

DOUTORAMENTO

CIÊNCIAS BIOMÉDICAS

glycoINSPIRER: A MULTIVALENT
GLYCOCODE-INSPIRED
NANOVACCINE FOR BLADDER
CANCER IMMUNOTHERAPY

Rui Filipe Freitas

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RUI FILIPE NEVES FREITAS

**glycoINSPIRER: A MULTIVALENT GLYCOCODE-INSPIRED
NANOVACCINE FOR BLADDER CANCER IMMUNOTHERAPY**

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“If your dreams do not scare you, they are not big enough”

Ellen Johnson Sirleaf

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Declaro que este trabalho é integralmente da minha autoria, não tendo sido utilizada previamente noutro curso ou unidade curricular, desta ou de outras instituições. As fontes de informação estão devidamente referenciadas e identificadas bem como as obras consultadas. Não contém, por isso, qualquer tipo de plágio de textos publicados ou trabalhos académicos, qualquer que seja o meio dessas publicações.

Os trabalhos de investigação presentes nesta tese foram realizados no Grupo de Patologia e Terapêutica Experimental do Centro de Investigação do Instituto Português de Oncologia do Porto, EPE e no grupo *Tumour and Microenvironment Interactions* do Instituto de Investigação e Inovação em Saúde.

Declaro ainda que o presente trabalho foi financiado pela Fundação para a Ciência e a Tecnologia, pela atribuição de uma bolsa individual de doutoramento, com a referência 2020.08708.BD. A Fundação para a Ciência e Tecnologia é co-financiada pelo Fundo Europeu de Desenvolvimento Regional da União Europeia, através do Programa Operacional Fatores de Competividade do Quadro de Referência Estratégico Nacional. Reconheço também o financiamento, pela Fundação para a Ciência e Tecnologia, do Centro de Investigação do Instituto Português de Oncologia do Porto: CI-IPOP-29-2014; CI-IPOP-58-2015; RESOLVE (<https://doi.org/10.54499/PTDC/MED-OUT/2512/2021>); REVERENT (<https://doi.org/10.54499/2022.03621.PTDC>). Mais declaro que o presente trabalho também financiado pelo projeto “The Porto Comprehensive Cancer Center Raquel Seruca” com a referência NORTE-01-0145-FEDER-0726678 – Consorcio PORTO.CCC – Porto.Comprehensive Cancer Center Raquel Seruca, apoiado pelo Programa Operacional Regional do Norte de Portugal (NORTE 2020), no âmbito do acordo de parceria PORTUGAL 2020, através do fundo de desenvolvimento regional europeu (FEDER), Portugal. Por último, delaro que o presente trabalho teve ainda o suporte financeiro do Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto.



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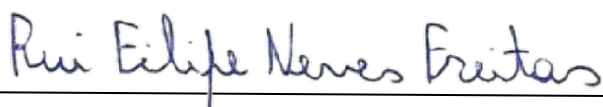


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- a. **Rui Freitas***, Andreia Peixoto*, Eduardo Ferreira, Andreia Miranda, Lúcio Lara Santos, José Alexandre Ferreira. Immunomodulatory glycomedicine: Introducing next generation cancer vaccines. *Biotechnology Advances* (Impact Factor in 2023: 16.0, Q1). 2023. 10.1016/j.biotechadv.2023.108144
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RESUMO

O cancro da bexiga é o segundo cancro do sistema geniturinário mais diagnosticado, comportando diversos desafios clínicos tais como altas taxas de recidivas, ausência de biomarcadores preditivos e de estratificação de doentes, e falta de terapias eficazes. A introdução de imunoterapias foi um passo importante para a gestão da doença; contudo, uma alta percentagem de doentes não responde ao tratamento. Além disso, as atuais abordagens terapêuticas são incapazes de prevenir a formação de metástases, levando à necessidade de novas terapias mais seguras e eficazes. As vacinas contra o cancro têm ganho um crescente interesse devido ao seu potencial de erradicar tumores e evitar recidivas. No entanto, estas ferramentas terapêuticas enfrentam alguns obstáculos, tais como falta de assinaturas moleculares específicas, especialmente em tumores primários e metástases com baixa carga mutacional. Como tal, explorar modificações pós-traducionais pode ser uma abordagem promissora como fonte de antígenos.

A glicosilação aberrante é frequentemente encontrada em células tumorais e, quando associada a alterações na abundância e composição de proteínas de membrana, origina assinaturas moleculares específicas de tumores com potencial para serem aplicadas em imunoterapia. De facto, a combinação de glicómica, glicoproteómica, transcriptómica e bioinformática tem permitido a identificação, pelo nosso grupo, de diversas glicoproteínas associadas ou específicas de tumores, duas delas exploradas, neste trabalho, no contexto de vacinas anti tumorais. Colocámos por hipótese que estes dois candidatos conseguem estimular especificamente o sistema imune, gerando imunogenicidade através do desenvolvimento de respostas celulares e humorais específicas.

Em primeiro lugar, desenvolvemos um método enzimático, usando a MUC16 como molde, combinando glicosiltransferases e nucleótidos de açúcares de forma gradual, consoante a glicoespécie a ser produzida. Depois, para enriquecer os glicopéptidos para espécies sialiladas, aplicámos um protocolo de enriquecimento com TiO_2 e caracterizámos todos os produtos glicosilados por espectrometria de massa. Além disso, conjugámos os glicopéptidos com duas proteínas imunogénicas, pela química de conjugação maleimida-tiol, para produzir conjugados multivalentes. Subsequentemente, aplicando a abordagem enzimática desenvolvida inicialmente, sintetizámos péptidos tumorais específicos CD44s-Tn/STn semelhantes às espécies identificadas em tumores de bexiga. Finalmente, conjugámos os péptidos de CD44s-Tn com a proteína imunogénica KLH e

avaliámos o conjugado *in vitro* e *in vivo*. O candidato a vacina não demonstrou toxicidade para células dendríticas derivadas de monócitos (mo-DCs) e estimulou estas células na presença do adjuvante monofosforil lípido A (MPLA). Notavelmente, a formulação demonstrou segurança *in vivo* e gerou resposta humoral e celular específica.

A conjugação de antígenos tumorais com proteínas imunogénicas desencadeia muitas vezes um excesso de resposta imune contra a proteína transportadora. Isto pode gerar supressão dos epítomos, prejudicando as respostas tumorais específicas o que pode culminar na falha das vacinas. Para evitar este efeito, glicopéptidos de MUC16-Tn foram combinados com nanopartículas de ácido poli(lático-co-glicolítico)-b-poli(etileno glicol) - maleimida (PLGA-PEG-MAL) para gerar um candidato a gliconanovacina multivalente. As nanoformulações demonstraram propriedades físico-químicas e morfológicas adequadas. De facto, as nanopartículas com MUC16-Tn foram especificamente reconhecidas pela lectina do tipo galactose de macrófagos (MGL), um recetor expresso em células dendríticas imaturas e em macrófagos com alta afinidade para o antígeno Tn. Além disso, o candidato a gliconanovacina foi especificamente internalizado por células dendríticas derivadas de monócitos (mo-DCs) sem sinais de toxicidade *in vitro*. Adicionalmente, o candidato a gliconanovacina estimulou as mo-DCs *in vitro*, através do aumento da expressão dos marcadores de maturação de superfície, como o co-estimulador to receptor das células TCD86 e o recetor do complexo de histocompatibilidade class II HLA-DR, e libertação de diversas citocinas pró-inflamatórias, realçando as suas propriedades de direcionamento e adjuvante, sem necessidade de adjuvantes adicionais. Por fim, o candidato a gliconanovacina foi testado *in vivo*, demonstrando segurança e resposta humoral específica, avaliada através da reatividade dos anticorpos IgG produzidos contra MUC16-Tn sintética, linhas celulares tumorais e tecidos com expressão de MUC16-Tn.

Em suma, esta tese aplicou assinaturas moleculares tumorais associadas/específicas anteriormente identificadas, MUC16 e CD44s, em candidatos a vacinas multivalentes contra o cancro de bexiga. No geral, este trabalho oferece as bases biotecnológicas para o desenvolvimento de candidatos a glicovacinas anti tumorais multivalentes inspiradas no glico-código celular.

ABSTRACT

Bladder Cancer (BLCA) is the second most diagnosed genitourinary tract cancer, facing clinical challenges such as high recurrence rates, absence of predictive and patient stratification biomarkers, and lack of effective therapies. The introduction of immunotherapies was a remarkable achievement in BLCA management; however, a high percentage of patients remains unresponsive to treatment. In addition, current therapeutic approaches are unable to prevent tumour relapse and progression, urging effective and safer therapeutics. Cancer vaccines have gained growing interest due to their potential to fully eradicate tumours and prevent relapse. However, these therapeutic tools have been hampered by the lack of tumour-specific antigens, especially in primary tumours and metastases with low mutational burden. In line with this, exploiting post-transcriptional modifications may be a promising source of tumour antigens. Aberrant glycosylation is frequently found in aggressive tumour cells and, when combined with alterations in protein abundance and composition at the cell surface, it gives rise to tumour-specific molecular signatures with potential for immunotherapy. In fact, combining glycomics, glycoproteomics, transcriptomics and bioinformatics has enabled the identification of several tumour-associated/specific glycoproteins by our group, two of which are explored, in this work, in the context of cancer vaccines. We hypothesized that these two constructs can specifically stimulate the immune system, leading to immunogenicity through the development of cellular and humoral specific immune responses.

An enzymatic method to produce BLCA-associated glycopeptides, using MUC16 as a scaffold and combining glycosyltransferases and sugar donors was developed. To further enhance the ratio of sialylated glycopeptides, a TiO₂ enrichment protocol was established. All synthetic products were characterized by mass spectrometry. Moreover, the synthesized glycopeptides were conjugated with two immunogenic proteins through maleimide-thiol chemistry, to produce multivalent glycoconjugates. Subsequently, by applying the enzymatic approach developed initially, CD44s-Tn/STn tumour-specific peptides were synthesized, resembling the species identified in bladder tumours. CD44s-Tn peptides were then conjugated with the immunogenic protein keyhole limpet hemocyanin (KLH) to yield a multivalent anti-tumour cancer vaccine candidate, following activity characterization *in vitro* and *in vivo*. The glycovaccine candidate showed no toxicity to monocyte-derived dendritic cells (mo-DCs) and stimulated them in the presence

of the monophosphoryl lipid A (MPLA) adjuvant. In addition, the formulation was proven non-toxic *in vivo* and stimulated cellular and humoral specific immune responses.

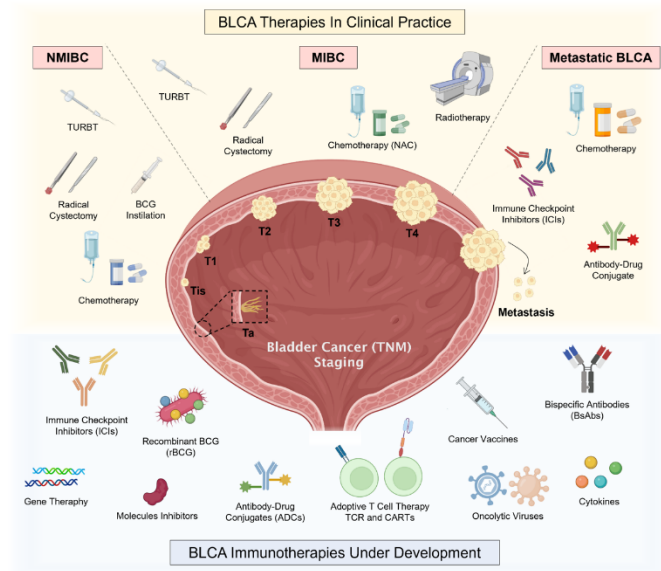
The classical conjugation of tumour antigens with protein carriers commonly fosters immune responses against the immunogenic carriers. This can generate epitope suppression, hampering tumour-specific immune responses and leading to vaccines failure. To circumvent this challenge, tumour associated antigen MUC16-Tn glycopeptides were combined with poly(lactide-co-glycolide)-b-poly-(ethylene glycol)-maleimide (PLGA-PEG-MAL) nanoparticles to generate a multivalent glyconanovaccine candidate. The nanoformulations showed adequate physicochemical and morphological properties for specific targeting of dendritic cells (DCs). MUC16-Tn nanoparticles were specifically recognized by macrophage galactose-type lectin (MGL), a receptor with high affinity for the Tn antigen, expressed in immature DCs and macrophages. Moreover, the glyconanovaccine candidate was specifically internalised by monocyte-derived dendritic cells (mo-DCs) and showed no toxicity *in vitro*. In addition, it stimulated mo-DCs *in vitro*, enhancing the expression of maturation surface markers, as the Tcell co-stimulatory receptor CD86 and the major histocompatibility complex class II HLA-DR, as well as the production of several pro-inflammatory cytokines. This reinforced the glyconanovaccine targeted and self-adjuvant nature, eliminating the need for additional adjuvants. Finally, the formulation was tested *in vivo*, demonstrating a favourable safety profile and IgG-mediated tumour-specific humoral responses against synthetic MUC16-Tn, and cell lines or tumour expressing MUC16-Tn.

Overall, this thesis employs previously identified MUC16 and CD44s cancer-associated/specific molecular glycosignatures into multivalent anti-bladder cancer vaccine candidates. Ultimately, it offers biotechnological grounds for the development of multivalent glycode-code inspired anti-tumoural glycovaccines candidates.

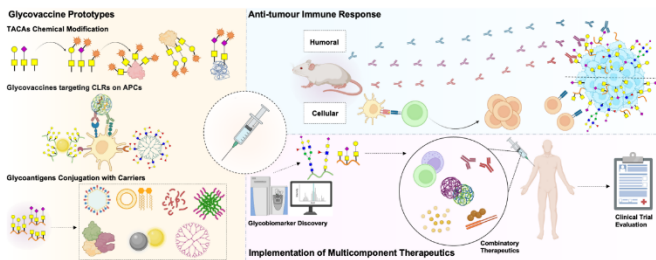
GRAPHICAL ABSTRACT

A. Introduction

Bladder Cancer Clinical Challenges and Therapies Development



Next-generation cancer glycovaccines



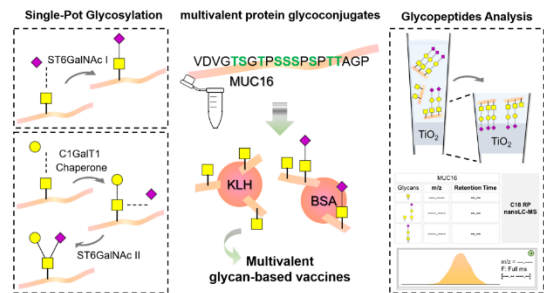
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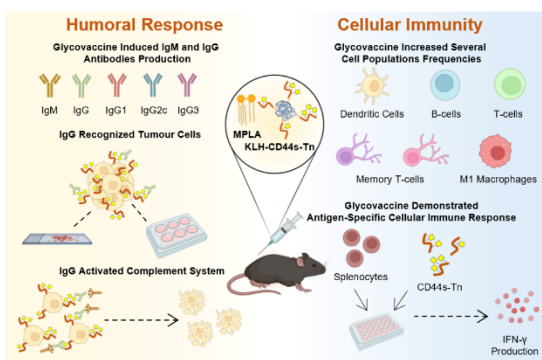
C. Chapter II

D. Chapter III

B. Enzymatic synthesis of cancer antigens



C. Development of a cancer vaccine candidate



D. Development of a cancer nanovaccine candidate

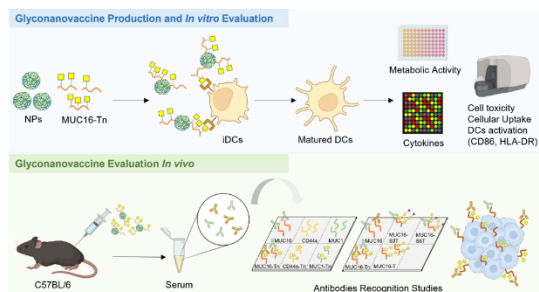


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ABBREVIATIONS & ACRONYMS

ABC: adenin-biotin complex

Abs: antibodies

ADC: antibody-drug conjugate

ADCC: antibody-dependent cellular cytotoxicity

APC: allophycocyanin

APCs: antigen presenting cells

ATCC: American Type Culture Collection

B3GNT6: beta-1,3-*N*-acetylglucosaminyltransferase 6

BCG: *Bacillus* Calmette-Guérin

BLCA: bladder cancer

BSA: bovine serum albumin

BsAbs: bispecific antibodies

C1GalT1: core 1 beta-1,3-galactosyltransferase

C1GALT1C1 or COSMC: core 1 beta-1,3-galactosyltransferase-specific molecular chaperone

C2GnT1: core 2 beta-1,6-*N*-Acetylglucosaminyltransferase

CAR: chimeric antigen receptor

CAR T: chimeric antigen receptor T cell

CD3: cluster of differentiation 3

CD4: cluster of differentiation 4

CD11c: cluster of differentiation 11c

CD19: cluster of differentiation 19

CD44: cluster of differentiation 44

CD44s: short CD44 standard splicing isoform

CD45: cluster of differentiation 45

CD62L: L-selectin

CD8: cluster of differentiation 8

CD86: cluster of differentiation 86

CD155: cluster of differentiation 155

CD206: cluster of differentiation 206

CD276: cluster of differentiation 276

CDC: complement dependent cytotoxicity

CEA: carcinoembryonic antigen

CFA: complete Freund's adjuvant

CHUSJ: Centro Hospitalar Universitário São João
 CID: collision-induced dissociation
 CLR: c-type lectin receptors
 CMP-Neu5Ac: cytidine-5-monophospho-*N*-acetylneuraminic acid
 Core 1: Gal β 1-3GalNAc-*O*-Ser/Thr
 Core 3: GlcNAc α 1-3GalNAc-*O*-Ser/Thr
 COSMC: Gal-transferase (β (1-3)-galactosyltransferase
 CpG: cytosine phosphoguanine
 CPMV: cowpea mosaic virus capsid
 CRISPR: clustered regularly interspaced short palindromic repeats
 CRM197: cross-reactive material 197
 CRP: C-reactive protein
 CTAs: cancer testis antigens
 CTLA-4: cytotoxic T-lymphocyte associated protein 4
 CTLs: cytotoxic T lymphocytes
 DAPI: 4',6- diamidino-2-phenylindole dihydrochloride
 DCs: dendritic cells
 dDcs: dermal dendritic cells
 DLS: dynamic light scattering
 DM1: maytansine derivative
 DMF: dimethylformamide
 DMFS: distant metastasis-free survival
 DMSO: dimethyl sulfoxide
 DOPC: dioleoylphosphocholine
 DPPC: dipalmitoyl-phosphatidylcholine
 DPPE: 1,2-dipalmitoyl-sn-glycero-3-phosphorylethanolamine
 dST: di-sialyl-T
 DT: diphteria toxoid
 DTT: dithiothreitol
 ECL: enhanced chemiluminescence
 EDTA: ethylenediaminetetraacetic acid
 EGFR: endothelial epidermal growth factor receptor
 EIC: extracted ion chromatograms
 EMT: epithelial-to-mesenchymal transition
 ER: endoplasmic reticulum
 ESI: electrospray ionization

EU: endotoxin units
EV: enfortumad vedontin-eivf
FACS: fluorescence-activated cell sorting
FBS: fetal bovine serum
FDA: food and drug administration
FFPE: formalin-fixed paraffin-embedded
FITC: fluorescein isothiocyanate
GA: Golgi apparatus
Gal: galactose
GalNAc: *N*-acetylgalactosamine
GalNAc-Ts: *N*-acetylgalactosaminyltransferases
GalNAc-T1: *N*-acetylgalactosaminyltransferase 1
GalNAc-T2: *N*-acetylgalactosaminyltransferase 2
GalNAc-T3: *N*-acetylgalactosaminyltransferase 3
GalNAc-T11: *N*-acetylgalactosaminyltransferase 11
GBRs: glycan binding receptors
Glc: glucose
GlcNAc: *N*-acetylglucosamine
GLP: glycolipopeptides
GM-CSF: granulocyte-macrophage colony-stimulating factor
HCD: higher-energy collisional dissociation
hCG- β : human chorionic gonadotropin beta-chain
HER2: human epidermal growth factor receptor 2
HG-NMIBC: high-grade non-muscle invasive bladder cancer
HILIC: hydrophilic interaction liquid chromatography
HLA: human leucocyte antigens
HLA-DR: human leucocyte antigens – DR isotype
HPLC: high performance liquid chromatography
HRP: horseradish peroxidase
ICIs: immune checkpoint inhibitors
IDAA: indel detection by amplicon analysis
IDO: indoleamine 2,3-dioxygenase
IFA: incomplete Freund's adjuvant
IFN- α : interferon alpha
IFN- γ : interferon gamma
IgG: immunoglobulin G

IgG1: immunoglobulin G1
 IgG2b: immunoglobulin G2b
 IgG2c: immunoglobulin G2c
 IgG3: immunoglobulin G3
 IgM: immunoglobulin M
 IHC: immunohistochemistry
 IL-1 β : interleukin 1 β
 IL-1RA: interleukin 1RA
 IL-4: interleukin 4
 IL-6: interleukin 6
 IL-8: interleukin 8
 IL-10: interleukin 10
 IL-12: interleukin 12
 IL-12(p40): interleukin 12(p40)
 IL-12(p70): interleukin 12(p70)
 IL-18: interleukin 18
 iNKT: invariant natural killer T cells
 IPO-Porto: Instituto Português de Oncologia do Porto
 ISO: International Organization for Standardization
 KLH: keyhole limpet hemocyanin
 KO: knock out
 Le^y: Lewis y
 LDA: laser doppler anemometry
 LG-NMIBC: low-grade non-muscle invasive bladder cancer
 LPS: lipopolysaccharides
 MDSCs: myeloid-derived suppressor cells
 MAG: multiple antigenic glycopeptide
 MGAT1: alfa-1,3-mannosyl-glycoprotein 2-beta-*N*-acetylglucosaminyltransferase
 MAL: maleimide
 MALDI: matrix-assisted laser desorption/ionization
 Man: mannose
 mBLCA: metastatic bladder cancer
 MBS: m-maleimidobenzoyl-*N*-hydroxysuccinimide ester
 MFI: mean fluorescence intensity
 MGL or CLEC10A/CD301: macrophage galactose-type lectin or C-type lectin domain family 10 member A/cluster of differentiation 301

MHC-I: major histocompatibility class I
 MHC-II: major histocompatibility class II
 MHCs: major histocompatibility complexes
 MIBC: muscle-invasive bladder cancer
 MMAE: monomethyl auristain E
 MPLA: monophosphoryl lipid A
 Mo-DCs: monocyte-derived dendritic cells
 mOS: median overall survival
 mPFS: median progression-free survival
 MR: mannose receptor
 MS: mass spectrometry
 MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
 MUC: mucin
 MUC1: mucin 1
 MUC4: mucin 4
 MUC16: mucin 16
 NAC: neoadjuvant cisplatin-based chemotherapy
 NanoLC: nano liquid chromatography
 NK: natural killer
 NMIBC: non-muscle invasive BLCA
 NMR: nuclear magnetic resonance
 NPs: nanoparticles
 Neu5Ac: *N*-acetylneuraminic acid
 OMPC: outer membrane protein complex
 OP: optical density
 ORR: objective response rate
 OS: overall survival
 OVA: ovalbumin
 P2: tetanus toxoid peptide
 P3CS: tripalmitoyl-*S*-glycerylcysteinylserine
 Pam: palmitic acid
 Pam2Cys: *S*-[2,3-bis(palmitoyloxy)propyl]cysteine
 Pam3Cys: *S*-[(*R*)-2,3-dipalmitoyloxy-propyl]-*N*-palmitoyl- (*R*)-cysteine
 Pam3CysSK4: *N*-Palmitoyl-*S*-[2,3-bis(palmitoyloxy) (2*RS*)-propyl]- [*R*]-cysteinyl-[*S*]-seryl-[*S*]-lysyl-[*S*]-lysyl-[*S*]-lysyl-[*S*]-lysine
 PAMAM: polyamidoamine

PB: pacific blue
 PBS: phosphate buffered saline
 PBMCs: peripheral blood mononuclear cells
 PD: *Haemophilus* protein D
 PD-1: programmed cell death protein 1
 PD-L1: programmed death-ligand 1
 PDI: polydispersity index
 PDVF: polyvinylidene difluoride
 PE: phycoerythrin
 PEG: poly(ethyleneglycol)
 PerCP: peridinin-chlorophyll protein complex
 PFA: paraformaldehyde
 PFS: progression free survival
 P(HPMA): poly(*N*-(2- hydroxypropyl)methacrylamide)
 PLGA: poly(d,l-lactic-co-glycolic acid)
 PMA: phorbol 12-myristate 13-acetate
 PNA: peanut agglutinin
 pNPP: *p*-nitrophenyl phosphate
 PO: pacific orange
 ppGalNAc-Ts: polypeptide *N*-acetulgalactosaminyltransferases
 PSM: peptide-spectrum match
 QS-21: *Quillaja Saponaria* 21 based adjuvante
 rBCG: recombinant *Bacillus Calmette-Guérin*
 RFS: relapse-free survival
 RNA: ribonucleic acid
 RT: room temperature
 S3T: sialyl-3-T (Neu5Ac α 2-3Gal β 1-3GalNAc-*O*-Ser/Thr)
 S6T: sialyl-6-T (Gal β 1-3[Neu5Ac α 2-6]GalNAc- α 1-*O*-Ser/Thr)
 scFv: single-chain variable fragment
 SDS-PAGE: sodium dodecyl-sulfate polyacrylamide gel electrophoresis
 SLITRK6: SLIT and NTRK-like protein 6
 ST: sialyl-T
 ST3Gal I: beta-galactoside alpha-2,3-sialyltransferase
 ST6GalNAc I: alpha-*N*-acetylgalactosaminide alpha-2,6-sialyltransferase 1
 ST6GalNAc II: alpha-*N*-acetylgalactosaminide alpha-2,6-sialyltransferase 2
 STn: sialyl-Tn (Neu5Ac α 2-6GalNAc-*O*-Ser/Thr)

SUVs: small unilamellar vesicles
 T: Thomsen-Friedenreich (Gal β 1-3GalNAc-*O*-Ser/Thr)
 TACAs: tumour-associated carbohydrate antigens
 TALENs: transcription activator-like effector nucleases
 TAMs: tumour-associated macrophages
 TCEP: tris-(2-carboxyethyl) phosphine
 TEM: transmission electron microscopy
 TH1: type 1 T helper
 TH2: type 2 T helper
 TILs: tumour-infiltrating lymphocytes
 TLR: toll-like receptor
 TMB: 3,3',5,5'-tetramethylbenzidine
 TMS: tetramethylsilane
 Tn: GalNAc (α -*O*-Ser/Thr)
 TNF- α : tumour necrosis factor alpha
 TNM: tumour-node-metastasis
 TOF: time-of-flight
 TolDCs: tolerogenic dendritic cells
 TR: tandem repeats
 Treg: regulatory T cell
 Trop2: trophoblastic cell surface antigen 2
 TT: tetanus toxoid
 TTP: time to progression
 TURBT: transurethral resection of the bladder tumour
 UDP-Gal: uridine 5'-diphosphogalactose
 UDP-GalNAc: uridine 5'-diphospho-*N*-acetylgalactosamine
 VEGF: vascular endothelial growth factor
 VNTRs: variable tandem repeats
 VVA: *Vicia villosa*
 ZFNs: zinc finger nucleases
 α -GalCer: alpha-galactosylceramide

INTRODUCTION

Introduction Outline

This introduction is organized into two main sections:

A first section on bladder cancer clinical challenges and on the novel immunotherapeutic approaches developed envisaging personalized medicine has been introduced. A general overview on bladder cancer (BLCA) aberrant *O*-glycosylation and glycan-based opportunities for the development of cancer vaccines has been also included.

A second section includes one review article addressing a comprehensive overview of emerging anti-tumour glycovaccines exploiting several strategies for vaccine design, including nanotechnology. In addition, it highlights a roadmap toward clinical implementation, including cancer-specific glycosignatures discovery, vaccine design, combinatory therapies, pre-clinical models and clinical trials. This is summarized in the manuscript:

Rui Freitas*, Andreia Peixoto*, Eduardo Ferreira, Andreia Miranda, Lúcio Lara Santos, José Alexandre Ferreira. Immunomodulatory glycomedicine: Introducing next generation cancer vaccines. *Biotechnology Advances* (Impact Factor in 2023: 16.0, Q1). 2023. 10.1016/j.biotechadv.2023.108144

Overview of Bladder Cancer Clinical Challenges and Novel Glycan-based Immunotherapeutic Opportunities Thereof

1.1. Bladder cancer clinical challenges

Bladder cancer (BLCA) is one of the most frequently diagnosed genitourinary tract cancers, accounting for approximately 614,000 new cases and 220,000 deaths in 2022 [1]. BC global incidence is higher in men than in woman, being the Southern Europe the region with more diagnosed cases. Tobacco smoking is the most frequent risk factor [2], followed by exposure to carcinogenic compounds such as aromatic amines and arsenic [3]. Infection with *Schistosoma haematobium* also contributes for BLCA development, mostly in African countries [4]. Its recurrence rates, surveillance strategies and high treatment costs make BLCA the most expensive cancer to treat. Therefore, improvements in BLCA care are necessary to avoid repeated interventions. Namely, improvement in early diagnostic tools might be a key necessity once advanced stage and metastatic BLCA (mBLCA) are associated with higher costs. Moreover, focus should be set in the development of more effective therapeutics [5].

The most frequently diagnosed BLCA cases are urothelial carcinomas [6], following squamous cell carcinoma, small cell carcinoma, adenocarcinoma, and sarcoma. Based on its prevalence, the information provided in this section is related to urothelial carcinoma. Bladder cancer is classified into non-muscle invasive BLCA (NMIBC) and muscle invasive BLCA (MIBC) according to the tumour-node-metastasis (TNM) staging. According to its histological grade NMIBC can be divided into high-grade NMIBC (HG-NMIBC, including carcinoma in situ pTis, pTa and pT1) and low-grade NMIBC (LG-NMIBC) [7]. On the other hand, MIBC is staged from T2-T4 depending on the extent of muscle invasion, constituting mostly high-grade cases [7]. Despite most NMIBC cases having a low probability of progression (10-20%) and a high survival rate (near 90% 5-year overall survival), lymph node metastases are found in 5% of pT1 tumours and 50% of pTis progress and disseminate [8, 9]. In addition, 10-20% of NMIBC cases progress to MIBC, and 30-70% of patients relapse within 5 years [10]. On contrary, MIBC present poor prognosis, rapid metastatic progression, and high

mortality rates (approximately 50%), especially in patients with early metastatic dissemination (near 10% 5-year overall survival).

Non-muscle invasive bladder cancer is diagnosed in 70-75% of patients [11], and is further divided into low, intermediate and high risk for recurrence and progression [12]. Low-risk patients are treated with transurethral resection of the bladder tumour (TURBT) following intravesical instillation of chemotherapy [13], while intermediate and high-risk patients are treated with *Bacillus Calmette-Guérin* (BCG) instillation [14, 15]. Of note, BCG increases the time to recurrence and decreases disease progression (**Figure 1**) [16]. BCG immunotherapy has a 55-65% positive response rate for high-risk tumours and 70-75% for Tis tumours [17]. However, BCG immunotherapy fails in approximately 40% of patients due to relapse, refraction or intolerance. The underlying mechanisms of BCG action are not fully understood. Disclosing the immunobiology of BCG may be a critical milestone for tailoring BCG treatment to improve efficacy and reduce intolerance. In refractory cases, radical cystectomy is usually applied. Nevertheless, the combination of BCG with immune checkpoint inhibitors has been considered, given the role of exhaustion markers in BCG resistance [13].

In turn, MIBC is detected in 25-30% of patients [11] and the standard of care treatment for non-metastatic tumours consists of neoadjuvant cisplatin-based chemotherapy (NAC) prior to radical cystectomy (**Figure 1**) [18]. Of note, chemotherapy treatment can be the combination of several drugs (doxorubicin, methotrexate, vinblastine and cisplatin) or a combination of cisplatin and gemcitabine. The NAC treatment frequently reduces tumour size before surgery, eradicates micrometastases and decreases metastasis formation [19]. However, 50% of patients are ineligible for NAC due to comorbidities and cystectomy is currently the preferred option [20], although immunotherapy can also be considered for patients with tumour expressing high levels of PD-L1 [21]. In addition, only 30-40% of patients show a positive response to chemotherapy, urging to establish effective methods to select and predict responders and non-responder patients. Nevertheless, patients who are ineligible for surgery receive chemotherapy or radiotherapy after TURBT. Patients treated this way present a 5-year overall survival rate of 56% and a 5-year bladder-intact survival rate of 42% [22]. Despite aggressive multimodal therapies, approximately 50% of MIBC patients will evolve to metastatic disease. Moreover, the survival rate of MIBC

patients has not suffered changes in the last 30 years, reinforcing the need to improve patient's stratification, envisaging correct treatment application for better outcomes.

Metastatic BLCA is detected in 10% of MIBC cases at diagnosis and 40-50% of patients present metastatic formation after recurrence [23]. For the latter, there are no effective therapies and palliative care is mostly applied. Until very recently, the first-line treatment for metastatic BLCA was NAC and second-line therapy consisted in immune checkpoint inhibitors (ICIs). The Food and Drug Administration (FDA) approved three ICIs targeting PD-L1 (atezolizumab, avelumab and durvalumab), two targeting PD-1 (nivolumab and pembrolizumab) and one targeting CTLA-4 (ipilimumab) (**Figure 1**) [24]. However, only a small percentage of patients respond to ICIs (20-25%). Since October 2023, after FDA approval, the new first-line standard of care for mBLCA consists of an antibody-drug conjugate (ADC) composed by enfortumab vedotin-eivf (EV) plus pembrolizumab [25]. The EV is a human IgG1 ADC against nectin-4, an adhesion membrane protein, conjugated to the microtubule-disrupting agent, monomethyl auristatin E (MMAE). The EV plus pembrolizumab showed a median OS of 31.5 months when compared to 16.1 months presented by platinum chemotherapy, which resulted in a significant improvement in patient's survival [26].

In summary, BLCA presents several clinical challenges such as high recurrence rates, rapid progression, and treatment failure [23]. The current BLCA classification system and therapeutic approaches do not account for tumour heterogeneity, contributing to treatment failure. In the last years, there have been several attempts to identify molecular subtypes to better understand tumour biology, select therapies and predict treatment response as recently reviewed by Minoli, *et al.* [23]. However, these methods are not currently used in clinical practice and require further improvements toward precision medicine. The lack of predictive biomarkers is also a major drawback in disease management. Moreover, the need for novel, safer and more effective therapeutics has encouraged great efforts in the development of several immunotherapeutic approaches envisaging personalized medicine.

1.2 Novel bladder cancer immunotherapies under investigation

To date, only BCG, ICIs and an ADC have been approved for BLCA treatment. Despite their positive results, these approaches frequently present adverse side effects and are unable to prevent tumour recurrence [23]. In addition, ICIs efficacy strongly depends on tumour genomics, immune checkpoint levels and other factors such as the gut microbiome [27, 28]. Thus, in the last few years, several improvements in existing therapies and the development of novel approaches have been attempted, envisaging more efficient BLCA management (Figure 1).

