal-1 mediated T cell apoptosis, which is associated with a more severe inflammation and a more effective immune response. In line, in DSS-induced colitis mice model, *St6gal1*^{-/-} T cells have been shown to induce a less severe colitis by exposing glycosylated ligands for Gal-1 [34]. However, the specific role of high-mannose glycans in T cell activation and function needs to be further explored.

Moreover, the tumor cells from *Mgat5*^{-/-} mice are covered with less complex glycans, which are enriched in mannose glycans [27]. M2-macrophages are characterized by high expression of C-type lectin MR in cell surface, and a recent study demonstrated that the binding of mannose receptor to mannose-decorated nanoparticles or soluble mannose are able to polarizes M2-macrophages into pro-inflammatory and anti-tumor M1-macrophages [35, 36]. Specifically in AOM/DSS model, the supplementation with mannose has been shown to attenuate tumorigenesis also by targeting macrophages polarization [37]. In contrast, the overexpression of GnT-V, and consequent overexpression of branched *N*-glycans in macrophages, leads to a decrease expression of IL-10 and increase of IL-1 β , increasing susceptibility to DSS-induced colitis and colitis-associated tumorigenesis [38]. These studies, together with our results, suggest that, similar to T cells, glycans also play a key role in macrophage function and polarization, mediating M2 to M1-macrophages shift in CAC model, which could be very interesting to further its application in anti-tumor immune therapy.

Altogether, these results disclose the glycosylation changes in the development of CAC, emphasizing the particular role of T cell glycosylation. We disclose the possible role of T cell glycosylation in the shift from inflammation to cancer, highlighting changes in β 1,6-GlcNAc branched N-glycans on T cells as a potential biomarker for prognosis and a target to prevent CAC development. However, further experiments need to be conducted in order to characterize specifically T cell function and polarization mediated by glycosylation in the transition from IBD to cancer. This is an ongoing work, and *in vitro* experiments with colonic organoids, fresh biopsies characterization, new *in vivo* model and a longitudinal study of IBD patients are planned to better describe the significance of all these features as a biomarker and a target in IBD-associated carcinogenesis.

Authors contributions:

M.C. Silva and E. Leite-Gomes: Conceptualization, software, formal analysis, validation, investigation, visualization, methodology, writing—original draft, writing—review and editing. A.M. Dias: Conceptualization, software, formal analysis, validation, investigation, visualization, methodology. G. Faria and Â. Fernandes: Software, formal analysis, validation, investigation, visualization, methodology. B. Santos-Silva, C. Azevedo and B. Oliveira: Investigation and methodology. M.J. Fernandez-Aceñero, C. Taxonera, R. Marcos-Pinto and P. Lago: Resources. S.S. Pinho: Conceptualization, resources, supervision, funding acquisition, investigation, writing—original draft, project administration, writing—review and editing.

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Chapter 5

Glycans as shapers of tumour microenvironment: A sweet driver of T-cell-mediated anti-tumour immune response

Glycans as shapers of tumour microenvironment: A sweet driver of T-cell-mediated anti-tumour immune response

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Funding information

European Regional Development Fund, Grant/Award Number: NORTE-01-0145-FEDER-000029; Fundação para a Ciência e a Tecnologia, Grant/Award Numbers: PD/BD/135452/2017, POCI-01-0145-FEDER-028772, SFRH/BD/07357/2021, SFRH/BD/136388/2018; Liga Portuguesa Contra o Cancro, Grant/Award Number: C 3035006C17

Abstract

Essentially all cells are covered with a dense coat of different glycan structures/sugar chains, giving rise to the so-called glycocalyx. Changes in cellular glycosylation are a hallmark of cancer, affecting most of the pathophysiological processes associated with malignant transformation, including tumour immune responses. Glycans are chief macromolecules that define T-cell development, differentiation, fate, activation and signalling. Thus, the diversity of glycans expressed at the surface of T cells constitutes a fundamental molecular interface with the microenvironment by regulating the bilateral interactions between T-cells and cancer cells, fine-tuning the anti-tumour immune response. In this review, we will introduce the power of glycans as orchestrators of T-cell-mediated immune response in physiological conditions and in cancer. We discuss how glycans modulate the glyco-metabolic landscape in the tumour microenvironment, and whether glycans can synergize with immunotherapy as a way of rewiring T-cell effector functions against cancer cells.

KEYWORDS

cancer immunology, cancer immunotherapy, glycosylation, T cells, tumour microenvironment

INTRODUCTION

One of the most widely occurring post-translational modifications of cells is glycosylation, an enzymatic process in which carbohydrate structures (glycans) are attached to proteins (or lipids), predominantly via nitrogen (N) and oxygen (O) linkages [1]. This pathway starts with an *en bloc* transfer to asparagine residues and entails a sequential addition/removal of carbohydrates, including N-acetylglucosamine (GlcNAc),

glucose (Glc), fucose (Fuc), galactose (Gal), Mannose (Man), N-acetylgalactosamine (GalNAc), 5-N-acetylneuraminic acid (Neu5Ac or sialic acid), among others, by a variety of glycosyltransferases and glycosidases enzymes. This culminates in the generation of intricate glycan structures, with α - or β -linkages between monosaccharides. Glycans then cover the surface of essentially all cells, creating a dense coat of sugar chains, collectively known as glycocalyx. These carbohydrate structures act as key mediators for the

control of several biological processes, including cell-cell adhesion, inflammation, host-pathogen interaction and immune recognition [2].

Alterations on the cellular glycan repertoire (glycome) are widely described as a hallmark of cancer [3, 4]. Specifically, aberrant expression of *N*-glycan structures in cancer cells have been associated with malignant transformation, invasiveness and metastatic potential of cancer cells [5–9]. Notably, this aberrant glycophenotype of malignant cells has a role in cancer immunosurveillance and immunoediting [10–12], which reinforces the major role of glycans in the interaction between cancer cells and immune system.

The immune system is known to be tightly regulated by glycosylation [13], with the development, trafficking, activation and overall function of T-cells being highly dependent on the glycosylation profile of their surface receptors [14]. Therefore, the glycan repertoire at T-cell surface constitutes an important player in the tumour microenvironment (TME) by regulating anti-tumour immunity. This highlights the functional relevance of T-cell glycome in neoplastic conditions, reinforcing the possibility of leveraging T-cell glycosylation as a new strategy to treat and prevent cancer.

In this review, we will discuss the role of T-cell glycosylation in physiological conditions and during malignant transformation, addressing how changes in T-cell glycosylation impact the effector functions of these cells against tumours. We will also highlight the latest advances concerning T-cell glycoreprogramming as a potential immunotherapeutic approach to enhance anti-tumour immune responses and to intercept cancer development and progression.

T-CELL GLYCOSYLATION: THE ROLE OF GLYCANS AS ORCHESTRATORS OF T-CELL DEVELOPMENT, FUNCTION AND ACTIVITY IN HOMEOSTASIS

The immune system is tightly regulated by glycosylation, in which glycans are vital tuners of the circuits that coordinate both innate and adaptive immune responses. Specifically, T-cell development, trafficking, antigen recognition and T-cell activation are highly dependent on the glycosylation profile of T-cell surface receptors [14, 15]. Disruption of this tightly regulated glycan-mediated system can lead to the formation of aberrant immunosuppressive networks

that foster malignant transformation and cancer progression.

T-cell development dynamics are regulated by glycans

The development of T-cells occurs in the thymus. Thymic T-cell development is a major physiological process, in which the formation of a diverse repertoire of T-cell receptors (TCRs) and the establishment of central tolerance are the main goals, as they ensure proper T-cell responses [16]. This process is dependent on both cell-intrinsic and -extrinsic factors, which points to a major role of glycans (Figure 1) [14, 15].

The study of T-cell development has deserved a lot of attention in the last decades, having shaped modern immunology [17]. However, the role of glycans in this complex process is far from being completely understood. The development of T-cells has its initial step in the bone-marrow, where lymphoid progenitors migrate into the thymus, through the bloodstream. Evidence have been suggesting that glycans are essential determinants needed to assure a proper T-cell development [14, 18]. Specifically, we have shown that *N*-glycans are key molecules in the crucial steps of T-cell development, ensure proper stage transition dynamics [18]. In the sialyltransferase *St8SiaIV*^{−/−} mouse model, deficient thymic seeding was observed, caused by inefficient progenitor bone-marrow egression [19]. The trafficking of thymus seeding progenitors (TSPs) requires the expression of P-selectin glycoprotein ligand-1 (PSGL-1) by those cells, and of its partner, P-selectin, by the thymic epithelium [20]. Being PSGL-1 a glycoprotein, it was observed that α1,3-fucosylation presence were required for its binding to P-selectin, and in its absence, by the genetic deletion of the *Fut4* and *Fut7* fucosyltransferases, TSPs homing was impaired [21]. Once TSPs enter the thymus, occurs their development into early thymocyte progenitors (ETPs), a subset of the CD4[−]CD8[−] double-negative 1 (DN1) population. These cells may give rise to multiple lymphoid lineages and their commitment to the T-cell lineage is dependent on Notch signalling [22]. The glyco-profile of Notch receptors was shown to regulate Notch-dependent intracellular signal transduction. Notch receptors are modified by the *O*-fucosyltransferase *Pofut1*, in the repeats of the consensus epidermal growth factor-like (EGF-like) sequences, present in the extracellular domain of Notch [23]. Mice lacking this enzyme in haematopoietic progenitors have severely decreased T lymphopoiesis [24]. These receptors are further modified by the lunatic, manic and radical Fringe glycosyltransferases, which transfer