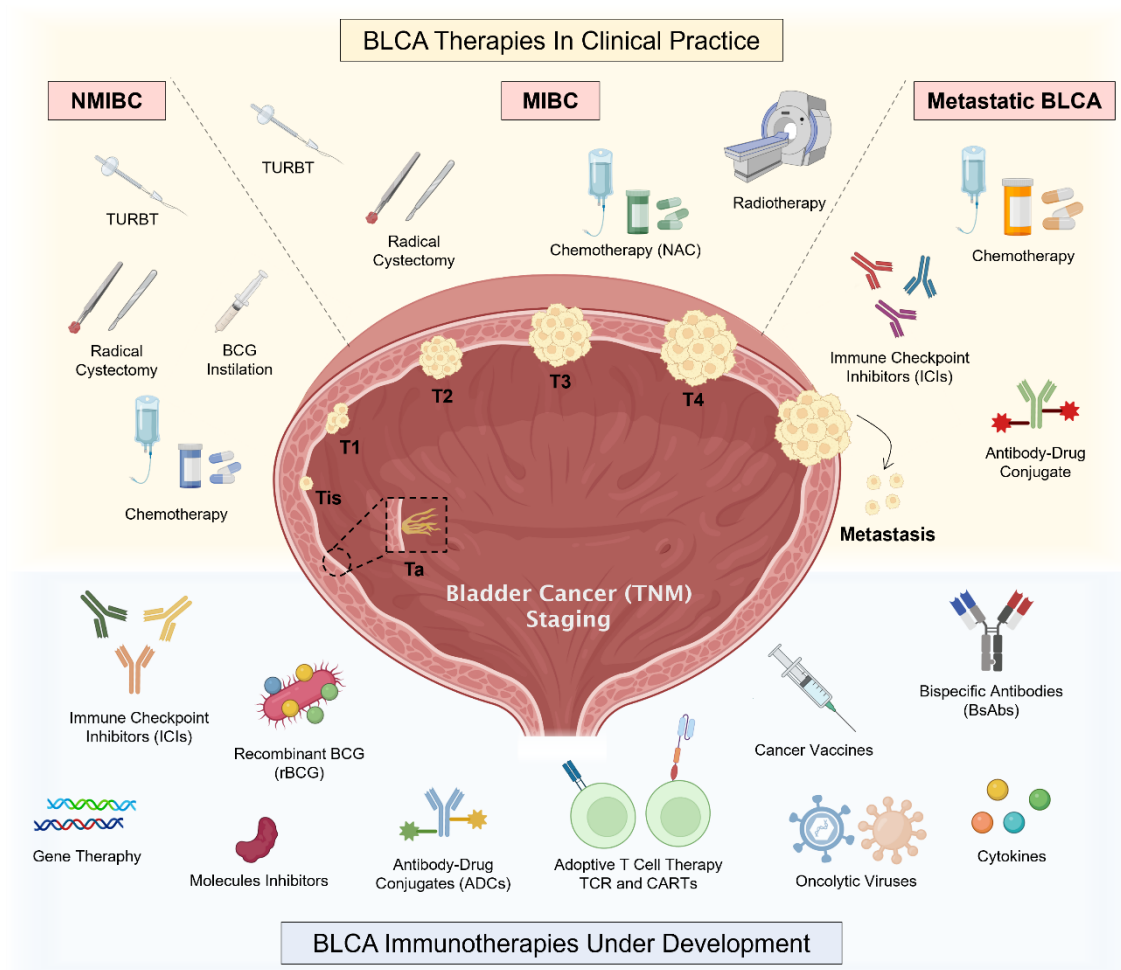


Figure 1. Bladder Cancer (BLCA) staging according to tumour-node-metastasis (TNM) classification, current applied therapies in clinical practice and novel immunotherapies under development. Non-muscle invasive BLCA includes Ta, Tis and T1 stages, which are managed through transurethral resection of the bladder tumour (TURBT), chemotherapy, *Bacillus Calmette-Guérin* (BCG) instillation, and, in some cases, radical cystectomy. Muscle invasive BLCA is divided in T2, T3 and T4 stages, and mainstay therapies include neoadjuvant cisplatin-based chemotherapy (NAC) prior to

radical cystectomy, and TURBT followed by radiotherapy. Finally, metastatic BLCA is treated with chemotherapy and immunotherapy, including immune checkpoint inhibitors and antibody-drug conjugates (ADC).

Several modifications and novel BCG formulations have been attempted to increase the effectiveness and decrease BCG associated toxicity. In particular, recombinant BCG (rBCG) expressing several pro-inflammatory cytokines (IL-2, IL-18, IFN- α or IFN- γ) has been developed [29, 30]. Despite promising results reported *in vitro*, few rBCG have shown antitumour activity *in vivo*. Notwithstanding, no clinical trials have been conducted to evaluate the safety and efficacy of these therapeutics, hampering the establishment of these approaches.

The development of novel antibodies targeting different immune checkpoints is currently ongoing. Several novel ICIs targeting PD-1, PD-L1 and CTLA-4 are currently being evaluated in clinical trials for mBLCA treatment, as recently reviewed by Patel *et al* [25]. The results of immunotherapy alone, or in combination with chemotherapy, are promising but not yet approved in routine practice. Other classes of immune checkpoint inhibitors are coming to light. One example is MGA271, an IgG1 monoclonal antibody against B7-H3 (CD276), which was tested in a phase I clinical trial (NCT01391143) [20]. However, no results have been published to date. CD276 is a member of the B7 family receptor, expressed in dendritic cells, macrophages, T cells, B cells and natural killer cells [31], being frequently found in bladder tumours [32]. Its reported functions include both stimulatory [33] and inhibitory immune functions [34]. In addition, the receptor for CD276 remains unidentified, which may hamper the development of a potentially more effective approach such as bispecific antibodies.

Another currently exploited approach for BLCA treatment is the use of antibody-drug conjugates (ADCs). ADCs combine the specific targeting of tumour antigens by monoclonal antibodies with the antitumour activity of cytotoxic drugs [35, 36]. This strategy ensures the selective targeting of tumour cells and decreases systemic drug toxicity. The recent FDA approval of EV for metastatic BLCA treatment confirmed the reliability and the potential of this approach [25]. In addition, several other ADCs are being developed, targeting cell membrane antigens overexpressed in BLCA, such as Nectin-4, human

epidermal growth factor receptor 2 (HER2), trophoblastic cell surface antigen 2 (Trop2), and SLIT and NTRK-like protein 6 (SLITRK6) [37-40]. Disitamab Vedotin is an ADC composed by an antibody targeting HER2 conjugated with MMAE. This ADC was tested in a phase II clinical trial in mBLCA patients showing an objective response rate (ORR) of 51.2% (95% CI 35.5-66.7%), median progression-free survival (mPFS) of 6.9 months (95% CI 5.6-8.9) and median overall survival (mOS) of 13.9 months (95% CI 9.1-NR) [41]. Trastuzumab Emtansine, an ADC combining trastuzumab (targeting HER2) conjugated with maytansine derivative (DM1), an inhibitor of microtubule assembly, was recently tested in a phase II clinical trial in patients with advanced or mBLCA (NCT02999672) [35]. A partial response was observed in 38.5% of patients, with a mOS of 7.03 months (95% CI 3.75-NE). Trastuzumab Deruxtecan is another HER2-targeting ADC that uses DX-8951 (DXd), a topoisomerase I inhibitor. This ADC is currently under clinical evaluation in combination with Nivolumab for advanced BLCA (NCT03523572) [42]. Sacituzumab Govitecan is an ADC combining an antibody that specifically targets Trop2, conjugated with the topoisomerase inhibitor SN-38. This ADC showed acceptable toxicity in phase I studies [43] and is currently being evaluated in phase II studies for mBLCA (NCT03547973) and in phase I/II studies in combination with chemotherapy (NCT03992131). Sirtratumab Vedontin is an antibody against SLITRK6 conjugated with MMAE. This ADC has been tested in a phase I clinical trial for mBLCA, showing an ORR of 33%, mPFS of 16 weeks and a median duration of response of 15 weeks [44]. Together, these studies demonstrate that with the approval and introduction of EV in clinical practice and growing interest in the development of new ADCs, these conjugates may gain a profound impact on the treatment of advanced and mBLCA.

The efficacy of anti-tumour immune response strongly depends on the activity of T cells. Therefore, adoptive T cell therapy has gained tremendous interest in cancer therapy. Adoptive T cell therapy describes a process in which circulating or tumour-infiltrating T cells are removed, manipulated ex vivo, and reinfused into the patient [45]. Two approaches can be used to generate specific T cells: TCR T cells or chimeric antigen receptor T cells (CAR T). TCR T cells are engineered to have a strong affinity for tumour antigens presented by major histocompatibility complexes (MHCs) on the tumour surface [46]. Currently, the safety of several TCR T cells targeting different antigens (MAGEAs, CTA, NY-ESO-1, HER2 and 4SCAR-PSMA/FR α) is being evaluated in the context of BLCA [47].

However, TCR T cells can only bind to MHC-presented peptides on tumour cells surface, and it is well known that several tumours downregulate MHC molecules to evade immune response [48]. To circumvent this limitation, CAR-T cells therapies have been developed. A CAR-T cell is an engineered T cell modified with a chimeric antigen receptor (CAR) formed by multiple domains, including a single-chain variable fragment (scFv) recognition domain that allows the recognition of cell surface antigens. CAR-T cells have been successfully used in clinical trials to treat B cell malignancies, which allowed their approval by the FDA [49]. The translation of CAR-T cells into solid tumours is ongoing, including the evaluation of two CAR T cells targeting 4SCAR-PSMA and 4SCAR-FR α in a phase I/II clinical trial for BLCA patients (NCT03185468) [50].

Cancer vaccines aim to activate innate and adaptive immune responses to effectively eradicate tumour cells and generate immunological memory to prevent tumour relapse. In cancer vaccine design, tumour antigens may consist in nucleic acids, synthetic peptides, recombinant proteins or viral vectors [51]. In addition, vaccine platforms include dendritic cell (DCs)-based vaccines (DCs generated and loaded with tumour antigens ex vivo and infused into patients) or in situ administration of tumour antigens [52]. Moreover, cancer vaccines can be designed using a multiplicity of carriers and adjuvants to enhance immune responses. The potential of this approach makes it an emerging strategy in cancer therapy, with several vaccine candidates being evaluated in pre-clinical and clinical trials [52, 53]. One example is Vesigenurtacel-L, a cell-based vaccine composed of an allogeneic BLCA cell line modified to secrete the endoplasmic reticulum chaperone protein gp96. This vaccine was tested in a phase I/II clinical trial in combination with BCG in patients with NMIBC (NCT02010203) [36]. However, no statistical differences in recurrence-free survival (RFS) were found between BCG alone and the vaccine combined with BCG [54]. Another example is Lapuleucel-T, a DC-based vaccine combined with a fusion protein, the tumour antigen HER2/neu and the cytokine GM-CSF [55]. This vaccine is currently being evaluated in a phase II clinical trial in patients with high-risk HER2-positive BLCA (NCT01353222) [55]. Another example is PANVAC, a recombinant poxviral vector-based vaccine engineered to express the tumour antigens carcinoembryonic antigen (CEA) and Mucin-1 (MUC1) and the costimulatory molecules B7.1, ICAM-1, and LFA-3. Currently, PANVAC is being tested in a phase II clinical trial in BCG-refractory NMIBC patients in combination with BCG [56].

Cancer testis antigens (CTAs), such as MAGE-A3 and NY-ESO-1, are also being pursued in anti-bladder cancer vaccines design due to their high expression in bladder tumours [57]. A MAGE-A3 based vaccine is currently being tested in a phase II clinical trial in patients that undergone radical cystectomy (NCT01435356) [20]. On another hand, a NY-ESO-1 based vaccine was evaluated in combination with BCG in a phase I clinical trial, resulting in cellular and antibody-specific immune responses [58]. Several vaccine candidates are also being evaluated in clinical trials in patients with mBLCA, as recently described by Patel *et al* [25]. Despite the growing interest in the development of cancer vaccines, this strategy has not yet reached its full potential. Only Sipuleucel-T, a therapeutic vaccine, has been so far approved by FDA for the treatment of castration-resistant prostate cancer, based on a 4.1-month improvement in mOS [59]. More studies are needed to evaluate cancer vaccine benefits for cancer treatment, especially in combination with other therapies. Notwithstanding, the potential of cancer vaccines to fully eradicate tumours and prevent them from relapse sets this approach as one of the most promising for clinical translation of BLCA antigens, bringing us closer to precision medicine.

In recent years, bispecific antibodies (BsAbs) have been extensively exploited for cancer treatment. Bispecific antibodies have two different binding domains that can target two distinct antigens or two epitopes of the same antigen simultaneously [60]. Two bispecific antibodies targeting CD3xEGFR and CD3xHER2 were developed and tested *in vitro* against BLCA cells. Both antibodies showed cytotoxic activity and mediated secretion of pro-inflammatory cytokines [61]. The same was observed for a CD3xCD155 bispecific antibody [62]. ATOR-1015, another BsAb targeting OX40xCTLA-4, was developed and showed impairment of tumour proliferation and prolonged survival *in vivo* [63]. Due to promising pre-clinical results, this BsAb is currently being evaluated in a phase I clinical trial (NCT03782467) [35]. Finally, PRS-343, a BsAb targeting HER2 and the 4-1BB costimulatory receptor on T cells 4-1BB, was developed and evaluated *in vivo*. The antibody promoted the recruitment of tumour-infiltrating lymphocytes (TILs) and inhibited tumour growth [64]. Currently, this BsAb is being evaluated in phase I clinical trials alone (NCT03330561) or in combination with Atezolizumab (NCT03650348) [35]. Despite the growing interest in this approach and the promising results, no BsAbs are yet approved for BLCA treatment. However, there are currently nine

FDA-approved BsAbs to treat malignancies [65], which confirms the potential of this approach and sets an opportunity for future BsAbs approval regarding BLCA treatment.

Alternatively, several other therapies have been developed and tested in pre-clinical and clinical settings for BLCA treatment, including cytokines, gene therapy [35, 66], oncolytic viruses [66], and inhibitors of several molecules. Molecular inhibitors mostly target tyrosine kinases [67], telomerases [68], vascular endothelial growth factor (VEGF) [69], and indoleamine 2,3-dioxygenase (IDO) [70, 71]. Vaccination with modified mouse BLCA MB-49 cell line expressing cytokines on the cell surface has demonstrated tumour growth inhibition *in vivo* in mouse models [72-74]. However, the efficacy of this approach needs to be proved for human tumours, envisaging clinical translation.

2.1 Bladder Cancer aberrant O-glycosylation

As stated above, several novel anti-tumour immunotherapies have been developed for BLCA treatment. These approaches still face several bottlenecks such as BLCA heterogeneity and a highly suppressive tumour microenvironment [23]. In addition, several primary lesions, tumours, and metastases present low tumour mutational burden [75, 76], which negatively impacts the amount of cancer-associated/specific molecular signatures to be used for immunotherapy development. As such, exploiting post-transcriptional modifications is a promising approach for the identification of novel cancer-specific targets.

Glycosylation is the most frequent post-transcriptional modification, consisting of the addition of one or more monosaccharides to polypeptide chains through glycosidic linkages [77]. This process occurs in the Golgi (GA) apparatus and endoplasmic reticulum (ER) through the synchronized activity of glycosyltransferases, glycosidases, and sugar nucleotides. Of note, the two main classes of glycans found in cell surface proteins are O-glycans and N-glycans. Importantly, glycans are responsible for essential biological functions in several development and physiological processes, including normal embryonic development, cellular differentiation, growth, protein folding, cell contact inhibition, cell-cell recognition, cell signalling, cell adhesion, molecular trafficking and clearance, receptor activation, signal transduction, host-

pathogen interaction, endocytosis, and host immune response [78, 79]. However, alterations in glycosylation patterns are frequently found in several diseases, such as cancer [79], generating tumour-associated carbohydrate antigens (TACAs) due to incomplete or neo-synthesis processes [80]. The next section will specifically detail the premature stop of *O*-glycosylation extension with the consequent overexpression of several short-chain *O*-glycans, frequently overexpressed in BLCA.

The first *O*-glycosylation step begins with the addition of an N-acetylgalactosamine (GalNAc) residue to Ser/Thr amino acids, rendering the simplest *O*-glycan Tn (GalNAc α -*O*-Ser/Thr) antigen. This reaction is catalysed by multiple UDP-GalNAc:polypeptide N-acetylgalactosaminyl transferases (ppGalNAc-Ts) in the ER [81]. The Tn antigen is generally extended with a galactose (Gal) moiety to form the Thomsen-Friedenreich antigen (T antigen or core 1) by C1Gal-T1 [Gal-transferase (β (1-3)-galactosyltransferase, T-synthase] and its chaperone (COSMC) [82]. *O*-glycan extension beyond the Tn or T antigens can be interrupted by sialylation, giving rise to sialyl-Tn (STn), sialyl-T (ST) or disialyl-T (dST) antigens. Namely, Tn can be sialylated by ST6GalNAc-I/II raising STn (NeuAc α 2-6GalNAc α 1-*O*-Ser/Thr) antigen, which prevents further monosaccharide addition and glycan elongation [83]. Alternatively, T antigen can be sialylated by α 2,3 sialyltransferase (ST3Gal-I) and α 2,6 sialyltransferase 2 (ST6GalNAc-II), rendering sialyl-3T (Neu5Ac α 2-3Gal β 1-3GalNAc-*O*-Ser/Thr, S3T) and sialyl-6T (Gal β 1-3 (Neu5Ac α 2-6)GalNAc-*O*-Ser/Thr, S6T) antigens, respectively, or dST (SA α 2-3Gal β 1-3(SA α 2-6)GalNAc α 1-*O*-Ser/Thr) [84, 85].

A recent *O*-glycomics approach showed that healthy bladder epithelial cells are characterized primarily by proteins carrying S3T, extended and sialylated core 1 structures as well as extended core 3 (GlcNAc β 1-3GalNAc α -*O*-Ser/Thr) and core 2 (Gal β 1-3GalNAc-*O*-Ser/Thr) structures (**Figure 2**) [86]. In contrast with healthy conditions, changes in glycosylation patterns can be found in disease. Aberrant glycosylation is a BLCA feature linked to dysregulations in glycosylation pathways and responsible for the expression of TACAs arising from incomplete or de novo synthesis of these antigens [80]. Under- and overexpression of glycosyltransferases, alterations on peptide backbone conformation, bioavailability of both acceptor substrates and sugar nucleotides as well as cofactors and miss-localization of glycosyltransferases in the GA and

ER are mechanisms that contribute to aberrant glycosylation [77]. *O*-glycomics and lectin-based assays disclosed that in BLCA the most expressed TACAs arising from incomplete synthesis or neo-synthesis are truncated *O*-glycans Tn, STn, T, ST, S3T, S6T and dST (**Figure 2**) [82, 87-89]. It is well known that these glycans play an important role in tumour progression and contribute to a malignant phenotype associated with migration, invasion, metastasis, and immune escape [90]. TACAs are absent or poorly expressed in healthy tissues [91, 92], which is an important feature for the development of anti-cancer therapies. In addition, the overexpression of TACAs is associated with decreased overall survival and patients' poor prognosis [80, 93].

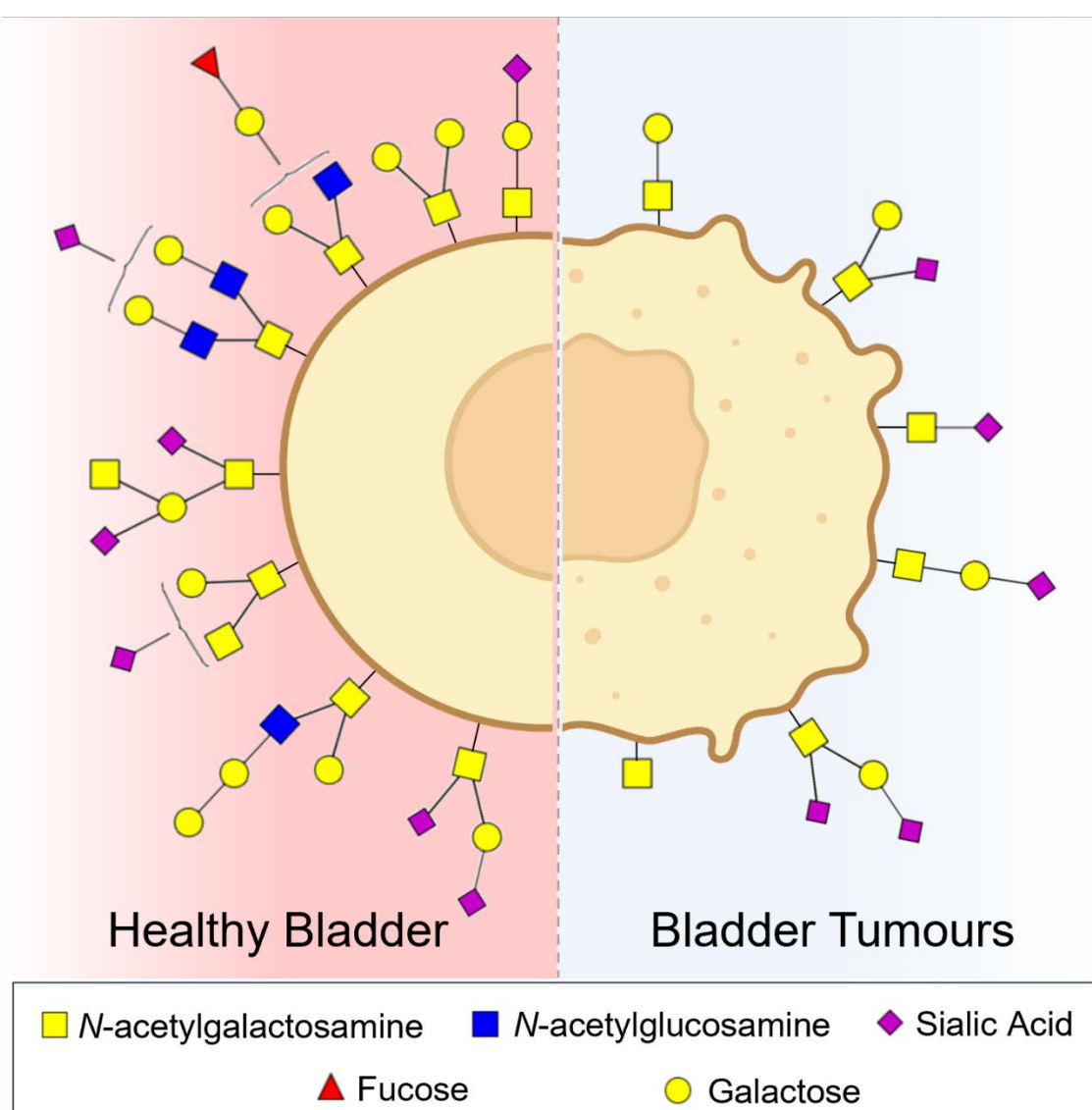


Figure 2. O-glycosylation profile in healthy bladder and bladder tumours. Healthy tissues are predominantly characterized by expression of S3T, extended and sialylated

core 1 structures as well as extended core 3 (GlcNAc β 1-3GalNAc α -O-Ser/Thr), and core 2 (Gal β 1-3GalNAc-O-Ser/Thr) structures. Contrastingly, tumour tissues overexpress truncated O-glycans such as Tn, STn, T, ST, S3T, S6T and dST.

Tn overexpression is normally associated with mutations [94]/epigenetic silencing of COSMC [95] (with loss of T-synthase activity), reduced expression of both C2GnT1 (responsible for core-3 synthesis) [96] and B3GNT6 (responsible for core-2 synthesis) [97], increased activity of ppGalNAc-Ts [98], and altered localization of ppGalNAc-Ts in ER and GA [99]. Tn antigen is found in several tumours, including BLCA [100], with low expression in healthy tissues [101]. Moreover, this glycan is frequently linked to membrane proteins that are per se overexpressed in cancer, such as mucins [102]. Biologically, Tn can modulate immune responses due to its recognition by immune cell lectins, such as C-type lectins. The interaction between Tn with macrophage galactose lectin (MGL), a C-type lectin found in both macrophages and dendritic cells (DCs) [103, 104], contributes to modulation of immunosuppressive M2-like tumour associated macrophages (TAMs) phenotype [104]. Moreover, Tn antigen is correlated with poor prognosis in many carcinomas, including BLCA [105].

Alternatively, Tn can be sialylated, raising STn (NeuAc α 2-6GalNAc α 1-O-Ser/Thr) [83]. STn is expressed by more than 80% of human carcinomas, including BLCA [83]. STn overexpression is correlated with altered expression of glycosyltransferase genes [106], mutations or hypermethylation in COSMC [107, 108], miss-localization of glycosyltransferases in the ER and GA [99, 109]. In addition, STn overexpression may be due to ST6GalNAc-I [110], nucleotide sugars or sugar transporters altered expression as result of tumour microenvironment deprivation of oxygen and nutrients [89, 111]. STn modulates both innate and acquired immunity [112]. Namely, the interaction of STn positive cells with siglec-15 expressed by macrophages leads to TGF- β production, a cytokine correlated with induction of anti-inflammatory M2-like macrophages phenotype. Also, STn seems to impair dendritic cells (DCs) maturation, inducing a tolerogenic phenotypes and incapacity to activate T cells [113]. STn antigen is also responsible for diminished cell-cell adhesion, invasion, chemotherapeutic resistance and metastatic potential in some tumours [114]. Moreover, STn is correlated with tumour progression and patients' poor prognosis [82, 92, 115].

Of note, this antigen is rarely or non-expressed by healthy tissues offering a promising opportunity for cancer-specific targeting [116, 117].

Regarding T antigen, reduced expression of C2GnT1[96] and increased availability of UDP-galactose appear to promote its biosynthesis [118]. This antigen is found in 90% of adenocarcinomas [119], including poor prognosis BLCA cases [120, 121]. The mechanisms of T antigen upregulation in cancers are not fully understood. T antigen is also expressed in germinal B cells [122] and parts of the central nervous system [123]. T antigen biological impact has been described for several cancers, acting as a promotor of tumour cell migration and survival [124, 125]. However, there is a lack of studies to evaluate its biological role in BLCA. Despite the existence of some reports that associate the expression of T antigen with immunosuppression in several tumours [126, 127], little is known about the molecular mechanisms responsible for immune modulation, especially in BLCA. Accordingly, there is a need to study the immunosuppressive function of T antigen to support the association between the expression of this antigen and immunosuppression in cancer patients.

ST and dST antigens are overexpressed in late stage BLCA and correlated with cancer malignancy, recurrence, and poor prognosis [88, 128]. A study conducted by Schneider *et al.* showed that the overexpression of both ST3Gal-I and ST6GalNAc-II enzymes may contribute to tumorigenesis, metastasis, and shorter overall survival of colorectal cancer patients [129]. ST antigen plays an important role on tumour microenvironment modulation and tumour progression. Namely, the interaction of MUC1 carrying ST glycan with siglecs, I-type lectins, (sialic acid binding Ig-like lectins) expressed by monocytes and macrophages, can promote tumour growth. A study conducted by Beatson *et al.* showed that the recognition of MUC1-ST by siglec-9 promotes (TAM)-like phenotypes, with increased expression of the immune checkpoint ligand PD-L1 and production of suppressive tumour microenvironment and disease progression factors [130]. However, the biological role of these sialylated structures in BLCA remains poorly understood.

In summary, glycomics-based approaches and lectin-based assays disclosed significant alterations in the O-glycome of bladder tumours when compared to the healthy bladder. These tumour-associated aberrant O-glycans hold promise for the development of novel non-invasive diagnostic tools and targeted immunotherapeutics, offering opportunities for precision oncology.

2.2 Glycan-based opportunities for the development of cancer vaccines

As stated above, targeting TACAs is a promising approach for immunotherapies development. Cancer patients' vaccination with tumour neoantigens has recently gained ground with the establishment of high-throughput genome sequencing technology [131-133]. This powerful analytical tool enables screening of patient's genome for unique cancer fingerprints, which have been successfully incorporated into vaccines. However, the low or scarce expression of these glycans in healthy tissues requires the addition of one extra layer of specificity for the selective target of tumour cells [83]. As such, targeting cancer cells surface glycoproteins may present the necessary means to improve treatment selectivity for cancer cells and avoid off-target effects. The introduction of glycomics, glycoproteomics, genomics, and bioinformatics tools has been a powerful approach for the identification of cancer-specific molecular glycosignatures with potential for immunotherapy design, namely cancer vaccines [76].

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Immunomodulatory glycomedicine: introducing next generation cancer glycovaccines

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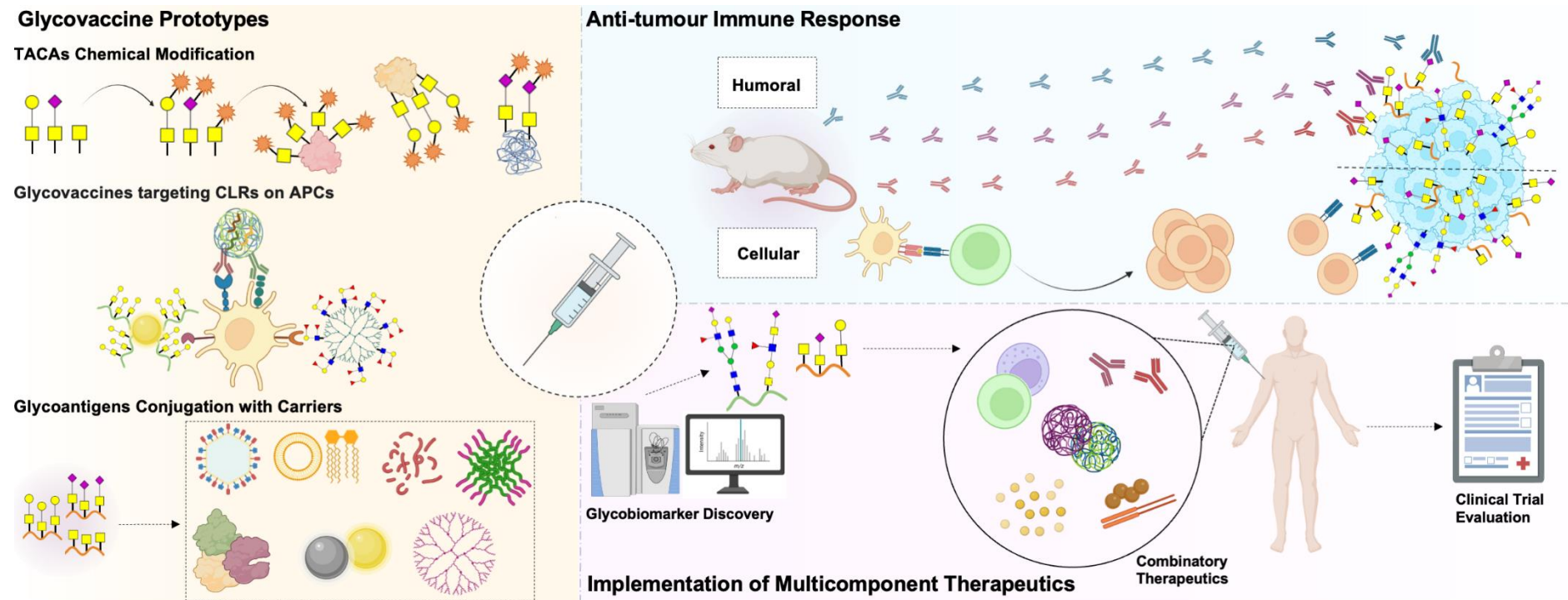
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ABSTRACT

Cancer remains a leading cause of death worldwide due to the lack of safer and more effective therapies. Cancer vaccines developed from neoantigens are an emerging strategy to promote protective and therapeutic anti-cancer immune responses. Advances in glycomics and glycoproteomics have unveiled several cancer-specific glycosignatures, holding tremendous potential to foster effective cancer glycovaccines. However, the immunosuppressive nature of tumours poses a major obstacle to vaccine-based immunotherapy. Chemical modification of tumour associated glycans, conjugation with immunogenic carriers and administration in combination with potent immune adjuvants constitute emerging strategies to address this bottleneck. Moreover, novel vaccine vehicles have been optimized to enhance immune responses against otherwise poorly immunogenic cancer epitopes. Nanovehicles have shown increased affinity for antigen presenting cells (APCs) in lymph nodes and tumours, while reducing treatment toxicity. Designs exploiting glycans recognized by APCs have further enhanced the delivery of antigenic payloads, improving glycovaccine's capacity to elicit innate and acquired immune responses. These solutions show potential to reduce tumour burden, while generating immunological memory. Building on this rationale, we provide a comprehensive overview on emerging cancer glycovaccines, emphasizing the potential of nanotechnology in this context. A roadmap towards clinical implementation is also delivered foreseeing advances in glycan-based immunomodulatory cancer medicine.

Keywords: cancer vaccines, glycomedicine, glycosylation, immune modulation, glyconeantigens

Graphical Abstract



1. Introduction

Cancer remains a worldwide healthcare concern, with 19.3 million new cases in 2020 and almost 10.0 million deaths [1]. Furthermore, global cancer incidence is expected to rise by 47% until 2040 [1], urging more effective management strategies. Cancer immunotherapy has been experiencing unprecedented bench-to-bedside clinical success, focusing on three major treatment modalities. Namely, checkpoint blockade, adoptive cellular therapy, and cancer vaccines [2]. Conceptually, cancer vaccine immunotherapy can be explored as a preventive, interceptive or therapeutic strategy. Preventive or prophylactic vaccines constitute most FDA approved approaches, usually targeting the oncogenic pathogens responsible for the development of cancerous lesions, such as hepatitis B virus or human papillomavirus, associated with liver and cervical cancer respectively [3]. However, the use of prophylactic vaccines against non-virally induced cancers is limited by lack of tumour specific antigens (neoantigens) [4]. Prophylactic vaccination against pre-malignant conditions may also be possible by targeting antigens linked to pre-cancerous lesions or oncodrivers [5, 6]. Finally, therapeutic vaccines aim to induce tumour regression, eradicate residual disease, and promote protective anti-tumour immunological memory [7]. Therapeutic cancer vaccines have been extensively tested in patients with advanced cancer. The currently limited clinical success of cancer vaccines has been mainly attributed to the immunosuppressive tumour microenvironment [8] and inability to elicit anti-cancer cellular responses [9]. In line with this, only one therapeutic vaccine, Provenge (Sipuleucel-T), has been so far approved by the FDA for the treatment of castration-resistant prostate cancer, based on a 4.1-month improvement in median survival [10]. Based on this successful implementation, novel cancer vaccines continue to be pursued, including glycan-based vaccines.

This review focuses on cancer glycovaccines exploiting tumour-associated carbohydrate antigens (TACAs) as immunogens. Novel strategies where TACAs target glycan binding receptors (GBRs) on the surface of antigen presenting cells (APCs) are also showcased. The latter strategy has been widely explored to facilitate the delivery of antigenic payloads to APCs, boosting immune responses. Several TACAs, including short-chain *O*-GalNAc glycans, branched *N*-glycans, and sialylated Lewis antigens, have been extensively implicated in tumour cells aggressiveness, including cancer cell invasion, metastasis, and immune suppression [11-19]. Amongst the most explored cancer-associated glycoforms are short-chain *O*-

GalNAc glycans, such as the Tn (GalNAc α 1-Ser/Thr), sialyl-Tn (STn; Neu5Ac α 2-6GalNAc-Ser/Thr), T (Gal β 1-3GalNAc-Ser/Thr), and sialyl-T (ST; Neu5Ac α 2-6Gal β 1-3GalNAc-Ser/Thr or Neu5Ac α 2-3Gal β 1-3GalNAc-Ser/Thr) antigens, resulting from premature stop in protein *O*-glycosylation [20, 21]. These glycans are commonly overexpressed at the cell-surface in more aggressive tumours, frequently associated with worst prognosis [22-26] and linked to higher metastatic potential [22, 27, 28]. Truncated *O*-glycans have been found to play a key role in cancer progression, driving epithelial-to-mesenchymal transition (EMT), migration, invasion, and immune escape [14, 29-32]. Of note, these are present in low abundance in healthy human tissues, mostly across the gastrointestinal and respiratory tracts [23, 33, 34]. Moreover, many changes in glycan biosynthesis or recognition are involved in other human diseases and non-malignant conditions, such as inflammatory states, and pregnancy [35]. Based on its negative functional impact on cellular features contributing to cancer progression, TACAs-based glycovaccines could potentially address aggressive cancer cells driving disease progression. However, glycovaccine development has been hurdled by several biotechnological obstacles, starting with the identification of cancer unique glycosignatures not observed in non-malignant conditions. Notwithstanding, glycomics, glycoproteomics, glycoproteogenomics, and immuno-peptidomics, supported by dedicated bioinformatic tools, have now reached the necessary technological readiness to render more cancer-specific glycosignatures to support vaccine development. Efficient synthesis of glycoconjugates also constitutes a relevant holdup in glycovaccine formulation. However, improvements in chemo-enzymatic and automated carbohydrate synthesis, as well as in cell-based synthesis platforms have been meeting pre-clinical studies needs for pure and well characterized glycoconjugates [36-42]. In addition, new versatile methodologies have been proposed to produce multi-gram amounts of nucleotide sugars (UDP-Gal, UDP-GlcNAc, and UDP-GalNAc) through stable Leloir-glycosyltransferases cascades [43]. Another relevant aspect is the poor immunogenicity of glycans, which are T-cell-independent antigens not eliciting protective immunoglobulin G (IgG) responses nor inducing immunological memory [44, 45]. As such, different molecular glycoengineering strategies have been explored to break TACAs self-tolerance, including synthesis of chemically modified TACAs [46], their conjugation with immunogenic carriers [47], and the use of adjuvants [48, 49]. In parallel, new strategies to engage APCs are being actively pursued, including the use of

antibody-mediated or natural ligand targeting of C-type lectin receptors on their surface to optimize activation and provide both humoral and cellular anti-cancer responses [50-52]. Multivalent carrier platforms that simultaneously target and stimulate APCs are also being explored in pre-clinical studies towards these objectives. This review attempts to provide a comprehensive vision on the outstanding progresses made in these fields over the last decade and their clinical readiness level. It delivers a thorough overview on different types of glycovaccine formulations (**Table 1** and **2**) and provides a roadmap for cancer glycovaccines clinical implementation.

2. Spontaneous TACA specific immune recognition in cancer

There are numerous demonstrations that cancer patients' immune system can mount immune responses against glycoconjugates. It has been long described that MUC1 glycopeptides can be processed through the major histocompatibility (MHC) class I antigen presentation pathway, showing strong MHC-I binding capacity and glycopeptide-specific CTL stimulatory ability [53]. The GalNAc residue plays a key role in this context, promoting high affinity anchoring of glycopeptides to the MHC groove [54]. Notably, this is not exclusive of typical extracellular glycosylation, as cytosolic O-GlcNAc glycopeptides may also be presented via MHC-I and elicit glycopeptide-specific cytotoxic T lymphocyte responses in mice (CTL) [55]. The presence of autoantibodies directed against glycoconjugates in the serum of cancer patients is another striking observation supporting the existence of glycan and glycopeptides-specific immune responses [56-58]. There are several reports of autoantibodies in the serum of cancer patients against mucin variable tandem repeats carrying immature *O*-glycans [56, 58]. High-throughput approaches exploiting mucin glycoarrays in colorectal cancer further suggest that the distinction between self- and non-self-molecular glycosignatures may be dictated by the density and distribution of glycosidic chains along the peptide backbone [59]. Interestingly, high autoantibody titers against core3-MUC1 (GlcNAc α 1-3GalNAc-MUC1) and STn-MUC1 (NeuAc α 2,6GalNAc-MUC1) glycoforms have been associated with reduced incidence and increased time to metastasis in breast cancer [56]. Notably, the induction of autoantibodies is not exclusive of *O*-glycans. In fact, higher autoantibody titers against *N*-glycans (Man9 oligomers and tri- or multi-antennary type II (Gal β 1 $\rightarrow$ 4GlcNAc) chains (Tri/m-II)) have been found in prostate cancer patients in comparison to men with benign prostatic hyperplasia

(Wang, 2012), shedding a light on a class of previously less studied immunological targets. Collectively, these findings support that cancer-associated glycans mounted on peptides may be recognized by the immune system. These can be successfully processed and presented through the MHC class I pathway, generating glycan-targeted cellular responses mediated by CTLs, which is a pivotal mechanism to achieve successful cancer glycovaccines. There is also strong evidence linking glycan-targeted immune responses and cancer protective effects, again supportive of the cancer glycovaccines rationale. However, there is also growing awareness that successful cancer vaccines will require a precise identification of disease-specific glycoproteoforms. Exploiting patient's autoantibodies repertoire to interrogate the cancer patient's glycoproteome poses as a promising strategy towards this objective.

Table 1. Pre-clinical glycovaccine studies involving TACAs conjugated to different carriers and/or immunogens.

Carrier	Antigen	Adjuvant	Model	Outcome	Reference
<i>Protein carriers</i>					
KLH	Tn, STn and T synthetic MUC1 TR glycopeptides.	CFA	BALB/c wild-type and MUC1.Tg mice. Human breast cancer MCF-7, T47D, MTSV1-7 cell lines and tissues.	IgG1 production directed to Tn and STn MUC1 glycoforms. Responses to STn 60-mer MUC1 also included IgG2A and IgG2B. Abs positive reactivity against MUC1 ⁺ breast cancer cell lines and tissues.	[291]
KLH	STn or clustered tri-STn.	QS-21	BALB/c-C57BL/6 FI mice. Human colon cancer cell lines LS-B (STn ⁻) and LS-C (STn ⁺).	IgM and IgG production against the immunizing antigens and ovine submaxillary mucin. Abs positive reactivity against STn ⁺ cell lines.	[292]
KLH	α -sp2 GalNAc-Thr glycopeptide	CFA in the first immunization. IFA in boost immunizations.	BALB/c MUC1.Tg mice. Human embryonic kidney HEK293T (MUC1 ⁻), and human breast cancer cells MCF7 (MUC1 ⁺) and T47D (MUC1 ⁺).	The engineered antigen mimics the conformational landscape of the natural Tn antigen and exhibits a good affinity towards anti-MUC1 antibodies VU-11E2 and SM3. Specific production of anti-MUC1 IgG antibodies that outnumber those obtained with the natural Tn analogue. Abs positive reactivity against MUC1 ⁺ cell lines.	[115]
BSA	Tn, STn, T and 2,6-ST synthetic MUC1 TR glycopeptides.	CFA in the first immunization. IFA in booster immunizations.	BALB/c wild-type mice. Human breast cancer cell line MCF-7.	Predominant IgG1 and IgM production. Minor IgG2a,b, IgG3, IgA isotypes production. Abs positive reactivity against MUC1 ⁺ breast cancer cell line.	[49]
BSA	Tn and T synthetic MUC1	CFA in the first immunization.	BALB/c wild-type mice.	IgG production directed to T or Tn MUC1 glycoforms at T9.	[293]

	TR glycopeptides.	IFA in boost immunizations.				
OVA ₃₂₃₋₃₃₉	Synthetic STn-MUC1 TR glycopeptides.	CFA in the first immunization. IFA in boost immunizations.	DO11.10 mice.	transgenic	IgG production directed to MUC1-STn glycoforms	[110]
OVA	Mono-Tn, di-Tn and tri-Tn synthetic STn-MUC1 TR glycopeptides	CFA in the first immunization. IFA in booster immunizations.	DO11.10 mice.	transgenic	IgG production directed to MUC1 glycoforms, except with the triglycosylated vaccine	[111]
TT	Synthetic MUC1 TR glycopeptides with: • one Tn antigen; • one STn antigen, and one Tn antigen side chain; • one STn and two Tn side chains; • one 2,6-sialyl-T-antigen; • one 2,3-sialyl-T-antigen; • one T-antigen side chain.	CFA in the first immunization. IFA in boost immunizations.	BALB/c wild-type mice.		IgG production directed to STn-MUC1, Tn-MUC1, STn, Tn-MUC1, STn, di-Tn-MUC1 and S6T-MUC1 glycoforms.	[112]
TT	Synthetic STn-MUC1 TR glycopeptide	IFA	PyMTxhuMUC1-tg, PyMT-tg, and C57BL/6-TG(MUC1)79.24GEND/J mice. T47D and MCF-7 (MUC1+) human breast		Preventive immunization inhibited tumour progression. A specific IgG1 humoral response against hu(TA)MUC1 in immunized mice was demonstrated. Mouse anti-sera containing IgG1 and IgG2b Abs reacted against PyMTxhuMUC1 tumour cells <i>ex vivo</i> .	[113]

						cancer cells. Mouse PyMTxhuMUC1 tumour cells. Triple-negative breast tumour tissues and healthy breast tissues.	
CRM197	TnThr	No adjuvant.	external	DCs derived from peripheral adherent mononuclear cells of healthy donors. 4T1-luc mouse mammary gland adenocarcinoma cell line. Immunocompetent syngeneic BALB/c mice. Chemotherapy naïve breast cancer patients' blood.	Induction of high frequency of CD4+ T cells and low frequency of M2-type macrophages. Reduction of tumour progression and lung metastasis growth. Activation of human dendritic cells and mime[4]CRM-specific T cells, in cancer tissue and peripheral blood of patients with breast cancer.	[116]	
Q11 domain	Synthetic MUC1 glycopeptides.	Tn-TR	No adjuvant.	external	BALB/c wild-type mice. MCF-7 human breast cancer cells.	IgG2a and IgM production with strong reaction against MUC1+ MCF-7 human tumour cells. [287]	
No carrier, KLH or Pam3Cys	Lewis Y or clustered tri-Lewis Y (Le ^Y) ₃ attached to Ser-Ser-Ser-Ala-Val-Ala-Val peptide	QS-21 formulations.	No adjuvant	external for KLH formulations.	BALB/c and C57/BL6 wild-type mice. Human ovarian cancer OVCAR-3 (LeY+) cell line.	(LeY)3 induced Abs against LeY-Cer, LeY-mucin and LeY -positive OVCAR3 tumour cells. [294] (LeY)3-Peptide-KLH induced IgG and IgM responses against the immunizing epitope. LeY-Peptide-KLH induced IgG and IgM responses against natural LeY-Cer, LeY-mucin.	

					LeY -Peptide-Pam3Cys induced Abs against the immunizing epitope and LeY -positive OVCAR3 tumour cells	
KLH or MPLA	GM2	For KLH formulations CFA was used in the first immunization, following IFA in boost immunizations.	No external adjuvant for MPLA immunizations.	C57BL/6 J mice Human breast cancer MCF-7 (GM2+) cell line. Human melanoma SKMEL-28 (GM3 and GD3+) cell line.	GM2-MPLA induced high levels of IgG3 Abs. GM2-KLH elicited mainly IgG1 Abs. Both conjugates elicited low to moderate levels of IgG2b and IgM Abs. Abs derived from both KLH and MPLA conjugates immunization bound GM2 positive MCF-7 tumour cells. Basal cross reactivity with GM3 and GD3 related structures was detected. Abs derived from both KLH and MPLA conjugates immunization mediated CDC to GM2+ tumour cells.	[126]
<i>Lipid carriers</i>						
α -GalCer	STn	No adjuvant	external	BABL/c wild-type mice	A robust specific anti-STn IgG1 and IgG3 antibody response was induced.	[139]
α -GalCer	Tn	No adjuvant	external	C57BL/6 mice	Isotype switching to IgG1 and affinity maturation against Tn was observed in the absence of peptidic T cell epitopes.	[140]
P3CS	di-Tn	No adjuvant.	external	Mouse strain not mentioned.	High anti-Tn IgG and IgM antibody titers were induced.	[142]
Pam	Clustered Tn-PADRE-OVA ₂₅₇₋₂₆₄	No adjuvant.	external	MO5/BALB/c mice. Breast cancer cell line MCF-7 (Tn+) and MO5 melanoma cells.	Elicited IgG Abs could recognize human MCF-7 tumour cell line expressing Tn. The vaccine prevented tumour growth after inoculation of MO5 cells.	[143]

Terminal Pam (linear construct) or middle Pam (branched construct)	Clustered Tn- PADRE- HER2 ₄₂₀₋₄₂₉	No adjuvant.	external	B10.D1 mice. Breast cancer cell line MCF-7 (Tn+). Lymphoma-derived T2 and Hodgkin lymphoma RS cell lines (Tn). NT2 mammary tumour cells.	Significant IgG levels were induced by both constructs against the glycan RAFT, particularly by the branched construct. The generated IgG specifically bound human breast tumour cell line MCF-7 expressing Tn. Both constructs induced HER420-429-specific CD8+ T cell responses, however the linear construct was a better immunogen. Vaccination with the linear construct reduced tumour progression and increased mouse survival after challenge with NT2 cells.	[144]
Pam3Cys	Tn-YAF	No adjuvant or QS-21.	external	BALB/c wild-type mice.	Vaccination elicited IgM and IgG antibodies against the Tn antigen, with increased titers upon QS-21 employment.	[138]
Pam2-CysSK4 or Pam3CysSK4	Tn glycopeptide- Polio peptide	No adjuvant or QS-21	external	BALB/c wild-type mice. Human breast cancer MCF-7 (MUC1+) cell line. Human melanoma SKMEL-28 (MUC1-) cell line. Human embryonic kidney (HEK) 293T cells and HEK 293T cells. RAW 264.7 γNO (-) murine macrophage cells.	Vaccination elicited IgM and IgG Abs. Vaccination with the Pam3CysSK4 formulation elicited exceptionally high titers of anti-MUC1 IgG aAbs, including anti-carbohydrate IgG3. QS-21 administration reenforced mixed Th1/Th2 responses with no increase in Abs titers. Both formulations induced Abs that could recognize MUC1+ cancer cells, induced the secretion of TNF-α and upregulation of costimulatory CD80, CD83 and CD86.	[146]

Pam3CysSK4	MUC1 glycopeptide-Th peptide epitope-	No external adjuvant, CpG as built-in adjuvant.	BALB/c wild-type mice, RAW 264.7 murine macrophage cells, and MCF-7 human breast tumour cells. C57BL/6 mice and B16F0 (MUC1+) tumour cells.	The formulation was successfully internalized by macrophage-like cells, upregulating CD40, CD80, and CD86 activation markers and inducing IL-6 and IL-12 production. [295] Abs against MUC1 glycopeptide were elicited, particularly IgG2a. Abs promoted CDC against MCF-7 tumour cells. Vaccination anti-tumour immune response did not inhibit mouse tumour growth in the absence of CpG but induced strong anti-tumour immune responses upon combination of Pam3CysSK4 and CpG.
Pam2Cys	Tn-MUC1(MeSer)-Th peptide	No external adjuvant	MUC1.Tg mice (C57BL/6; H-2b). MCF-7 human breast cancer and C57mg.MUC1 mammary gland tumour cells (MUC1⁺).	A specific IgG1 and IgG2a,b Abs response was induced against glycosylated and non-glycosylated MUC1. [145] Abs recognised MUC1+ tumour cells. High titers of anti-carbohydrate IgG3 were produced. The unnatural vaccine did not improve the effectiveness of a previously reported natural threonine-containing derivative.
Synthetic liposome	Chol-Tn	CpG	BALB/c wild-type mice. 4T1 cells. Bone marrow-derived DCs (BMDCs).	The glyco-liposome vaccines exhibit strong binding ability with an anti-Tn specific antibody. [148] Vaccination enhanced antigen presentation of both BMDCs and spleen B cells. Vaccination generated a Th1-dependent cellular immune response, evidenced by IFN-γ secretion.
<i>Dendrimeric carriers</i>				

Tetravalent lysine core dendrimeric MAG	Tn-Pv peptide	Aluminum hydroxide or CFA	BALB/c wild-type mice. Murine adenocarcinoma TA3/Ha cells and human T lymphocyte Jurkat cells (Tn ⁺). Murine colorectal carcinoma CT26 cell line (Tn ⁻).	MAG:Tn3-PV vaccination induced IgG1, IgG3 and IgM Tn-specific Abs. [150] Positive binding of both IgM and IgG Abs to murine and human tumour cells was observed. MAG:Tn3-PV vaccination protected 70–90% of TA3/Ha challenged mice in a Tn specific manner. Prophylactic MAG:Tn3-PV vaccination warranted 35–40% survival of TA3/Ha challenged mice. Cyclophosphamide co-administration could improve the efficacy of the MAG:Tn3-PV treatment.
Tetravalent lysine core dendrimeric MAG	Tn3-PV, Tn(S)3-PV, and Tn(hS)3-PV in mouse studies. Tn(S)3-PADRE, and Tn3-TT in non-human primate studies.	Aluminum hydroxide or QS21 plus CpG 1826 in mice. Aluminum hydroxide plus ODN 2006 in primate immunization.	BALB/c wild-type mice. African Green Monkeys, Chloerecebus sabaeus. Macaca mulatta. T lymphocyte Jurkat and human breast cancer MCF-7 (Tn ⁺) cell lines. Lymphoma T2 and human breast cancer MDA-231 (Tn ⁻) cell lines.	Natural serine and threonine immunogens induced a broad spectrum of polyclonal Abs recognizing natural Tn. [151] T-Cell help is required to induce anti-Tn Abs with MAG glycoconjugates. MAG:Tn3-PV induced higher titers of IgG Abs specifically directed against Tn, while KLH-Tn induced cross reactivity with T antigen Both humanized MAG induced IgG Abs against Tn in primates, with particularly exacerbated TT-specific T-cell responses. Primate MAG:Tn3-TT immunization sera contained Abs able to recognize human cancer-associated mucin peptides and Tn positive cancer cells, mediating killing of these cells

MAG	Tn3- P2 (TT ₈₃₀₋₈₄₄)	AS15	Inbred C57BL/6J (H-2^b), BALB/c(H-2^d), and C3H/HeN (H-2^k), as well as outbred NMRI and CD-1 mice. Cynomolgus monkeys. T-lymphoma Jurkat cell line, mammary carcinoma cell line MCF-7, ovarian carcinoma cell lines OVCAR and SHIN3, and murine mammary carcinoma TA3Ha cell line (Tn⁺).	The TT peptide enabled the production of anti-Tn Abs in mice expressing various H-2 haplotypes. The anti-Tn Abs production induced by MAG-Tn3 vaccination is associated with a T cell response to TT, mainly oriented toward a TH1 profile. Both anti-Tn IgM and IgG responses were detected in all monkeys immunized with MAG-Tn3-TT, and these Abs were able to recognize Tn expressing human tumour cell lines. Sera of cynomolgus monkeys immunized with MAG-Tn3 were able to mediate CDC in Jurkat and SHIN3 cells. Anti-Tn Abs produced in response to MAG-Tn3 vaccination demonstrated survival protective capacity in mice grafted with the Tn-expressing TA3Ha murine breast cancer cells.
<i>Peptide-free carriers</i>				
P(HPMA) polymer	MUC1 glycopeptide-P2 (TT ₈₃₀₋₈₄₄)	CFA	BALB/c wild-type mice. human breast cancer MCF-7 cells	P(HPMA-MUC1-P2) vaccination induced IgG Abs [155] production with notable binding to human tumour cells.
PLGA	MUC1 glycopeptide or MUC1 glycopeptide-MPLA	No adjuvant	external C57BL/6J mice	PLGA-MUC1-MPLA microspheres induced increased T cell proliferation compared to PLGA-MUC1 particles. [156] Both formulations induced IFN-γ production, with a 20-fold increase in the presence of MPLA. PLGA-MUC1 vaccination did not induce Abs production, while PLGA-MUC1-MPLA fostered a minimal IgG2a,b and IgG3 response without IgM production.

<i>Nanoparticles</i>							
Gold nanoparticles	PEG-Tn	No adjuvant	external	New Zealand White rabbits		Optimal Tn-antigen glycan density was 20–25 units per polymer chain, regardless of chain length. Detectable levels of IgG Abs specific for naturally occurring mucin glycans were found.	[296]
	MUC4-di-T-C3d or MUC4-T-C3d	No adjuvant	external	BALB/c wild-type mice		All particle vaccines bearing the T antigen induced small IgG and IgM responses. Vaccines with one carbohydrate antigen gave more robust responses than those with two contiguous T disaccharides.	[297]
Iron Nanoparticles	Oxide	DPPE-MUC1-Tn	No adjuvant or built-in MPLA.	external	Pathogen-free mice, with no indication of strain. Human MUC1-Ag104 and MCF-7 (MUC1 ⁺) tumour cell.	NPs alone were not able to activate DCs, suggesting good biocompatibility of the NPs. The addition of MPLA to NPs enhanced the expression of costimulatory molecules CD40, CD80, CD86, and MHC class II on DCs. NPs could effectively drain into local lymph nodes to interact with immune cells. Vaccines elicited IgG1, IgG2b, and IgM anti-MUC1 Abs, able to recognize Tn-MUC1 glycopeptides. Antibodies from immunized mice showed binding and CDC against MUC1-expressing tumour cells.	[163]

Cationic nanohydrogel particles	MUC1-Tn-P2 (TT ₈₃₀₋₈₄₄)	Built-in CpG	BALB/c wild-type mice	CpG containing nanogels upregulated costimulatory factors for T-cell proliferation. [298] Vaccination elicited anti-MUC1 Abs.
Nap-G ^P F ^D F ^D Y ^D K self-assembling peptide	MUC1-Tn	No external adjuvant or CFA.	BALB/c wild-type mice MCF-7 (MUC1 ⁺) breast cancer cells.	Nap-G ^P F ^D F ^D Y ^D K is poorly immunogenic. [165] Covalent binding of Nap to MUC1(Tn) enhanced Nap adjuvant properties, making it equivalent to complete Freund's adjuvant. Vaccination elicited similar titers of IgG1, IgG2b and IgG3 Abs, with higher titers of IgG2a as well as IL-4 and IL-5 production by T cells. Elicited Abs recognize MUC1 positive human cell lines and are capable of inducing CDC.
<i>Virus-like particles</i>				

Q β virus-like particles	Tn	CFA in the first immunization, and IFA in boost inoculations.	C57BL/6 mice. Human T-lymphocyte Jurkat cell line.	All Qβ-Tn immunized mice showed high anti-Tn IgG titers. [299] In some cases, competitive immune responses to the capsid or the triazole linker were observed, suppressing anti-glycan immunity. Increasing of Tn antigens on Qβ did not affect IgM titers but had great influence on potentiating T helper cell dependent IgG responses. Organized epitope display is crucial for induction of Abs isotype switching, but not for early B cell activation. Anti-Tn antibodies reacted with native Tn epitopes on human tumour cells.
Q β virus-like particles	MUC1 glycopeptide-T or MUC1 glycopeptide-STn	MPLA	MUC1.Tg mice B16-MUC1 cells Human breast cancer MCF-7 cells Normal breast MCF-10A cells	Qβ-MUC1-Tn and Qβ-MUC1-STn elicited exceptionally high anti-MUC1 IgG antibodies. [171] IgG antibodies recognized a wide range of MUC1 glycopeptides bearing Tn, T, or cores 1-4 glycans at various locations of serines and threonines. Antibodies induced by Qβ-MUC1-T showed strong binding with MUC1-expressing melanoma B16-MUC1 cells, and effectively killed these cells <i>in vitro</i>. In a lung metastasis model, vaccination with Q-MUC1-T followed by tumour challenge resulted in significantly decreased tumour foci in the lungs of vaccinated mice compared to control animals.

Q β virus-like particles	MUC1- β -T	MPLA		MUC1.Tg mice B16-MUC1 cells	The sera from immunized MUC1.Tg mice showed strong anti-MUC1-β-T IgG responses, suggesting the induction of helper T cell activation and antibody isotype switching. [172] Antibodies induced by Qβ-MUC1-β-T conjugates recognized multiple MUC1 glycoforms in glycopeptide microarray screening. Antibodies elicited by Qβ-MUC1-β-T could recognize and promote complement dependent cytotoxicity in MUC1-expressing cancer cells.
Fd Filamentous bacteriophages	α -GalCer	No adjuvant	external	C57BL/6 mice CD1d-restricted V α 14i NKT hybridoma FF13 OTI hybridoma cell line B16-OVA melanoma cells (H2Kb)	fd/α-GalCer was successfully internalized by DCs. [173] fd/α-GalCer was presented by BMDCs, triggering activation of iNKT hybridoma <i>in vitro</i>. fd/α-GalCer activates CD3+NK1.1+ cells and FN-γ production <i>in vivo</i>. OVA antigen and α-GalCer co-delivered by phage particles are presented by DCs. Immunization with fdOVA/α-GalCer particles developed an OVA257-264-specific IFN-γ-secreting T cell response <i>in vivo</i>. fd/α-GalCer treatment of B16 melanoma cells challenged mice resulted in a significant delay in the tumor growth compared to while administration of free α-GalCer or fd wild-type bacteriophages. Mice engrafted with B16-OVA cells after fdOVA/α-GalCer inoculation showed 80% survival rate, in comparison with 40% survival of mice treated with fd/α-GalCer in the absence of the specific OVA257-264 peptide.

<i>Carrier-free approaches</i>					
Carrier-free	GD3-replica peptide	No adjuvant	external	CD-1 mice	The immunogenic GD3P4 GD3-replica peptide is [188] exposed on APCs with a similar molecular shape to that GD3 epitope and induces anti-GD3 Abs.

3. Strategies to overcome TACAs self-tolerance

3.1. Synthesis and chemical modification of TACAs

Carbohydrate-based vaccine production is first and foremost dependent on large-scale synthesis of pure and well-defined glycan derivatives at high yields, including natural glyco-epitopes, glyco-analogues, and glycomimetics. Natural sources of glycans are highly heterogeneous, hindering single glycan purification. The complexity in isolating unique TACA structures from natural sources has boosted the development of alternative approaches. Conventional carbohydrate synthesis protocols, and optimized chemical and biochemical strategies have been employed to obtain structurally diverse oligosaccharides, such as Globo-H, Lewis^{x,y}, KH-1, MUC-1, Tn, STn and T antigens [36-41, 60]. Classical carbohydrate chemical synthesis encompasses several expensive and time-consuming protection and deprotection steps to ensure the desired carbohydrate sequences and glycosidic linkages [50, 61]. This can be surpassed by enzymatic or chemo-enzymatic synthesis, using glycosidases and glycosyltransferases that provide adequate regio- and stereo-specificities. However, biochemical approaches are limited by the availability and high cost of the required enzymes [50, 62, 63]. Accordingly, constant optimization of mainstay protocols and the pursue for more effective synthesis methods is an ever-evolving field. In this context, genetically engineered cell factories have also been explored to produce glycoengineered vaccines [64, 65], building on genetically modified mammalian and bacterial platforms for large-scale production of human glycoconjugates.

Glycan-based vaccine formulations are commonly subjected to *in vivo* enzymatic degradation by endogenous glycosidases, drastically reducing vaccine stability and tumour-associated antigens immunogenicity [50]. To tackle stability issues, several tumour TACA analogues have been developed to replace native antigens in vaccine formulations. These include C- and S-oligosaccharides [66-69], fluorinated [70-72] and desialylated mimetics [73], as well as thioether-bridged analogues [74] (**Figure 1**). These modifications aim to provide resistance to enzymatic degradation, while preserving the immunogenicity and structure-selectivity of the vaccine. The most explored strategy is to derivatize cancer-associated short O-GalNAc glycans into fluorinated analogues. Namely, fluorinated Tn [75], sialyl-Tn (STn) [76], and T antigens [77] have been developed and conjugated to immunogenic proteins (**Figure 1**). These formulations induced

strong IgG production *in vivo*, and these antibodies were able to recognize tumour cells expressing the targeted glycans [71]. In addition, some formulations containing fluoro-modified STn antigens could elicit both humoral and cellular immunity, lymphocyte proliferation and pro-inflammatory cytokines production *in vivo* [46]. Moreover, immunized mice antisera successfully recognized STn-positive cancer cells, promoting antibody-dependent and complement-dependent cellular cytotoxicity [46]. These findings reinforce the relevant role of TACA analogues in enhancing vaccine stability and immunogenicity. However, clinical studies employing such strategies are lacking.

3.2. Glycovaccine carriers

In general, carbohydrates are T cell-independent antigens, promoting short-life B-cell responses lacking affinity maturation and directed toward the production of IgM and IgG2 in humans and IgM and IgG3 in mice [78]. To overcome this restricted response, carbohydrate antigens can be coupled to carriers of different natures to generate T-cell epitopes, and effectively achieve T-cell engagement (detailed in **Table 1**, **Figure 1**). Carbohydrates conjugation to different carriers (proteins, lipids, dendrimers, biocompatible polymers, nanoparticles, and virus-like particles) also frequently generates high affinity IgGs against the glycan moiety and long-lasting antibody-mediated protection promoted by memory B cells [47] (**Figure 1**). Concerns that prior or concurrent exposure to a specific carrier may lessen the anti-carbohydrate immune response has driven research of novel platforms. Moreover, the selectivity of conjugation sites of glycans to carriers has been pursued in the form of synthetic vaccines, envisaging increased immunogenicity and standardization of formulations. In addition, synthetic self-adjuvating and self-assembled vaccines have been offering increasingly simple and reproducible systems, paving the way for clinical implementation.

Table 2. Glycovaccine clinical studies involving TACAs conjugated to different carriers and/or immunogens.

Trial	Carrier	Antigen	Adjuvant	Cohort	Outcome	Reference
<i>Protein carriers</i>						
Phase I	KLH	STn or T	Detox or QS-21	22 Dukes' B, C or D colon cancer patients free from disease after resection.	IgG and IgM Abs production reactive against the respective synthetic disaccharide epitopes but unreactive toward natural antigens.	[117]
Phase III (NCT00003638)	KLH	STn	Enhanzyn™ (previously called Detox B stable emulsion)	1,028 women with metastatic breast cancer with no evidence of disease (10%) or no progressive disease (90%) following first-line chemotherapy.	No significant differences in the median time to progression, OS, disease status or use of hormone therapy was observed between the treatment groups.	[118]
Phase I	KLH	Clustered tri-T	QS-21	20 biochemically relapsed prostate cancer patients.	IgG and IgM Abs were produced, reactive against the synthetic trimer T epitope. IgM Abs were reactive against prostate cancer cell line DU145. Antibody-mediated complement lysis of MCF-7 breast cancer cells was observed. Anti-tumour effect was observed, translated by a decrease in logPSA slopes post treatment.	[120]
Phase I	KLH	Lewis Y	QS-21	25 patients with epithelial ovarian, fallopian tube, or peritoneal cancer of any stage or grade at diagnosis.	Primarily IgM production was detected. Abs reacted with Ley-positive cell line OVCAR-3 (particularly with Ley-ceramide) inducing CDC.	[121]

Ph II/III trial	KLH	Globo H	OPT-821	349 metastatic breast cancer patients who had ≤ 2 events of progressive disease and who achieved at least stable disease after ≥ 1 anticancer regimen.	No improvement in PFS were observed. However, PFS and interim OS were significantly improved in patients who developed an immune response to the vaccine.	[123]	
Phase (NCT01516307)	II	KLH	Globo H	OBI-821	348 hormone receptor-positive (HR+)/human epidermal growth factor receptor 2-negative (HER2-) (70.4%), triple negative (12.9%), and HER2+ (16.7%) metastatic breast cancer patients.	No improvement in PFS of previously treated patients was observed. However, humoral immune response to Globo H correlated with improved PFS.	[124]
Phase I		KLH	GM2	QS-21	52 melanoma patients free of disease after resection but at high risk for relapse.	High-titer IgM and IgG Abs against GM2 were produced.	[127]
Phase (NCT00005052)	III	KLH	GM2	QS-21	1314 stage II melanoma patients with a primary tumour > 1.50 mm in thickness (T3-4N0M0).	No improvements in RFS, DMFS, and OS were observed.	[125]
Phase I		KLH	GM2	QS-21	970 stage II melanoma patients of the EORTC18961 trial.	Prognostic serum Abs responses were observed, associating a significant dismal (GM2, Rhod_E2, SSX2) or good prognosis (CyclinB1, SCYE1v1) for RFS, DMFS and OS.	[128]
Phase I		KLH	Unimolecular peptide-Globo-H-GM2-STn-T-Tn	QS-21	24 advanced-stage (92% high-grade serous; 75% stage III and 25% stage IV disease), poor-risk, first-remission ovarian cancer patients.	Unimolecular pentavalent vaccine construct was shown to be safe and immunogenic, with IgG and/or IgM responses recorded against several immunizing epitopes.	[129]

<i>Dendrimeric carriers</i>						
Phase I (NCT02364492)	MAG	Tn3- 844	TT830-	AS15	7 patients with localized breast cancer at high-risk of relapse.	MAG-Tn3/AS15 vaccination did not significantly modify the total concentration of circulating IgG1, IgG2, IgG3, or IgG4 in most patients. [154] All vaccinated patients developed IgG and IgM Tn-specific Abs that could recognize Tn positive Jurkat and MCF-7 cells and mediate CDC of Jurkat tumour cells.
<i>Carrier-free approaches</i>						
Phase I	Carrier-free P10s mimitope	LeY, GD2		MONTANIDE™ ISA 51 VG	6 metastatic breast cancer patients.	Immunization with P10s-PADRE induced anti-P10s antibody response. [185] P10s-PADRE-immunized serum reacted with both GD2 and LeY antigens. Postimmune serum suppressed the viability of HCC1954 (LeY+), MDA-MB-231 (GD2+), and ZR-75-1 (LeY+) breast cancer cells <i>in vitro</i>. No reactivity was observed with normal epithelial cell line MCF-10A.

						The effect of P10s-PADRE-induced immune serum on cell viability was caspase-3-dependent. P10s-PADRE-induced immune serum sensitized 3D spheroid cultures to Paclitaxel treatment.
Combined I/II (NCT02229084)	Phase	Carrier-free P10s mimitope	LeY, GD2	MONTANIDE™ ISA 51 VG Plus NAC (doxorubicin, cyclophosphamide or docetaxel/paclitaxel).	61 stage I, II or III HR+/HER2– breast cancer patients	P10s-PADRE immunization in combination with chemotherapy induced Abs response. [186] Combination therapy affected the expression levels of NK-cell markers and cytokine production. Combination therapy resulted in a significant reduction in the primary size of tumours regardless of treatment schedules.

3.2.1. Protein carriers

Covalent linking of glycans or mucin-derived glycopeptides (MUC) presenting TACAs to different proteins has been one of the most adopted strategies to generate chimeric glycovaccines. Mucins are natural carriers of short-chain *O*-GalNAc glycans, which compose more than 50% of the mucin molecular by weight [79]. These highly glycosylated glycoproteins are extensively altered in epithelial cancers and provide a highly relevant scaffold for glycovaccines [80, 81]. As result, a wide number of glycovaccine formulations have been prototyped and tested in pre-clinical settings. The most explored protein carriers are the diphtheria toxoid (DT) [82], tetanus toxoid (TT) [83], CRM197 [84], Haemophilus protein D (PD) [85], keyhole limpet hemocyanin (KLH) [86], and the outer membrane protein complex of serogroup B meningococcus (OMPC) [87] (**Table 1, Figure 1**). Synthetic peptides, including segments of the primary sequence of CRM197 [88], and recombinant proteins, as the N19 polyepitope (comprising a sequence of universal human CD4⁺-T-cell epitopes) [89], have also demonstrated to be a valid option for improved glycoconjugate vaccines.