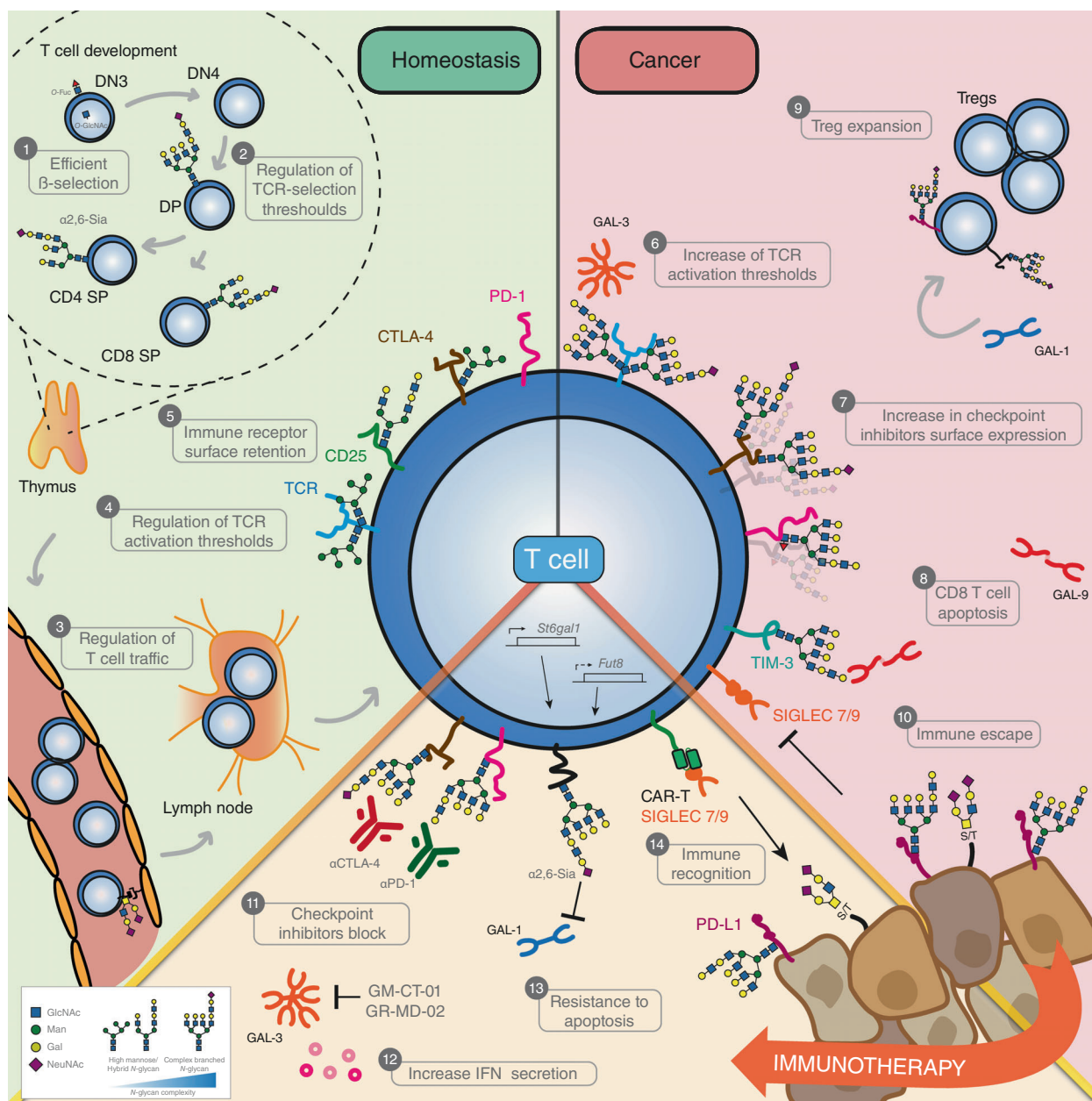


FIGURE 1 Glycans as chief molecules in the T-cell-mediated immune response both in physiological conditions and in cancer. Glycans are master regulators in T-cell function, having a key role in thymocyte developmental progression. In homeostasis, in the initial $\alpha\beta$ T-cell lineage commitment step, β -selection of DN3 cells (1), O-GlcNAc and notch glycans are key determinants for normal development. On other hand, branched N-glycans regulate the thresholds of TCR-selection (2), occurring in the DP stage, as well as TCR repertoire diversity found in SP thymocytes. On T-cell activation, these migrate to the lymph nodes, via expression of core 1 O-glycans (3). At the periphery, activated T-cells express less complex N-glycans both on their TCR (4) and their co-receptors, such as CD25 (allowing its surface retention (5)) and CTLA-4, guaranteeing a controlled immune response and homeostasis. Nevertheless, changes in the cellular glycosylation pattern expression occur at the tumour microenvironment during the process of malignant transformation at the tumour microenvironment, contributing to cancer immunoediting. In fact, the overexpression of complex branched N-glycans that occurs in cancer, is described to increase the TCR activation thresholds (6) leading to increased surface expression of the inhibitory CTLA-4 and PD-1 (7). In addition, expression of galectins on the tumour microenvironment have profound effects on T-cell function, such as Gal-9 binding to TIM-3 (8), associated with the promotion of CD8⁺ T-cell apoptosis as well as the expansion of Tregs by Gal-1 (9). Sialic acids expressed on tumour cells have been described to inhibit T-cell activity through siglec-9 binding (10). Importantly, from the clinical point of view, T-cell glycans and the correspondent glycan-binding proteins can be modulated to improve T-cell effector functions against tumour cells. Specifically, antibodies against glycosylated forms of CTLA-4, PD-1 (11) and anti-Gal-3 promote T-cell function with increased IFN γ secretion (12). Moreover, the increase of α 2,6 sialic acid (and not α 2,3 sialic acid) on T-cells abrogates Gal-1 binding, avoiding T-cell apoptosis (13). CAR-T-cells expressing siglecs-7/9 receptor exodomain also promote immune recognition of sialic acids on tumour cells (14). Overall, these biological processes highlight T-cell glycoengineering as a promising immunotherapeutic strategy for cancer treatment

N-acetylglucosamine to O-linked fucose glycans [25]. In a mouse model of triple Fringe deficiency, the lack of such glycans in the Notch receptors led to reduced binding to Delta-like ligands (DLL), namely DLL4, altering the frequencies of several T-cell subsets in the thymus [26]. The DN3 thymocytes perform the decisive commitment to the T-cell lineage, by the generation of a pre-TCR signalling complex [27]. Together with Notch and interleukin (IL)-7, the pre-TCR signalling initiates β -selection by inducing the transition to DN4 cells (Figure 1/1). We have shown that DN3 thymocytes lacking the *Mgat1* gene fail to efficiently transit to the DN4 stage [18]. These cells then begin a round of multiple divisions, giving rise to the most represented thymocyte population, the CD4⁺CD8⁺ double-positive (DP) cells. It was observed that intracellular O-GlcNAc is required for DN4 expansion, induced by Notch signalling [28].

In the DP stage, mature TCRs are screened by specificity and binding strength for the major histocompatibility complex (MHC) ligands presented by thymic epithelial cells [27, 29]. The initial step of selection is named 'positive selection', where the DP population is enriched for cells that express immunocompetent TCRs. Cells that exhibit high levels of activation, indicative of self-reactive potential, are targeted for apoptosis, a process called 'negative selection'. Finally, cells that survive these selection checkpoints develop into CD4⁺CD8⁻ or CD4⁻CD8⁺ single positive (SP) cells. One of the major contributions of glycans is represented by the ones of the CD4 and CD8 co-receptors. It was shown that chemical desialylation of CD8 mature cells increases CD8/MHC-I interactions [30], and in fact, *ST3Gal1*^{-/-} mice present significantly altered TCR repertoires, suggesting a role for sialylation in thymocyte selection [31]. Moreover, *ST6Gal1*^{-/-} mice show rescued thymic cellularity, which may be a result of selection impairments [32]. It was also demonstrated that branching N-glycosylation expands the range of TCR signalling of positive selection by differentially controlling both the lower and upper limits of positively selected TCR-MHC-antigen interactions (Figure 1/2). This was pointed out to result from decreased surface expression of CD4/CD8 co-receptors, in *Mgat1*- and *Mgat2*-deficient DP cells [33]. Finally, we have shown that *Mgat1*-deficient DP cells display an impairment on TCR repertoire diversity, suggesting that the regulation of selection thresholds by N-glycans is a major determinant on the overall goal of DP selection, the generation of TCR-diverse mature T-cells [18].

Once mature, SP thymocytes egress from the thymus to the periphery, up-regulating sphingosine-1-phosphate receptor-1 (S1P1), which is required for the cells to reach the bloodstream, where the concentration of S1P1 ligand is higher [34]. It was demonstrated that *Cosmc*-deficient

thymocytes, containing only truncated O-glycans, are able to fulfil thymic developmental programs, but fail to populate the periphery, indicating a role for O-glycans in thymic egress [35].

It is worth noting that the majority of the findings regarding the role of glycans in T-cell development has relied on murine models, highlighting an urgent need for validation in human thymocytes. Nevertheless, the body of evidence in this section showcases glycans as master regulators in T-cell immunology, starting in the development of this key immune cell type.

Glycans as modulators of T-cell trafficking

Following their development and egress from the thymus, recent thymic emigrants need to undergo post-thymic maturation to enter the peripheral naïve T-cell pool. It was shown that the expression of sialyltransferase genes, regulated by the transcriptional repressor NF κ B activating protein (NKAP) and the histone deacetylase 3 (HDAC3), was essential for overall sialylation of recent thymic emigrants. In the absence of such sialylated glycans, peripheral T-cells were bound by IgM and complement proteins, leading to their elimination [36, 37]. Once matured, the trafficking of lymphocytes to lymph nodes is mediated by L-selectin ligands, that are constitutively expressed in endothelial cells in high endothelial venules (HEVs) (Figure 1/3) [38]. The ligands for L-selectin, such as GlyCAM-1 and CD34, rely on the synthesis of sulfated core 1 O-glycans [39, 40]. Interestingly, it was found that L-selectin ligands could also be formed on complex N-glycans [41]. Although sulfated core 1 O-glycans are the primary ligands for L-selectin, in their absence, N-glycans decorated with sulfated sialyl Lewis X (sLeX) can promote the binding of T-cells on HEVs, as demonstrated by β -1,3-N-acetylglucosaminyl transferase 3 (β 3GnT-3) deficiency and the blocking of sulfated core 1 O-glycans present on HEVs [41–43].

On activation, T-cells can synthesize core 2 O-glycans and this post-translational modification converts proteins, such as PSGL-1 and CD43, into P- and E-selectin ligands, which allows their trafficking into non-lymph nodes tissues during inflammatory processes (Figure 1) [44]. Different from recently activated effector T-cells, memory T-cells do not actively synthesize core 2 O-glycans, which limit their capacity to leave the circulation during homeostatic/non-inflammatory conditions. Memory CD8⁺ T-cells, however, acquire the capacity of trafficking into sites of inflammation or tissue injury [45]. In fact, a study showed that memory CD8⁺ T-cells that traffic into the lung or skin after an inflammatory event or a viral infection express core 2 O-glycans and the blocking of

P- and E-selectin impaired the traffic of this population to the local of inflammation [46].

Glycans as modulators of T-cell activation and function

Glycosylation, particularly *N*-glycosylation of the TCR and co-receptors, is also involved in the regulation of T-cell activation/function (Figure 1). T-cell response starts by the recognition of the antigen presented in the context of MHC-I/II. On MHC-I, *N*-glycans are important for protein folding, trafficking to the cell surface and peptide loading [47–50]. In fact, it was demonstrated that blocking MHC-Ia *N*-glycosylation leads to a significant increase in intracellular misfolded protein along with a decrease in cell surface expression [51]. Interestingly, a single conserved site for *N*-glycosylation across all known alleles in the heavy chain of MHC-I was described [52]. Moreover, the knockout of the *Mgat2* gene, by compromising the synthesis of complex branched *N*-glycans, decreases antigen presentation by MHC-II and leads to loss of T-cell stimulatory activity [53].

N-glycans decorating TCRs play an important role in stabilizing molecules at the immunological synapse, protecting against the action of proteases and restricting nonspecific protein–protein interactions, helping to orient the interactions of the proteins in the central clusters [54]. We and others have demonstrated that T-cell activity is particularly regulated by β 1,6-GlcNAc branched *N*-glycans (catalysed by *N*-acetylglucosaminyltransferase V—GnT-V enzyme; encoded by the gene *Mgat5*) on the TCR, by modulating the threshold of T-cell activation and signalling (Figure 1/4) [55, 56]. Additionally, these complex branched *N*-glycans on T-cells can be extended with poly-*N*-acetylglucosamine structures, which are ligands for galectins. Specifically, Galectin (Gal)-3 establishes a molecular lattice with TCR and the CD45 receptor, through the binding to these polylactosamine structures in branched *N*-glycans, preventing TCR clustering, and in this manner controlling T-cell activation and signalling [56, 57]. Additionally, it was demonstrated that the presence of β 1,6-GlcNAc branched *N*-glycans on the TCR inhibits Th1 cell differentiation and promotes Th2 cells polarization [58]. Moreover, a decrease in UDP-GlcNAc and consequently on complex branched *N*-glycans promotes a pro-inflammatory T helper (Th)17 over anti-inflammatory-induced regulatory T-cell (iTreg) differentiation [59]. The anti-inflammatory cytokine IL-10 was also described to induce the expression of *Mgat5*, thus reducing the antigen recognition capacity of CD8⁺ T-cells [60].