Given that the conjugation sites impact on immunogenicity, the selectivity of conjugation sites has been a major objective to progress over the mainstay random display of carbohydrates onto the carrier proteins surface [90]. In standard chemical conjugation protocols, protein carriers or glycans are usually linked to spacers containing hydrazides, azido, bromoacetyl or maleimido groups that react with carboxyl, alkynes, cyano-esters or thiol groups naturally present or introduced in the protein carrier [90-93]. To improve batch-to-batch reproducibility and generate neo-glycoconjugates with consistent biological activity, specific amino acids can be targeted for conjugation. For instance, computational analysis of solvent accessibility to each amino acid residue (based on the carrier's crystal structure) can establish a correlation between reactivity and surface exposure. As such, the reactivity of residues in the protein can be mapped and ranked, and bioconjugation can be controlled [94]. In this process of bioconjugation, lysine [94], cysteine [95], and tyrosine [93, 96] can be selectively targeted. The insertion of unnatural amino acids into the protein carrier primary structure, as homoserine, α -methylserine or β 3-homothreonine may also allow selective coupling through click chemistry protocols [97-100]. Bioconjugation protocols for site-selective glycan conjugation can be further optimized by introducing *in vitro* enzymatic modification of amino acids in opposition to chemical modification [90]. Moreover,

the careful selection of polypeptide N-acetylgalactosaminyltransferases supported by dedicated bioinformatics [101] may be explored to tailor O-GalNAc glycosylation of serine and threonine along a given polypeptide chain [63]. However, the production of glycoconjugate vaccines using chemical or chemoenzymatic methods is still a laborious and multi-step process, requiring extensive purification steps for defined products and retrieving low yields. Accordingly, cost-effective exploitation of cell factories building on recombinant *Escherichia coli* has been suggested as a more straight forward method for producing conjugate vaccines [102]. Even though this approach may be of great promise for producing glycoconjugate vaccines against bacterial pathogens, humanization of the glycosylation machinery could be required to produce cancer-associated glycoepitopes [64, 103]. To address this limitation, genetic glycoengineering of mammalian cells has been employed in the generation of fusion proteins decorated with high-mannose N-linked glycans (MGAT1 knockout), Tn antigens (COSMC knockout), and O-6 sialylation (ST6Gal1 knock-in) [104] in mammalian cells. Genetic modifications have been mainly produced through zinc finger nucleases (ZFNs) [105], transcription activator-like effector nucleases (TALENs) and CRISPR-Cas9 [106] technologies, rendering readily usable glycoproteins.

MUC-derived glycopeptides carrying TACAs

This section addresses formulations exploiting TACAs linked to endogenous peptide backbones in closer mimicry of cancer cells glycoepitopes. Due to its overexpression on several epithelial cancers, tumour-associated MUC1 has been by far the most attractive target antigen for vaccine formulations [107, 108]. Moreover, allied to abnormal glycosylation with short-chain O-GalNAc glycans, MUC1 glycopeptides provide cancer specificity for immunotherapy, being extensively explored in glycovaccines. The peptide moiety offers the necessary scaffold for more effective immune system activation and immunological memory development. Most MUC1 glycovaccines developed to this date build on multiple variable tandem repeats (VNTR) [HGVTSAPDTRPAPGSTAPPA], containing five potential O-glycosylation sites (T4, S5, T9, S15, and T16), with PDTRP being the immunodominant motif. MUC1 VNTR glycopeptides with different densities of cancer-associated Tn, STn and T antigens conjugated to KLH have been used as immunogens to investigate an optimal vaccine design in BALB/c and human MUC1 transgenic mice [109]. O-glycan occupancy had a significant impact in antibody

responses. Namely, full *O*-glycosites occupancy rendered the most robust antibody response against MUC1 positive breast cancer cell lines. Of note, no reactivity with the T glycoform was found, suggesting that *O*-glycans extension toward the normal glycosylation pattern (ST and core 2 structures) masks the epitope. Both peptide sequence and carbohydrate structures contributed to the elicited humoral immune response, highlighting that the glycopeptide design ultimately dictates antibody specificity [109]. On the same note, synthetic MUC1 glycopeptides bearing the STn or 2,6-ST at position S15 and Tn or T antigen at T9 of the VNTR were conjugated to bovine serum albumin (BSA) carrier protein and tested as vaccines [48, 49] (**Figure 1**). Complete Freund's adjuvant was used for the first immunization, incomplete Freund's adjuvant for each subsequent booster in BALB/c mice. IgG1 isotype antibodies were predominantly produced for all vaccine designs, indicating the establishment of immunological memory. Moreover, antibodies from mice sera were able to specifically bind to tumour-associated MUC1 glycoproteins expressed on MCF-7 tumour cells. The glycosylation site T9 within the immunodominant PDTRP epitope has also been demonstrated to be determinant for the immunogenicity of the synthetic vaccines [49]. On a similar note, a MUC1-STn glycopeptide construct connected to a Th-cell peptide epitope from ovalbumin (OVA323–339) was described and tested in combination with complete Freund's adjuvant in DO11.10 transgenic mice, whose T cells express a specific receptor for the ovalbumin T-cell epitope OVA323–339 (**Figure 1**). Mice immunization resulted in highly specific humoral responses toward the targeted glycopeptide, but not the corresponding peptide sequence alone or the STn structure coupled to a different peptide sequence [110]. These results highlight the potential of fully synthetic vaccines in achieving selective activation of the immune system against glycoprotein structures. Following a similar strategy, MUC1-STn-OVA glycopeptide constructs were compared with MUC1-STn-OVA glycopeptides mono-, di- or tri-glycosylated with the Tn antigen in DO11.10 mice. Complete Freund's adjuvant was used in parallel [111]. Mice immunized with the monoglycosylated and diglycosylated vaccines displayed high antibody titers, all specific to the MUC1-glycopeptide antigens, including the triglycosylated MUC1-glycopeptide, while the triglycosylated vaccine did not induce immune response. Notwithstanding, the antibodies induced by monoglycosylated STn-MUC1 exhibited the highest binding affinity to all three tumour-associated MUC1-glycopeptide antigens. Furthermore, boost immunizations did not increase antibody titers in any experimental group

[111]. In line with previous studies, this work evidenced that specific immune responses can be induced by synthetic glycovaccines. However, a useful scope of recognition directed to several aberrant glycopeptide structures present on a tumour cell can be maintained. To explore the selectivity of immune responses against structurally similar glycoepitopes, STn-MUC1 glycopeptides were coupled to tetanus toxoid (TT) carrier protein (A) (**Figure 1**) and compared to MUC1 glycopeptides having Tn antigen (B), one STn antigen and one Tn antigen side chain (C), one STn antigen and two Tn antigen side chains (D), one 2,6-sialyl-T antigen (E), one 2,3-sialyl-T antigen (F), one T-antigen side chain (G), as well as the non-glycosylated MUC1 eicosapeptide (H). All formulations were tested with incomplete Freund's adjuvant in BALB/c mice [112]. The results showed that the STn-MUC1-TT vaccine induced a strong immune response in all mice. Neither the saccharide nor the peptide alone were recognized by the antibodies elicited by STn-MUC1-TT, highlighting the high selectivity of the triggered immune response. However, the closely related glycopeptide structures B-E were also bound by the induced antibodies to a reduced, but distinct degree, corroborating a basal level of glycan cross reactivity [112]. Also, a human MUC1 peptide (huMUC1) glycosylated with STn on Ser17 of the immune dominant region was coupled to tetanus toxoid and tested in humanized double-transgenic breast cancer mouse models [113]. Furthermore, prophylactic vaccination with the synthetic huMUC1-STn-TT vaccine inhibited progression of breast tumours in preclinical PyMTxhuMUC1 mice, while inducing humoral immune responses selectively targeting aberrantly glycosylated huMUC1 [113]. Reduction in tumour burden was also observed. A different approach involved using as epitope the full-length MUC1 VNTR domains expressing the Tn antigen conjugated to a self-assembly peptide sequence (Q11 domain), which aggregates into fibers to serve as both adjuvant and vaccine carrier [114]. This synthetic self-adjuvating and self-assembled vaccine aggregated into fibrils and displayed multivalent B-cell epitopes, providing high levels of antibodies that recognized human breast tumour cells. Moreover, these vaccines appeared to act through a T-cell independent pathway and were associated with the activation of cytotoxic T cells [114], holding promise as novel anticancer therapy.

TACAs linked to protein carriers

Herein we describe studies involving formulations where TACAs are covalently linked to non-endogenous biocompatible protein carriers. Many of which

have progressed from early prototypes into phase I clinical trials (**Table 2**). Amongst the most explored glycans are truncated Tn, STn and T *O*-glycans; terminal Lewis y epitopes (Le^y); Globo H - a globo-series glycosphingolipid antigen; and GM2 gangliosides.

In pre-clinical settings, a cancer vaccine candidate characterized by a threonine decorated with a sp2-iminosugar-type α -GalNAc (Tn) mimic conjugated to KLH has been synthesized and tested in mice. This vaccine elicited high levels of specific anti-MUC1 IgG antibodies in comparison to the natural Tn antigen analogue. Moreover, these antibodies recognized human breast cancer cells with high selectivity, highlighting the potential of exploiting non-natural glycan mimetics for efficient cancer vaccines [115]. In a similar approach, a conformationally constrained mimetic of the Tn antigen TnThr (GalNAc-Thr) was conjugated to the carrier CRM197 (mime[4]CRM) and tested as vaccine in a triple-negative breast cancer mouse model [116]. *In vivo*, the mime[4]CRM vaccine had an anti-tumorigenic effect and impaired distal dissemination. This was possibly due to the recruitment of DCs and T helper CD4⁺ cells, as well as reduction of immunosuppressive Tregs and pro-tumorigenic M2 tumour-associated macrophages in the tumour microenvironment. An IgG1 humoral response was also elicited by the candidate vaccine as well as IL-17 immunomodulatory activity on T cells [116]. Moreover, the mime[4]CRM vaccine provided an overall survival therapeutic benefit. In addition, tumour-infiltrating lymphocytes isolated from breast cancer patients were found to target mime[4]CRM [116]. Future studies should focus on assessing the candidate vaccine's impact on Tn-positive cancer lymphocytes.

In clinical settings, the serological response of high risk for recurrence colon cancer patients was monitored after immunization with T-KLH without adjuvant, T-KLH plus the immunological adjuvant Detox, STn-KLH plus Detox, or STn-KLH plus the immunological adjuvant QS-21 [117]. T-KLH and STn-KLH conjugates plus immunological adjuvants Detox and QS-21 induced high IgM and IgG antibody titers against the respective synthetic disaccharide epitopes. However, IgM antibodies were only weakly reactive against natural antigens, while IgGs were found nearly non-reactive [117]. Of note, a synthetic STn-KLH vaccine (Theratope) was tested in a phase III clinical trial assessing time to progression (TTP) and overall survival in metastatic breast cancer patients [118] (**Figure 1**). Although STn-KLH was well tolerated, no overall benefit in TTP or survival was observed in this largest

to date metastatic breast cancer vaccine trial [118]. However, lessons were learnt regarding the design of future clinical trials. Namely, it became clear that patients should be enrolled in clinical cohorts based on the validated expression of the targeted antigen, and vaccines should be tested in combination with mainstay therapies to engage immune responses and enhance clinical results. Furthermore, future designs should look at selecting individuals with low tumour burden, and longer vaccination periods with concomitant Treg suppression could be explored. Also, these trials highlighted the need to pursue antigen modifications toward optimized IgG responses, including exploring mono-epitopic cluster vaccines versus multi-epitopic vaccines. In line with this, clustered STn-KLH formulations seem to generate increasingly higher antibody affinities compared to non-clustered STn, fostering new scaffolds for STn-based glycovaccines [119]. Still on mono-epitopic cluster vaccines, clustered T-KLH constructs (three T antigen disaccharides attached to three sequential threonines on a peptide backbone) plus the immunological adjuvant QS-21 were tested in prostate cancer patients with rising PSA in the absence of radiographic disease [120]. In this dose escalating trial, all dosages induced IgM and IgG antibodies against T antigen, without increased antibody production with escalating doses of vaccine. An anti-tumour effect in the form of a change in post-treatment versus pre-treatment logPSA slopes was observed, vowing for the inclusion of minimal dose clustered T antigen in a multivalent phase II vaccine trial in patients with high-risk minimal disease. However, to our knowledge, a follow up study was not initiated. A Lewis y (Le^y) pentasaccharide-KLH conjugate vaccine plus the QS-21 adjuvant was also tested in ovarian cancer patients in a phase I trial [121]. Most patients (16/24) produced anti-Ley antibodies capable of inducing complement-dependent cytotoxicity, particularly reacting with glycolipids but not glycoproteins [121]. Notwithstanding, most of the antibody responses were modest and mainly restricted to immunoglobulins of the IgM class. Moreover, the glycovaccine was well tolerated with no adverse effects related to autoimmunity. Based on this study, Ley-KLH offers potential as a polyvalent vaccine targeting epithelial cancers. Building on this trial, the impact of epitope clustering, carrier structure, and adjuvant modification on IgM and IgG production was revisited in pre-clinical settings. It was demonstrated that clustered Ley structures were more efficient at eliciting antibodies reacting with natural forms of Ley and tumour cells than single Ley oligosaccharides [122]. The immunogenicity of clustered constructs in which the

oligosaccharides were linked to the peptide through the natural α -O-GalNAc serine linkage was then compared with those linked through an unnatural β -O-GalNAc serine linkage. Both antigen species were equally efficient at producing antibodies to natural forms of Ley and Ley-expressing cells [122]. Interestingly, the (Le^y)₃-KLH construct tested in this study has not provided reactivity with any of the natural forms of Ley. Thereby, the substitution of KLH for Pam3Cys- glycolipopeptides was also tested [122]. This more potent antigenic construct induced antibody production, particularly IgG, which were highly specific for the immunizing structure and had little or no reactivity with related structures, including Le^y-Cer and Le^y-mucin. The induced antibodies were able to react with cancer cells expressing Le^y, therefore further clinical evaluation might be needed to assess the potential of glycolipopeptide substitution. In summary, this study highlights that both epitope clustering and carrier structure influence antibody responses to Ley conjugates in mice.

Globo H is a prominently expressed glycolipid in breast cancer. In a phase II/III trial, vaccination with OPT-822 (Globo H-KLH plus OPT-821 adjuvant) induced antibodies mediating cytotoxicity toward Globo H expressing breast cancer cells [123]. On the same note, a phase II study assessed the effectiveness and tolerability of adagloxad simolenin (AS/OBI-822; a Globo H epitope covalently linked to KLH) with adjuvant OBI-821 in metastatic breast cancer patients [124]. The glycovaccine was well tolerated, however, no improvements in progression-free survival (PFS) were observed in previously treated metastatic breast cancer patients. However, early specific humoral immune responses to Globo H correlated with improved PFS in 50% of AS-KLH/OBI-821 recipients, irrespectively of the breast cancer subtype [124], vowing for therapeutic scheme optimization trials.

The cancer specific GM2 ganglioside has emerged as a promising TACA for developing melanoma targeted glycovaccines [125]. For instance, a GM2 derivative was covalently linked to KLH, eliciting robust GM2-specific IgG antibody responses in mice as well as complement-dependent cytotoxicity in GM2-expressing cancer cell lines, showing potential as therapeutic cancer vaccine [126]. Given the preclinical success of GM2 derivatives, melanoma patients who were free of disease after resection but at high risk of relapse were immunized with a GM2-KLH/QS-21 vaccine in a dose escalating trial, resulting in both IgM and IgG anti-GM2 antibodies [127] (**Figure 1**). The GM2-KLH/QS-21 vaccine was then investigated in a randomized phase III trial in stage II melanoma patients versus observation after

resection of the primary tumour. The GM2-KLH/QS-21 vaccine also induced high IgM and IgG antibody responses; however, without any outcome improvements for patients [125]. More recently, the prognostic and predictive significance of tumour associated antigen -specific serum antibodies in stage II melanoma patients from a GM2-KLH/QS-21 vaccination phase III trial was assessed by multiplex serology. Spontaneous antibody responses were correlated with a significant poor (GM2, Rhod_E2, SSX2) or favourable prognosis (CyclinB1, SCYE1v1) regarding relapse-free survival (RFS), distant metastasis-free (DMFS) and overall survival (OS) [128]. As such, spontaneous humoral immune responses against TACAs may not have a therapeutic effect but could point out patients' subgroups that may benefit from immunotherapy. Moreover, multiple target approaches and combination therapies could offer the necessary boost to induce multi-faceted responses that improve anti-cancer efficacy. In line with this, a pentavalent (Globo-H-GM2-STn-T-Tn) vaccine carried on a peptide support, conjugated with KLH and mixed with QS-21 adjuvant, was tested in patients with epithelial ovarian, fallopian tube, or peritoneal cancer in first remission [129]. The vaccine was found safe at all doses, and 96% of vaccinated patients had antibody responses against at least one of the antigens, while 83% responded against at least three antigens. Clinical outcome determination was not a primary objective of this phase I trial, but the patient with the longest progression-free survival (PFS) mounted an IgM response against all vaccine antigens, while the single patient who did not generate a response to any of the antigens had the shortest PFS [129]. Furthermore, such unimolecular vaccine design greatly simplifies both regulatory requirements and manufacturing hurdles, while facilitating scalability. This vows for multivalent approaches as a feasible tactic to mimic tumour microheterogeneity. Notwithstanding, the possibility that exposure to a given carrier can lead to immune interference (carrier epitope suppression and bystander effect [130]) has increased interest in the dual role of proteins as carrier and protective antigens. Moreover, the appeal of the employment of self-adjuvating platforms as driven the search for novel carriers

[131], as well as carrier-free approaches.

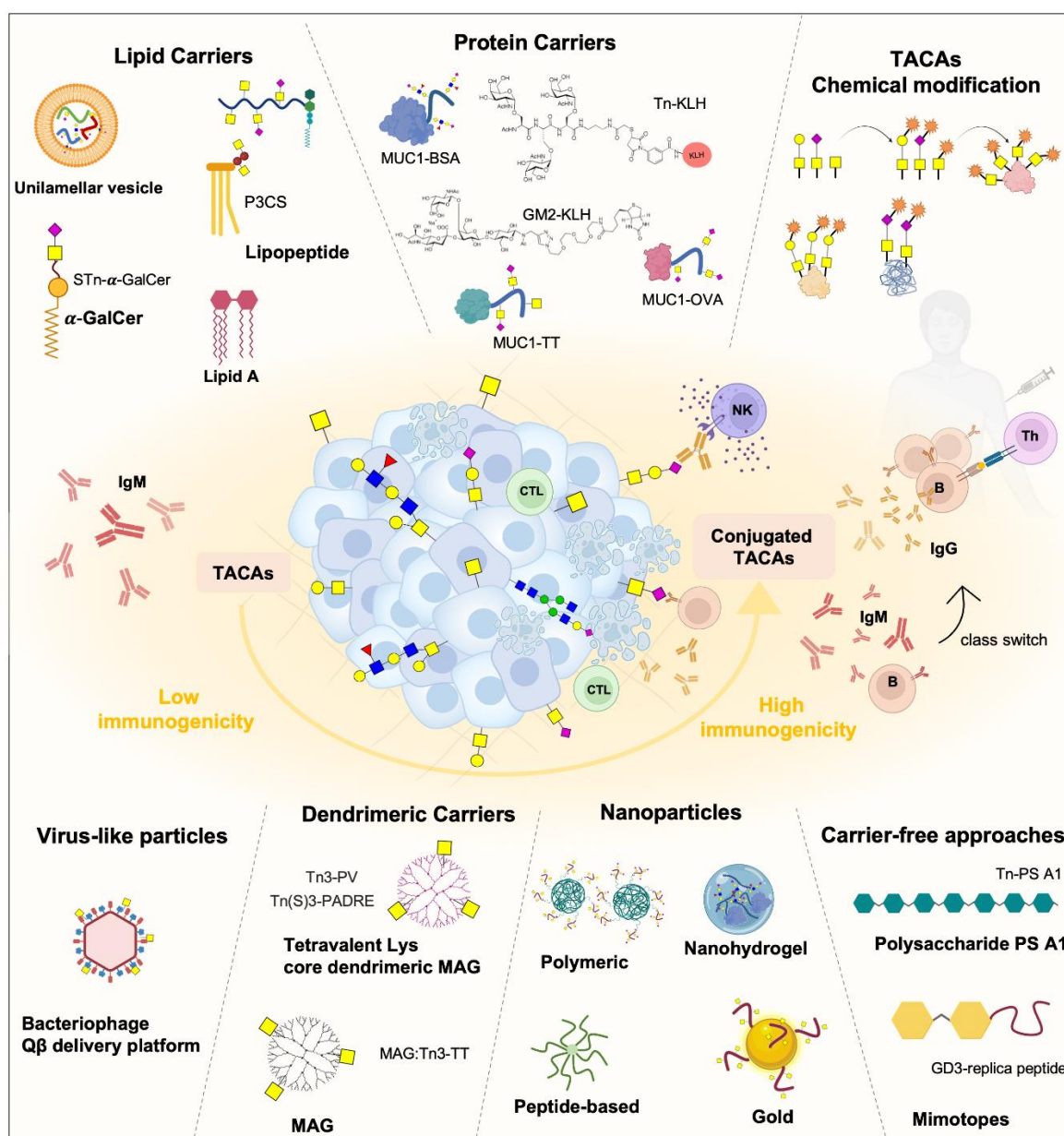


Figure 1. Approaches to break tumour associated carbohydrate antigens (TACAs) self-tolerance. Chemical modification of TACAs gives rise to increasingly stable and standardized glycovaccine formulations, with fluorinated carbohydrate analogues being the most common forms found in glycovaccines. In addition, carrier-based (protein, lipid, dendrimeric and peptide-free carriers), and carrier-free approaches as well as glycoconjugates combination with nanoparticles and virus-like particles are commonly used strategies to increase TACAs immunogenicity. Namely, MUC1 VNTR glycopeptides attached to protein carriers as KLH, BSA, OVA, and TT constitute some examples of immunoreactive protein-based glycovaccines. Lipids are commonly used in self-adjuvating vaccines, where they contribute to vaccine immunogenicity as adjuvants, while providing a carrier. In the context of glycovaccines, glycolipid carriers, such as α -galactosylceramide (α -GalCer), MPLA,

lipopeptides, and small unilamellar vesicles are the most explored tools. To reduce the risk of undesired immune reactions against carrier epitopes, peptide-free approaches have also emerged, and synthetic polymer glycopeptides conjugates constitute relevant components explored in this context. Viral particles comprise extremely versatile carriers for vaccines, with the delivery platform bacteriophage Q β being one of the most employed in glycovaccines. Dendrimeric multiple antigenic glycopeptides (MAG) are biocompatible polymers extensively explored in synthetic glycovaccines, constituting useful scaffolds for molecularly defined glycovaccines. Nanoparticle systems of polymeric, metallic or nanohydrogel natures offer multivalent delivery platforms, allowing both surface functionalization and encapsulation of cargo for multifaceted vaccine designs. Carrier-free approaches have emerged to overcome potential undesirable off-target immunity promoted by carriers and linkers. These platforms provide full carbohydrate-based glycovaccines with potential to unleash targeted immune responses. Overall, these approaches enable T-cell engagement, generating protective B cell memory and stimulating activation of NK cells; thereby triggering an effective cellular and humoral immune responses.

3.2.2. Lipid carriers

Carbohydrate-driven immune responses have also been achieved by using glycolipid carriers, such as α -galactosylceramide (α -GalCer) [132-134], synthetic lipid A derivatives like monophosphoryl lipid A (MPLA) [135-137], lipopeptides [138], and small unilamellar vesicles [138] (**Table 1**, **Figure 1**). These amphiphilic carriers can assemble into liposomes displaying carbohydrate antigens in its outer leaflet; thereby, providing the necessary multivalency for B cell stimulation [138-140]. Moreover, these carriers often provide self-adjuvating elements via activation of alternative immunological pathways such as invariant natural killer T (iNKT) cells activation or Toll-like receptors (TLR) engagement [138, 139]. Accordingly, the lipid components of glycovaccines are often mentioned as formulation adjuvants and not carriers. Notwithstanding, the following text will address to these elements as carriers. Taking advantage of these spontaneous interactions, several fully synthetic and well-defined self-adjuvating cancer vaccine candidates were proposed in pre-clinical studies. Namely, a STn- α -GalCer vaccine showed remarkable efficacy in inducing antibody class switch from IgM to STn-specific IgG1 and IgG3 in mice [139] (**Figure 1**). Antibody class switch indicated a mixed Th1/Th2 response, which is consistent with the “Th0-like” profile of cytokine response initiated by α -GalCer activation of iNKT cells [139]. Similarly, a liposome-formulated self-adjuvating Tn- α -GalCer vaccine consistently induced strong anti-Tn IgG antibody responses and

affinity maturation in immunized mice compared to stearylated Tn liposomes lacking the α -GalCer moiety [141]. Contrastingly, a semi-synthetic Tn-CRM197 proteoglycoconjugate induced anti-glycan IgGs only in a subset of immunized mice, highlighting the relevance of lipids self-adjuvancy in the formulation of minimalistic glycovaccines [141]. Furthermore, modulating the size of liposomes promoted distinct immune responses, with larger liposomes promoting Th1-type IgG2a antibodies and smaller ones favouring IgG1 associated with Th2-mediated immunity [141]. Collectively, these findings support the importance of finetuning lipid carrier dimensions for tailored immune responses.

Glycolipopeptides (GLP) are also under intense investigation as B- and T-cell based vaccine prototypes for several cancers. For instance, synthetic di-Tn coupled to tripalmitoyl-S-glycerylcysteinylserine (P3CS) lipopeptide elicited immune responses against Tn-expressing glycoproteins, however with limited isotype switching from IgM to IgG [142] (**Figure 1**). More recently, a vaccine composed of a cluster of Tn analogues as B cell epitope, a CD4⁺ Th peptide epitope (PADRE), a CD8⁺ CTL peptide epitope (OVA257-264), and the built-in immunoadjuvant palmitic acid was proposed [143]. This fully synthetic, self-adjuvating, and multivalent GLP cancer vaccine elicited IgG antibodies capable of recognizing human tumour cell lines expressing the Tn antigen. In addition, the GLP vaccine induced strong and specific CD4⁺ T cell and CD8⁺ T cell responses, suggesting appropriate APCs processing and T cell presentation of both Th and CTL epitopes displayed by the candidate vaccine. Finally, this multivalent B-Th-CTL GLP vaccine prevented tumour formation in mice challenged by MO5 tumour cells, resulting in a 100% survival rate of immunized animals compared to only 30% survival rates of non-immunized mice [143]. Using a similar vaccine scaffold, but making use of HER-2 (HER420–429) peptide as a CD8⁺ T-cell epitope, the impact of the position of the lipid moiety in GLP vaccine immunogenicity was explored [144]. The palmitic acid moiety was either attached at the N-terminal (linear HER-GLP-1) or between the CD4⁺ and CD8⁺ T cell epitopes (branched HER-GLP-2). Significant differences were observed between the structural analogues, with HER-GLP-1 inducing a stronger and more durable HER420-429-specific CD8⁺ T cell response, while the branched HER-GLP-2 induced a stronger tumour-specific IgG response. Moreover, while tumour regression was induced by both linear and branched HER-GLP, the inhibition of tumour growth was significantly higher in linear HER-GLP-1 immunized mice [144]. These findings highlight the relevance of glycovaccines multivalency in

achieving optimized anti-cancer immune responses, while uncovering important implications of vaccine design and carrier position in anti-cancer immune responses.

Building on the success of lipoglycopeptide-based formulations, a vaccine candidate composed of the tumour-associated Tn antigen, the T epitope peptide YAF from *Neisseria meningitidis*, and the lipopeptide Toll-like receptor 2 agonist Pam3Cys was designed [138]. This molecular construct and further incorporated into phospholipid-based liposomes to form small unilamellar vesicles (SUVs) [138]. Liposome formulations elicited both IgM and IgG antibodies against the Tn antigen, showing that the built-in adjuvant Pam3Cys had triggered appropriate signals for the maturation of DCs, while providing Th cells engagement in BALB/c mice. Moreover, the incorporation of a peptide T epitope was critical for class switch to IgG antibodies, since previous immunizations with a saccharide coupled to Pam3Cys resulted in absence or very low titres of IgG antibodies [138]. On a different approach, a MUC1-Tn vaccine containing a quaternary amino acid (α -methylserine) in the most immunogenic domain of MUC1 was coupled to Pam2Cys, originating a three-component cancer vaccine [145]. Interestingly, despite being more resistant to enzymatic degradation than the natural counterpart, the immune response elicited by the unnatural vaccine was not improved compared to a vaccine candidate containing natural threonine [145]. Notwithstanding, these findings highlight that the rational design of engineered vaccines needs to ensure that unnatural amino acids mimic the conformational properties of the glycosidic linkage, since the presentation and dynamics of the sugar moiety plays a critical role in immune recognition. On a similar note, three-component vaccine candidates composed of covalently linked MUC1-Tn, a Th epitope derived from the polio virus, and a built-in adjuvant lipopeptide (Pam2CysSK4 or Pam3CysSK4) were synthesised [146]. Both candidates were incorporated into phospholipid-based SUVs and administered with or without QS-21 to BALB/c mice. The anti-sera elicited by both vaccines reacted strongly with MUC1 positive tumour cells, whereas no binding was observed in MUC1 negative cells [146]. Interestingly, the Pam3CysSK4 formulation provided exceptionally high antibody titers with no improvements upon QS-21 adjuvant co-administration. Furthermore, it has been demonstrated that for optimal antigenic responses the three vaccine components should be covalently linked. Moreover, the lipidic vaccine fraction facilitates antigen presentation and retention into liposomes [146]. Overall, the encouraging performance of such

vaccine design was assigned to its capacity to induce local cytokine production, upregulation of co-stimulatory proteins and APCs uptake, as well as prevention of epitope suppression. The promising Pam3CysSK4 has also been explored in other multicomponent self-assembly approaches, as MUC1-Pam3CSK4/CpG-Th epitope peptide candidates. These effectively activate macrophages *in vitro* and elicit strong antibody immune responses as well as anti-tumour immune responses *in vivo* [147], vowing for further development of multivalent lipid-based self-adjuvating cancer glycovaccines.

Non-self-adjuvating lipids, as cholesterol, can also be explored for liposome-based carbohydrate vaccines. For instance, the cholesterol-modified monosaccharide Chol-GalNAc was synthesised and integrated with synthetic DOPC (dioleoylphosphocholine), DPPC (dipalmitoylphosphatidylcholine), and adjuvant CpG to form self-assembled glycoliposome formulations [148]. The Chol-GalNAc/CpG vaccine elicited humoral immunity in immunized mice, significantly enhancing anti-Tn antigen antibody titers. IFN- γ secretion in an immune coculture of immunized T cells with Tn-expression 4T1 cancer cells also evidenced the induction of Th1-dependent cellular immune responses [148].

Despite encouraging preclinical results, to our knowledge, none of these formulations was tested in human clinical trials.

3.2.3 Dendrimers

Dendrimers are monodispersed and frequently biocompatible polymers with well-defined chemical and biological properties, which display multiple reactive surface groups that allow heterofunctionalization [149]. These multivalent scaffolds have emerged as useful tools for molecularly defined glycovaccine design, often mimicking the cancer-associated clustered form of short-chain O-glycans (**Table 1**). For instance, a dendrimeric multiple antigenic glycopeptide (MAG) with a lysine core was synthesised and tested as vaccine in mice challenged with Tn-expressing tumour cells [150]. This MAG carried four copies of a CD4+ T cell epitope (PV peptide) extended with a tri-Tn glycoepitope (MAG:Tn3-PV) (**Figure 1**). The synthetic MAG:Tn3-PV was highly immunogenic, generating IgG1 antibodies able to recognize both human and murine Tn positive cell lines. Also, the tri-Tn glycotope was proven more immunogenic than monomeric Tn. Furthermore, prophylactic vaccination with MAG allowed rejection of implanted Tn-expressing

tumour cells in mouse models [150]. To investigate the influence of the aglyconic backbone on the Tn glycotope recognition, mice were immunized with MAG:Tn3-PV, MAG:Tn(S)3-PV (a glycotope cluster containing three α -GalNAc on a STT backbone), and MAG:Tn(hS)3-PV (a glycotope cluster containing three α -GalNAc on a non-natural homoserine hShShS backbone). No significant difference was found in the recognition of Tn-positive Jurkat cells by antibodies elicited by MAG:Tn3-PV or MAG:Tn(S)3-PV, whereas MAG:Tn(hS)3-PV induced antibodies with low reactivity for native Tn. These findings highlight that the aglyconic moiety influences Tn antigen/antibody interaction. However, if immunogens include natural serine and threonine, sufficient diversity of the polyclonal IgG response should enable the efficient recognition of native forms of the Tn antigen. Furthermore, the MAG:Tn3-PV formulation was compared to the conventional KLH-Tn(c) design, showing qualitative and quantitative superior efficacy in inducing an anti-Tn immune response [151]. In addition, two humanized MAG were designed by introducing CD4 T-cell peptides PADRE or TT830-844 (MAG:Tn(S)3-PADRE and MAG:Tn3-TT), following non-human primates inoculation together with alum and CpG oligonucleotide as adjuvants. Both MAG elicited Tn-specific IgG antibodies in non-human primates, which were able to recognize glycosylated human mucin peptides and Tn-positive human tumour cells. Of note, MAG:Tn3-TT induced increased titers. Generated antibodies specific for tumour-associated Tn were able to induce antibody-dependent cellular cytotoxicity in tumour cells in the presence of human NK cells, demonstrating their anti-tumoral potential [151]. Ultimately, these pre-clinical studies reinforce the relevance of fully synthetic vaccine targeting of Tn for immunotherapeutic purposes in humans, setting the grounds for future clinical trials (**Table 2**). A novel solid-phase synthetic process for production of MAG-Tn3-TT at gram-scale for human use was reported [152], envisaging its readily application in phase I clinical trials. The MAG-Tn3-TT vaccine (**Figure 1**) was formulated with the GSK-proprietary immunostimulant AS15, composed of CpG7909, MPL, and QS-21. MAG-Tn3-TT plus adjuvants induced an anti-Tn antibody response in mice of various H-2 haplotypes and in cynomolgus monkeys, which targeted Tn-expressing tumour cells and mediated tumour cell death both *in vitro* and *in vivo* [153]. Subsequently, in a phase I clinical trial in breast cancer patients with high-risk of relapse, the MAG-Tn3-TT plus AS15 vaccine induced high levels of Tn-specific antibodies and complement-dependent cytotoxicity in Tn-expressing human tumour cells in all patients [154]. Overall, this study highlighted

the ability of a synthetic cancer glycovaccine to induce specific immune responses in humans.

3.2.4 Peptide-free carriers

Several molecular glycoengineering strategies have been employed in the development of simple platforms for multivalent display of TACAs and boosting anti-TACA immune responses without the need for a typical protein carrier. Contrasting with carrier proteins, carrier-free approaches may reduce the risk of undesired immune reactions against carrier epitopes. The most adopted strategy exploits polymeric glycoconjugates (**Table 1**). For instance, a synthetic polymer glycopeptide conjugate composed of MUC1-Tn-TT was coupled to a poly(N-(2-hydroxypropyl)methacrylamide) [P(HPMA)] polymer and administered to wild-type BALB/c mice together with complete Freund's adjuvant [155] (**Figure 1**). The novel polymer-based glycopeptide vaccine formed micelle-like nanostructures and induced significant MHC-II-mediated immune responses in mice, eliciting IgG antibodies capable of recognizing MCF-7 breast tumour cells [155]. Given the numerous degrees of freedom for structure modification, this novel vaccine concept may offer new opportunities for anti-tumour glycovaccines. Other polymers, as poly(D,L-lactic-co-glycolic acid) (PLGA), can also be used as vaccine carriers, producing highly biocompatible particulate suspensions. Accordingly, PLGA microspheres containing MUC1 peptides with incorporated MPLA adjuvant were tested in mice [156]. Specific Th1 responses were elicited; however, barely minimal IgG responses were achieved and only in the presence of MPLA [156]. Notwithstanding, these preliminary results highlight the potential of biodegradable and biocompatible PLGA vaccine formulations to harness the protective capacity of the immune system.

3.2.5 Nanoparticles

Nanoparticles are emerging as novel attractive delivery vehicles for cancer vaccines, offering potential for multiplex functionalization and site directed delivery, maximizing immune system stimulation [57, 157, 158]. (**Table 1**). In cancer vaccines, nanoparticles have mainly been explored as scaffolds for surface functionalization. Notwithstanding, nanoparticle encapsulation of antigenic cargo can potentially minimize antigen degradation in circulation, while allowing prolonged release of antigens, which remains to be explored in vaccines. In line

with this, polymerizable PEG-Tn conjugated to gold nanoparticles (**Figure 1**) was tested for its efficacy to induce *in vivo* immune responses in New Zealand White rabbits [159]. These multicopy and multivalent nanoparticles generated a significant immune response *in vivo*, with promising indications that the antibodies generated can recognize natural Tn-antigen glycans and mammalian mucins [159]. While the absolute titers reported were lower than those obtained with glycoconjugates based on protein toxins, these fully synthetic and multivalent peptide-free glycoconjugate vaccines provided novel platforms for cancer glycovaccines. On a similar approach, spherical gold nanoparticles were coated with tumour-associated MUC4-di-T antigen glycopeptides attached at different sites, and a molecular adjuvant peptide from the complement derived protein C3d [160]. Both IgM and IgG antibody isotypes were detected in mouse sera; however, vaccines with one carbohydrate antigen gave more robust responses than those with two contiguous disaccharides [160]. Accordingly, these platforms may be a viable delivery system for tumour-associated glycopeptide antigens, warranting further studies. Gold glyconanoclusters (**Figure 1**) comprising mixed monolayers of STn and Ley antigens in various proportions, a peptide from tetanus toxoid (TT89-105), and glucose (inert component to control the density of the antigens in the final construct) were also explored as cancer glycovaccines in mice [161]. Preliminary data indicated that mouse anti-sera detected STn/Ley epitopes on the surface of the nanoparticles; even so, further study reports are lacking [161]. On a different approach, multivalent lipopeptide-coated iron oxide nanoparticles were also tested as potential glycoconjugate-based synthetic anti-cancer vaccines [162]. These MUC1-Tn magnetic nanovaccines provided significantly higher IgG antibody responses in mice compared to those induced by MUC1 peptide or lipopeptide in soluble forms. Moreover, a single Tn gave the highest anti-MUC1 IgG titers compared to full glycopeptide glycosylation. These antibodies also bound more strongly and exhibited higher cytotoxicity toward Tn overexpressing cells than heterogeneously glycosylated tumour cells. Furthermore, the magnetic nanoparticle itself was almost nonimmunogenic, inducing a low amount of anti-carrier antibodies [163]. Besides MUC1, this strategy can be readily applied to vaccines targeting other glycopeptides, glycolipids, or gangliosides. Hence, magnetic nanoparticles (NPs) are valuable tools to be employed in glycoconjugate-based anticancer vaccines, adding to other platforms as gold and polymer NPs. In addition, nanohydrogel particles also emerged as alternative delivery vehicles.

Nanohydrogel particles conjugating MUC1-Tn glycopeptide (**Figure 1**), tetanus toxin α -T-cell epitope P2, and CpG, as internal adjuvant for TLR stimulation, were tested as a self-adjuvating vaccine [164]. *In vitro* studies showed that only the CpG containing nanogels upregulated costimulatory factors for T-cell proliferation. Co-delivery of B- and T-cell epitopes with CpG was necessary to induce antibodies with binding affinity to human tumour cells [164]. Considering the numerous degrees of freedom for structure modification, functionalization, and complexation, the novel self-adjuvating nanogel vaccine concept opens new perspectives for anti-tumour immunization without external adjuvants. Additionally, peptide-based nanoparticles, exploiting the self-assembly properties of peptides, constitute an appealing platform for physical carriers without the reliance on a typical protein carrier or external adjuvant [165] (**Figure 1**). In this context, nanovectors composed of MUC1-Tn glycopeptide TSAPDTRPAP and the peptide Nap-GDFDFDYDK (DF stands for D-Phenylalanine, DY for D-tyrosine, DK for D-lysine non-natural amino acids) were tested as a multivalent and self-adjuvating vaccine (C-MUC1(Tn)-Nap) [165]. C-MUC1(Tn)-Nap induced humoral and cellular immune responses marked by specific anti-MUC1 IgG1, Th1 plus Th2 cytokine production, and complement-dependent cytotoxicity in cancer cells [165]. Overall, these results show that Nap-GDFDFDYDK could be used as an efficient multivalent nanovector with adjuvant properties in MUC1-based anti-tumour vaccines, offering a suitable alternative to the non-clinically approved complete Freund's adjuvant.

3.2.6 Virus-like particles

Virus-like particles constitute an extremely versatile vaccine platform (**Table 1**), consisting of highly repetitive immunogenic but non-pathogenic particles resembling parental viruses. Its main advantages lay on their morphological similarity to viruses and ability to display antigens in an organized and high-density manner. This impacts positively on the efficiency of APCs uptake and immune processing of particulate vaccines [166]. Based on these premises, the delivery platform bacteriophage Q β has been explored to boost immune responses to the cancer associated Tn (**Figure 1**). Given its small size and self-like nature, the monomeric Tn antigen often does not encompass strong immune responses, even when coupled to immunogenic carriers [138, 151, 167]. Clustering of consecutive Tn antigens has rendered more promising responses; however, the biosynthetic challenge is troublesome and time-consuming [151, 167]. Icosahedral Q β scaffolds

allow the one-step attachment of multiple copies of the monomeric Tn, providing high-density multivalent antigen display. Q β -Tn conjugates produced high IgG titers in mice, showing specificity for Tn expressing cells [168]. Interestingly, passing a threshold level of Tn density, the pattern of Tn antigen display, rather than the total amount of Tn antigen administered, appeared to be crucial for the induction of high anti-Tn IgG titers [168]. Cowpea mosaic virus capsid (CPMV) and tobacco mosaic virus capsid (TMV) viruses were also used as Tn carriers, although inducing more modest yet specific anti-Tn IgG production [168-170].

MUC1 glycopeptides SAPDT*RPAP bearing T and STn antigens were conjugated with bacteriophage Q β virus-like particles, and the immunogenicity of these conjugates were evaluated in immunotolerant MUC1.Tg mice [171]. Exceptionally high IgG antibody titers were generated *in vivo*, showing robust binding capabilities to tumour cells. Q β -MUC1-T induced antibodies bound to B16-MUC1 melanoma cells with increased affinity compared to antibodies induced by unglycosylated MUC1 peptide or glycopeptides with various glycoforms. In addition, Q β -MUC1-T vaccination of MUC1.Tg mice substantially defended against B16-MUC1 cells challenge in a tumour metastasis model [171]. Overall, this study demonstrates Q β -MUC1 glycopeptide conjugates are a promising tool as anti-cancer glycovaccines. Of note, the O-glycosyl linkage between the glycan and the peptide backbone on native MUC1 glycopeptides is prone to glycosidase activity, leading to vaccine instability. Accordingly, novel MUC1 glycopeptide mimetics with the T antigen linked to threonine through unnatural β -glycosyl bonds were conjugated to Q β and tested as vaccine [172]. MUC1- β -T was less susceptible to glycosidase degradation than MUC1- α -T. IgG antibodies generated against the MUC1- β -T glycopeptide in MUC1.Tg mice could cross-react with native MUC1- α -T glycopeptides as well as with a broad range of tumour associated MUC1 glycoforms. In addition, anti- MUC1- β -T antibodies could kill tumour cells via complement mediated cytotoxicity [172]. In summary, this study has demonstrated that glycomimetics with an artificial anomeric stereochemistry can be a strategy for increasingly stable anti-cancer glycovaccines. On a similar approach, filamentous bacteriophage fd was conjugated to α -GalCer (bacteriophage fd/ α -GalCer) and tested as vaccine [173]. Bacteriophage fd/ α -GalCer conjugates were able to trigger iNKT cells *in vitro* and *in vivo*, boost adaptive CD8+ T cell responses and efficiently delay tumour progression in mice [173]. Altogether, conjugation of fd

bacteriophages and lipids provided new self-adjuvating vaccine platforms capable of efficient priming and expansion of T cells with relevant anti-cancer effect.

3.2.7. Carrier-free approaches

Carrier-free approaches have also been introduced to overcome potential undesirable off-target immunity promoted by carriers and linkers (**Table 1**). An entirely carbohydrate-based immunogen consisting of a zwitterionic polysaccharide PS A1 (MHC-II elicitor for a T-cell-dependent immune response) and the Tn antigen has been designed [174] (**Figure 1**). Tn-PS A1 conjugates elicited high anti-carbohydrate IgG3 antibody titers in mice even in the absence of immune stimulants. Moreover, ELISA confirmed immunoglobulin specificity toward Tn [174], vowing for the possibility of fully carbohydrate-based vaccines. Furthermore, it has been observed that peptides mimicking carbohydrate structures, termed mimotopes, can induce antibodies with differing carbohydrate specificities [175, 176] (**Figure 1**). Carbohydrate mimicking peptides have been described for GD2 [177-179], GD3 [180], sialyl Lewis^{A/X} [181], and Le^Y [177, 182, 183]. Taking advantage of the poly-specific nature of antibodies, a pan-immunogen carbohydrate mimicking peptide that mimics both Le^Y and the ganglioside GD2 (termed P10s) was developed [184, 185]. A Phase I dose-escalation trial of P10s-PADRE (T cell epitope) plus adjuvant MONTANIDE™ ISA 51 VG was conducted in patients with metastatic breast cancer [185] (**Table 2**). The P10s-PADRE vaccine induced anti-P10s, Le^Y and GD2 antibodies in all patients. Moreover, serum antibodies mediated caspase-3-dependent apoptosis of Le^Y and GD2 expressing breast cancer cells. Immunization with the P10s-PADRE vaccine was found safe and induced functional antibodies, suggesting potential clinical benefit [185]. More recently, a combined phase I/II clinical trial tested the P10s-PADRE vaccine in combination with neoadjuvant chemotherapy (Doxorubicin + Cyclophosphamide + Docetaxel (or Paclitaxel)) in HR+/HER2– early-stage breast cancer patients [186] (**Table 2**). Glycovaccine immunization in combination with standard-of-care chemotherapy was feasible and well-tolerated, inducing antibody responses, activation of NK cells, and potentiating infiltration of T cells in the tumour microenvironment [186]. These results support the need for a randomized phase II trials focused on long term survival to validate current findings and evaluate clinical efficacy. As an alternative to mimotopes for vaccination, glycoreplica peptides, arising from phage display peptide libraries, have been proposed [187,

188]. The produced peptides mimic original glycoconjugate antigens configuration but also some of their functions [187, 188]. For instance, disialoganglioside GD3-replica peptides were able to generate anti-GD3 antibodies in mice, which were able to recognize GD3 positive human melanoma SK MEL-28 cells and melanoma tissue sections [188] (**Figure 1**). This study constituted the first proof of principle that antibodies can be successfully obtained through an immune response to glycoreplica peptides. In summary, carbohydrate mimicking peptides, as mimotopes and glycoreplica peptides, are xenoantigens of TACAs that can induce antibodies against glycans on tumour cells and target shared carbohydrate residues on different carbohydrate structures. Ultimately, this has been demonstrated to inhibit tumour growth *in vitro* and *in vivo* [189]. Thus, these represent a promising tool to increase the efficiency of the immune response to glycan antigens.

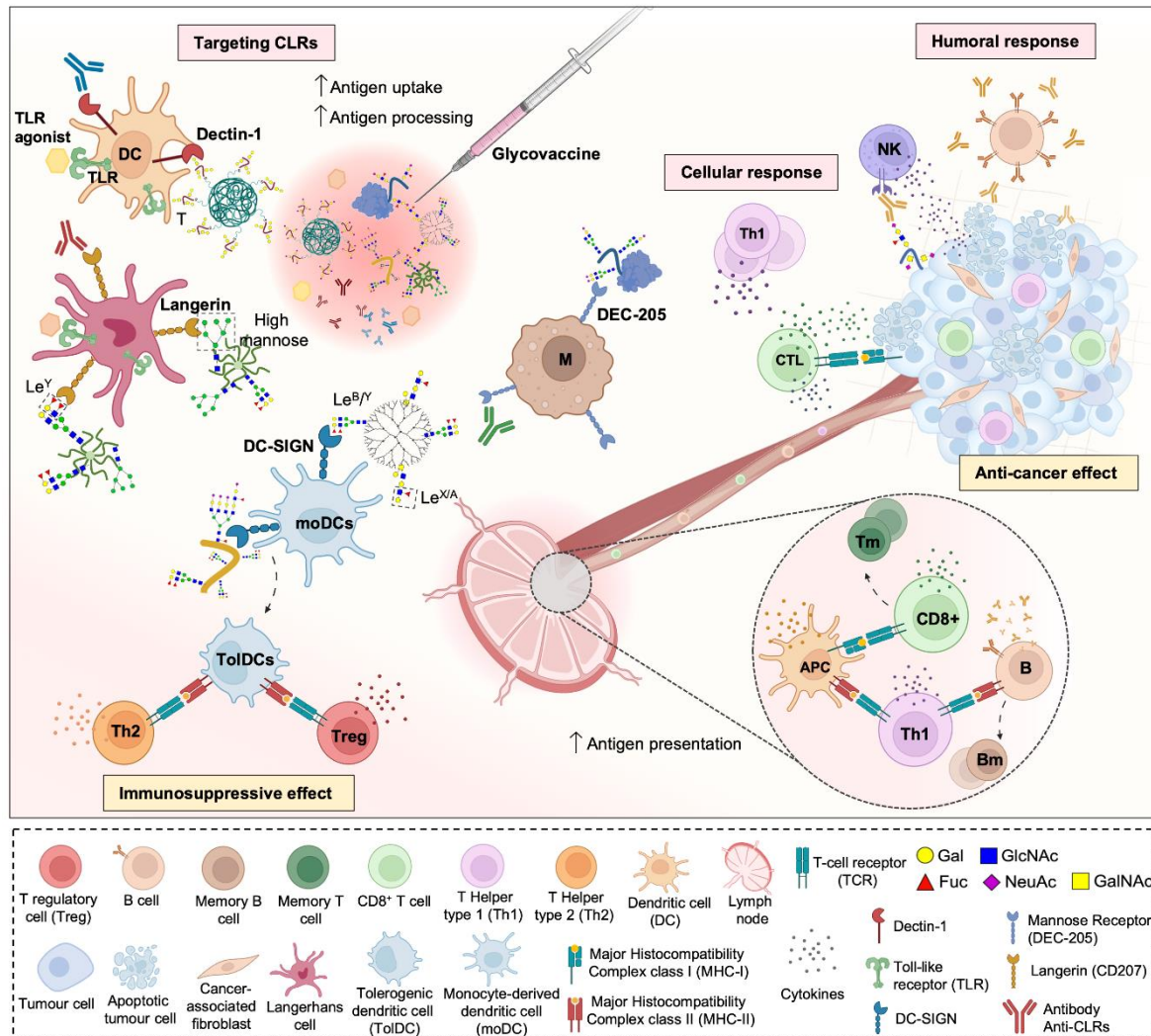


Figure 2. Targeting C-type lectin receptors (CLR) on antigen presenting cells surface.

Current strategies include the incorporation of anti-CLR antibodies and CLR natural carbohydrate ligands (e.g. high mannose, lewis type carbohydrates) on glycovaccines to directly target multiple APCs subpopulations (e.g. monocyte-derived DC (moDCs), dermal DC (dDCs), Langerhans cells). This strategy intends to selectively engage APCs, increase antigens uptake, processing, and presentation to T-cells. The selective target of CLRs (e.g. Dectin-1, Langerin, DC-SIGN, DEC205) on APCs is expected to trigger species and tissue specific anti-tumour cellular and humoral responses. Anti-tumour T cell responses are engaged through MHC I receptors on DCs and the T cell receptor of naïve CD8⁺ T cells. This interaction leads to T cell maturation and the establishment of CD8⁺ cytotoxic and memory T phenotypes. Humoral responses are mainly induced through the interaction of MHC II receptors on DCs and the T cell receptor of naïve CD4⁺ T cells. This crosstalk ensures the establishment of T helper cells (Th1) which stimulate B cell maturation and antigen-specific antibody production, while stimulating CD8⁺ cytotoxic T cells expansion and memory B cell differentiation. Of note, glycans engagement with CLRs can also lead to immunosuppressive effects. Namely, the induction of tolerogenic DCs, promotion of Th2

and regulatory Treg cells differentiation, and the formation of immunosuppressive microenvironments. However, glycovaccines targeted to APCs generally induce anti-tumour pro-inflammatory responses. Nanoparticles and dendrimers have been extensively explored as vehicles for selective target of APCs in pre-clinical studies, while the mannose receptor family appears to be one of the most targeted CLRs. Targeting C-type lectin receptors has opened an avenue for improved humoral and cellular anti-cancer responses.

4. Targeting glycovaccines to C-type lectin receptors on antigen presenting cells

Most cancer glycovaccines have been demonstrated successful in the production of humoral responses against one or more antigens included in the vaccine design. However, cellular responses against the targeted antigens are usually lacking, restricting the anti-cancer effect of glycovaccines. Engagement of antigen presenting cells, as dendritic cells (DCs) and macrophages (M), is essential to trigger and maintain effective anti-tumour responses provided by cytotoxic T cells and resident memory T cells [190-192]. Accordingly, several therapeutic glycovaccine designs specifically target APCs, envisaging efficient antigen uptake and activation toward the development of anti-tumour cellular responses. Most of these approaches engage APCs through C-type lectin receptors (CLRs), which recognize glycosylated antigens and mediate their uptake, processing, and presentation to T cells [193-196] (**Figure 2**). These targeted approaches rely on the incorporation of anti-CLR antibodies on vaccine formulations or simply CLR natural carbohydrate ligands [197] (**Figure 2**).

4.1. Pre-clinical glycovaccines targeting APCs

Preliminary works envisaging APC targeting in a tissue and species-specific manner involved antigen-coupled antibodies against surface endocytic CLRs (DECTIN-1, DCIR2, DEC205, CLEC12A, CLEC9A, MR, MGL, DC-SIGN, and Langerin), Siglec-1, and non-lectin receptors [193, 198-209]. Vaccine nanovehicles targeting distinct DC subsets have also been developed [197]. For instance, PLGA nanoparticles functionalized with biotinylated antibodies to BDCA3 and DC-SIGN were demonstrated to engage different DC subclasses [210]. This resulted in significantly greater activation of T cells, in response to complex peptide mixtures from viral and tumour antigens, compared to targeting single DC subgroups [210]. This study demonstrated that combinatorial targeting of multiple DC populations

may improve cancer vaccines by engaging DC crosstalk (**Figure 2**). In a simpler approach, MUC4-T-coated nanoparticles targeted to dectin-1 were shown to induce strong *in vivo* immune responses, generating high-titer antibodies and priming of antigen-recognizing T-cells [211]. Interestingly, targeting vaccine carriers to DC-specific CLRs, as DC-SIGN, via receptor-specific antibodies instead of natural receptor carbohydrate ligands has been proven more efficient in mediating DC antigen uptake and activation [212]. However, targeting vaccine carriers via carbohydrate ligands that have a broader specificity for several CLRs expressed on several cell types may result in a similar or even stronger effect on DCs activation compared to antibodies (**Figure 2**). Based on this rationale, branched polyamidoamine (PAMAM) dendrimers were used as scaffolds for the melanoma-specific gp100 antigen and the common DC-SIGN and Langerin ligand Le^x [213]. The *in situ* targeting of human dermal DC and Langerhans cells through intradermal injection in a human skin explant model was elucidated [213]. Glyco-dendrimers targeting of DC-SIGN and Langerin lead to enhanced internalization and gp100 specific CD8⁺ T cell activation, vowing for the relevance of dual CLR targeting to reach multiple DC subsets and improve anti-tumour CD8⁺ T cell responses.

Of note, *N*-glycans have not been as explored as *O*-glycans as immunogens in glycovaccines. Still, typical *N*-glycan monosaccharides as mannose and mammalian glycoproteins with *N*-linked high-mannose are known ligands of the mannose receptor (MR or CD206), which has been explored for decades in vaccine studies to target APCs [214-218]. Mannosylated structures may bind the C-type lectin CD209 on macrophages and DCs as well, also constituting a target for vaccines addressed to DCs [219, 220]. For instance, a MUC1 vaccine containing covalently linked divalent mannose ligands provided increased uptake and presentation of the formulation by MR⁺ macrophages and DCs compared to non-mannosylated controls [214]. Vaccination induced strong IgG production with affinity for breast cancer cells in mice, while boosting the numbers of APCs and CD4⁺T cells in proximal lymph nodes. Of note, the tetravalent version of the vaccine displayed improved binding to MR compared to divalent versions, highlighting the relevance of multivalency when targeting MR [214]. On a similar note, high-mannose containing glycopeptides yielded by enzymatic glycosylation have also provided enhanced targeting to human APCs through MR when compared to non-glycosylated controls [215]. Ultimately this enhanced formulation uptake

while preserving the immunogenicity of peptide epitopes within the glycopeptides and allowed activation of a T cell clone [215]. It is important to point that O-linked oligomannose structures can also bind MR and DC-SIGN, opening an avenue to explore O-linked mannosylation as APC targeting tools. In line with this, mannosylated mucin-type fusion proteins were explored as versatile APC-targeting molecules for vaccines, resulting in enhanced humoral and cellular immune responses in mice [216]. In this context, binding of glycans to CLRs increased antigen uptake, processing, and MHC presentation, ultimately improving effector T-cell responses. However, CLRs can also mediate tolerogenic responses and even be exploited for the induction of tolerance in the context of autoimmune diseases [194, 221, 222]. For instance, in pre-clinical studies, fucosylated ligands that target DC-SIGN often favour Th2 immune responses and regulatory T cell phenotypes, having an immunosuppressive net effect and mitigating several autoimmune diseases [221-224]. Thus, CLRs targeted cancer vaccines seem to have a positive impact on optimizing antigen presentation and uptake, despite the inherent immunosuppressive potential of some natural carbohydrate ligands of CLRs.

4.2. Clinical studies of glycovaccines targeting APCs

The targeting of APC CLRs for improved anti-cancer immune responses has also been explored in clinical trial settings. For instance, a vaccine composed of human chorionic gonadotropin beta-chain (hCG- β) fused to a mannose receptor (MR)-specific monoclonal antibody (CDX-1307) was tested in patients with advanced breast, colorectal, pancreatic, ovarian or bladder cancer [225]. CDX-1307 was well tolerated and induced significant hCG- β -specific cellular and humoral immune responses when co-administered with GM-CSF, the Toll-like receptor agonist resiquimod, and poly-ICLC. Based on these promising preliminary results, a phase II trial was set to evaluate combination therapy of CDX-1307 vaccine with chemotherapy [226]. The CDX-1307 vaccine was tested as neoadjuvant and adjuvant therapy in combination with gemcitabine and cisplatin in patients with newly diagnosed, muscle-invasive, hCG- β -expressing bladder cancer amenable to resection [226]. However, this trial was shut down by lack of funding and, to our knowledge, no preliminary results have been published. On a different approach, a MUC1 fusion protein chemically conjugated to mannan (M-FP) was designed and tested in a phase I trial in adenocarcinoma patients, envisaging targeting of mannose receptors on APCs. M-FP was found highly immunogenic in humans, since

patients generated significant levels of high titered, high-binding anti-MUC1 antibodies [227]. To a less extent, some patients exhibited HLA-restricted T cell proliferation and CTL responses. It remained to be determined whether such responses had anti-tumour effect. On this basis, three phase I trials took place to evaluate safety and immunologic responses to mannan MUC1 in patients with metastatic or locally advanced carcinoma of the breast (trial 1), colon (trial 2), and various adenocarcinomas (trial 3). M-FP was found non-toxic and capable of inducing immune responses. The co-administration of cyclophosphamide with M-FP had no impact on outcomes. Interestingly, approximately 60% of patients had high-titer anti-MUC1 IgG1, with the intraperitoneal route providing around 10-fold higher responses. Still, cellular responses (cytotoxic T cells proliferation or production of tumour necrosis factor-alpha and interferon-gamma in response to MUC1 stimulation *in vitro*) were generated in only 28% of patients. No objective responses were found [228]. Subsequently, a phase III double-blind placebo-controlled trial was performed in early-stage breast cancer patients with no evidence of disease. Oxidised mannan-MUC1 immunization induced humoral and cellular responses, protecting these breast cancer patients against recurrence [229, 230]. Interestingly, activating and maturing DCs with oxidized mannan-MUC1 did not require any additional treatment, possibly due to the fact that oxidized mannan not only binds to the MR but also activates DCs through TLR-4, serving a dual purpose. In summary, targeting cell surface receptors on DCs, such as the MR, can lead to immune responses to otherwise poorly immunogenic self-antigens, which might be a suitable strategy to improve the effectiveness of cancer immunotherapy trials employing self-antigens. Moreover, these trials emphasised the importance of patient's disease status at the time of immunotherapy, since immunotherapy may not be clinically relevant in advanced cancer patients compared to early-stage individuals.

The MR family of C-type lectin endocytic receptors DEC-205 is also at the basis of preclinical research and several trials. For instance, the CDX-1401 vaccine, composed of a human anti-DEC-205 mAb fused to the full-length tumour antigen NY-ESO-1 (anti-DEC-205-NY-ESO-1), was explored in a phase I trial [231]. This trial intended to assess CDX-1401 tolerability, immunogenicity, and clinical activity when co-administered with the TLR agonists resiquimod (TLR7/8) and Hiltonol (poly-ICLC, TLR3) in refractory patients with advanced malignancies [231]. The vaccine was proven safe, and the treatment induced humoral and cellular immunity

to NY-ESO-1 in patients with confirmed NY-ESO-1-expressing tumours. Objective tumour regression was observed in 2/45 patients and in 6/8 patients who received immune-checkpoint inhibitors within 3 months after CDX-1401 administration, providing the rationale for combination immunotherapy [231]. More recently, a phase II trial assessed the effect of CDX-1401 vaccine given with or without CDX-301, a fms-like tyrosine kinase 3 ligand (Flt3L), in treating patients with stage IIB-IV melanoma [232]. Flt3L enhanced peripheral monocytes, conventional DCs, and plasmacytoid DCs responses. Significant increases in humoral and T-cell responses as well as NK cells engagement was also elicited [232]. This trial provided insights on an immunogenic vaccine regimen, vowing for further studies. Of note, DEC-205 presents a differential expression pattern in humans versus mice. It is predominantly expressed in mice CD8⁺ DCs, dermal DCs and LCs, while being found in human myeloid blood DCs, monocytes, B cells, pDCs, T cells, and NK cells [233, 234]. Accordingly, this differential distribution needs to be considered while translating preclinical findings to human trials, since different sets of immune cells would be preferably targeted by DEC205-vaccines.

5. A roadmap toward clinical implementation of cancer glycovaccines

The glycovaccines discussed in previous sections frequently displayed encouraging results in pre- and early-stage clinical studies. Strikingly, only the synthetic sialyl-Tn KLH Theratope vaccine has reached phase III trials for metastatic breast cancer but was later discontinued due to modest therapeutic benefits [118]. Yet, one may argue that it was not explored to its full potential, as the results known to public are not completely elucidative on whether treatment responses were stratified according to the STn-tumour status. Also, targeting patients with disseminated disease could have fogged potential therapeutic benefits. As such, precise identification of therapeutic windows and patient selection should be rationally considered in future studies. Even though cancer glycovaccines are yet to fulfil their claimed potential, trials conducted to this date emphasise the acceptable toxicity and capacity to engage T-cell mediated immunity presented by formulations. Moreover, important lessons have been taken to support novel vaccine prototyping and improve study designs. Building on this knowledge, the following sections establish key milestones for bringing glycoconjugate vaccines closer to the bed side.

5.1. Glycoantigens discovery

Selecting functionally and clinically relevant glycoantigens remains amongst the main challenges towards effective cancer glycovaccines. Thus far, the main sources of cancer antigens have been immature *O*-GalNAc glycans. Even though elevated in more aggressive tumours and intimately linked to cancer aggressiveness [12, 14], these glycans are not completely cancer specific, as low levels can be found across the respiratory and gastrointestinal tracts as well as in non-malignant conditions [23, 33, 35, 235]. The comprehensive interrogation of cancer cells' glycoproteome for unique molecular signatures, backed by a deeper understanding of the glycome by high-throughput mass spectrometry, may provide the necessary means for precise cancer targeting [23, 34, 236] (**Figure 3A**). Conducting glycobiomarker discovery in personalized settings will be required towards increased therapeutic efficacy. This will also be key to identify common molecular grounds to support off-the-shelf solutions serving many patients. Ensuring vaccine multivalency by including multiple cancer-specific glycans/glycoconjugates, reflecting the heterogenous molecular nature of tumours and metastases, should also be considered (**Figure 3A**). However, portraying protein glycosylation remains a daunting enterprise due to significant micro- and macro-heterogeneity, i.e variation and occupancy of glycans at a given site [237]. In addition, proteins may present high meta-heterogeneity due to variations in glycosylation across multiple glycosites [237]. Also, thorough characterization of the cancer glycome will be required to support glycoproteomics; however, glycans' isomeric and isobaric nature further contributes to structural complexity. Nevertheless, outstanding progresses have been made in glycomics and glycoproteomics, as recently reviewed [238-240]. As the field progresses towards standardization and automatization [238-242] it will be possible to obtain more comprehensive characterizations of the cancer glycoproteome. The future incorporation of genomics and transcriptomics in glycomics-oriented glycoproteomic workflows will materialize further the emerging concept of glycoproteogenomics [34, 236], greatly expanding the capacity to identify cancer unique glycoproteoforms (**Figure 3A**). The emerging field of immuno-peptidomics also provides an opportunity to prioritize glycopeptides for vaccine development, accelerating antigens discovery [243, 244] (**Figure 3**).

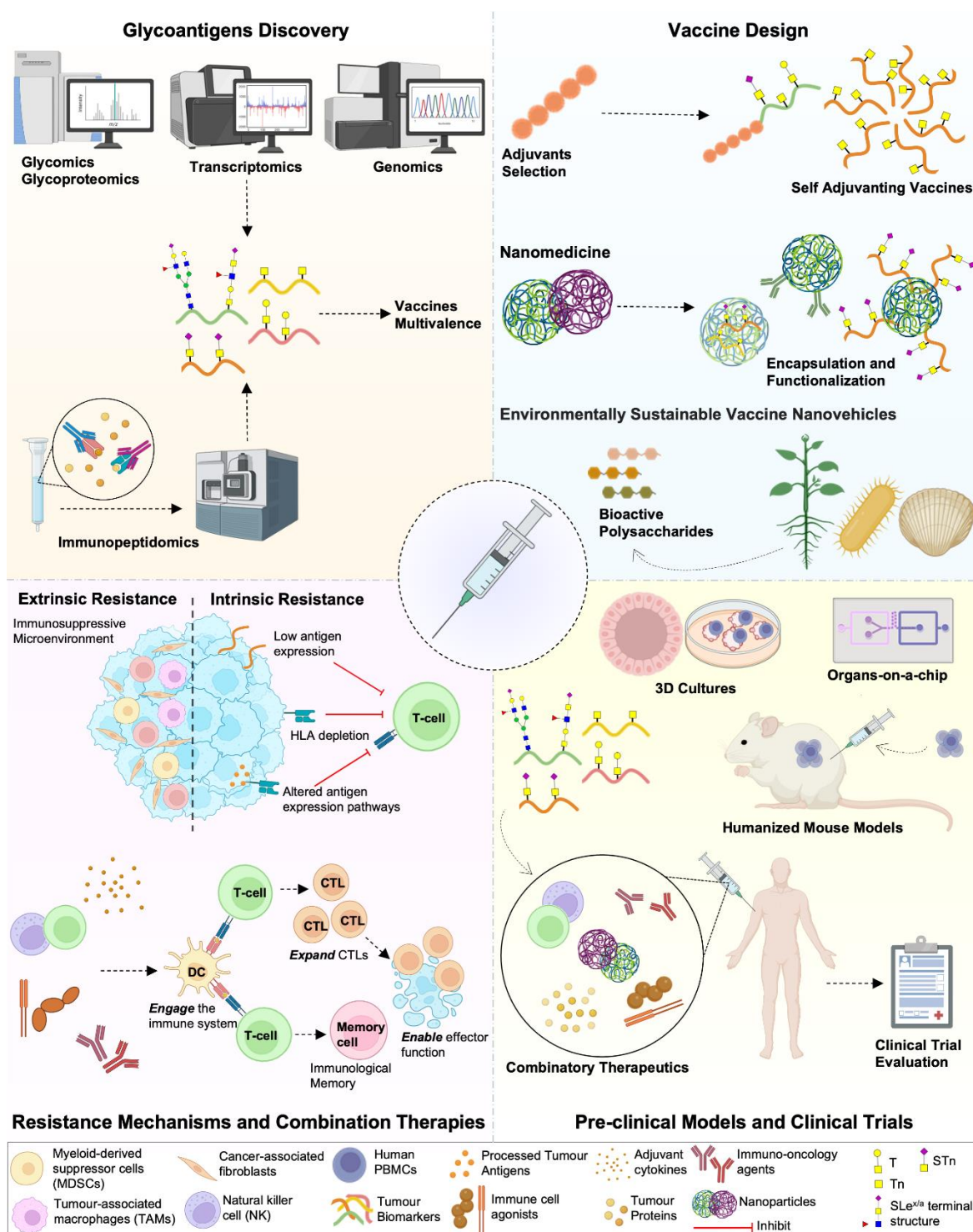


Figure 3. Roadmap toward clinical implementation of cancer glycovaccines. (A) Omics approaches integrating genomics, transcriptomics, glycomics, and glycoproteomics data have been driving glycoantigens discovery, required for establishing relevant glycovaccine designs. Immunopeptidomics could provide the necessary means to prioritize relevant glycopeptides for glycovaccines. Glycovaccine multivalency, reflecting tumour heterogeneity, is also emerging as a useful tool for generating crossed reactivity and improving anti-cancer responses. (B) Besides glycoantigens selection, adjuvants are the

next milestone for effective glycovaccines. Self-adjuvating vaccine formulations have been at the center of newly proposed approaches. Biocompatible nanoparticles and bioactive natural polysaccharides (marine, plants, bacteria) constitute some of the next generation glycovaccine vehicles. (C) To finetune clinical implementation of glycovaccines, a comprehensive interrogation of intrinsic and extrinsic resistance mechanisms to vaccination therapy is required. Extrinsic resistance mechanisms come mainly from the tumour microenvironment, while intrinsic pathways derive from tumour cell adaptation, including HLA depletion, low cell-surface antigen expression, and altered antigen presentation pathways. In this context, combination of cytokines, immune checkpoint inhibitors and adoptive cellular therapies with cancer glycovaccines may help circumvent resistance mechanisms while potentiating anti-tumour immune responses. (D) Relevant humanized models should speed translation of pre-clinical data to clinical trials. Moreover, clinical trials design evolution to speed clinical outcomes assessment of multicomponent therapies constitute the next logical step for glycovaccine clinical implementation.