N-glycosylation also targets co-stimulatory and co-inhibitory receptors present on T-cells, such as CD28 and

cytotoxic T-lymphocyte protein 4 (CTLA-4) respectively, highlighting its critical role in regulating T-cell activation/function [14]. Co-stimulation through the interaction of CD28 with CD80/86 and CD28-mediated T-cell adhesion can be negatively regulated by *N*-glycans on human CD28. In fact, mutation of all potential *N*-glycosylation sites of CD28 leads to enhanced binding to CD80 expressed on antigen-presenting cells (APCs) [61]. On the other hand, Lau et al. demonstrated that CD28 surface levels are not affected by the absence of branched *N*-glycans [62]. CTLA-4 also exhibits two *N*-glycosylation sites that are involved in the modulation of its cell surface retention on T-cells and consequently in its affinity to CD80/86 on APCs and function (further discussed in section ‘Glycosylation of immune checkpoints at the cross-talk between tumour and immune cells’) [63–65].

Other T-cell receptors are also highly dependent on *N*-glycosylation. For instance, CD45 is decorated with *N*-glycans that are involved in T-cell activation, differentiation, migration, signalling and apoptosis [66]. Of note, all of the different isoforms of CD45 present distinct glycosylation profiles [67, 68]. The branching *N*-glycosylation of the CD25 receptor also modulates its cell surface expression and retention, thus controlling T-cell differentiation with an impact in immune tolerance (Figure 1/5) [59, 61]. Moreover, when complex and hybrid *N*-glycans were depleted on CD25 through *Mgat1* silencing, a reduction on CD25 surface expression and on IL-2 signalling was observed [59, 69].

Taken together, these evidence highlight *N*-glycans as essential players in T-cell activation and functions in homeostatic conditions.

THE ROLE OF T-CELL GLYCOSYLATION IN ANTI-TUMOUR IMMUNE RESPONSE

Besides the well-known glycosylation alterations in cancer cells [4–6, 10, 70, 71], the glycocalyx of T-cells in the TME may also undergo glycosylation changes, with impact on how the immune system perceives and fights malignant transformation. In fact, alterations of T-cell glycosylation patterns are involved in immunosuppressive pathways and have crucial implications for tumour immune evasion.

T-cell-mediated anti-tumour immunity

To prevent tumour development, the immune system contains a vast library of processes involving different immune cell types. Among them, T-cell responses are crucial for a proper tumour immune surveillance [72].



The successful recognition and elimination of tumours proceeds through the cancer-immunity cycle. The first step consists in the release and exposure of neoantigens/tumour-associated antigens to the intrinsically complex TME, as a consequence of the inherent genomic instability of cancer cells. These tumour-specific antigens are recognized by APCs, which activate T-cells in lymph nodes. Then, activated T-cells traffic to the tumour site and infiltrate the TME, eliminating tumour cells. On the other hand, CD8⁺T-cells are also able to recognize tumour-antigens presented through MHC-I on the tumour cell surface, resulting in cytotoxic T-cell activation [73–76].

CD8⁺ T lymphocytes are directly associated with tumour eradication, being involved in the elimination of cancer cells through granzyme-, TRAIL- and FAS-induced apoptosis, as well as through the cytokines release, including INF γ [73, 77]. CD4⁺ T-cells can also participate in the elimination of tumour cells by modulating TME through the release of cytokines and chemokines [78]. Thus, successful T-cell-mediated cancer immune surveillance relies on the synergistic effect of CD4⁺ and CD8⁺ T-cell responses. However, despite this intricate well-oiled process, tumours can progress, as malignant cells acquire several mechanisms to evade immune recognition, hampering an effective anti-tumour immune response. This includes the expression of immune inhibitory checkpoints that, associated with T-cell dysfunction, lead to malignant transformation [79].

In fact, although T-cells recognize transformed cells, they are often rendered dysfunctional, leading to an immunosuppressive environment that fosters malignant transformation. Prolonged T-cell activation due to antigen exposure in nascent tumours—in combination with the changing cytokine milieu—leads to a progressive accumulation of exhausted CD8⁺ T-cells. These cells exhibit reduced responsiveness, decreased cytotoxic potential and diminished production of key anti-tumoral cytokines, such as IFN- γ [80–84]. In fact, T-cell exhaustion has been shown to be a determinant of cancer progression and response to therapy with prognostic value for many cancer types [85–91]. Moreover, increased abundance of tumour-infiltrating regulatory T-cells (Tregs) is also known to favour tumour development, as Tregs contribute to immunosuppressive environments [92–96]. Furthermore, cancer-associated imbalances between Th populations have also been described [97–100].

Altogether, T-cell dysfunction is driven by various TME factors [82]. However, there is still a lack of knowledge on whether and how the glyco-metabolism on the TME contributes to renders T-cells dysfunctional associated with malignant transformation. The understanding of how glycosylation is implicated in such alterations and how it can be leveraged to rewire an effective T cell-

mediated anti-tumour immunity may open up new possibilities for preventing and treating cancer.

The role of galectins and siglecs within the tumour microenvironment

Galectins, a family of glycan-binding proteins, are crucial for the regulation of the immune system by modulating TCR signalling and T-cell death, acting either as positive or negative regulators of T-cell function. In cancer context, galectins have a key role in the TME by controlling T-cell activity and inflammation (Figure 1) [101].

Gal-1 was described to be involved in T-cell function, controlling activation, differentiation, survival and cytokine production [102–104]. In cancer, Gal-1 has an immunosuppressive role [105], as its overexpression is inversely correlated with the number of tumour infiltrating lymphocytes (TILs) [106]. Additionally, in a breast cancer model, it was demonstrated that Gal-1 leads to the expansion of Tregs in the TME (Figure 1/9) [107]. In ovary cancer, Gal-1 secretion by $\gamma\delta$ T-cells was described to promote malignant progression [108, 109]. Gal-1 was also associated with exclusion of T-cells from the tumour in head and neck squamous carcinoma [110]. This galectin can specifically exert its inhibitory function on Th1 and Th17 cells due to their differential glycosylation profile, rich in α 2,3-sialic acids, which promotes the binding of Gal-1, inducing apoptosis [111]. In this line, Th2 cells are protected from the inhibitory function of Gal-1 because they express α 2,6-sialic acids on their surface receptors, which inhibit Gal-1 binding [111].

Additionally, Gal-3 binding to glycoproteins can also have anti- or pro-apoptotic effects on T-cells, depending on its cellular localization. Intracellular Gal-3 blocks apoptosis while extracellular Gal-3 binds to glycosylated receptors on the T-cell surface, leading to activated T-cell inhibition [103, 112–114]. Gal-3 produced by activated T-cells interacts with glycoproteins associated with the TCR, negatively regulating T-cell activation through the destabilization of the immunological synapsis, precluding TCR clustering (Figure 1/6) [56, 115, 116]. In tumours, Gal-3 establishes glycan-dependent lattices with the TCR and CD8, impairing cytotoxic T-cell function and IFN- γ secretion [117]. With the use of galectin disaccharide ligands, such as *N*-acetyl-lactosamine (LacNAc), which inhibits Gal-3 binding to cellular glycans, this effect was abrogated, reversing the anergy of human TILs [117]. Furthermore, Gal-3 was described as capable of binding to *N*-glycans on immune checkpoints, such as CTLA-4, maintaining its inhibitory signal [62] and lymphocyte-Activation Gene 3 (LAG-3), suppressing cytotoxic T-cell function within the TME [114].

Gal-9 is another galectin known to negatively regulate T-cell immune response through the binding to mucin-domain-containing molecule-3 (Tim-3), its preferential and more studied receptor [118, 119]. In accordance, through glycosylation-dependent binding to Tim-3, Gal-9 abolishes Th1 and Th17 cell development and activity, promoting apoptosis of tumour-infiltrating CD8⁺ T-cells (Figure 1/8) [120–122]. Interestingly, it was described that the interaction of Gal-9 with Tim-3 on T-cells in the TME is involved in programmed cell death protein 1 (PD-1) immunotherapy resistance in patients with cancer, suggesting that this interaction may be targeted for improving effectiveness of immunotherapy [123] (further discussed in section ‘Glycans as fine tuners of cellular immunotherapy in cancer: T-cells glycoengineering as a promising strategy to rewire effectiveness against cancer progression’). Additionally, Gal-9 was described to promote the stabilization and function of Tregs through its association with CD44 [124].

Sialic acid-binding immunoglobulin-type lectins (siglecs) are another family of glycans-recognizing receptors that also exert an immunosuppressive role in the TME, triggered by the abnormal expression of sialylated glycans on tumour cells (Figure 1/10) [125]. Particularly, Siglec-9 was demonstrated to be expressed on tumour infiltrating CD8⁺ T-cells in several cancers, being associated with poor survival [126, 127]. Furthermore, these cells feature an effector memory phenotype and co-express a variety of inhibitory T-cell receptors, such as PD-1, CTLA-4 and Tim-3 [126, 127]. Surprisingly, Siglec-9⁺ CD8⁺ T-cells, albeit presenting an inhibitory phenotype, exhibit better functional responses on co-stimulation with anti-CD3 and anti-CD28 than Siglec-9[−] CD8⁺ T-cells, in vitro [126]. In addition to Siglec-9, increased Siglec-10 expression on T-cells was found to inhibit T-cell activity in the TME in human cancer patients [128].

Together, these evidence highlight the biological relevance of glycan-recognizing receptors on the TME, such as galectins and siglecs, as key players in the promotion of immunosuppressive networks that dampen an effective anti-tumour immune response, and thus as potential targets for immunotherapy (Figure 1).

Glycosylation of immune checkpoints at the cross-talk between tumour and immune cells

Evasion of the immune system by cancer cells is facilitated by the expression of immune checkpoint inhibitors in the TME. T-cells express different immune checkpoint receptors at their surface, such as PD-1, CTLA-4, Tim-3,

among others, that are regulated by glycosylation and responsible for providing inhibitory signals (Figure 1) [14, 129].

T-cell exhaustion mediated by PD-1/programmed cell death 1 ligand 1 (PD-L1) is a widely characterized mechanism by which the anti-tumour immune response is impaired. The primary functions of the PD-1/PD-L1 axis between immune cells and cancer cells are to maintain immune tolerance and to control T-cell activation [130]. However, tumours take advantage of this mechanism to escape from anti-tumour immune response by inhibiting T-cell proliferation, cytokine production and cytolytic activity [130]. It has been described that PD-1 relies on *N*-glycosylation to preserve its cell surface [131], namely with core-fucose *N*-glycans stability (Figure 1/7) [132]. Moreover, glycosylation of the PD-1 receptor was shown to be altered, with increased levels of poly-LacNAc and core fucose, on T-cell activation [131]. It was also described that PD-1 glycosylation is important in immunotherapy, for the binding of antibodies against PD-1, which improves anti-tumour immunity [133, 134]. Importantly, the glycosylation of PD-1 controls its interactions with its primary ligand PD-L1, which is also glycosylated to maintain its stability at the membrane [131, 135]. Likewise, PD-L1 glycosylation also determines the binding to its receptor PD-1, influencing its immunosuppressive activity [135–137]. The β 3GnT-3 was identified as a crucial glycosyltransferase in PD-L1 *N*-glycosylation and in the interactions with PD-1. Indeed, down-regulation of *B3GNT3* improves cytotoxic T-cell-mediated anti-tumour immunity [138]. Thus, targeting the glycosylated forms of PD-1/PD-L1 could provide an effective immunotherapeutic strategy.

CTLA-4, another immune checkpoint molecule, is also regulated by *N*-glycosylation [65]. Indeed, on TCR signalling, there is an increase in branched *N*-glycans in CTLA-4, resulting in its retention at the cell surface, with a consequent suppression of T-cell function (Figure 1/7) [62, 139–141]. In accordance, it was demonstrated that in *Mgat5*^{−/−} T-cells the expression levels of CTLA-4 on the T-cell surface decrease on low TCR stimulation [62]. Moreover, *N*-acetylglucosamine (GlcNAc; a precursor of UDP-GlcNAc and the substrate for GnT-V enzyme) supplementation was shown to increase CTLA-4 surface levels, leading to immunosuppression by inhibiting T-cell activation [142, 143]. Together, these evidence highlight the importance of the CTLA-4 glycosylation in the regulation of its inhibitory functions in cancer and thus a potential target for improving immunotherapy.