5.2. Vaccine Design

Increasingly better understanding of tumour immunology, supported by high-dimensional flow and mass cytometry at single-cell level, together with spatial transcriptomics and proteomics, has been significantly aiding vaccine development and treatment personalization [194, 236]. These high-throughput approaches are still in the infancy of implementation in standard of care, mostly due to high cost of action. However, patient-tailored approaches are expected to gain grounds in future clinical practice. The gathered molecular data will be of key importance for both rational design of more effective vaccines as well as for characterization of underlying immune responses. This will allow to finetune and elect more effective vaccine formulations, as well as characterize mechanisms of action and resistance. It may further aid precise identification of vaccine adjuvants capable of unleashing compromised immune responses. Notably, several studies are conducted with adjuvants that are not approved for human vaccination, such as complete Freund's adjuvant, hurdling human translation [245]. Thus, new self-adjuvating vaccines are highly needed to elicit immune responses in absence of external adjuvants [246].

Finally, advances in nanomedicine provide opportunities to introduce the next generation vaccine nanovehicles, delivering antigens to APCs with reduced toxicity and increased therapeutic efficacy (**Figure 3B**). Biocompatible nanoparticles of different natures (liposomes, polymers) have been superficially grafted with biomolecules, including glycans recognized by immune system lectins

[247], that specifically target immune cells, promoting their activation. These innovations have been comprehensively reviewed by different authors, also providing grounds to improve on glycovaccines designs [247, 248]. Exploitation of novel bioactive biomolecules, namely natural polysaccharides from different sources (marine, plants, bacteria) [246, 249], is expected to pave new avenues for improved and environmentally sustainable vaccine nanovehicles (**Figure 3B**). For instance, chitosan nanoparticles offer potential because of its ease of production, abundant availability, hydrophilic nature, biocompatibility, and biodegradability [250, 251]. Chitosan nanoparticles have been extensively used for drug delivery, particularly for administration through mucosal and parenteral routes potentiated by their strong mucoadhesive properties. These have also been employed in anti-cancer therapeutics and immunotherapy, gene delivery, anti-HIV therapy, theranostics, and multistage delivery systems [251-253], highlighting the versatility of polysaccharide-based nanovehicles. Other plants, algae, yeast and bacterial polysaccharides of distinct natures such as glucans, mannans, pectic polysaccharides, arabinogalactans, fucoidans, galactans, hyaluronans, fructans, and xylans have also been shown to bind immune receptors and present immunostimulatory properties [254-258] (**Figure 3B**). Many of these polysaccharides are by-products of food and other industries, warranting close attention foreseeing next generation vaccine vehicles and the context of green circular economy.

5.3. Resistance mechanisms and combination therapies

While much effort is being devoted to finetuning glycovaccine formulations, there is also a growing body of knowledge on intrinsic and extrinsic mechanisms of cancer resistance to vaccine immunotherapy [259]. Classically, this has been linked to low neoantigens expression, resulting from low tumour mutational frequency [260, 261] (**Figure 3C**). However, altered antigen processing pathways [262] and decreased human leukocyte antigens (HLA) expression by immune cells [263, 264] may also contribute to diminished display of tumour antigens and compromised recognition of tumour cells by T cells (**Figure 3C**). Resistance to vaccine-based immunotherapy is also favoured by the establishment of a systemic or in tumour immunosuppressive microenvironment [265]. This is marked by the expansion of regulatory T cells (Tregs) [266], myeloid-derived suppressor cells (MDSCs) [267], tumour-associated macrophages (TAMs) [268], and cancer-

associated fibroblasts [269] (**Figure 3C**). Tumour-infiltrating lymphocytes apoptosis is also a relevant mechanism of immunotherapy resistance [270]. Immunosuppressive tumour microenvironments may also be supported by deregulation of both cancer and immune cells glycosylation. Namely, cancer cells display several immunomodulatory glycans, including neutral and sialylated short-chain *O*-GalNAc glycans and terminal Lewis antigens. These glycans, also explored in cancer vaccines formulations, are known to induce tolerogenic phenotypes in infiltrating immune cells by targeting different carbohydrate binding receptors on immune cells, decisively contributing to disease spread [194]. Tolerogenic immune cells in the tumour are also characterized by altered glycosylation, many times mimicking cancer cells glycosylation as result of yet not fully characterized microenvironmental cues [271, 272]. Such findings provide an opportunity for glycan-targeted therapeutics against tumour resident immune cells, which should be explored in future studies in potential combination with glycovaccines for improved therapeutic efficacy [194].

In recent years, there has been a push towards the development of cancer vaccines [273]. Amongst the driving forces are recent advancements in proteogenomics, enabling rapid cancer neoantigens identification under personalized settings [194, 236]. There has been a crescent awareness of the importance of delivering vaccination in combination with different therapies for improved outcomes. As such, cancer vaccines have been so far tested in clinical trial settings in combination with: i) adjuvant cytokines [NHS-IL-12 (fusion of two IL-12 homodimers fused to the NHS76 antibody), and ALT-803 (IL-15/IL-15R α superagonist); ii) multifunctional immuno-oncology agents [Bintrafusp alfa (monoclonal antibody targeting PD-L1 with the receptor for TGF- β fused to its C-terminus); iii) adoptive cell therapy (immortalized NK cell therapeutic products and transfer of autologous *ex vivo* stimulated T cells), and iv) immune cell agonists (OX40 Agonist BMS-986178) [274] (**Figure 3C**). Preclinical data also suggests that combination therapy with cancer vaccines allows sustained vaccine-induced immune responses by modulating suppressive TME features and blocking the negative modulation of anti-tumour responses [259, 275-277]. The underlying concept builds on inducing tumour antigen-specific T-cell populations and potentiate their migration to the tumour microenvironment, where they may exert prolonged effector functions [278] (**Figure 3C**). Nevertheless, as multicomponent immunotherapeutic approaches gain ground, treatment-induced toxicities may

arise, requiring specific management according to the immunomodulator's mechanisms of action [279]. In this context, multifactorial targeted nanosystems could be of great promise for multitargeted approaches by allowing systemic toxicity management [280, 281].

5.4. Pre-clinical models and clinical trials

Careful election of pre-clinical models to assess the efficacy of glycan-based immunotherapies is also of outmost importance. For instance, a substantial number of developed glycovaccines exploit MUC1 VTR glycopeptides. However, subsequent pre-clinical validation studies have mostly been conducted in common laboratory animal strains instead of transgenic mouse models expressing human MUC1 in a tissue specific manner (**Table 1**). Accordingly, efforts should be made to employ models closer to clinical translation and in mimicry of humans [113, 282]. Nevertheless, elegant models envisaging clinical translation have been proposed. Namely, Stergiou *et al.* explored a human MUC1 double-transgenic breast cancer mouse model for validation of MUC1-based glycovaccines. This model expresses the human MUC1 gene in all epithelial cells, while displaying morphologic similarities and biomarkers associated with poor outcome in humans [113]. More details on available experimental models for glycoimmunology, including tremendous progresses observed in the development of organs-on-a-chip, 3D cultures of cancer and immune cells, and 3D humanized models can be found in our recent review on cancer glycoimmunology [194, 236] (**Figure 3D**).

Progression to clinical trials requires precise definition of targeted populations and therapeutic windows, and could strongly benefit from high-throughput molecular and clinical characterization of patients' responses. The generated molecular data would be key for patient stratification and identification of potential resistance mechanisms. Early clinical trials frequently assess safety and efficacy of candidate therapies, which becomes progressively more critical for combinatory regimens (**Figure 3D**). As such, clinical trial design must evolve to accommodate increasingly complex therapeutic approaches (**Figure 3D**). In this context, a recent adaptive-design combination clinical trial named quick efficacy seeking trial (QuEST1) was designed for rapid clinical signal assessment of combination immunotherapy. Namely, combination of a brachyury-targeted antitumour vaccine, a TGF- β TRAP/anti-PD-L1 antibody, an IL-15 agonist, and an IDO1 inhibitor was tested in metastatic castration-resistant prostate cancer patients

[283]. This novel “fail early or win early” strategy can timely identify inactive combinations in the treatment process and allow immediate interrogation of the next combination, posing the most efficient strategy for clinical testing of combination therapy [283].

6. Concluding remarks and Future Perspectives

Aberrant tumour glycosylation constitutes a source of TACAs, such as short-chain O-GalNAc glycans, with sufficient cancer specificity to support immunotherapy, including glycovaccines. However, glycans are usually poorly immunogenic, eliciting T-cell independent B cell activation, leading to IgM production but not cytotoxic responses. Facing these challenges, several biotechnological strategies have been put in place to optimize anti-TACAs immune responses. These include conjugating glycans and glycoconjugates to carriers of different molecular natures, introducing chemical modifications, and employing adjuvants. The most common approach is to covalently link glycans and glycopeptides to immunogenic proteins. In addition, several vehicles such as PLGA nanoparticles and liposomes, nanocrystals, virus-like particles and 3D-scaffolds have been employed in delivering antigens and adjuvants to APCs [208, 284-286]. Notwithstanding, the field has been progressing towards fully synthetic and self-adjuvating vaccines with potential for multivalent formulations [287, 288]. Namely, self-assembly nanovaccines have been used to avoid uncontrolled immune activation caused by carrier proteins and linkers. Moreover, these versatile vectors allow multivalency and multicomponent features, including combination of different immunostimulants. Targeting of glycovaccines to APCs with antibodies against cell-surface receptors or through glycans with affinity for different immune-lectins has also gained ground over the last decade.

Even though the molecular foreground for glycovaccines has been set, translation of pre-clinical proof-of-concept studies to human trials has been limited, requiring the accomplishment of relevant milestones. Amongst the major challenges will be the implementation of glycoproteogenomics workflows for decisive glyconeantigens identification and precise cancer targeting [236], which has been one of our main research topics over the past decade [23, 33, 34, 235, 289, 290]. The development of cost-effective and environmentally friendly glycopeptides synthesis methods is also pressing to support future translation to the clinics. Improving vaccine design through nanotechnology, with introduction

of novel and sustainable targeting vehicles that decrease toxicity while increasing therapeutic efficacy, is also a relevant future research topic. Overcoming the immunosuppressive tumour microenvironment remains a challenge which would require precise immunophenotyping and tailored election of combinatory therapies, rather than single treatment modalities. In fact, it becomes consensual that glycovaccines efficacy would benefit from combination with immune modulating agents, such as immune checkpoint inhibitors, able to surpass cancer immunosuppressive mechanisms. In this context, nanomedicine may offer the necessary framework towards multitargeted systems, while offering manageable toxicity profiles. Finally, we augment that cancer glycovaccines would better serve early-stage patients showing competent immune systems and tumours where immunosuppressive microenvironments are not yet major drivers of disease progression. High throughput immunophenotyping and deep omics characterization of tumours will be major assets for comprehensive patient stratification and election of cohorts for treatment. Overall, novel, more effective and patient tailored therapies will stem from synergistic crossdisciplinarity efforts from cancer immunology, cellular and molecular glycoengineering, glycomics and glycoproteomics, nanomedicine, and oncology.

AUTHOR CONTRIBUTIONS

Rui Freitas: conceptualization, writing, review, editing and illustration; **Andreia Peixoto:** conceptualization, writing, review, editing, and funding acquisition; **Eduardo Ferreira:** review, editing and illustration; **Andreia Miranda:** review, editing and illustration; **Lúcio Lara Santos:** funding acquisition, review and editing; **José Alexandre Ferreira:** conceptualization, funding acquisition, writing, review, and editing.

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CONFLICTS OF INTEREST

The authors declare no potential conflict of interests.

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AIMS & STUDY OUTLINE

Aims & Study Outline

Bladder cancer (BLCA) is one of the deadliest and most frequently diagnosed genitourinary malignancies, encompassing several clinical challenges [1]. These include the lack of biomarkers for patient stratification, clinical outcome prediction and targeted therapeutics, as well as the need for safer and more effective therapeutics. Immunotherapy, such as cancer vaccines, holds great potential for tumour eradication and relapse prevention. However, the frequently low mutational burden presented by tumours and metastases has hampered the identification of antigens for vaccine construction [2, 3]. Exploiting aberrant tumour cell-surface glycosylation patterns is a promising approach for the identification of novel tumour antigens, envisaging their application for cancer vaccines development. Showcasing this, several glycoantigens have been recently pinpointed in BLCA, offering an opportunity for cancer vaccine development [4-7]. Accordingly, this thesis aims to explore the potential of two BLCA glycosignatures, CD44s and MUC16, for anti-tumour vaccines design. We hypothesized that these constructs can stimulate the immune system to generate cancer-specific cellular and humoral immune responses.

This study outline starts with Chapter 1 description of an enzymatic method to produce *O*-glycosylated MUC16 synthetic peptides, following their conjugation with immunogenic proteins to yield the construction of a multivalent glycovaccine. It consists of a published research paper addressing the:

- i) Production of MUC16 glycopeptides through combination of glycosyltransferases and sugar nucleotides in a stepwise manner, using a single-pot approach;
- ii) Application of a TiO_2 enrichment approach for sialoglycopeptides enrichment and characterization of all glycospecies by mass spectrometry;
- iii) Conjugation of synthetic glycopeptides with immunogenic proteins to design multivalent cancer vaccine candidates.

In summary, Chapter 1 establishes the biotechnological foundations for obtaining immunogenic synthetic glycoantigens for cancer vaccine construction.

Chapter 2 describes the construction of CD44s-Tn based glycovaccine candidate and its pre-clinical evaluation *in vitro* and *in vivo*. It consists of a published research paper addressing the:

- i) Application and optimization of the enzymatic strategy reported in chapter 1 to produce and characterize CD44s-Tn/STn synthetic glycopeptides;
- ii) Conjugation of CD44s glycopeptides with immunogenic proteins and process optimization to produce immunogenic glycoconjugates;
- iii) Evaluation of the glycovaccine candidate safety and capability to induce the maturation of dendritic cells *in vitro*;
- iv) Evaluation of the glycovaccine candidate safety and capacity to induce anti-tumoural humoral and cellular specific responses *in vivo*.

Taking together, Chapter 2 describes a roadmap for the production and evaluation of safety as well as immunogenicity of a glycovaccine candidate, both *in vitro* and *in vivo*.

Building on lessons learnt from the previous classical vaccine constructs, Chapter 3 illustrates the design of a cancer glyconanovaccine candidate surpassing the need for conventional carriers. It exploits MUC16-Tn antigen in combination with poly(lactide-*co*-glycolide)-*b*-poly(ethylene glycol)-maleimide (PLGA-PEG-MAL) nanoparticles, evaluating glyconanovaccine profile *in vitro* and *in vivo*. Chapter 3 consists of a published research paper addressing the:

- i) Application and optimization of the enzymatic strategy reported in chapter 1 to produce and characterize MUC16-Tn synthetic glycopeptides;
- ii) Conjugation of MUC16-Tn glycopeptides with PLGA-PEG-MAL nanoparticles and physicochemical characterization of the formulation;
- iii) Evaluation of the glyconanovaccine candidate safety and capability to induce maturation of dendritic cells *in vitro*;
- iv) Evaluation of the glyconanovaccine candidate safety and capacity to induce anti-tumour humoral response *in vivo*.

In conclusion, Chapter 3 describes a roadmap for the production and characterization of the safety and immunogenic profile of glyconanovaccine candidates, both *in vitro* and *in vivo*.

Finally, the Concluding Remarks and Future Perspectives section addresses the overall conclusions of the thesis and discusses future directions and critical milestones for the developed glycovaccines.

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CHAPTER I

**Single-pot enzymatic synthesis of cancer-associated MUC16
O-glycopeptides libraries and multivalent protein
glycoconjugates: a step towards cancer glycovaccines**

The information provided in this chapter is based in the following publication:

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*Equal contribution.

Single-pot enzymatic synthesis of cancer-associated MUC16 O-glycopeptides libraries and multivalent protein glycoconjugates: a step towards cancer glycovaccines

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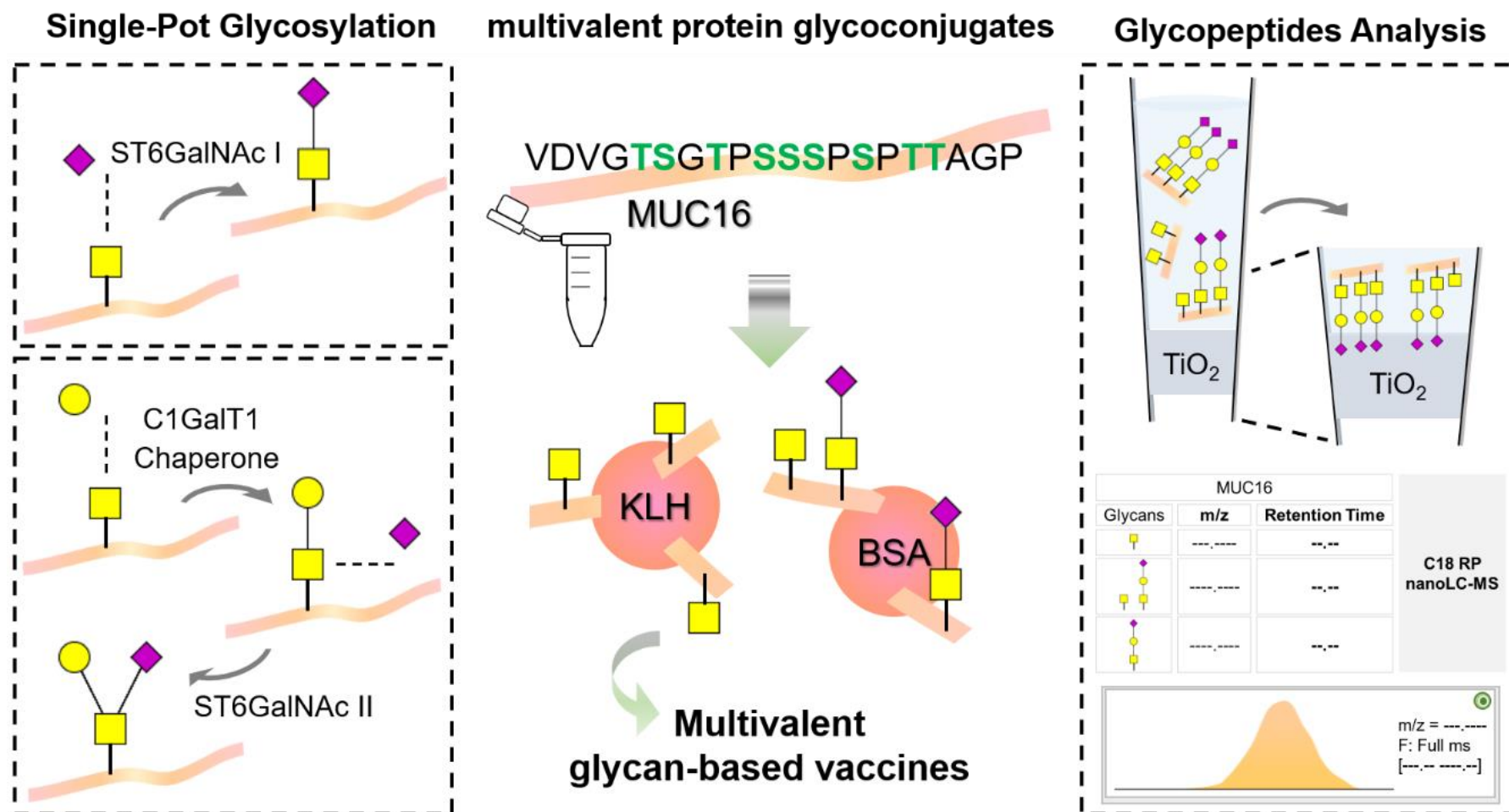
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ABSTRACT

Cancer cells often overexpress and/or express *de novo* glycoproteins modified with short-chain sialylated *O*-glycans, sialyl-Tn (STn), sialyl-3-T (S3T) and sialyl-6-T (S6T) antigens, that hold potential for carbohydrate-based vaccines. However, the generation of glycopeptides libraries composed by structurally defined standards remains a critical step for carbohydrate metrology and vaccine development. A simple and fast multi-enzymatic single-pot method was applied for generating a wide array of STn, S3T and S6T glycopeptides. A 20-mer tandem repeat peptide from cancer-associated glycoprotein MUC16 was used as scaffold towards this end. Different glycosyltransferases and nucleotide sugars were combined in a stepwise manner to generate the glycopeptide libraries. TiO₂ enrichment was further used to isolate sialoglycopeptides and the glycopeptide libraries were characterized by nanoLC-MS. Multiple glycopeptides were conjugated with protein immunogens, bovine serum albumin (BSA) and keyhole limpet hemocyanin (KLH), foreseeing multivalent glycoepitopes that may pave the way for immunotherapy. We have synthesized MUC16-Tn antigens containing up to 5 glycosites and mapped the main occupied glycosites. We have also successfully synthesized MUC16-STn, S3T and S6T glycopeptides and hybrid MUC16-Tn/STn, MUC16-Tn/S3T, MUC16-T/S6T and MUC16-Tn/T/S6T glycoproteoforms. As proof of concept, we have coupled MUC16-Tn/STn glycopeptides to immunogenic proteins. This sets the molecular background for future development of multivalent glycan-based vaccines exploiting MUC16 glycoepitopes.

Keywords: enzymatic synthesis, glycopeptides, protein glycoconjugates, cancer glycovaccines

Graphical Abstract



1. Introduction

Glycan-based therapeutics face some drawbacks such as lack of pure and structurally well-defined carbohydrates and glycoconjugates to develop analytical methods and therapeutics applications [1, 2]. Chemical and enzymatic methods have been developed for oligosaccharide synthesis to be explored in many applications, such as vaccines and antibodies [3, 4]. However, chemical synthesis could be hard to perform due to multiple steps of protection, deprotection and purification, being expensive and time consuming [5]. The need for more efficient approaches for synthesis of oligosaccharides and glycoconjugates led to the development of chemo-enzymatic methods. Briefly, synthetic oligosaccharides and/or peptides act as substrates for glycosyltransferases to give rise to more complex glycans and glycoconjugates, allowing the preparation of relevant and structurally defined glycopeptides and the establishment of glycoconjugates libraries [6, 7]. Some advantages of employing enzymes in biosynthetic processes include high regio- or stereospecificity with no need for protection and deprotection reactions to obtain chemo-selectivity. As such, enzymatic methods have been successfully applied to the biosynthesis of *O*- and *N*-glycosides [8, 9]. Moreover, they are considered more environmentally sustainable approaches in comparison to chemical methods [10].

The exploitation of chemoenzymatic synthesis of immunogenic glycopeptides and glycoconjugates to develop antibodies and carbohydrate-based vaccine prototypes has significantly increased in recent years [11, 12]. The possibility of acquiring highly pure synthetic peptides at accessible prices to use as scaffolds for enzymatic glycosylation, greatly contributed for this interest. In addition, the increasing availability of a wide array of recombinant glycosyltransferases with distinct substrate specificities and well-defined kinetic properties enables a highly controlled glycopeptide synthesis [13, 14]. However, ensuring the desired glycosylation pattern of *O*-GalNAc glycopeptides remains challenging. This requires the precision setting of the initial *O*-glycosylation step, consisting of the covalent link of a GalNAc residue to serine or threonine amino acids, catalysed by polypeptide *N*-acetylgalactosaminyltransferases (GalNAc-Ts) in the presence of the nucleotide sugar donor UDP-GalNAc [15]. Many GalNAc-Ts show both complementary and overlapping activities, making it possible to be conjugated in a coordinated manner for defining the *O*-GalNAc glycosites. Moreover, most GalNAc-Ts target multiple glycosites in the peptide chain

producing a wide array of glycopatterns, which poses a significant challenge for producing uniform products. Notably, bioinformatics tools, such as ISOGLYP [16] and NetOGlyc [17], have been developed to predict the better suited GalNAc-Ts for generating glycopeptides of interest, as well as predicting the diversity and abundance of potential glycosites. After the first glycosylation step, *O*-GalNAc glycans can be further elongated by several glycosyltransferases to produce glycans ultimately presenting sialylated terminal structures. As such, these methods have been successfully applied to the synthesis of STn- and S3T-MUC1 glycopeptides commonly overexpressed by cancer cells [12, 18]. In addition, many studies explored the coupling of these glycopeptides with immunogenic proteins such as KLH, foreseeing the development of targeted therapeutics and cancer vaccines [19, 20]. Envisaging similar goals, this work describes the enzymatic synthesis of STn-, S3T- and S6T-MUC16 glycopeptides using synthetic a MUC16 peptide from the variable number tandem repeat (VNTR) region as starting point. It further exploits purification methods to tackle the wide array of glycosides synthesized. Finally, it focuses on developing multivalent MUC16-Tn glycopeptide-KLH conjugates, envisaging novel therapeutics for aggressive ovarian, bladder and other solid tumours known to overexpress abnormally glycosylated MUC16 glycoforms. It is anticipated that the synthesis and purification methods developed herein may be transferable to other targets, ultimately providing the necessary epitopes to support carbohydrate-based theragnostic applications for cancer.

2. Materials and Methods

2.1. Bioinformatics-assisted selection of MUC16-glycopeptide epitopes

The MUC16 protein sequence from NCBI database (NP_078966.2) presents 60+ tandem repeats (TR) of approximately 156 amino acids each. Accordingly, antigen synthesis was based on these highly repeated and *O*-glycosylated epitopes in MUC16. Specifically, the 156 amino acids repeats were analysed using the *O*-glycosylation site determination bioinformatics tool NetOGlyc (<http://www.cbs.dtu.dk/services/NetOGlyc/>) [17], followed by the selection of the 20 amino acids sequence within the TR comprising more *O*-glycosylation sites. Subsequently, GalNAc-T1 expression in bladder cancer was evaluated using transcriptomic data available in Oncomine (www.oncomine.org) [21]. This analysis was performed in relation to expression in healthy tissue and a p-value ≤ 0.05 , a 2

fold change and all gene rank were considered for this study [22]. The elected MUC16 sequence was run using the ISOGlyP (Isoform Specific *O*-Glycosylation Prediction) (<https://isoglyp.utep.edu/>) [16] bioinformatics tool to determine the fitness of human GalNAc-T1 (EC 2.4.1.41) to *O*-glycosylate this particular sequence of amino acids.

2.2. Enzymatic synthesis of MUC16-glycopeptide epitopes

A MUC16 20-mer peptide (VDVGTSGTPSSSPSTTAGP) from the TR region and its cysteine tagged form were purchased from GenScript Biotech. Synthesis of glycopeptides carrying the Tn-epitope (GalNAc- α -*O*-Ser/Thr) was performed in 100 μ L reaction mixtures containing glycosylation buffer (125 mM sodium cacodylate, 50 mM MnCl₂, pH 7.4; Sigma-Aldrich), 0.5 mM UDP-GalNAc (Sigma-Aldrich), 10 μ g of MUC16 20-mer peptide and 0.025 μ g GalNAc-T1 (R&D Systems). Tn-epitope sialylation, rendering STn-peptides, was performed through the overnight incubation at 37 °C of the Tn-peptides in glycosylation buffer, 0.5 mM CMP-Neu5Ac (Merck Millipore) and 0.020 μ g ST6GalNAc I (R&D Systems), a human α -*N*-acetylgalactosaminide α -2,6-sialyltransferase (EC 2.4.99.3). Synthesis of glycopeptides carrying the T epitope was performed in 100 μ L reaction mixture, containing the Tn-peptides in glycosylation buffer, 0.5 mM UDP-Gal, 0.015 μ g C1GalT1/C1GalT1-specific chaperone 1 (the core 1 β 1,3-galactosyltransferase 1 (EC 2.4.1.122) and its chaperone; R&D Systems) [23, 24]. T epitope sialylation, rendering S6T-peptides, was performed by overnight incubation at 37 °C of T-peptides in glycosylation buffer, 0.5 mM CMP-Neu5Ac (Merck Millipore) and 0.024 μ g ST6GalNAc II (R&D Systems), an α -*N*-acetylgalactosaminide α -2,6-sialyltransferase (EC 2.4.99.3) preferentially acting upon T antigens over Tn epitopes [25, 26]. S3T-peptides were synthesized following the same strategy but in the presence of 0.025 μ g ST3Gal I (R&D Systems), a human β -galactoside α -2,3-sialyltransferase (EC 2.4.99.4). The glycosyltransferases and sugars nucleotides were added in a sequential manner to the same reaction vial, as depicted in **Figure 1**. MUC16 sialoglycopeptides were purified using the High-Select™ TiO₂ Phosphopeptide Enrichment Kit (Thermo Fisher Scientific), according to the manufacturer instructions.

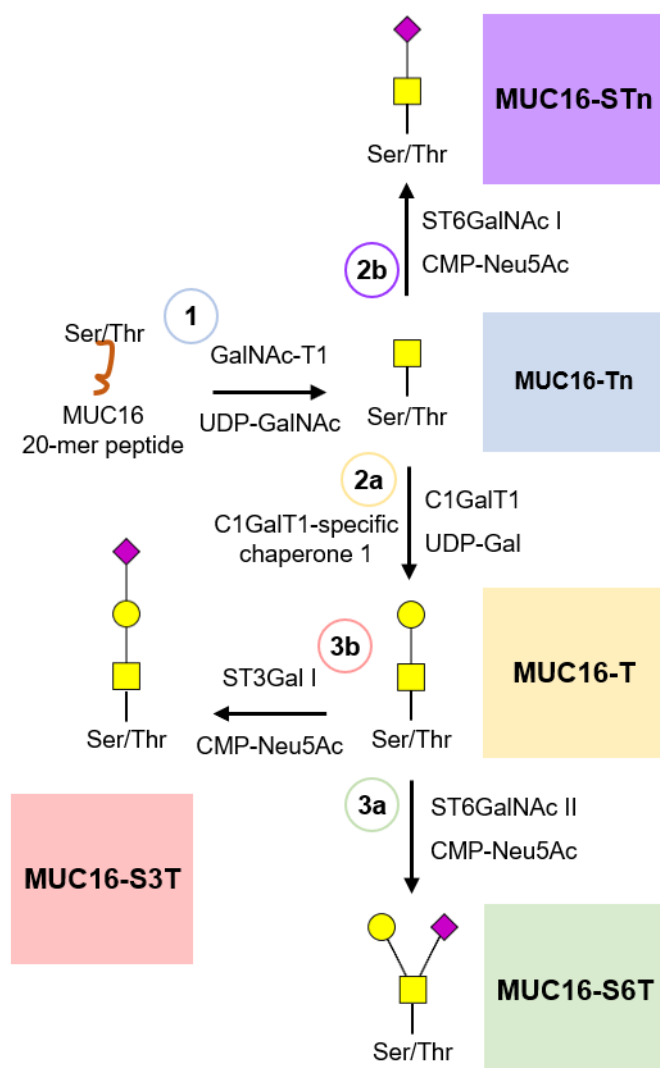


Figure 1. Enzymatic synthesis workflow for MUC16-Tn, MUC16-T, Muc16-STn, MUC16-S3T and MUC16-S6T glycopeptides. Polypeptide GalNAc transferases (GalNAc-Ts) are responsible for catalysing the transfer of a GalNAc residue from UDP-GalNAc to Ser or Thr residues in a peptide chain originating Tn antigens. Therefore, this step will define the number and distribution of glycosites that will serve as scaffold for further O-glycans elongation. Herein, we have elected GalNAc-T1 of 15 possible active enzymes as starting glycosyltransferase given its clinical relevance in bladder cancer (**step 1**). We anticipate that this may help generating glycopeptides that more closely resemble the structures produced by cancer cells. From that point on, further extension to generate the T antigen can only be achieved by C1GalT1 in the presence of the C1GALT1-specific chaperone 1 (*COSMC*) (**step 2a**). Regarding sialylated glycopeptides, two enzymes may be used for O-6 GalNAc sialylation, ST6GalNAc I or II. Nevertheless, ST6GalNAc I, which shows higher affinity for the Tn antigen, was elected to generate STn (**step 2b**). ST6GalNAc II showing more affinity for T antigen was used to generate S6T (**step 3a**). Alternatively, for O-3 Gal sialylation we have

used ST3Gal I (**step 3b**), based on its high expression and association with poor prognosis in bladder cancer.

2.3. BSA-MUC16-STn and KLH-MUC16-Tn glycoconjugates

Cysteine-tagged MUC16-STn and MUC16-Tn glycopeptides were conjugated to BSA (Sigma-Aldrich) and KLH (Sigma-Aldrich) [27] respectively, to render immunogenic glycoepitopes. Briefly, 0.01mg/ μ L KLH or BSA in 0.01 M PBS were incubated for 30 min at room temperature under agitation with 0.015mg/ μ L MBS (Sigma-Aldrich) in dimethylformamide (DMF, Sigma-Aldrich). Posteriorly, KLH-MBS and BSA-MBS solutions were passed through a PD-10 desalting column (Sigma-Aldrich) conditioned with 0.05 M phosphate buffer (pH 6). Desalted KLH-MBS and BSA-MBS solutions were incubated with 10 μ g of glycopeptides in 25 μ L DMF pH 7.0 and incubated at 4 °C overnight under rotation, followed by evaporation in a centrifugal vacuum concentrator (Labconco). The products of conjugation with BSA were characterized by SDS-PAGE on 4–20% precast polyacrylamide gels (Bio-Rad) and stained with Coomassie blue (Thermo Fisher Scientific). In parallel, the products were analysed by Western blot in 0.45 μ m nitrocellulose membranes (Amersham). The membranes, previously blocked with Carbo-free Blocking Solution (1x; Vector Laboratories), were blotted for the Tn antigen with biotinylated VVA lectin (1:5000) (Vector Laboratories) for 1 hour at room temperature and incubated with vectastain elite ABC reagent, Peroxidase, R.T.U. (Vector Laboratories). In parallel, membranes were subjected to digestion *in situ* with 0.2 U neuraminidase from *Clostridium perfringens* (Sigma-Aldrich) overnight at 37 °C before incubation with VVA lectin for analysis of STn antigen. The products of conjugation with KLH were analysed by Dot blot in a 0.45 μ m polyvinylidene difluoride membrane (PVDF; Merck Millipore). According to the protocol used above, the membrane was blotted for the Tn antigen with biotinylated VVA lectin (1:5000) (Vector Laboratories) for 1 hour at room temperature, followed by incubation with vectastain elite ABC reagent, Peroxidase, R.T.U. (Vector Laboratories).