The Tim-3 receptor was also described to be *N*-glycosylated with core-fucose [132, 144]. Interestingly, treatment of Tim-3 with PNGase F, which cleaves *N*-linked glycans from glycoproteins, impairs the binding



of this immune checkpoint molecule to Gal-9, which can also regulate the regulatory function of Tim-3 [120].

Regarding Treg function, the expression of tri- and tetra-antennary *N*-glycans was described to be correlated with the expression of immunosuppressive molecules on these cells, including Glucocorticoid-Induced TNFR-Related (GITR), PD-1, PD-L1, CD73, CTLA-4 and inducible T-cell Co-stimulator (ICOS). This was associated with an improved in vitro Treg immunosuppressive function [145].

Altogether, these data pinpoint the importance of immune checkpoints glycosylation, which may contribute to the development of resistance to an effective anti-tumour immune response, leading to a suppressive TME.

GLYCANS AS FINE TUNERS OF CELLULAR IMMUNOTHERAPY IN CANCER: T-CELLS GLYCOENGINEERING AS A PROMISING STRATEGY TO REWIRE EFFECTIVENESS AGAINST CANCER PROGRESSION

The manipulation of the immune system has revolutionized cancer treatment. Indeed, the basis for the 2018 Nobel Prize to Allison and Honjo showed how different strategies for inhibiting the brakes on the immune system can be used in the treatment of cancer [146, 147]. Among the different strategies to boost the immune response, the ability to remodel glycans at the T-cell surface can constitute an appealing strategy to improve the efficacy of cancer immunotherapy, an issue that remains largely unexplored [148]. In this line, different engineering approaches have been applied for the T or chimeric antigen receptor (CAR)- T-cell glycodeSIGN, showing promising results on the bolstering T-cell glycosylation to improve its effector function in the TME (Table 1; Figure 1) [159, 160]. In fact, the utilization of CAR-T-cells has been a major emerging strategy in the field of cancer immunotherapy.

Glycan-binding proteins as potential therapeutic targets in the tumour microenvironment

The potential of CAR-T-cells to target specific tumour-associated glycans has been suggested in several studies. The use of CAR-T-cells against glycosylated cancer-associated antigens has been described, including the recognition of Lewis Y antigen or Tn and sialyl(S)-Tn antigens on Mucin 1 (MUC1) [161, 162]. Different ongoing

clinical trials are focused on the efficacy of CAR-T-cells to recognize these abnormal glycoforms (NCT03633773; NCT03525782), highlighting its potential for a future cancer immunotherapy [163, 164]. Importantly, glycoengineered-CAR-T-cells are also beginning to be studied as a promising approach to enhance its efficacy in vivo. However, one of the major challenges in CAR-T-cells therapy is the poor trafficking and reduced infiltration of these cells to tumour sites [165]. As previously mentioned, the E-selectin plays a key role in T-cell extravasation from circulation to the TME. In fact, when E-selectin ligands (sLeX antigens) are increased, by the addition of a soluble fucosyl transferase on CAR-T-cells, their homing to the tumour site in vivo is improved, thus reducing the required cell dosage and tumour immune burden [166]. This mechanism was first demonstrated in vivo in Tregs in the context of inflammation, with an increase of Tregs number in inflamed sites [149], representing a promising strategy for the use of glycoengineered-CAR-T-cells in cancer treatment.

As above-described, galectins and siglecs play a fundamental role in the creation of immunosuppressive networks in the TME, which advances the inhibition of these lectins as another potential therapeutic strategy.

The metabolic inhibition of Gal-1 binding motifs, through treatment of T-cells with peracetylated 4-fluoro-glucosamine (4-F-GlcNAc), an inhibitor of *N*-acetylglucosamine (GlcNAc) biosynthesis, leads to an improvement of the anti-tumour immune response in melanoma- and lymphoma-bearing mice models, attenuating tumour growth by increasing tumour-specific CD8⁺ T-cells and IFN- γ production [167]. In a similar way, in an orthotopic cancer model, intratumoral treatment with thiodigalactoside, a non-metabolizable disaccharide, was shown to prevent Gal-1 binding to T-cells, promoting CD8⁺ T-cell-mediated anti-tumour immune response [150]. Interestingly, genetic deletion of the Gal-1 gene (*Lgals1*) in a model of pancreatic cancer promotes tumour T-cell infiltration, increasing mice survival and reducing metastasis [151]. Accordingly, targeting Gal-1 in a melanoma model leads to the improvement of Th1 and cytotoxic T lymphocytes responses, enhancing tumour killing [168]. The overexpression of the ST6Gal1 sialyltransferase on T-cells, and consequently the increase of α -2-6 sialylated *N*-glycans expression on the CD45 receptor, was also demonstrated to be efficient in impairing Gal-1 binding, preventing T-cell death, which can represent an effective anti-tumoral strategy (Figure 1/13) [169]. An anti-Gal-1 drug (OTX008) was also tested as an immunotherapeutic agent, but the results from the clinical trial were inconclusive [152], a thus further research is still needed.

TABLE 1 T-cell glycosylation as a potential target for immunotherapy: Molecular mechanism and effects on anti-tumour immune response

Target	Therapeutic approach	Molecular mechanism	Anti-tumour immune response	References
E-Selectin	sLeX-CAR-T-cells—soluble fucosyl transferase treatment	Increase of sLeX antigens and E-Selectin binding	Improves CAR-T-cell homing to TME and its function	[149]
Gal-1	LacNAc biosynthesis inhibitor (4-F-GlcNAc)	Reduction of Poly-LacNAc structures and Gal-1 binding	Increases tumour-specific CD8 ⁺ T-cells and IFN- γ production	[150]
	Thiodigalactoside	Binds to Gal-1 and blocks its binding to T-cells	Increases levels of tumour infiltrated CD8 ⁺ T-cells	[151]
	ST6Gal1 overexpressing	Increase of α 2,6-sialylated <i>N</i> -glycans on CD45 receptor, impairing Gal-1 binding	Avoids T-cell death	[152]
Gal-3	Galactomannan (GM-CT-01)	Binds to Gal-3 and blocks its binding to T-cells	Improves anti-tumour CD8 ⁺ T-cell response, through increased IFN- γ secretion	[153]
Gal-9/Tim-3	Anti-Tim-3 + anti-PD-1 antibodies	Impairs Gal-9 and PD-L1 binding	Reverses CD8 ⁺ T-cell exhaustion	[154]
Siglec-7 and -9	Siglec-7/9-based CAR-T-cells— with siglec-7/9 exodomain	Increase of T-cell recognition against tumour sialylated glycans	Improves CAR-T-cell efficacy (enhanced tumour recognition)	[155]
PD-1	Gene editing of N74 residue of <i>PDCD1</i>	Reduction of PD-1 <i>N</i> -glycosylation and its expression on CAR-T-cell surface	Prevents CAR-T-cell exhaustion	[156]
	Knockout of <i>FUT8</i>	Abolishes core fucosylation on PD-1	Decreases PD-1 expression surface and enhances T-cell activation	[133]
	Anti-glycosylated-PD-1 antibody (STM418)	Impairs PD-1/PD-L1 interaction	Avoids T-cell exhaustion	[132]
CTLA-4	Anti-glycosylated CTLA-4 antibody (mAb146)	Blocks CTLA-4-CD80/CD86 binding	Enhances T-cell function	[157]
IL-2R	2-deoxy-D-glucose (2DG), glucose analog	Impairs <i>N</i> -glycosylation, increasing IL-2R retention and reducing Gal-3 binding	Higher production of perforin/granzyme	[158]

The *in vitro* treatment of patient-derived TILs with galactomannan, GM-CT-01, an inhibitor of Gal-3, was also described to be efficient in improving the anti-tumour CD8⁺ T-cell response, through increased IFN- γ secretion (Figure 1/12) [170]. Notably, the disruption of lectin-T-cell binding has been studied as a possible adjuvant therapy of chemo- and/or immunotherapeutic strategies. Studies using Gal-3 inhibitor as an adjuvant of the chemotherapeutic drug 5-fluorouracil were performed against different advanced/metastatic solid tumours [153]. Unfortunately, the results were unfavourable and some of them were withdrawn [153]. Interestingly, Gal-3 inhibition (using GR-MD-02), combined with antibodies against immune checkpoint inhibitors, including anti-Tim-3, anti-PD1 or/and anti-CTLA-4, appears to be a promising strategy with ongoing clinical

trials for several solid tumours (e.g., NCT02575404; NCT02117362) [153].

The use of an antibody against Gal-9 was also described as a promising immunotherapy in a tumour mouse model, since this galectin is associated with T-cell death and consequently with poor prognosis in several human cancers [171]. Gal-9 is the main ligand of Tim-3 on T-cells, leading to T-cell exhaustion. In fact, T-cells which co-express Tim-3 and PD-1 are highly exhausted and represent the most abundant TILs population in solid tumours [172, 173]. Interestingly, it was demonstrated that PD-1 had the capacity to interact with Gal-9 and Tim-3, in PD-1⁺Tim-3⁺ exhausted T-cells, forming Gal-9/Tim-3/PD-1 molecular lattices, which protect this population from Gal-9/Tim-3-induced apoptosis [171].



Regarding siglecs, Siglec-7 and -9 have been also highlighted as promising targets in cancer immunotherapy. The use of blocking antibodies to Siglec-7 and -9 leads to a significant reduction of lung and ovarian tumour burden in mice [154, 174]. In addition, taking advantage of the glycan-binding capacity of siglecs, CAR-T-cells composed by the exodomain of human Siglec-7 and -9 have been demonstrated as efficient in the recognition of sialylated glycans on tumour cells, both in vitro and in vivo, consequently improving anti-tumour CAR-T-cell target function (Figure 1/14) [175]. Moreover, similarly to anti-galectin agents, siglec blockers are now gaining momentum as novel adjuvant therapies. In fact, the co-inhibition of siglecs, including Siglec-7 and -9, and immune checkpoint molecules (Tim-3) was also proposed as a promising treatment for patients with glioma [155]. On the other hand, alterations in ligands of siglecs on tumour cells could also be a strategy to impair siglec-glycans interactions. For instance, the treatment of tumours with sialic acid analogues reduces cell surface sialylated glycans, precluding siglecs recognition and improving the activation of intratumoral T-cells [176].

All of these evidence highlight the major roles of selectins, galectins and siglecs in TME as promising therapeutic targets, single or in combination with other immunotherapeutic strategies, to enhance an effective anti-tumour immune response and to improve cancer patients' survival.

Reprogramming the glycosylation of T-cells in cancer immunotherapy

PD-1 expression in CAR-T-cells has been described as a major inhibitory molecule, constituting a hurdle for effective anti-tumour surveillance [177]. The removal of PD-1 *N*-glycosylation, by editing the coding sequence of the N74 residue of *PDCD1* gene, was shown to reduce PD-1 expression at the cell surface, improving overall survival and increasing anti-tumour activity in vivo [178]. In this line, the abolition of core fucose in T-cells was also able to decrease surface PD-1 expression and enhance T-cell activation, leading to more efficient tumour eradication in a melanoma murine model [132]. Importantly, it was highlighted that some therapeutic antibodies are only functional against the glycosylated form of PD-1 (Figure 1/11) [133]. The monoclonal antibody STM418 (an anti-PD1 antibody) was specifically developed to recognize the N58-glycan present in this molecule, inducing an improved anti-tumour effect when compared to other anti-PD-1 antibodies [131]. Moreover, the use of an antibody targeting glycosylated PD-L1 was described to promote the internalization and degradation of this

molecule, resulting in T-cell induced tumour cell death [138]. Additionally, antibodies against glycosylated epitopes of CTLA-4 (mbAb146) are also being considered to increase its therapeutic efficacy against tumours (Figure 1/11) [156].