2.4. nanoLC-ESI-LTQ-Orbitrap mass spectrometry

Synthesized glycopeptides were characterized by nanoLC-ESI-MS/ MS on a nanoLC system (Dionex, 3000 Ultimate nano-LC) coupled online to an LTQ-Orbitrap XL mass spectrometer (Thermo Fisher Scientific) equipped with a nano-electrospray

ion source (EASY-Spray source, Thermo Fisher Scientific). Eluent A was aqueous formic acid (0.2%) and eluent B was formic acid (0.2%) in acetonitrile. Samples were injected into a trapping column (C18 PepMap 100, 5 μ m particle size) and washed with an isocratic flux of 95% eluent A and 5% eluent B at a flow rate of 30 μ l/min. After 3 minutes, the flux was redirected to the analytical column (EASY-Spray C18 PepMap, 100 Å, 150 mm $\times$ 75 μ m ID and 3 μ m particle size) at a flow rate of 0.3 μ l/min. Column temperature was set at 35°C. Glycopeptide separation occurred using a linear gradient of 5–40% eluent B over 30 min. Column wash and re-equilibration was warranted before the following injection. The mass spectrometer was operated in the positive ion mode, with a spray voltage of 1.9 kV and a transfer capillary temperature of 250°C. Tube lens voltage was set to 120 V. MS full scans were acquired at an Orbitrap resolution of 60,000 for an m/z range from 300 to 2000. Tandem MS data were acquired in the linear ion trap using a data dependent method with dynamic exclusion. CID settings were 35% normalized collision energy, 2 Da isolation window, 30 ms activation time and an activation Q of 0.250. Data were recorded with Xcalibur software version 2.1 (Thermo Fisher Scientific). Reported ions are presented in terms of relative abundance in relation to the total glycopeptides abundance, calculated based on chromatogram peak area and considering the occurrence of double and triple charged ions. Glycosite annotation was performed manually using database searches with the Sequest HT (Proteome Discoverer 1.4, Thermo Fisher Scientific) and Byonic (Byonic 3.8.13, Protein Metrics) search engines to identify possible peptide spectrum matches. The FASTA sequence of the MUC16 20-mer peptide was defined as the protein database. Variable modification of serine and threonine residues with HexNAc (+203.0794, GalNAc, Tn antigen) was considered. Artefact, on source, deamination was observed [28] and best PSMs were commonly obtained for the deaminated peptide. Based on chromatographic retention time, no evidence of pre-ESI peptide deamination was found [28].

2.5. MALDI-TOF mass spectrometry

BSA-MBS conjugation was also monitored by MALDI-TOF-MS on a Bruker UltrafeXtreme mass spectrometer (Bruker Corporation). Samples were previously passed through a HyperSep Tip C-04 (Thermo Fisher Scientific) and 1:1 matrix:sample mixes were spotted onto a MTP 384 polished steel target plate (Bruker Corporation) and air dried. The matrix used was α -cyano-4-

hydroxycinnamic acid (5 mg/ml in 50% acetonitrile and 0.1% trifluoroacetic acid in water; Sigma-Aldrich). Spectra were acquired in positive ion linear mode, for a mass range from 50 to 100 kDa. Laser attenuation was set to 95% and about 4000 shots were accumulated.

2. Results and Discussion

MUC16 glycoforms presenting short-chain sialylated *O*-glycans are frequently expressed by more aggressive bladder, ovarian and other tumours which, due to its cell-surface nature, hold potential for targeted therapeutics and immunotherapy [29, 30]. These glycoforms include the STn antigen, associated with poor prognosis and chemoresistance and, possibly, different forms of sialylated T antigens, namely S3T and S6T. Therefore, this work has been devoted to the establishment of rapid and easy enzymatic synthesis and purification of STn-, S3T- and S6T-MUC16 derived glycopeptides. For proof-of-concept, we have elected a MUC16 20-mer target peptide from the variable tandem repeat region, containing approximately 9 potential *O*-glycosylation sites (T5; S6; T8; S10; S11; S12; S14; T16; T17); however according to NetOGlyc, a web-based tool for glycosites prediction, only 7 present high probability of glycosylation (S6; S10; S11; S12; S14; T16; T17). GalNAc-T1 was elected as the initiation enzyme to mimic the *O*-glycosites presented by cancer cells given its abundant expression in bladder tumours and association with more aggressive forms of bladder cancer (according to Oncomine; <https://www.oncomine.org/resource/login.html>). Based on these observations, we further highlight the utility of web-based repositories for guiding the election of glycosyltransferases for enzymatic synthesis. Bioinformatics prediction using ISOGlyP further suggested that GalNAcT1 preferentially targets the T17 and, to less extent, but still with a high probability of glycosylation are T8, T16, T5 and S12. This explorative study also suggested that mixtures of peptides presenting distinct combinations of glycosylation patterns were likely to occur using the adopted strategy. While the effect is desirable foreseeing multivalent vaccines mimicking the structural diversity observed in solid tumours, it also requires precise characterization envisaging pharmaceutical applications. As such, emphasis was also set on exploring TiO₂-based enrichment and characterization of products by nanoLC-MS/MS.

3.1. Characterization of MUC16-Tn glycosites

The initial step in MUC16 *O*-glycosylation is crucial for defining glycosites before downstream *O*-glycans extension and sialylation. GalNAc-T1 and UDP-GalNAc were used for glycosylation of MUC16, leading to the formation of MUC16-Tn antigens with a combined reaction yield of approximately 70% (**Figure 2**). The reaction products were later analysed by nanoLC-ESI-MS/MS. According to **Figure 2** mono-glycosylated peptides were predominant in relation to di-glycosylated forms (38% vs 20% of total glycosylated species), followed by the production of tri-glycosylated species (12%). Lower amounts of MUC16 with up to 5 glycosylated sites could also be detected. NanoLC analysis further highlighted acceptable chromatographic separations for neutral species, suggesting it may be used as a starting point for isolation/analysis of these molecules. In particular, the used chromatographic conditions, namely the C18 reverse phase column, the linear gradient of elution and instrumental conditions, enabled a reasonable isolation of single and double glycosylated MUC16-Tn glycopeptides (**Figure 2**).

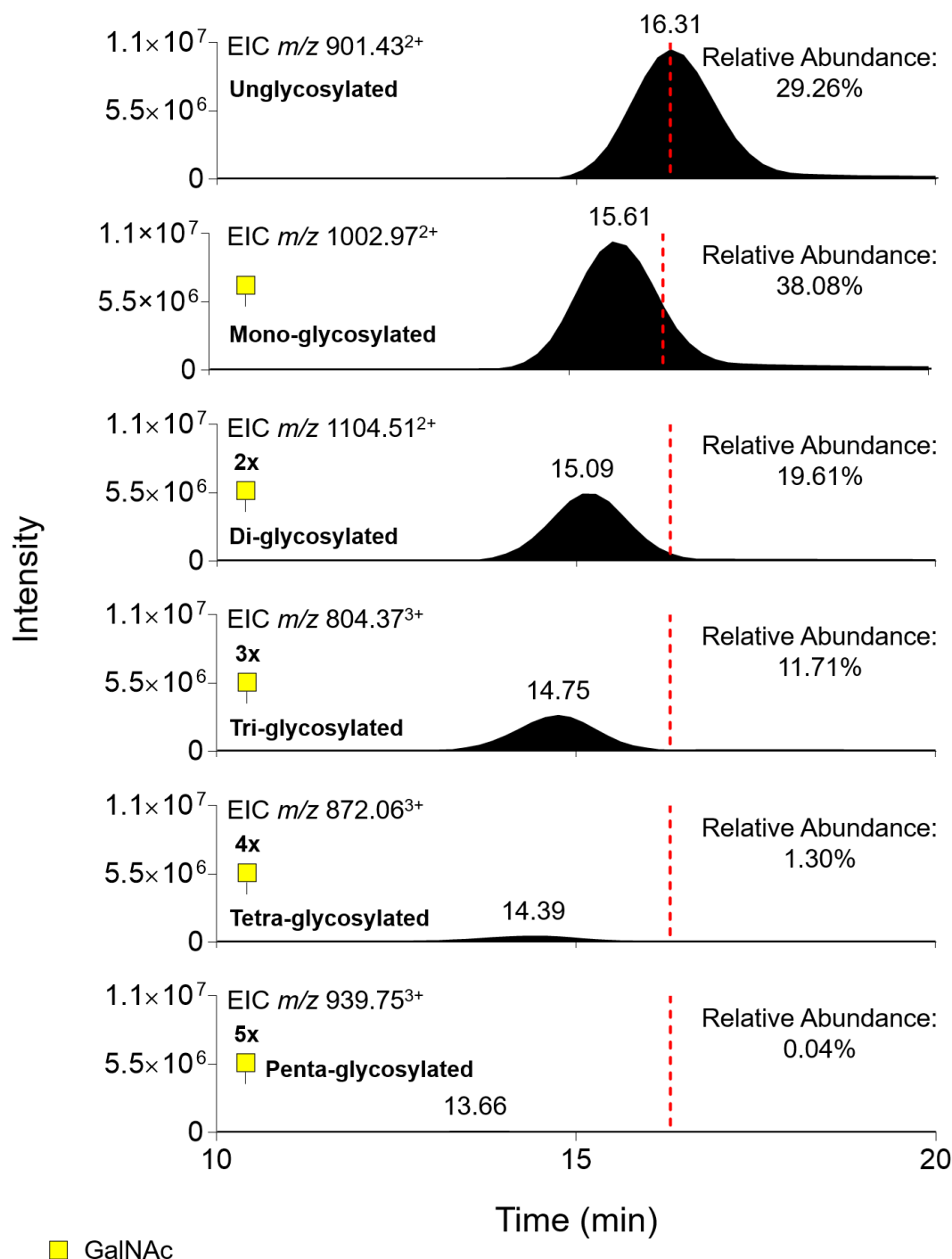


Figure 2. C18 reverse phase nanoLC-ESI-MS profiles for reaction products resulting from the glycosylation of the 20 mer MUC16 peptide by GalNAc-T1. The main products correspond to the unglycosylated form and mono-glycosylated peptides, followed by di- and tri-glycosylated species. MUC16 glycopeptides with up to five glycosylated sites could also be detected, however in much lower abundance. Reaction products mass is shown in Table S1-Supporting Information.

We then carried out a comprehensive characterization of MUC16-Tn main glycosites, which will serve as preferential scaffolds for O-glycan chains elongation based on characteristic b and y glycopeptide fragments from MS/MS spectra. Glycosite assignments were performed using the SEQUEST HT algorithm, analysis with Byonic software was also used for confirmation. This showed T16 as the preferential modification site, with vestigial amounts of glycopeptides (<5%) showing glycosylation at S6, 11, 12, and 14 and T5 and 17 (**Figure 3A**). Interestingly, these experimental observations confirm the initial bioinformatics predictions based on ISOglyP that pointed to T16 as one of the preferential glycosites. Finally, we observed a predominance of doubly glycosylated glycopeptides in which the second glycosylation occurred at T17 (**Figure 3B**), in perfect accordance with bioinformatics predictions, suggesting that this may constitute a reliable strategy for estimating the nature of synthesis products. Much less abundant glycospecies showing simultaneous glycosylations at S6 and S10, S12 and S14, T5 and S14, and T8 and S14 or T16 were also present. Regarding tri-glycosylated peptides, it was not possible to precisely identify the third preferential glycosite, due to their lower overall abundance when compared to mono and di-glycosylated species (**Figure 2**). Collectively these findings support the predominance of glycopeptides with a single glycosite at T16 and, to a lesser extent, a second glycosylation at T17. Other low abundant species were also synthesized; however, future studies should devote to combine different GalNAc-Ts towards the synthesis of a more diversified array of glycopeptides.

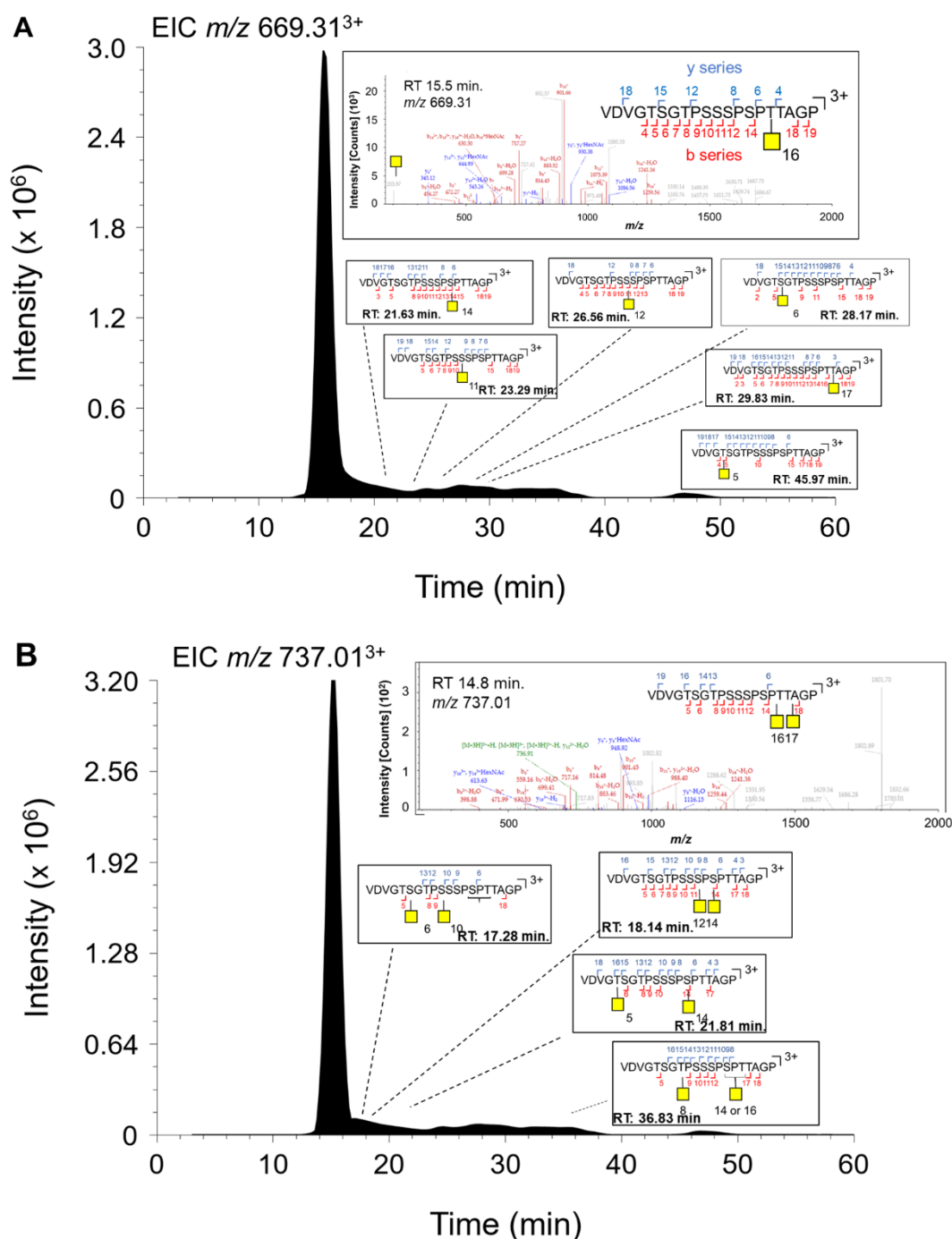


Figure 3. Glycosite identifications for 20 mer MUC16 peptide after glycosylation by GalNAc-T1. A) Identification of T16 as the main glycosite in MUC16-Tn glycopeptides. T16 appears as the preferential glycosite, with low abundant glycopeptides substituted at T5, S6, S11, S12, S14, T17. **B) Identification of T16 and T17 as the main glycosites in MUC16-2Tn glycopeptides.** The synthesis of S6/10, S12/14, T5/S14 and T8/S14 or T16 were also observed, although with low abundance.

3.2. Synthesis and purification of MUC16-STn glycopeptides

Efforts were also devoted to the synthesis of MUC16 carrying the STn antigen using a combination of GalNAc-T1 and ST6GalNAc I, the main STn synthase and strongly associated to the overexpression of STn in cancer cells [25, 31], in the presence of UDP-GalNAc and CMP-Neu5Ac in a stepwise manner. Notably, building on previous reports [18, 32], we have opted to conduct the reaction at pH 7.4 and not at sialyltransferases ideal buffer at pH 6-6.5. Even though this may, to some extent, compromise sialyltransferases activity [12, 33], it allowed to comply with the adopted single-pot strategy, avoiding the introduction of additional buffer exchange steps that could impact negatively in the reaction yields. This led to the formation of a wide array of glycosides presented in **Table 1**. Twenty-six species were identified, which can be classified in three groups: i) neutral glycopeptides, presenting the Tn antigen; ii) hybrid glycopeptides, bearing both STn and Tn antigens; and iii) sialylated glycopeptides, exhibiting STn as sole type of glycosylation. Notably, unglycosylated MUC16 peptides were not observed, demonstrating the efficiency of the reaction, while neutral and hybrid glycopeptides corresponded to almost 90% of the total amount of identified species (**Table 1**). However, both groups were highly heterogeneous (8 neutral species, 15 hybrid species) in comparison to sialylated glycopeptides (3 species). Moreover, experimental conditions favoured the formation of neutral glycopeptides with one or two glycosites, which correspond to approximately 55% of total glycopeptides. Furthermore, it favoured the formation of hybrid glycopeptides with two glycosites (Tn/STn) and sialylated glycopeptides with a single glycosite, which were present in similar proportions. The prevalence of single over multiple sialylation in both hybrid and sialo groups suggests that multiple glycosites may reduce the efficiency of sialylation under the described experimental conditions. Nevertheless, approximately 70% of the identified species presented more than two glycosites; however, in very low abundance (<3%). In summary, the most abundant glycopeptides arising from this synthesis corresponded to the neutral precursors MUC16-Tn and MUC16-2Tn, MUC16-Tn/STn and the sialoglycopeptide MUC16-STn. Therefore, TiO₂ chromatography was introduced to improve the isolation of sialylated glycopeptides [34]. TiO₂ is often used for enrichment of phosphorylated peptides; nevertheless, sialoglycopeptides have also shown high affinity for TiO₂. This is mainly due to the presence of the carboxyl group and the multiple hydroxyl groups of sialic acids at the nonreducing end of glycoepitopes, which allows a

multipoint binding between the sialic acids and the TiO₂ resin [35]. TiO₂ enrichment significantly decreased the abundance of neutral glycopeptides, while enriching it for hybrid glycopeptides (now composing 80% of the sample; **Table 1** and **Figure 4**) by reducing the dynamic range of glycopeptides concentrations.

Table 1. Products from the single-pot enzymatic synthesis of MUC16-STn glycopeptides, without purification and after TiO₂ enrichment, identified by C18 reverse phase nanoLC-ESI-MS.

O-glycans	Glycopeptides Monoisotopic mass [M+2H] ²⁺ / [M+3H] ³⁺	Retention time (min)	Without enrichment	Enrichment with TiO ₂
			Relative abundance (%)	Relative abundance (%)
Neutral glycopeptides				
Unglycosylated	901.43 / 601.29	----	0.00	0.00
1 Tn	1002.97 / 668.98	16.29	31.65	6.38
2 Tn	1104.51 / 736.67	15.73	23.73	3.36
3 Tn	1206.05 / 804.37	14.83	3.28	1.01
4 Tn	1307.59 / 872.06	14.45	3.18	0.49
5 Tn	1409.13 / 939.75	14.07	1.05	0.34
6 Tn	1510.66 / 1007.45	13.73	0.92	0.48
7 Tn	1612.20 / 1075.14	13.53	0.55	0.30
8 Tn	1713.74 / 1142.83	----	0.07	0.00
Hybrid glycopeptides				
1 Tn + 1 STn	1250.05 / 833.71	16.10	14.08	9.71
1 Tn + 2 STn	1497.14 / 998.43	15.56	0.45	3.97
1 Tn + 3 STn	1744.23 / 1163.16	15.02	0.06	3.86
2 Tn + 1 STn	1351.59 / 901.40	15.21	3.03	5.15
2 Tn + 2 STn	1598.68 / 1066.12	14.83	0.85	9.50
2 Tn + 3 STn	1845.77 / 1230.85	14.64	0.03	2.92
2 Tn + 4 STn	2092.86 / 1395.57	14.64	0.00	1.07
3 Tn + 1 STn	1453.13 / 969.09	14.64	2.24	7.32
3 Tn + 2 STn	1700.22 / 1133.82	14.45	0.26	5.04
3 Tn + 3 STn	1947.31 / 1298.54	14.25	0.04	4.51
3 Tn + 4 STn	2194.40 / 1463.27	14.45	0.00	0.37
4 Tn + 1 STn	1554.67 / 1036.78	14.25	1.01	3.47
4 Tn + 2 STn	1801.76 / 1201.51	14.07	0.26	8.09
4 Tn + 3 STn	2048.85 / 1366.23	14.07	0.01	1.84
5 Tn + 1 STn	1656.21 / 1104.48	13.89	0.00	5.52
5 Tn + 2 STn	1903.30 / 1269.20	13.89	0.10	4.41
6 Tn + 1 STn	1757.75 / 1172.17	13.73	0.43	3.01
6 Tn + 2 STn	2004.84 / 1336.90	13.53	0.00	0.23
7 Tn + 1 STn	1859.29 / 1239.86	----	0.03	0.00
Sialylated glycopeptides				
1 STn	1148.51 / 766.01	16.67	12.02	4.90
2 STn	1395.60 / 930.74	16.29	0.61	1.30
3 STn	1642.69 / 1095.46	15.73	0.03	0.80
4 STn	1889.78 / 1260.19	15.40	0.00	0.63

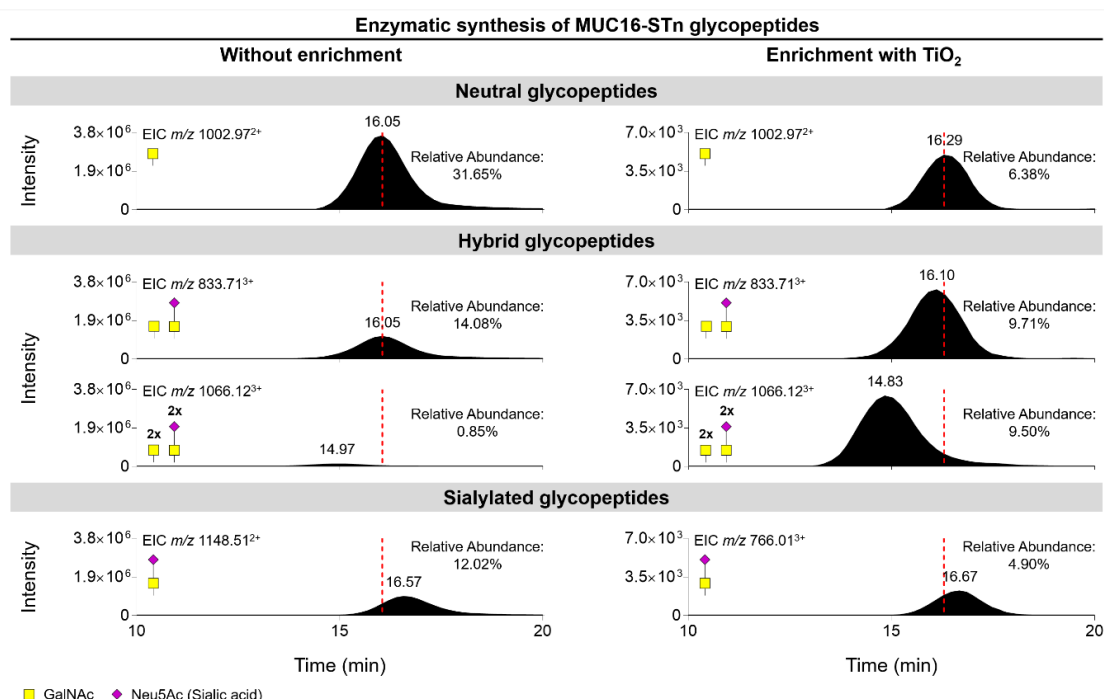


Figure 4. C18 reverse phase nanoLC-ESI-MS profiles for the main reaction products resulting from single-pot enzymatic synthesis of MUC16-STn glycopeptides without purification and after TiO₂ enrichment. Single-pot enzymatic synthesis of MUC16-STn led to the formation of a wide array of glycoforms, including neutral, hybrid and sialospecies. Most abundant glycopeptides correspond to the neutral precursor MUC16-Tn (as well as MUC16-2Tn), MUC16-Tn/STn and MUC16-STn. TiO₂ enrichment enabled a significant decrease in the abundance of neutral glycopeptides and increase in hybrid glycopeptides, namely in tetra-glycopeptides containing 2 STn residues.

3.3. Characterization of MUC16-T glycosites

Frequently, cancer cells accumulate high amounts of T antigen, which also serve as scaffold to form sialylated structures. Envisaging rapid synthesis of these antigens we maintained one-pot settings whereby all glycosyltransferases and nucleotide sugars necessary for T antigen synthesis were added to the reaction medium in a stepwise manner. In this case overall reaction yields were approximately of 60% (**Table 2**), but similar results to Tn antigen synthesis were obtained. Namely, a predominance of single glycosylated MUC16-T glycopeptides (36%) and, to less extent, di-glycosylated species (10%) was found. Lower amounts of MUC16-T with 3 glycosites and trace amounts of glycopeptides with 4 glycosites could also be detected, as well as glycopeptides showing both Tn and T antigens. Notably, the abundance of MUC16-T antigens decreased with glycosite occupancy, as previously observed during MUC16-Tn synthesis.

Table 2. Products from the single-pot enzymatic synthesis of MUC16-T glycopeptides analysed by C18 reverse phase nanoLC-ESI-MS.

O-glycans	Glycopeptides Monoisotopic mass [M+2H] ²⁺ / [M+3H] ³⁺	Retention time (min)	Relative abundance (%)
Neutral glycopeptides			
Unglycosylated	901.43 / 601.29	16.09	41.36
1 Tn	1002.97 / 668.98	15.39	3.06
2 Tn	1104.51 / 736.67	14.68	0.13
1 T	1083.99 / 723.00	15.02	36.49
2 T	1266.56 / 844.71	14.16	10.13
3 T	1449.13 / 966.42	13.48	3.37
4 T	1631.69 / 1088.13	12.54	0.26
1 Tn + 1 T	1185.53 / 790.69	14.50	3.17
1 Tn + 2 T	1368.10 / 912.40	13.82	1.61
1 Tn + 3 T	1550.66 / 1034.11	13.10	0.28
2 Tn + 1 T	1287.07 / 858.38	13.99	0.12
2 Tn + 2 T	1469.64 / 980.09	13.48	0.02

3.4. Synthesis of MUC16-S3T glycopeptides

MUC16-S3T glycopeptides were synthesized using a combination of GalNAc-T1, C1GalT1, C1GalT1-specific chaperone 1 and ST3Gal I, which was correlated with the high expression of sialylated T antigen in bladder cancer [36], in the presence of UDP-GalNAc, UDP-Gal and CMP-Neu5Ac. **Table 3** shows that 80% of the total amount of glycopeptides identified in the final reaction mixture were solely glycosylated with the Tn antigen, the initial synthesis intermediate. The second most abundant class of glycopeptides corresponded to those carrying both Tn and ST antigens, whereas a small percentage (2%) corresponded to glycopeptides presenting only the ST antigen. The di-glycosylated peptides were found to be the most abundant, irrespectively of glycosylation type, according to the observations for STn synthesis. Notably, no intermediates carrying the T antigen were observed, strongly suggesting that the rate limiting step of reaction was core 1 synthesis. The elevated number of Tn precursors emphasizes the low reaction efficiency. It is possible that C1GalT1 could be inhibited by either products and/or high substrate concentrations, which should be subjected to comprehensive evaluation to improve reaction yields [37]. Nevertheless, C18 reverse phase chromatography enabled the resolution of MUC16-S3T glycopeptides in comparison to other neutral and hybrid species present in the mixture, suggesting it may be a useful purification tool in this context (**Figure 5**). Finally, we explored the possibility of TiO₂ enrichment to improve purification. This significantly enriched the sample for sialylated glycopeptides, which corresponded to over 50% of the total amount of identified

glycopeptides (**Figure 5, Table 3**). Moreover, TiO₂ enrichment removed the neutral species and also enriched the sample for a wider array of hybrid glycopeptides, with emphasis on glycospecies containing 2 S3T and a Tn glycosite.

Table 3. Products from the single-pot enzymatic synthesis of MUC16-S3T glycopeptides, without purification and after TiO₂ enrichment, identified by C18 reverse phase nanoLC-ESI-MS.

O-glycans	Glycopeptides Monoisotopic mass [M+2H] ²⁺ / [M+3H] ³⁺	Retention time (min)	Without Enrichment	Enrichment with TiO ₂
			Relative abundance (%)	Relative abundance (%)
Neutral glycopeptides				
Unglycosylated	901.43 / 601.29	----	0.00	0.00
1 Tn	1002.97 / 668.98	----	15.37	0.00
2 Tn	1104.51 / 736.67	----	33.66	0.00
3 Tn	1206.05 / 804.37	----	17.90	0.00
4 Tn	1307.59 / 872.06	----	9.50	0.00
5 Tn	1409.13 / 939.75	----	2.78	0.00
6 Tn	1510.66 / 1007.45	----	0.97	0.00
7 Tn	1612.20 / 1075.14	----	0.17	0.00
1 Tn + 1 T	1185.53 / 790.69	----	0.07	0.00
2 Tn + 1 T	1287.07 / 858.38	----	0.01	0.00
3 Tn + 1 T	1388.61 / 926.08	----	0.01	0.00
Hybrid glycopeptides				
1 Tn + 1 ST	1331.08 / 887.72	16.12	10.77	2.34
1 Tn + 2 ST	1659.19 / 1106.47	16.12	0.00	20.55
1 Tn + 3 ST	1987.31 / 1325.21	16.30	0.00	8.44
1 Tn + 4 ST	2315.42 / 1543.95	16.30	0.00	0.27
2 Tn + 1 ST	1432.62 / 955.42	15.60	2.38	0.66
2 Tn + 2 ST	1760.73 / 1174.16	15.60	0.17	5.64
2 Tn + 3 ST	2088.85 / 1392.90	15.41	0.00	1.17
3 Tn + 1 ST	1534.16 / 1023.11	14.84	2.90	0.09
3 Tn + 2 ST	1862.27 / 1241.85	14.84	0.05	0.35
4 Tn + 1 ST	1635.70 / 1090.80	----	0.82	0.00
5 Tn + 1 ST	1737.24 / 1158.50	----	0.19	0.00
6 Tn + 1 ST	1838.78 / 1226.19	----	0.02	0.00
1 T + 1 ST	1412.11 / 941.74	15.60	0.00	1.80
1 T + 2 ST	1740.22 / 1160.48	15.76	0.00	3.21
1 T + 3 ST	2068.33 / 1379.23	15.60	0.00	0.55
2 T + 1 ST	1594.67 / 1063.45	15.04	0.00	0.14
1 Tn + 1 T + 1 ST	1513.65 / 1009.43	15.23	0.00	1.97
1 Tn + 1 T + 2 ST	1841.76 / 1228.18	15.23	0.00	1.06
1 Tn + 1 T + 3 ST	2169.87 / 1446.92	15.04	0.00	0.12
2 Tn + 1 T + 1 ST	1615.19 / 1077.13	14.65	0.00	0.23
2 Tn + 1 T + 2 ST	1943.30 / 1295.87	14.47	0.00	0.17
Sialylated Glycopeptides				
1 ST	1229.54 / 820.03	16.66	0.77	2.75
2 ST	1557.65 / 1038.77	16.66	1.48	22.18
3 ST	1885.77 / 1257.51	16.83	0.00	24.69
4 ST	2213.88 / 1476.26	17.19	0.00	1.60
5 ST	2542.00 / 1695.00	17.19	0.00	0.03

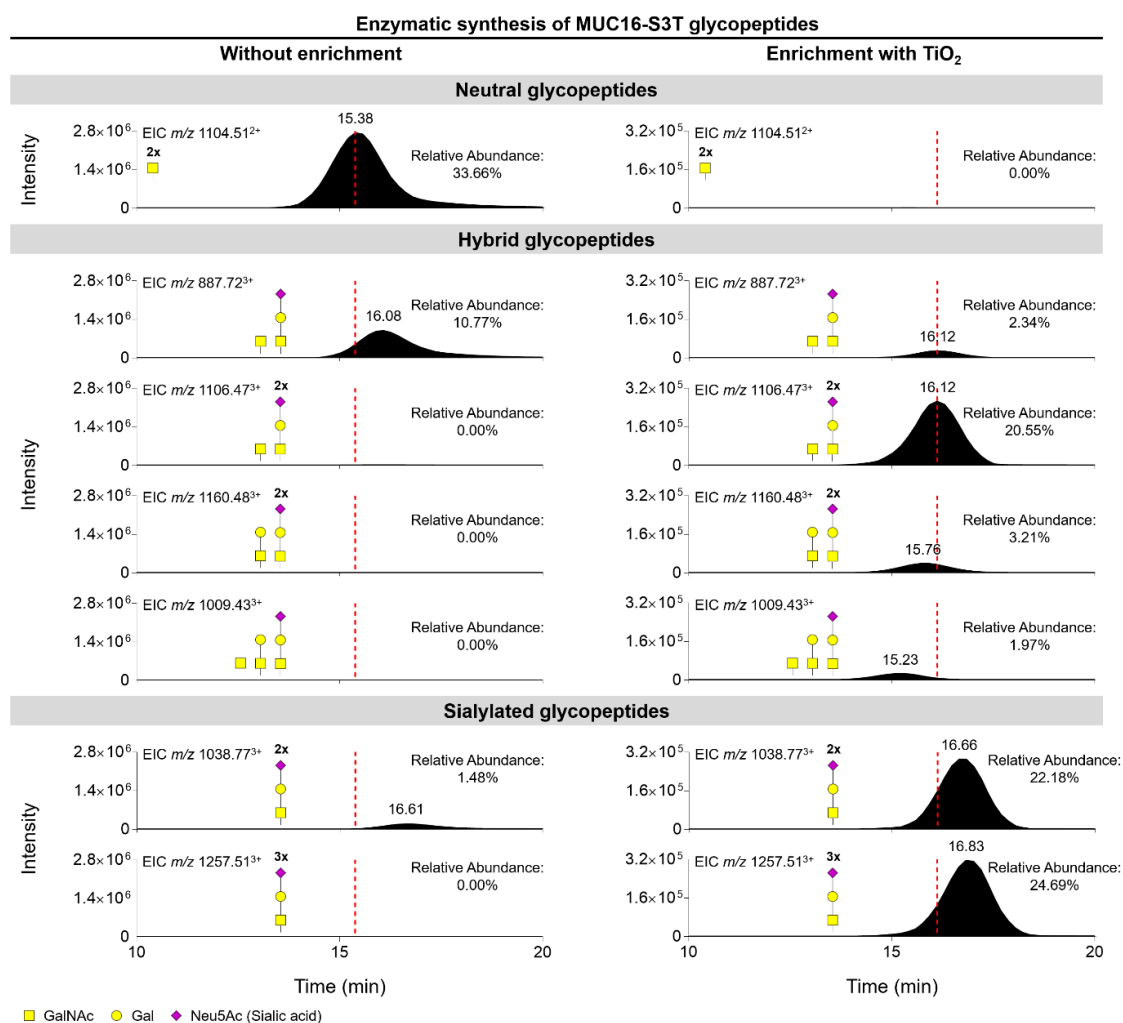


Figure 5. C18 reverse phase nanoLC-ESI-MS profiles for the main reaction products resulting from single-pot enzymatic synthesis of MUC16-S3T glycopeptides without purification and after TiO₂ enrichment. According to the presented extracted ion chromatograms, enzymatic synthesis produces mostly di-glycosylated neutral peptides and mono-sialylated glycopeptides. TiO₂ enrichment successfully removed neutral species and enabled a significant enrichment for sialylated glycopeptides, mainly for those containing 2 S3T and 3 S3T, which corresponded to almost 50% of the total amount of identified species. Moreover, it enriched the sample for a wide array of hybrid glycopeptides, with emphasis for glycospecies containing 2 S3T and a Tn antigen. Overall, TiO₂ enrichment was significantly effective in the removal of neutral species, while enriched the sample for glycopeptides containing S3T.