As mentioned above, T-cell signalling is highly regulated by branched glycosylation, in which the abolishment of *N*-glycans on the TCR, by removing the conserved *N*-glycosylation sites on TCR α and β chains, was associated with increased TCR activation [157]. In fact, when branched *N*-glycans are absent, on *Mgat5*^{-/-} mice, an increased TCR clustering and T-cell activation was observed [56]. Moreover, a deficiency on T-cell branched glycosylation was detected in patients with inflammatory bowel disease and multiple sclerosis with an active pro-inflammatory activity [55, 143, 179]. These evidence highlight the implications of *Mgat5*-derived *N*-glycan depletion specifically on T-cells, as a potential strategy to enhance anti-tumour immune response [14].

In addition, the treatment of T-cells with 2-deoxy-D-glucose (2DG), an analogue of glucose, has also been studied as a possible anti-tumour therapy. 2DG is able to regulate the *N*-glycosylation pathway, once it is converted to 2-deoxy-D-mannose and blocks *N*-glycan biosynthesis [180]. An in vitro study showed that alterations on *N*-glycosylation patterns of human T-cells, promoted by 2DG, enhance its anti-tumour immune activity through the retention of cell surface IL-2R, increasing the production of perforin/granzyme and the prevention of Gal-3 binding [181]. On the other hand, deglycosylation of PD-L1 induced by 2DG, in conjugation with other inhibitors of tumour immune evasion, was shown to promote anti-tumour immune response in breast cancer models [158, 182].

Taken together, these evidence highlight the power of glycans on T-cells and the correspondent glycan-binding proteins as attractive targets to improve T-cell effector functions against tumour cells, an issue that deserves further exploitation. The data gathered herein foster great hope in T-cell glycoengineering as a promising immunotherapeutic strategy to be considered as next generation of glycan-based immunotherapeutic approaches for cancer prevention and treatment (Figure 1).

CONCLUSION

Glycans are pivotal biological components that regulate each pathophysiological step of malignant transformation and progression. Dynamic alterations on the cellular glycosylation pattern of T-cells can lead to changes in the cross-talk between tumour cells and the immune system, with critical consequences for cancer development and progression.

Despite the clinical success of cancer immunotherapies focused on immune checkpoint inhibitors, a large number of cancer patients fails to respond or develop resistance to this therapeutic modality. The identification of new immune modulators in the TME is therefore crucial for the improvement of the clinical and therapeutic management of patients with cancer. Here, we provide a novel perspective on T-cell glycosylation and T-cell glycoreprogramming as a next-generation strategy to improve effectiveness of cancer immunotherapy.

FUNDING INFORMATION

This article is a result of the project NORTE-01-0145-FEDER-000029, supported by the Norte Portugal Regional Programme (NORTE 2020) under the PORTUGAL 2020 Partnership Agreement through the European Regional Development Fund. This work was also funded by *Fundo Europeu de Desenvolvimento Regional* (FEDER) funds through the COMPETE2020—Operacional Programme for Competitiveness and Internationalization (POCI), Portugal 2020, and by Portuguese funds through the Portuguese Foundation for Science and Technology (FCT) in the framework of the project POCI-01-0145-FEDER-028772. SSP acknowledges Liga Portuguesa Contra o Cancro LPCC and MULTICARE for funding (C 3035006C17). SSP also acknowledges Porto Comprehensive Cancer Center (P. CCC Beacon Project) for the support. CMA [SFRH/BD/07357/2021]; MSC [SFRH/BD/136388/2018]; MMV [PD/BD/135452/2017] received funding from the FCT

CONFLICT OF INTEREST

Authors have no conflicts of interest to declare.

DATA AVAILABILITY STATEMENT

Data openly available in a public repository that issues datasets with DOIs.

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How to cite this article: Fernandes Â, Azevedo CM, Silva MC, Faria G, Dantas CS, Vicente MM, et al. Glycans as shapers of tumour microenvironment: A sweet driver of T-cell-mediated anti-tumour immune response. *Immunology*. 2022. <https://doi.org/10.1111/imm.13494>

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Chapter 6

General Discussion

Concluding Remarks and Future Perspectives

General Discussion

The era of immunotherapy has revolutionized cancer treatment and given hope to thousands of patients with advanced disease. The first report associated with immunotherapy dates back to the late 19th century, when William Coley, a surgeon, noticed that some of his cancer patients who had contracted bacterial infections had better outcomes. He began to inject the patients with a mixture of killed bacteria, which he called Coley's Toxins, in an attempt to stimulate their immune systems to fight cancer [1]. Later, in the 1970s, researchers began to explore the use of monoclonal antibodies, targeting specific proteins on cancer cells. The first monoclonal antibody, named rituximab, which targets CD20, was approved for the treatment of lymphoma in 1997 [2]. In 2010, the first cellular vaccine, sipuleucel-T, was approved for the treatment of prostate cancer, and in 2011 immune checkpoint inhibitors were approved for the treatment of advanced melanoma [3]. This was the turning point for the therapies of several types of cancer, targeting the immune response. In fact, the discovery of immune checkpoint molecules and their applicability in immunotherapies resulted in Tasuku Honjo and James Allison being awarded with the 2018 Nobel Prize in Physiology or Medicine.

Despite all these advances, immunotherapies are far from being available to all patients, and effective for all types of cancer, either due to the lack of effector immune cells in the TME or due to the deficient identification of immune reactive tumor-associated antigens to improve the immune response. In this line, the present thesis aims to characterize the role of glycosylation, both in tumor cells and immune cells, as an important player in immune evasion, and to underline the importance of glycans in the design of immunotherapies.

In Chapter 2, we demonstrated that the aberrant expression of complex β 1,6-GlcNAc branched *N*-glycans was masking important neoantigens and impairing both innate and adaptative immune responses in CRC. The aberrant expression of complex β 1,6-GlcNAc branched *N*-glycans and its implication in cancer progression and metastasis have been well described in several types of cancer during the past decades [4-9]. Several studies have uncovered the potential of modulating *N*-glycan expression as a mechanism to impair tumor growth. Accordingly, tumor burden drastically decreases in the absence of *Mgat5*-derived *N*-glycans, as previously described [10, 11]. In fact, the *Mgat5* gene knockout mouse model was initially established for the study of cancer development [11], which also turned out to be a very interesting model for the study of autoimmune diseases, due to its characteristic T cell hyperreactivity (as discussed below) [12].

Taking this into consideration, we further investigated the role of complex β 1,6-GlcNAc branched *N*-glycans in the modulation of anti-tumor immune responses and tumor immune evasion. We demonstrated that the overexpression of complex branched *N*-glycans has impact in immune recognition and promoted an immunosuppressive tumor microenvironment. On the other hand, the removal of β 1,6-GlcNAc branched *N*-glycans increased tumor immune surveillance and the anti-tumor immune response, leading to a decreased tumor burden. Similar results were described using *Mgat5* knockdown breast cancer cells, which showed an improvement of pro-inflammatory responses by increasing CD4⁺ T cell proliferation and TNF- α and IFN- γ production, as well as increasing macrophage phagocytosis [10].

However, with the elimination of the expression of tetra-antennary β 1,6-GlcNAc branched *N*-glycans, the expression of less complex *N*-glycans, tri-, bi-antennary, hybrid and high-mannose glycans is increased at the cell surface. In fact, we have described that tumor cells lacking *Mgat5*-derived *N*-glycans exhibited increased expression of mannose rich glycans. We hypothesized that one of the main reasons for the decrease in tumor growth and increase of immune surveillance was the consequent increase of mannosylated antigens. The modulation and augmentation of mannosylated antigens have been explored for the treatment of cancer and to enhance the immune response. In fact, the treatment with mannose or with 2-deoxy-d-glucose (2DG) (a mannose analog, that blocks further complex *N*-glycosylation) disturbs PD-L1 glycosylation and its cell surface stabilization, leading to a more effective anti-tumor immune response [13-15]. Moreover, an interesting study has shown that the barrier formed by complex *N*-glycans has an impact on increasing the CAR-T cell activation threshold and promoting CAR-T cell exhaustion. Consequently, treatment with 2DG inhibits *N*-glycan synthesis and enhances CAR-T cell activity [16]. On the other hand, treatment with mannose not only disturbs tumor *N*-glycosylation, but also impacts in tumor metabolism. Accordingly, mannose treatment has been shown to impair tumor growth and enhance chemo- and radio-sensitivity by inhibiting glucose uptake and glycolysis [17, 18]. While many studies have described the effects of mannose in metabolism and signaling, they did not explore its effect on *N*-glycosylation, which, in our understanding, could be a confounding factor in these studies. For instance, several studies have shown that mannose can impair tumor metastasis and evasion by affecting various proteins involved on EMT and cell adhesion, such as β -catenin and E-cadherin [19, 20]. However, our group has described that β -catenin/E-cadherin function is highly dependent on *N*-glycosylation, and the presence of β 1,6-GlcNAc branched *N*-glycans leads to its dysfunction [21-23]. Therefore, the role of mannose supplementation on the impact in protein glycosylation should be further investigated.

Targeting GBPs is another strategy used to increase tumor cell recognition and improve the anti-tumor immune response. We have also shown that mannosylated-glycans in CRC tumor cells are recognized by C-type lectins DC-SIGN and MR, and the overexpression of complex N-glycans impairs its recognition. In line with this, a very recent study has shown that different tumor glyco-codes dictate different clinical outcomes by modulating DCs function. The presence of GlcNAc, sialic acids and fucose motifs is associated with poor prognosis, promoting DCs inhibition, whereas mannose and glucose-containing glycans elicit better survival [24]. Additionally, the stimulation of APCs with mannose-expressing antigens or nanoparticles, through DC-SIGN and MR, have been shown to induce tumor recognition and improve the anti-tumor immune response. For instance, DC-SIGN-mediated antigen recognition leads to antigen internalization and T cell presentation [25]. In addition, mannosylated tumor peptides have been used to increase DCs uptake and cross-presentation, thereby enhancing the anti-tumor immune response [26, 27], while nanoparticles covered with mannose have been shown efficiently deliver antigens to DCs [28-30]. These results support the idea that exposing mannose epitopes in tumor cells by removing complex glycans could be an efficient strategy to increase APCs-mediated anti-tumor immune responses and improve patient prognosis.

Altogether, these discoveries highlight the importance of tumor glycosylation in the control of the tumor microenvironment and immune response, and its potential for the development of novel immunotherapies. Moreover, the modulation of glycosylation, by exposing more immunogenic glycans, rich in mannose, have an enormous potential to enhance the anti-tumor immune response.

Based on previous results, in [Chapter 3](#), we aimed to find new insights for the design of new targeted glycan-based immunotherapies. During my training at LUMC, Leiden, The Netherlands, we explored how glycans could affect the T cell recognition of neoantigens. We started by searching for N-glycosylation sites in putative bioinformatic generated-neoantigens. Neoantigens (tumor mutated antigens) are antigens that could develop an efficient and specific anti-tumor immune response since they did not appear in normal tissue. The analysis and identification of these neoantigens are very relevant to generate targeted immune responses against cancer. For instance, therapeutics against specific neoantigens have increasing, including adoptive T cell therapy, nucleic acid, peptide or DCs vaccines, and bispecific antibodies [31]. However, neoantigen-based therapeutics are not accessible to the majority of patients and their response is variable. To increase their efficacy, we are exploring the effect of glycans in neoantigen uptake and MHC presentation.

The presentation of glycans through MHC molecules can affect the recognition of the antigen by T cells. The glycan may act as an epitope itself, or it may modify the peptide

epitope, altering its binding affinity to the MHC molecule and/or its recognition by T cells. Therefore, the presence of glycans on the antigen can significantly influence the immune response to the antigen. For instance, some studies have described the presence of glycosylated antigens in MHC-I/II [32, 33]. It has been shown that some T cell clones recognize specifically glycosylated-antigens, and not naked or modified-peptides, reinforcing the importance of glycans in T cell presentation [34-36]. Specific in cancer, tumor-associated Tn-O-glycosylated peptides have been shown to bind to MHC-II molecule and promote T cell reactivity [37, 38]. O-glycopeptides were found in both MHC-I and MHC-II, while N-glycopeptides have only been described in MHC-II so far [32]. This is explained by the processing of peptides, which are different in MHC-I and MHC-II. MHC-II typically presents exogenous glycoproteins and the processing occurs in endocytic pathway, where glycans can be removed, truncated, or maintained through the process and presented to T cells. In case of MHC-I, the protein targeted for degradation is processed in proteasome, and the peptide is deglycosylated by a cytosolic N-glycanase before going to proteasome [39]. Due to this process, Asn is often deamidated and converted to Asp [40]. This issue may be very problematic since in *N*-glycosylated peptides are not often found in MHC-I. Therefore, this study should be focused on MHC-II-presented *N*-glyco-peptides, through APCs.

The discovery of *N*-glycosylation sites could be a starting point for the identification of glycosylated neoantigens in CRC. However, we recognized some weaknesses in our preliminary study which need to be refined and clarified in further research. For instance, the neoantigens generated were 51-mer in length, with the mutation in the middle, and in some cases the *N*-glycosylation motifs identified were too far from the mutation and they could not be presented in HLA. The length of generated peptide sequence needs to be reduced (to approximately 31 amino acid (aa) length, 15 aa for each side of mutation), since HLA-II generally present peptides of 13-25 aa length. Also, we did not exclude nuclear and cytoplasmatic proteins for the analysis, and we know that these proteins could not be *in vivo* *N*-glycosylated (only *O*-GcNAcylated). Moreover, we need to confirm if identified glycosylation sites have in fact glycans attached. Mass spectrometry and glycomics need to be performed to confirm the presence of glycans in protein and also in HLA-presented peptides.

In the second part of the study, we investigated the influence of glycans in the antigen-independent immune response. Of note, all microsatellite instable (MSI) tumors in our cohort had defects in HLA, which could potentially limit the effectiveness of neoantigen-based therapy in these patients. In line with our previous results (Chapter 2), the removal of complex *N*-glycans in patient-derived tumor cells increases natural killer (NK) cell degranulation and effector response. This supports the hypothesis that complex *N*-glycans

are masking the immunogenic epitopes, thereby preventing recognition by innate immune cells, including DCs and NK cells. However, the mechanism of activation of NK cells mediated by glycans, as well as the recognition of glycosylated epitopes, needs to be elucidated.

The glycosylation of immune cells also plays a crucial role in their function and response. Therefore, in [Chapter 4](#), we explored the role of glycosylation in immune cells, with focus on T cells, in the transition from inflammatory bowel disease (IBD) to CRC. Our group has extensive experience in IBD, especially in the study of the role of T cell and Immunoglobulin G (IgG) glycosylation in IBD patients [41-45]. In this study, we aimed to characterize *N*-glycosylation patterns in IBD patients at risk of colitis-associated cancer (CAC), and to identify a potential biomarker for CAC risk. Our results demonstrated a decrease of branched *N*-glycans in the stroma that was correlated with an increased risk of developing CAC, and a significant increase in CRC. Consistent with these findings, our group has previously described that decreased expression of β 1,6-GlcNAc branched *N*-glycans on T cells is associated with greater severity of IBD [45]. This may be relevant to our current results since prolonged disease and greater severity have been linked to increased CAC risk.

Using the *Mgat5*^{-/-} mouse model of CAC (AOM/DSS), we found a delay in tumor development, with fewer tumors that were smaller size. These results align with our first publication (Chapter 2), where we showed that spontaneous models of CRC in *Mgat5*^{-/-} background, *Apc*^{min/+} and *VCMsh2*^{fl/fl}, also presented fewer and smaller lesions [46]. Accordingly, an immune modulation has been observed in all these models, consistent with an enhanced anti-tumor immune response, including a decrease in M2-macrophages and an increase in effector T cells. However, it needs to be noted that none of these models are in fact sporadic CRC, with important genetic defects (*Apc*^{min/+} and *VCMsh2*^{fl/fl}), and an inflammatory trigger with AOM/DSS. Testing a sporadic CRC model in *Mgat5*^{-/-} mice triggered with only 6 consecutive injections of AOM [47], could be a better strategy to search for new therapies and biomarkers, translating the results to sporadic CRC.

Nevertheless, in each of these CRC models *Mgat5* is knocked out in all cells, which makes it challenging to discriminate the role of the immune compartment or the epithelial compartment. For instance, the use of a conditional intestinal epithelial-specific or immune/T cell-specific *Mgat5* knockout mouse line, and an adoptive cell transfer (ACT) mouse model, should be considered to clarify the individual role of glycosylation in the immune system, as well as in the intestinal mucosa. In our study of the role of T cell glycosylation in CAC, T cells from *Mgat5*^{-/-} mice are transferred to ACT mice. Additionally, a conditional model for *Villin*-expressed cells has been generated and characterized to

verify the specific role of intestinal epithelial cell glycosylation. This model lacks hybrid or complex *N*-glycans, and only expresses high-mannose *N*-glycans at the intestine mucosa, as a result of crossing *Villin-cre* mice with *Mgat1^{fl/fl}* (*VCMgat1^{fl/fl}*) mice ([Appendix 1](#)). Interestingly, it also presents increased susceptibility to DSS-induced colitis compared to WT mice, which may be a good indicator of enhanced immunosurveillance. To investigate cancer development, we crossed this strain with *VCMsh2^{fl/fl}* mice and analyzed spontaneous tumor development. We are now currently awaiting the results of this analysis.

Regarding the field of T cell glycosylation in cancer, we published a review in the journal *Immunology* ([Chapter 5](#) of present thesis) which describes the role of glycans in T cell development and function in CRC [48]. In this chapter, we extensively explored how β 1,6-GlcNAc branched *N*-glycans, among others, shape the function and stability of several receptors in cancer immunity. Moreover, immunotherapies that involve T cell glycosylation or target glycans were also reviewed in this publication, highlighting the significance of glycans in modulating the immune response against cancer.

Taken together, the results presented here support the proposal of remodeling glycans as a strategy for immunotherapy in CRC and targeting GBPs in immune cells as a mechanism to increase antigen uptake and presentation. Additionally, we emphasize the importance of T cell glycosylation and the modulation of its function in the anti-tumor immune response.

Concluding Remarks and Future Perspectives

Overall, this PhD thesis provides evidence that β 1,6-GlcNAc branched *N*-glycans play an important role in cancer immune evasion by masking immunogenic epitopes in cancer and impairing APCs recognition and T cell activation. We demonstrated that the removal of this complex layer of glycans is able to improve the anti-tumor immune response and prevent cancer development, which were mediated by DC-SIGN recognition, DCs activation, reduced T regulatory cells infiltration and macrophage switch to M1-macrophage. Moreover, we uncovered a novel glyco-based strategy for the design of cellular immunotherapies, to improve both neoantigen-dependent and -independent immunity, by pointing HLA presentation and NK cell cytotoxicity, respectively. On the other hand, in the immune compartment, we showed that T cell glycosylation mediates anti-cancer immunity in colitis-associated CRC, and its glycoprofile completely changes from inflammation to cancer. These findings revealed an opportunity for a new biomarker and also a target for immunotherapy.

However, several questions need to be addressed, including: 1) How are *N*-glycans influencing neoantigen T cell recognition and the adaptive immune response? 2) Can we improve the efficacy of immunotherapies, such as CAR-T cells therapies and cancer vaccines, if glycans are considered in their design? 3) Can we predict the response to immunotherapies based on the tumor and T cell glycosylation profile? 4) What is the specific role of T cell glycosylation in cancer, and can its modulation impair tumor development? 5) Can we prevent inflammation-associated CRC development by modulating T cell glycosylation in IBD risk patients?

All these questions are part of the research pipeline of our group, and we are excited for the new advances in the field of glycosylation in cancer immunology.

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APPENDIX 1

A new glycoengineered mouse model for the study of Colorectal Cancer and Intestinal Inflammation – *Villin-Cre Mgat1^{LoxP/LoxP}* mice

A new glycoengineered mouse model for the study of Colorectal Cancer and Intestinal Inflammation – *Villin-Cre Mgat1^{LoxP/LoxP}* mice

1. Homeostasis colon characterization

Following the results obtain in Chapter 2 and Chapter 4, we decide to go further and generate a mouse model with mutation restrict to epithelial cells, in order to evaluate the impact of glycosylation of tumor cells in tumor development. The model was defined as *Villin-Cre Mgat1^{LoxP/LoxP}* (*VCMgat1^{fl/fl}*) and it is characterized by a knockout of *Mgat1* gene, coding GnT-I enzyme, responsible to generate branched *N*-glycans, and consequently increase mannose enriched *N*-glycans, specifically in *Villin*-expressing cells (intestinal epithelial cells). Of note, mice full knockout for *Mgat1* did not survive, having several problems in embryony development [1]. However, conditional knockout in intestinal cells had no development problems, or other physical issue. At 8-9 weeks old, no differences in body weight were observed in KO mice, comparing to the controls (*Mgat1^{fl/fl}*) (Figure 1A). Interestingly, we found that KO mice present an increase in small intestine length, while in colon no differences were observed (Figure B-C). The causes for increased small intestine length need to be explored, in order to understand the impact of *N*-glycans in intestine homeostasis. We also tested the intestinal permeability, performing Dextran-FITC permeability assay, that consists in gavage of Dextran FITC-labeled and, after 2 hours, address the concentration of FITC-dextran in the serum of the mice. This test demonstrated no differences in intestinal permeability of the mice when compared to the controls (Figure 1D). It means that the absence of highly branched and less complex glycans seems have no implications in permeability of the intestine, at a young age. However, a recent study demonstrates differences in intestine permeabilization depending on lean body mass, and the body weight dose-dependent could not be the better variable to study this mice [2]. These parameters will be monitored across the aging of the model, in order to clarify differences in phenotype of this model.

Regarding molecular characterization of intestinal epithelial cells, we confirm a decrease in branched *N*-Glycans, using L-PHA lectin, and an increase in mannose-glycans, by GNA lectin binding (Figure 2A-B). Moreover, we observed a tendency to increase O-GlcNAcylation, as a compensatory mechanism (Figure 2C). In sialic acids, a decrease in α 2,3- sialic acids was observed (Mal-II binding), but not differences in α 2,6- sialic acids (SNA lectin) (Figure 2D-E). No differences were observed in fucosylation (Figure 2F). Moreover, EpCam⁺ and MHC-I⁺ cells are also decreased (Figure 2G-H), what goes in

accordance, once these receptors depend on hybrid and complex glycans *N*-Glycans for it stablish in cell surface [3, 4].

We also look to immune infiltration in colon of these mice, and we found a strong tendency to decrease CD3⁺ cells infiltration, with a significant decrease in CD8⁺ T cells (Figure 3A-D). Indeed, there is an imbalance of CD4⁺ and CD8⁺ T cells within CD3⁺ population, with an increase in CD4⁺ cell frequency and a decrease in CD8⁺ percentage (Figure 3E).

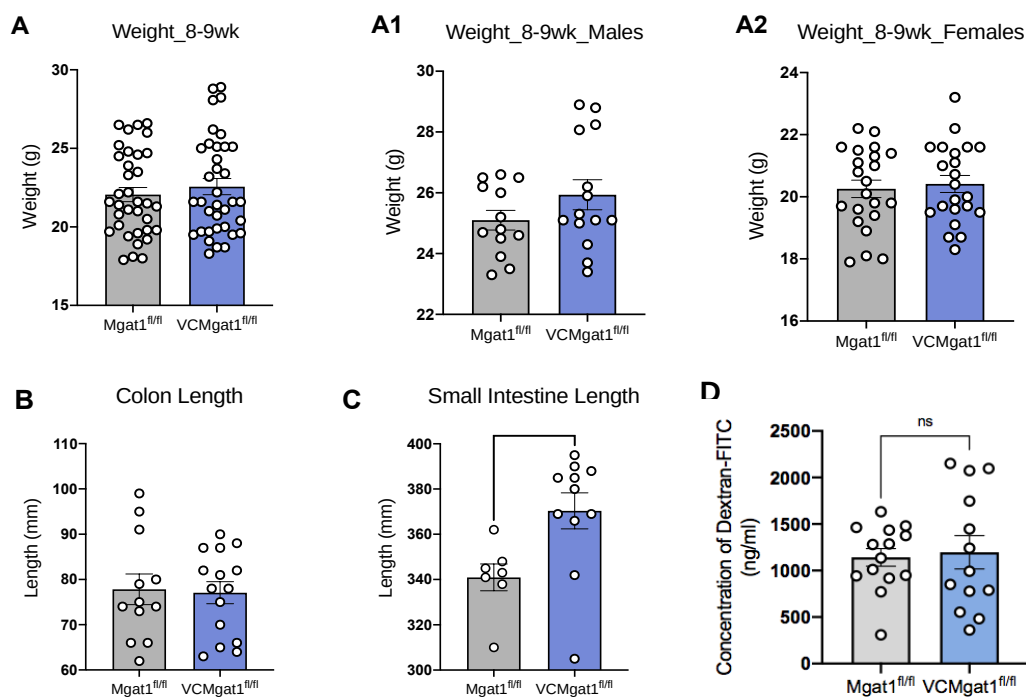


Figure 1 Macroscopic characteristic of VCMgat1^{fl/fl} mice compared to control (Mgat1^{fl/fl}) (A) Body weight, (B) Colon Length, (C) Small intestine length and (D) FITC-dextran test for intestinal permeabilization. Error bars represent Standard deviation (SD). Student t-test was performed. *, P ≤ 0.05

Besides that, we know that glycosylation of T cells has a key role in T cell function [5]. Therefore, we also looked to the glycosylation of T cells, and it was observed an increase in *N*-glycan branching in CD3⁺ T cells, that was mainly influenced by the increase observed in CD8⁺ T cell complex *N*-glycosylation (Figure 4A-C). In accordance, we found a decreased in high-mannose *N*-glycans in CD3⁺, CD4⁺ and CD8⁺ T cells (Figure 4D-F). No differences were observed in sialylation (Figure 4G-I).

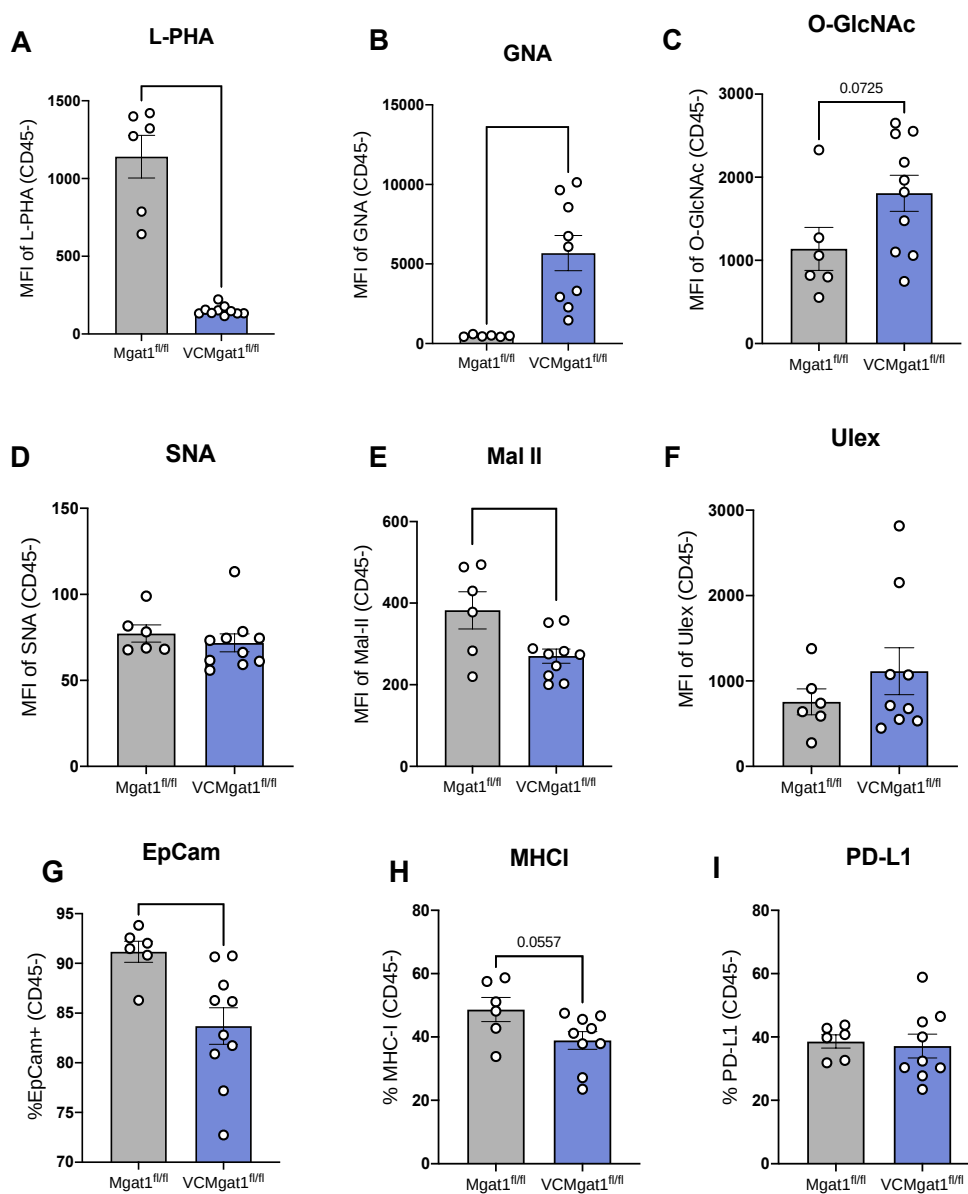


Figure 2 Colon epithelial characterization of glycans and cell surface receptors. (A) L-PHA, (B) GNA, (C) O-GlcNAc, (D) SNA, (E) Mal-II and (F) ULex lectin binding. Percentage of positive cells to (G) EpCam, (H) MHC-I and (I) PD-L1. Error bars represent Standard deviation (SD). Student t-test was performed. *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.0001$.

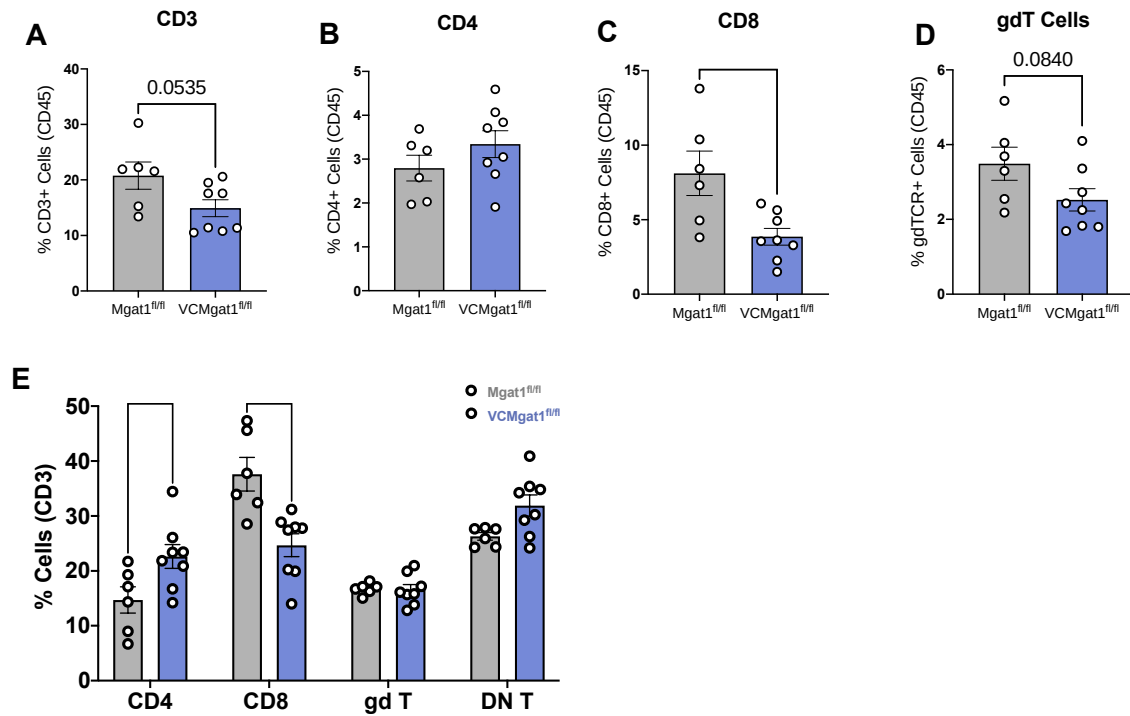


Figure 3 Immune infiltration profile of colon of VCMgat1^{fl/fl} mice, compared to WT mice. Percentage of (A) CD3+, (B) CD4+, (C) CD8+ and (D) $\gamma\delta$ TCR+ cells, within CD45+ cells. (E) Percentage of T cell populations within CD3+ T cells. Error bars represent Standard deviation (SD). Student t-test was performed. *, $P \leq 0.05$; ***, $P \leq 0.001$.

It is described that increased branched *N*-glycans in T cells was associated to a decrease on T cell activation [5]. CD8⁺ T cells not only are less infiltrated in colon, as they seem to be less activated. This diminished infiltration of CD8⁺ T cells could be related to a decrease in MHC-I that we observed on epithelial cells, promoting less activation and less recruitment of cells to the tissue. In fact, it could have a strong impact in tumor development since the deficiency of cytotoxic T cells is associated with tumor immune evasion [6]. On the other hand, the decrease of branched *N*-glycans in epithelial/tumor cells is associated with an improvement of anti-tumor immune response, as we described in our previous publication [7]. To test this dual effect in VCMgat1^{fl/fl} mice, we are now crossing these mice with VCMsh2^{fl/fl} and *Apc*^{min/+} mice, two colorectal cancer spontaneous models.

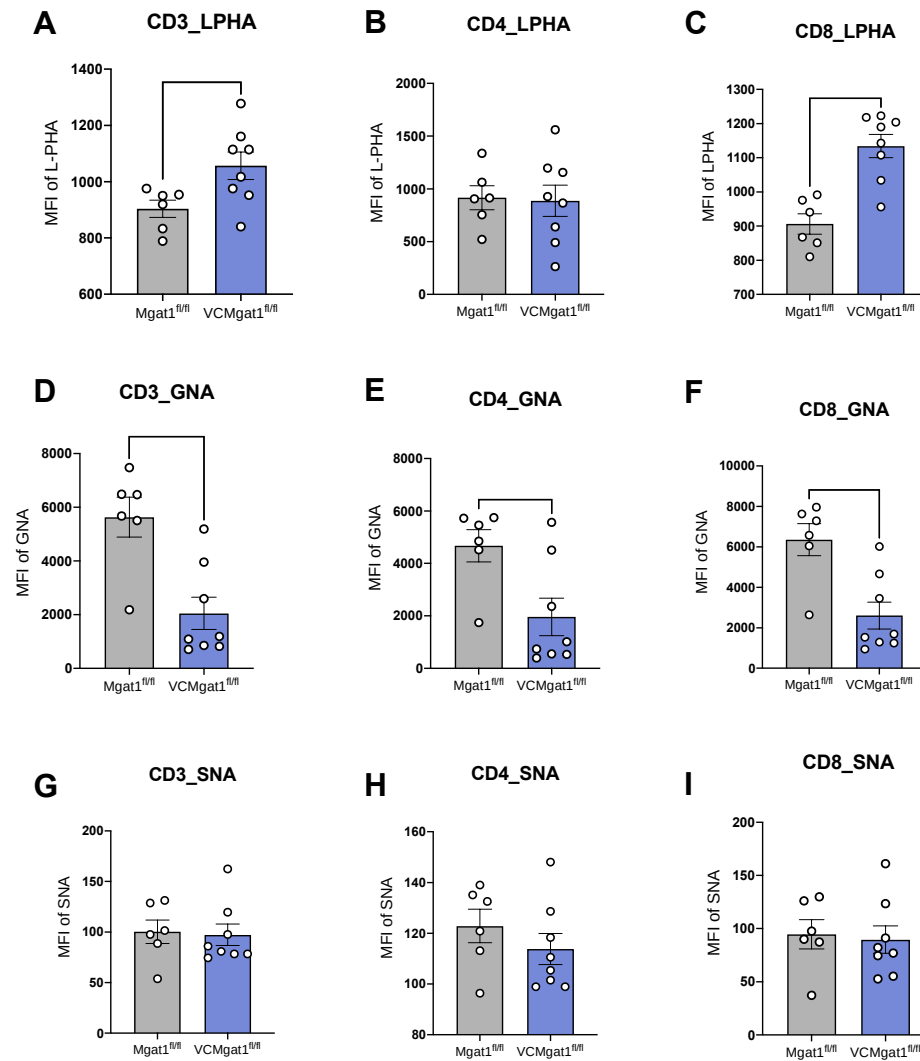


Figure 4 Glyco profile of T cell population. (A-C) L-PHA binding, (D-F) GNA binding and (G-I) SNA binding in T cell (CD3⁺, CD4⁺ and CD8⁺) population. Error bars represent Standard deviation (SD). Student t-test was performed. *, P ≤ 0.05; **, P ≤ 0.01; ***, P ≤ 0.001.

2. DSS-induced model of colitis

In order to evaluate the response of *VCMgat1^{fl/fl}* mouse model in the development of gut inflammation, DSS-induced colitis was performed. We found that *VCMgat1^{fl/fl}* mice had an increased susceptibility to develop colitis, with higher body weight loss and DAI score (Figure 5A-B). Also, a decrease in colon length, but no differences in colon weight and spleen weight, were observed (Figure 5C-E). In line, we found an increase in immune infiltration in KO mice compared to WT mice (Figure F-G). Now we are exploring the role of immune cell glycosylation in the pathogenesis of colitis in this model. According to literature, the truncation of *N*-glycosylation pathway causes high levels of endoplasmic reticulum stress, leading to activation of several cell death mechanisms [8]. It could exacerbate the inflammation induced by DSS, but we need to better dissect the mechanism. Of note, aged

APPENDIX 1

VCMgat1^{fl/fl} mice with more than 360 days and with any stimuli do not develop inflammation spontaneously inflammation or cancer in the intestine (Data not shown).

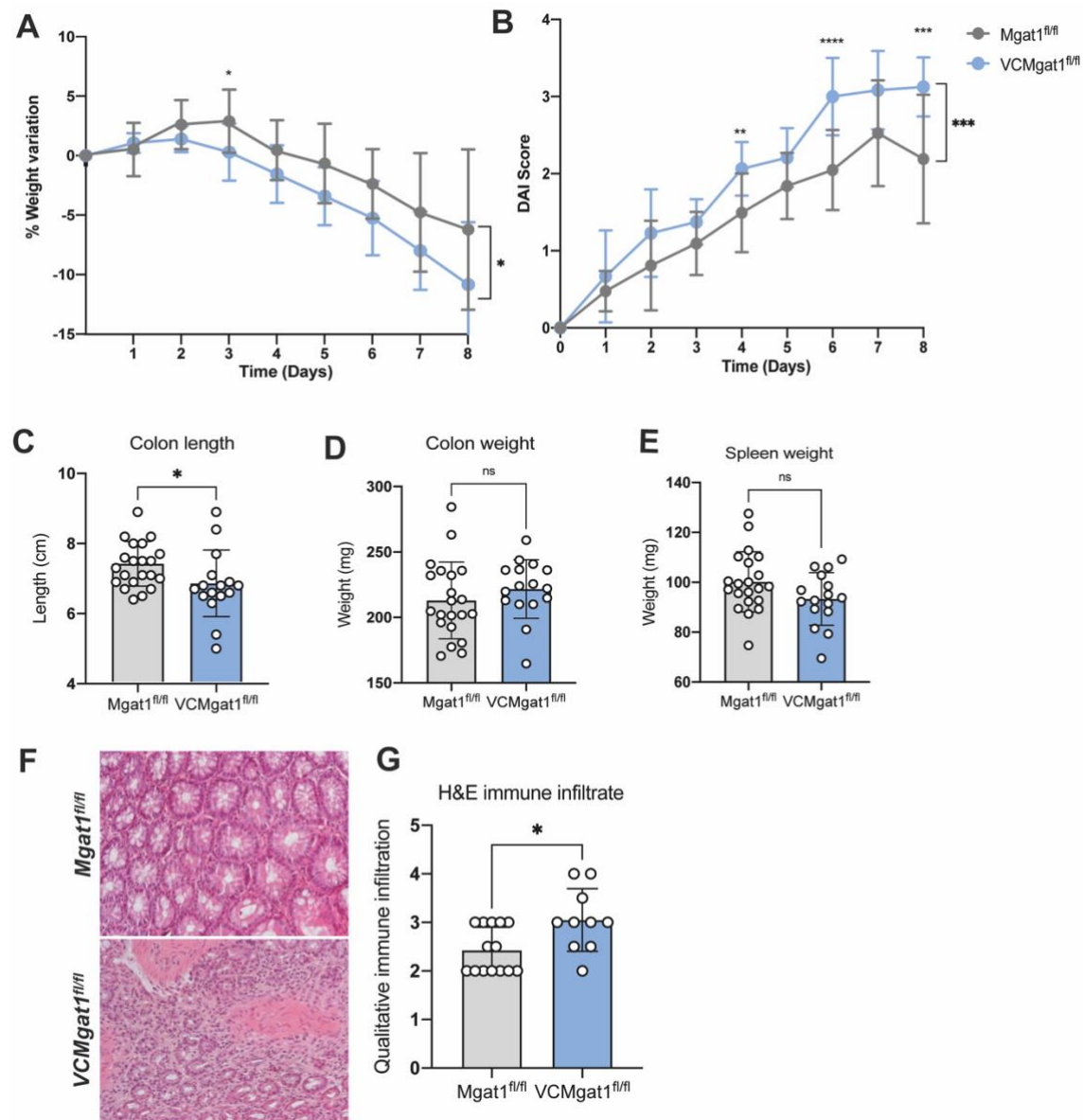


Figure 5 *VCMgat1^{fl/fl}* mice are more susceptible for DSS-induced colitis. **(A-B)** (A) % Body weight variation and (B) DAI score in *VCMgat1^{fl/fl}* mice vs *Mgat1^{fl/fl}* (WT) mice during DSS cycle. 2way ANOVA with multiple comparisons was performed. **(C-E)** (C) Colon length, (D) Colon weight and (E) Spleen weight at day 8 of the cycle (endpoint). **(F)** H&E histologic images of colon tissue at day 8 of the cycle (endpoint). Points represent each animal in in study. **(G)** Qualitative quantification (1-4) of immune infiltration. Points represent the mean of 3 independent individuals of each sample. Error bars represent Standard deviation (SD). Student t-test was performed. *, $P \leq 0.05$; ***, $P \leq 0.001$.

These results shown that the absence of complex *N*-glycans and highly expression of high-mannose *N*-glycans in intestinal epithelial compartment leads to a more severe inflammatory disease in intestine. Regarding cancer development, we have not sure what expect, since this model seems to be more susceptible to inflammation and tissue damage,

than together with less homeostatic CD8⁺ T cells infiltration could lead to tumor development, although pro-inflammatory immune response and less epithelial complex N-glycans could also be synonymous of proficient immunosurveillance.

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APPENDIX 2

Comprehensive Glycoscience, 2nd edition:

5.6. The Role of Glycans in Chronic Inflammatory Gastrointestinal and
Liver Disorders and Cancer

5.26 The Role of Glycans in Chronic Inflammatory Gastrointestinal and Liver Disorders and Cancer

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Glossary

Complex N-glycans Processed N-glycan with two, three or four antennas and possibly with bisecting N-acetylglucosamine.

C-type lectins Calcium-dependent glycan-binding proteins (GBPs) that share primary and secondary structural homology in their carbohydrate-recognition domains.

Glycocalyx A cell surface layer enriched in glycoproteins, glycosaminoglycans and proteoglycans that extends far from the cell membrane.

Glycoforms Different molecular forms of a glycoprotein, resulting from different glycan structure and/or glycan attachment site occupancy.

Glycome The total collection of glycans synthesized by a cell, tissue or organism under specified conditions of time, space and environment.

Glycomics Scientific discipline that defines the structures and functional roles of glycans in biological systems.

Mucins Heavily glycosylated proteins with high amount of serine (Ser), threonine (Thr) and proline residues and abundant O-linked glycans.

N-glycans Oligosaccharides covalently linked to an asparagine residue of a protein (via a nitrogen atom, beginning with GlcNAcβ1-Asn) at the consensus sequence Asn-X-Ser/Thr, in which X is any amino acid except proline. N-glycans are classified as high-mannose, complex and hybrid types.

O-glycans Oligosaccharides that are linked to a polypeptide via an oxygen atom. O-glycans are classified according to the initiating monosaccharide (GalNAc) linked to the hydroxyl of a Ser or Thr residue.

Truncated O-glycans Result from an incomplete synthesis of O-glycans. Includes Tn and T antigens and their sialylated forms.

*These authors contributed equally to this work.

Abstract

Carbohydrate structures (glycans) cover the surface of all mammalian cells, being fundamental for inter- and intracellular signaling, immune defense, inflammation, cell adhesion and pathogen invasion, both in health and disease. Alterations in the glycome (repertoire of glycans structures in an organism) are known to occur in several gastrointestinal and liver diseases, including inflammatory and autoimmune disorders as well as in cancer. The lack of effective (early) diagnosis, accurate prognostic markers and targeted treatments for these gastrointestinal pathologies highlights the unmet need to develop novel biomarkers and therapeutic strategies. In this context, protein glycosylation has emerged as a groundbreaking approach to mechanistically understand the pathogenesis of the different gastrointestinal diseases with the promise to reveal novel glycan-based biomarkers and treatments.

This chapter encompasses updated evidences on the relevance of cellular glycosylation in the immunopathogenesis of gastrointestinal disorders, with a focus on inflammatory bowel disease, inflammatory hepatic disorders, gastrointestinal and liver cancers, highlighting the potential of glycans as effective biomarkers and innovative therapeutic and preventive strategies with clinical applications.

APPENDIX 3

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Licensed Content Author	Salomé S. Pinho, Manuel M. Vicente, Carolina S. Dantas, et al
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Licensed Content Volume	168
Licensed Content Issue	2
Licensed Content Pages	16
Type of Use	Dissertation/Thesis
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Title	Glycans as novel immunomodulators in Colorectal cancer: an opportunity for innovative immunotherapeutic strategies
Institution name	University of Porto
Expected presentation date	Jun 2023
Requestor Location	i3S - Institute for Research and Innovation in Health Rua Alfredo Allen 208 Porto, other 4200-135 Portugal Attn: i3S - Institute for Research and Innovation in Health
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