3.5. Synthesis and purification of MUC16-S6T glycopeptides

MUC16-S6T glycopeptides were synthesized using a combination of GalNAc-T1, C1GalT1 and its chaperone as well as ST6GalNAc II, in the presence of UDP-GalNAc, UDP-Gal and CMP-Neu5Ac. ST6GalNAc II was elected and not the ST6GalNAc I used for STn synthesis, based on previous reports supporting its higher affinity for the T antigen [25, 26]. Overall, 24 glycospecies were identified, 7 of them corresponding to Tn precursors that together comprehended approximately 85% of the total amount of glycopeptides in the sample, as previously observed for S3T. The remaining 14 glycopeptide species derived from different combinations of MUC16 with Tn/T, Tn/STn, Tn/S6T were present in low to vestigial amounts ($\leq 3\%$) (**Table 4**). This group also include MUC16 solely glycosylated by T antigen or combined with S6T, which could be observed in vestigial amounts. Sialylated glycopeptides were also found in vestigial amounts ($\leq 1\%$). Such observations suggest the low efficiency of *O*-6 sialylation under the described experimental conditions. Nevertheless, the introduction of TiO₂ purification steps tremendously enriched the sample for hybrid glycopeptides containing the S6T antigen (MUC16-Tn/2ST, MUC16-T/ST, MUC16-T/2ST, MUC16-2T/ST and MUC16-Tn/T/ST), mostly at the expenses of MUC16-Tn glycopeptides (**Figure 6**, **Table 4**). Future studies should devote to improving the efficacy of T antigen *O*-6 sialylation and the isolation of pure glycospecies.

Table 4. Products from the single-pot enzymatic synthesis of MUC16-S6T glycopeptides, without purification and after TiO₂ enrichment, analysed by C18 reverse phase nanoLC-ESI-MS.

O-glycans	Glycopeptides Monoisotopic mass [M+2H] ²⁺ / [M+3H] ³⁺	Apex Retention time (min)	Without enrichment	Enrichment with TiO ₂
			Relative abundance (%)	Relative abundance (%)
Neutral glycopeptides				
Unglycosylated	901.43 / 601.29	----	6.16	0.00
1 Tn	1002.97 / 668.98	----	31.99	0.00
2 Tn	1104.51 / 736.67	15.21	40.52	0.13
3 Tn	1206.05 / 804.37	----	6.57	0.00
4 Tn	1307.59 / 872.06	----	4.45	0.00
5 Tn	1409.13 / 939.75	----	1.00	0.00
6 Tn	1510.66 / 1007.45	----	0.44	0.00
7 Tn	1612.20 / 1075.14	----	0.04	0.00
1 T	1083.99 / 723.00	15.40	0.84	1.91
2 T	1266.56 / 844.71	14.66	0.01	3.99
3 T	1449.13 / 966.42	13.91	0.00	1.43
4 T	1631.69 / 1088.13	12.98	0.00	0.21
1 Tn + 1 T	1185.53 / 790.69	15.02	3.33	1.54
1 Tn + 2 T	1368.10 / 912.40	14.30	0.00	6.27
1 Tn + 3 T	1550.66 / 1034.11	13.17	0.00	0.72
1 Tn + 4 T	1733.23 / 1155.82	12.46	0.00	0.09
2 Tn + 1 T	1287.07 / 858.38	14.30	0.23	0.55
2 Tn + 2 T	1469.64 / 980.09	13.72	0.00	0.79
2 Tn + 3 T	1652.20 / 1101.81	12.81	0.00	0.19
3 Tn + 1 T	1388.61 / 926.08	----	0.34	0.00
3 Tn + 2 T	1571.18 / 1047.79	13.17	0.00	0.12
4 Tn + 1 T	1490.15 / 993.77	----	0.05	0.00
Hybrid glycopeptides				
1 Tn + 1 STn	1250.05 / 833.71	----	0.28	0.00
3 Tn + 1 STn	1453.13 / 969.09	----	0.11	0.00
4 Tn + 1 STn	1554.67 / 1036.78	----	0.01	0.00
1 Tn + 1 ST	1331.08 / 887.72	15.40	1.92	1.44
1 Tn + 2 ST	1659.19 / 1106.47	15.21	0.00	7.20
1 Tn + 3 ST	1987.31 / 1325.21	15.02	0.00	0.30
2 Tn + 1 ST	1432.62 / 955.42	15.02	0.24	0.37
2 Tn + 2 ST	1760.73 / 1174.16	14.83	0.00	0.92
3 Tn + 1 ST	1534.16 / 1023.11	----	0.21	0.00
4 Tn + 1 ST	1635.70 / 1090.80	----	0.03	0.00
1 T + 1 ST	1412.11 / 941.74	15.21	0.10	15.08
1 T + 2 ST	1740.22 / 1160.48	15.02	0.00	6.26
2 T + 1 ST	1594.67 / 1063.45	14.47	0.00	10.30
2 T + 2 ST	1922.79 / 1282.19	14.11	0.00	0.58
3 T + 1 ST	1777.24 / 1185.16	13.53	0.00	1.00
3 T + 2 ST	2105.35 / 1403.90	13.17	0.00	0.06
1 Tn + 1 T + 1 ST	1513.65 / 1009.43	14.83	0.00	21.57
1 Tn + 2 T + 1 ST	1696.21 / 1131.14	13.91	0.00	4.36
1 Tn + 3 T + 1 ST	1878.78 / 1252.85	12.98	0.00	0.61
1 Tn + 1 T + 2 ST	1841.76 / 1228.18	14.66	0.00	2.62
1 Tn + 2 T + 2 ST	2024.33 / 1349.89	13.53	0.00	0.39
2 Tn + 1 T + 1 ST	1615.19 / 1077.13	14.30	0.00	2.31
2 Tn + 2 T + 1 ST	1797.75 / 1198.84	13.35	0.00	1.03
2 Tn + 1 T + 2 ST	1943.30 / 1295.87	13.91	0.00	0.26
3 Tn + 1 T + 1 ST	1716.73 / 1144.82	13.72	0.00	0.46
Sialylated glycopeptides				
1 STn	1148.51 / 766.01	----	0.06	0.00
1 ST	1229.54 / 820.03	16.15	1.03	0.64
2 ST	1557.65 / 1038.77	15.78	0.03	3.39
3 ST	1885.77 / 1257.51	15.58	0.00	0.92

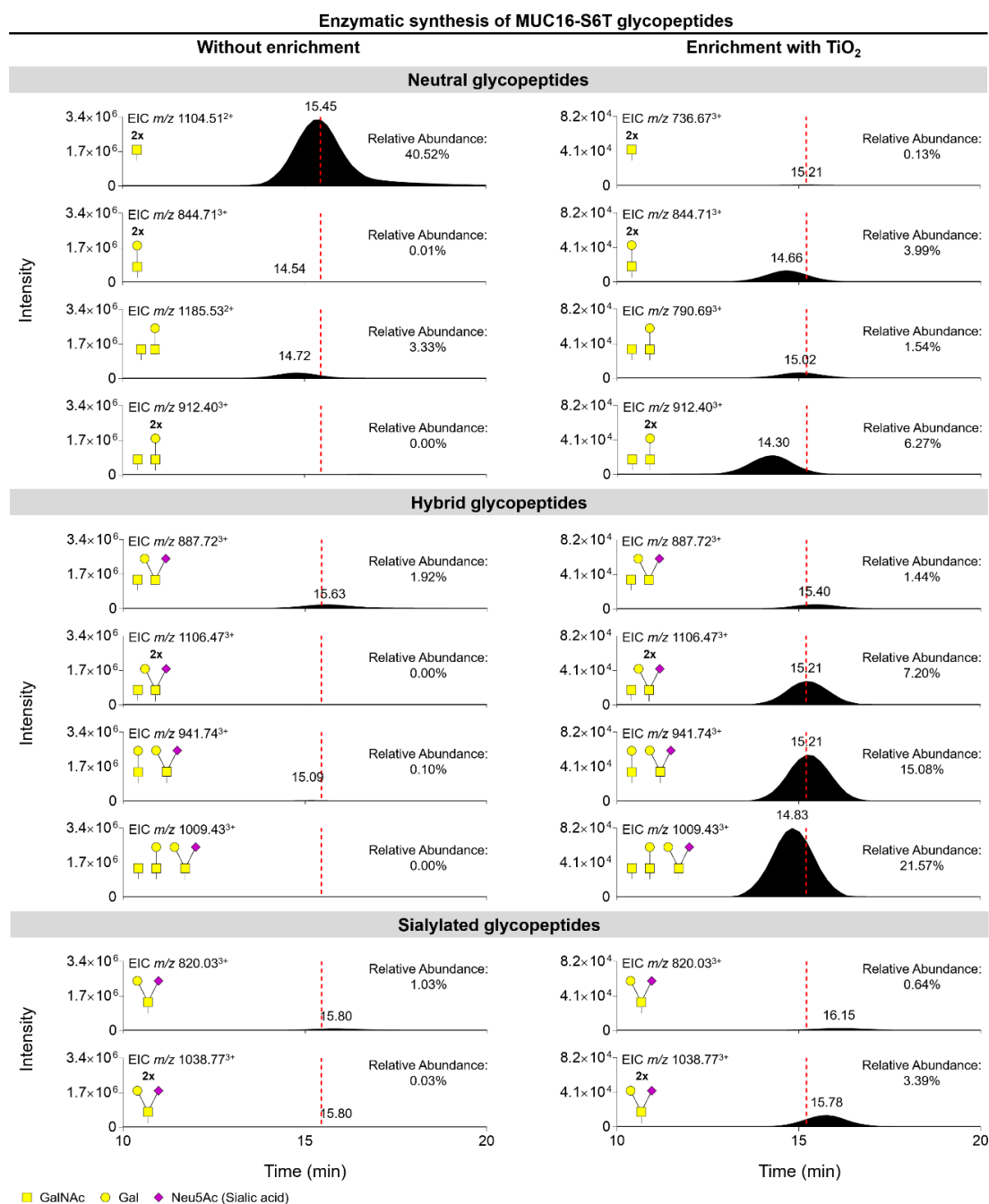


Figure 6. C18 reverse phase nanoLC-ESI-MS profiles for the main glycosylated reaction products resulting from single-pot enzymatic synthesis of MUC16-S6T glycopeptides without purification and after TiO₂ enrichment. The enzymatic synthesis of MUC16 glycopeptides with S6T modifications presented very low yields, as translated by the high amount of neutral species in relation to hybrid and sialoglycopeptides, mostly presenting one S6T glycosite. TiO₂ enabled the enrichment for a wider number of hybrid glycopeptides, containing one or two glycosites occupied with the S6T antigen.

3.6. Synthesis of BSA-MUC16-STn and KLH-MUC16-Tn glycoconjugates

MUC16-Tn glycoforms have been associated with worst cancer prognosis and recently with decreased bladder cancer response to cisplatin [29]. As such, we have devoted to the production of glycoepitopes aiming towards the development of novel MUC16-STn/MUC16-Tn targeting antibodies and cancer vaccines. To overcome their poor immunogenicity, MUC16-STn glycopeptides were covalently linked to BSA, a strategy that has been proven capable of inducing antibody production against relevant glycopeptides in animal models [12, 19]. As such, BSA was incubated with the amino-to-sulphydryl crosslinker MBS (m-maleimidobenzoyl-*N*-hydroxysuccinimide ester), which contains an NHS-ester and a maleimide reactive groups separated by a short aromatic spacer arm. The maleimide linker was subsequently used to link MUC16-STn cysteine-tagged glycopeptides. Efforts were set on the development of protein-glycopeptide conjugates presenting a diversified array of hybrid and sialylated glycoepitopes, in an attempt to translate the structural diversity of glycoepitopes presented by cancer cells [17, 29, 38]. The efficacy of the conjugation reaction was later confirmed by observing the migration of BSA's major band in SDS-PAGE gels near 70 kDa upon introduction of the MBS linker. MALDI-TOF-MS analysis later confirmed the presence of a major signal at 66 kDa corresponding to BSA that shifted to 70 kDa, suggesting the incorporation of approximately 21 MBS linker residues. The addition of the glycopeptides to the reaction media promoted a shift in the BSA-MBS band to approximately 150 kDa, strongly suggesting glycopeptide conjugation, which was validated by western blot. In fact, as shown in **Figure 7A**, BSA and BSA-MBS conjugates showed no reactivity to the VVA lectin, which has affinity for the Tn antigen. On the other hand, two major bands above 150 and 200 kDa were observed in the lanes corresponding to BSA-MUC16-STn/Tn hybrid glycoconjugates. These signals were further enhanced after sialidase digestion that exposes cryptic Tn epitopes by sialylation. Taken together, these observations support the successful conjugation of both hybrid and sialoglycoepitopes to BSA. Notably, bands at molecular weights above 150 kDa could also be observed for BSA, which may relate with protein DTT-irreversible multimerization, as previously reported [39]. SDS-PAGE and western blot analysis suggests that these bands may also be experiencing linker functionalization and glycoconjugation, which warrants future validation.

After performing a conjugation of MUC16-STn with BSA as a proof of concept, we conjugated MUC16-Tn with a classical immunogenic carrier, namely

KLH, using the same methodology [40]. KLH is a high-molecular-weight glycoprotein that is known by inducing both cell-mediated and humoral responses in animals and humans. Because of its high immunogenicity, low toxicity associated and its availability as a clinical grade product, this carrier is used extensively as a natural immunostimulant for basic research and clinical applications [41]. The efficacy of the conjugation reaction was later confirmed by Dot Blot. In fact, as shown in **Figure 7B**, KLH and KLH-MBS conjugates showed no reactivity to the VVA lectin. On the other hand, one major dot was observed in the area corresponding to KLH-MUC16-Tn glycoconjugates. These observations support the successful conjugation of both hybrid and sialoglycoepitopes to KLH. In summary, this sets the bases for producing different protein-glycopeptide conjugates for biomedical applications, with emphasis on antibody production.

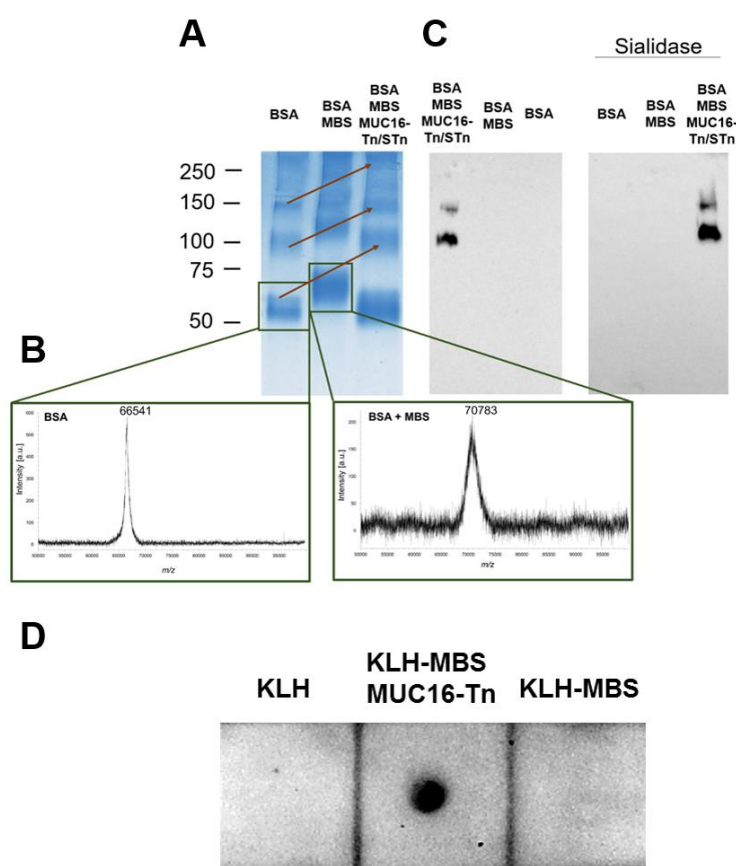


Figure 7. A) Monitoring reaction products of BSA-MBS-MUC16-Tn/STn biosynthesis by SDS-PAGE. SDS-PAGE shows a main band above 55 kDa corresponding to BSA. Higher molecular weight bands of lower intensity could also be observed above 100 kDa, most likely resulting from BSA multi-dimers. The addition of MBS to the reaction pot drifted the main band to approximately 70 kDa. A mass increase could also be observed for the molecular bands above 100 kDa. The conjugation of hybrid MUC16-Tn/STn glycopeptides

to BSA-MBS increased the molecular weight to above 100 kDa. In this case, a higher amount of BSA in relation to MBS was used for conjugation, explaining the presence of unconjugated protein in the SDS-PAGE gel. The arrows attempt to highlight the increase in molecular weight during the different conjugation steps. **B) Monitoring BSA-MBS biosynthesis by MALDI-TOF-MS.** The band at m/z 66541 in the left MS spectrum corresponds to BSA, while the right MS spectrum confirmed the conjugation of BSA-MBS. **C) Monitoring reaction products of BSA-MBS-MUC16-Tn/STn biosynthesis by Western blot.** Conjugation of the glycopeptides to BSA-MBS was confirmed using the VVA lectin targeting the Tn antigen. The left side western blot supports the presence of proteins containing MUC16-Tn (bands above 100 and 150 kDa). Membrane incubation with sialidase increased their affinity for VVA, also supporting the presence of STn antigen. Bands corresponding to BSA and BSA-MBS were not recognized by the lectin, confirming signal specificity. **D) Monitoring coupling of KLH to MUC16-Tn glycopeptides by Dot Blot.** KLH and KLH-MBS showed no reactivity to the VVA lectin. On the other hand, dots were observed in the area corresponding to KLH-MUC16-Tn glycoconjugates.

3. Concluding remarks

The synthesis of O-glycopeptides for bioanalytical and biomedical applications has been a challenging research topic. Exploiting human recombinant glycosyltransferases for glycosite definition and glycan chain extension offers significant advantages over organic synthesis, enabling an easy, rapid, less expensive, and sustainable production of glycan chains with desired conformations. Nevertheless, it requires a fine tuning of the myriad of initial GalNAc-Ts to ensure the correct definition of glycosites. Herein, we have exploited the enzymatic synthesis of short-chain sialylated peptides mimicking cancer-associated epitopes by a stepwise one-pot combination of glycosyltransferases, chaperone and nucleotide sugars. Similar approaches have been used in the past to generate MUC1-Tn, -STn, -T, -S3T antigens with relative success [12, 18]. However, even though tremendously straightforward, these procedures produce complex glycopeptide mixtures with distinct glycosite patterns and different combinations of glycan chains, often presenting all synthesis intermediates. Therefore, mixtures of different epitopes likely translating the *in vivo* context may be desirable, envisaging multivalent carbohydrate-based vaccines. However, its translation into pharmaceutical applications requires precise characterization of the generated glycoepitopes. In this work we have combined enzymatic synthesis with a TiO₂ enrichment method to prepare several libraries of MUC16 glycoepitopes

and addressed these limitations, providing a roadmap for their characterization. Briefly, we have successfully explored one-pot enzymatic synthesis to produce a wide array of MUC16-Tn, -T, -STn, -S3T and, in our understanding, for the first time, -S6T glycopeptides. These glycopeptides are expressed in bladder cancer and may also be present in other solid tumours overexpressing MUC16 as ovarian [30], breast [42], and pancreatic cancer [43]. The efficacy of the synthesis reaction varied between 60-70% for neutral species and was higher than 90% for sialylated glycopeptides. This suggests that single-pot settings may help increasing glycosylation yields, potentially driven by sialylation of particular glycosites, which warrants confirmation. Nevertheless, T16 and T17 emerged as main substituted glycosites using this technique and GalNAC-T1 as initial glycosyltransferase, in perfect agreement with bioinformatics predictions by ISOGlyP. Nevertheless, a myriad of other less abundant glycosites (T5, T8, S12) were also observed, as initially predicted. These observations suggest that analysis *in silico* may constitute a good starting point to predict single pot *O*-glycosylation products. These glycosites served as scaffolds to generate the myriad of glycopeptides showing different glycan combinations.

A TiO₂ enrichment strategy has been introduced to isolate hybrid and sialylated glycopeptides that are more frequent in some solid tumours in comparison to neutral glycosides. Downstream analysis of the products by reverse phase chromatography showed reasonable separation between neutral and fully sialylated glycopeptides, irrespectively of their nature. Moreover, it enabled a good separation of glycopeptides with different Tn-glycosites, which may be introduced at initial stages as precursors for downstream glycosylation. Moreover, MS and tandem MS analysis allowed further estimation of glycosites, nature of the glycosylation for each molecule and, to some extent, glycosites annotation. This showed predominant glycosylation at T16 and, to less extent, also T17; however low abundant glycopeptides showing a myriad of glycosites could also be detected, fulfilling the goal of multivalency desired for vaccine construction. Notably, the combination of different GalNAC-Ts with complementary affinities for different glycosites may further expand the number of synthesized glycospecies, which will be explored in future studies. The introduction of phosphatases may also be used to prevent inhibition of glycosyltransferases by the nucleotides generated during the enzymatic synthesis of glycopeptides [44]. Finally, there is still room to improve chromatographic settings since reverse phase chromatography has demonstrated

limited capacity to fully resolve hybrid glycopeptides presenting both neutral and sialoglycans. This limitation may be significantly improved with the introduction of hydrophilic interaction liquid chromatography (HILIC), which enables good separation between neutral and sialoglycopeptides [34]. A possible combination with reverse phase chromatography in 2D settings may significantly increase the quality of these procedures and should be tested in the future, envisaging glycopeptides purification and more detailed structural characterization.

The second part of this work described the generation of MUC16-STn/Tn glycopeptides-protein glycoconjugates. MUC16-STn and MUC16-Tn were elected for proof-of-concept studies, given its clinical relevance for targeting chemoresistant bladder tumours [29]. No purification steps were introduced at this stage to provide a multivalent construct presenting a wide myriad of glycoepitopes. The future construction of glycoconjugates with distinct glycoepitopes and immunogenic proteins with tailored properties to improve immunogenicity, half-life in circulation, amongst other critical aspects may be now envisaged. This will pave the way for formulations capable of eliciting antibody production in different animal models and may ultimately be tested as carbohydrate-based vaccines.

In summary, this work sets important foundations for construction of glycan libraries capable of aiding biomedical research and the development of glycan-inspired targeted therapeutics, namely immunotherapy. The methodology may be explored as a tool to investigate further different glycosyltransferases activity. Moreover, important observations have been made to support carbohydrate metrology, a crucial milestone for fostering carbohydrate-based biotechnological applications.

AUTHOR CONTRIBUTIONS

Conceptualization: JAF; **Data curation:** RF, MRS, JS, RA, EF, AMNS, JAF; **Formal Analysis:** RF, MRS, JS, RA, BT, EF, AMNS, JAF; **Funding acquisition:** LLS, JAF; **Investigation:** RF, MRS, JS, RA, BT, EF; **Methodology:** AMNS, JAF; **Project administration:** AMNS, JAF; **Resources:** LLS, AMNS, JAF; **Software:** JS, AMNS, JAF; **Supervision:** AMNS, JAF; **Validation:** RF, MRS, JS, RA, BT, AMNS, JAF; **Visualization:** RF, MRS, JAF; **Writing – original draft:** JAF; **Writing – review & editing:** RF, MRS, LLS, AMNS

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CONFLICTS OF INTEREST

There are no conflicts to declare.

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CHAPTER II

**A Multivalent CD44 Glycoconjugate Vaccine Candidate for
Cancer Immunotherapy**

The information provided in this chapter is based in the following publication:

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A Multivalent CD44 Glycoconjugate Vaccine Candidate for Cancer Immunotherapy

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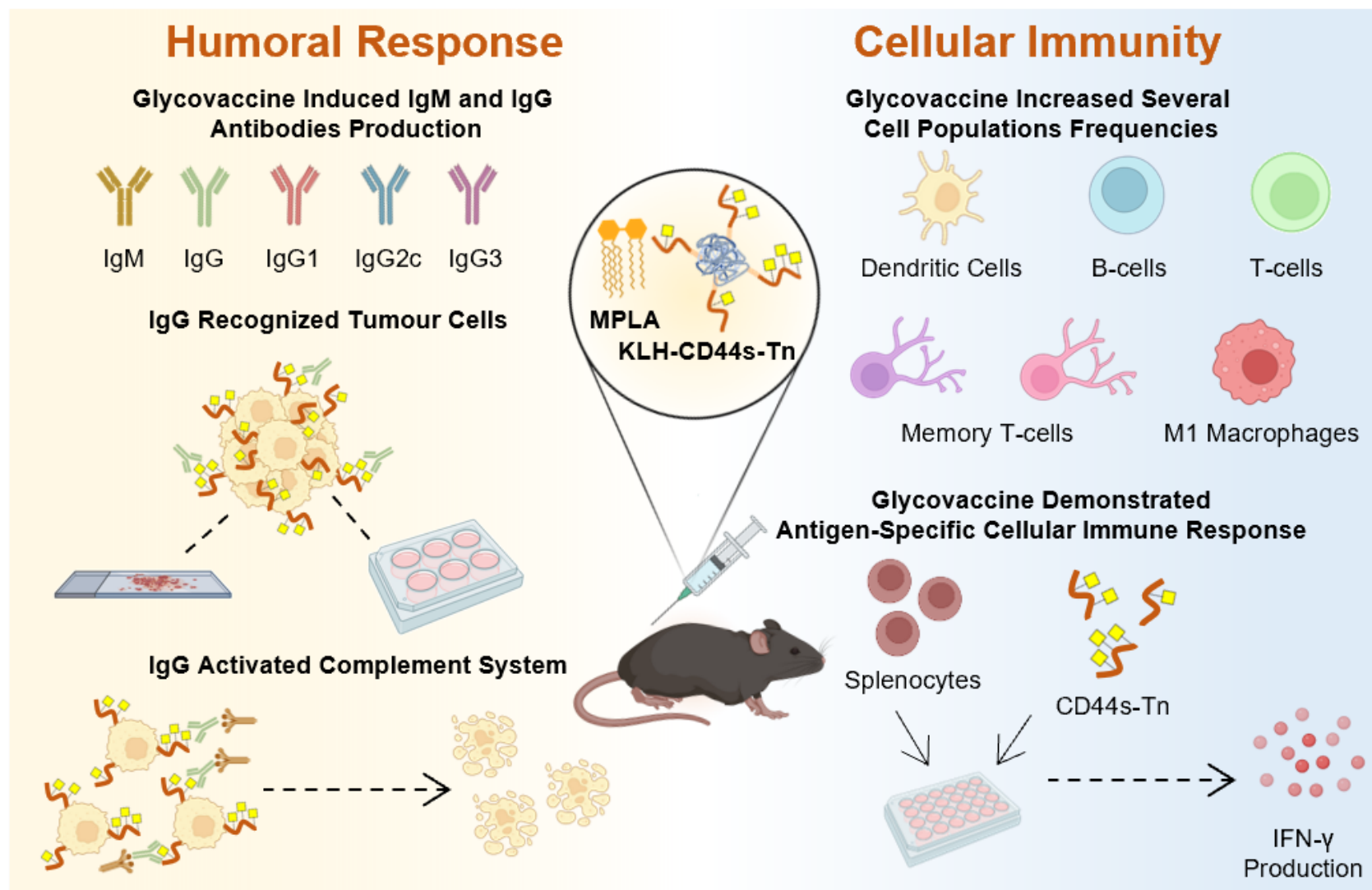
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Abstract

Cancer presents a high mortality rate due to ineffective treatments and tumour relapse with progression. Cancer vaccines hold tremendous potential due to their capability to eradicate tumour and prevent relapse. In this study, we present a novel glycovaccine for precise targeting and immunotherapy of aggressive solid tumours that overexpress CD44 standard isoform (CD44s) carrying immature Tn and sialyl-Tn (sTn) O-glycans. We describe an enzymatic method and an enrichment strategy to generate libraries of well-characterized cancer-specific CD44s-Tn and/or sTn glycoproteoforms, which mimic the heterogeneity found in tumours. We conjugated CD44-Tn-derived glycopeptides with carrier proteins making them more immunogenic, with further demonstration of the importance of this conjugation to overcome the glycopeptides' intrinsic toxicity. We have optimized the glycopeptide-protein maleimide-thiol conjugation chemistry to avoid undesirable cross-linking between carrier proteins and CD44s glycopeptides. The resulting glycovaccines candidates were well-tolerated *in vivo*, inducing both humoral and cellular immunity, including immunological memory. The generated antibodies exhibited specific reactivity against synthetic CD44s-Tn glycopeptides, CD44s-Tn glycoengineered cells, and human tumours. In summary, we present a promising prototype of a cancer glycovaccine for future therapeutical pre-clinical efficacy validation.

Keywords: glycovaccines, cancer immunotherapy, glycoproteoforms, chimeric vaccines, CD44

Graphical Abstract



1. Introduction

Cancer remains one of the deadliest diseases worldwide, with conventional chemotherapy, radiotherapy, and surgery being frequently ineffective against advanced-stage disease and unable to prevent recurrence and metastasis [1]. Although the introduction of immunotherapy, particularly of immune checkpoint inhibitors (ICIs), has modestly improved the survival of relevant patient subsets showing high number of mutations and tumour lymphocytes infiltration, it also has several relevant side effects [1, 2]. These limitations highlight the need for novel, safer, and more efficient therapeutics.

Emerging approaches such as cancer vaccines, which exploit protein neoantigens derived from genetic mutations, dysregulated RNA splicing, disordered post-translational modifications, and integrated viral open reading frames [3], hold great promise in simultaneously eradicating tumours and preventing relapse [4]. However, the low mutational burden of several lesions presents a challenge, requiring the identification of novel neoantigen sources and improved vaccine design [5, 6]. One potential source is the altered glycosylation patterns that accompany cancer progression, resulting in the accumulation of immature Tn and sialyl-Tn (sTn) glycoantigens on the cell surface. These glycoantigens contribute to several cancer hallmarks such as cell motility, invasion, and metastasis and are overexpressed by most advanced solid tumours linked to worst prognosis [7-9]. However, the sparse expression of these glycoantigens in healthy tissues remains an obstacle for precise cancer targeting and vaccine implementation [10, 11]. On the other hand, recent glycoarray-based studies have highlighted the presence of autoantibodies in cancer patients' serum against glycoprotein domains carrying abnormal glycosylation [12]. These seminal observations support the notion that tumour cells express cancer-specific glycoproteoforms that may provide the necessary grounds towards precision oncology. The density and distribution of glycosidic chains in the peptide backbone appear to be key for distinguishing self from non-self, and high autoantibody titers against glycoconjugates have been associated with increased patient survival, suggesting a protective effect [13]. Moreover, glycopeptides can be processed and presented through major histocompatibility class I (MHC-I) with high affinity mediated by glycosylation, eliciting cytotoxic lymphocytes [14, 15]. These findings

support the potential of glycoconjugate vaccines building on the precise identification of cancer unique glycoproteoforms.

Historically, the first chimeric glycovaccine to reach clinical trials resulted from conjugating synthetic sTn motifs to keyhole limpet hemocyanin (KLH) (Theratope vaccine) [16]. However, despite being well-tolerated by patients, the incapacity to significantly improve survival in phase III clinical trials resulted in its cancellation, possibly related to inadequate study design [17]. In recent years, numerous second and third generation glycovaccines capable of inducing more robust immune responses have been synthesized, primarily exploiting abnormally glycosylated Mucin 1 (MUC1) variable number tandem repeats (VNTRs) peptides linked to protein carriers and/or protein carriers plus T cell agonists and/or adjuvants [18, 19]. However, MUC1 is highly overexpressed across human epithelia, making the implementation of glycovaccines based on true neoantigens a pressing need. Another major bottleneck is the difficulty posed by chemical methods to accurately install multiple glycans on the peptide chain, mimicking heterogeneous glycosylation patterns found in tumours [20, 21].

This work proposes a novel approach to cancer vaccine design based on aberrant glycosylation patterns found in cancer cells, with a specific focus on the short CD44 standard splicing isoform (CD44s) [22]. CD44s is overexpressed in several types of cancer and carries abnormal short-chain O-glycosylation [10, 23]. Using a mass spectrometry-based glycoproteogenomics workflow, we comprehensively addressed the CD44s glycoproteome and identified potentially targetable Tn and sTn-glycoepitopes. Herein, we propose an enzymatic method to synthesize libraries of well-characterized CD44s cancer glycoepitopes found in tumours and describe the synthesis of multivalent CD44s glycopeptide-protein chimeras capable of inducing both humoral and cellular immunological responses *in vivo*, foreseeing the pre-clinical bases for a novel, safer and more efficient immunotherapies for cancer patients.

2. Material and Methods

2.1. Enzymatic synthesis of CD44s-glycopeptides epitopes

A CD44s 30-mer peptide (DSPWITDSTDRI PATRDQDTFHPSGGSHHT) and its cysteine-tagged form (at C-terminal) were purchased from GenScript Biotech.

Synthesis of glycopeptides carrying the Tn-epitope (GalNAc- α -O-Ser/Thr) was performed in a 100 μ L reaction mixture containing glycosylation buffer (125 mM sodium cacodylate, 50 mM MnCl₂, pH 7.4; Sigma-Aldrich), 50 μ g UDP-GalNAc (Sigma-Aldrich), 50 μ g of CD44s 30-mer peptide and a polypeptide N-acetylglactosamine transferases (GalNAc-T) mix (0.18 μ g GalNAc-T1, 0.36 μ g GalNAc-T2, 0.36 μ g GalNAc-T3, 1.69 μ g GalNAc-T11; R&D Systems) by overnight incubation at 37 °C. CD44s-Tn glycopeptides were loaded on 80 μ L of agarose-bound *Vicia villosa* agglutinin (VVA) column, for enrichment. The column was washed with 10 column volumes of LAC A buffer (20 mM Tris/HCl pH 7.4, 150 mM NaCl, 1 M urea, 1 mM CaCl₂, MgCl₂, MnCl₂ and ZnCl₂) at 1200 g for 30 s at room temperature (RT). The glycopeptides were then eluted with 3x100 μ L 3% acetic acid. Tn-epitope sialylation, rendering sTn-peptides, was performed by overnight incubation at 37 °C of the Tn-peptides in glycosylation buffer (50 mM MES, 20 mM EDTA, 2 mM DTT, pH 6.5; Sigma-Aldrich) with 0.5 mM CMP-Neu5Ac (Merck Millipore) and 1.41 μ g of a α -N-acetylgalactosaminide α -2,6-sialyltransferase (ST6GalNAc I; R&D Systems). CD44s sialoglycopeptides were purified using the High-SelectTM TiO₂ Phosphopeptide Enrichment Kit (Thermo Fisher Scientific), according to the manufacturer's instructions. Synthesis of CD44s carrying the T-epitope was performed by overnight incubation at 37 °C of the CD44s-Tn peptides in glycosylation buffer (125 mM sodium cacodylate, 50 mM MnCl₂, 10 mM MgCl₂, pH 7.4; Sigma-Aldrich) with 50 μ g UDP-Gal (Sigma-Aldrich) and 0.05 μ g of core 1 β -1,3-galactosyltransferase 1 and its chaperone (C1GalT1/C1GalT1C1; R&D Systems). CD44s-T glycopeptides were loaded on 80 μ L of agarose-bound Peanut agglutinin (PNA) column, for enrichment. The column was washed with 10 column volumes of LAC B buffer (10 mM HEPES pH 7.5, 150 mM NaCl, 1 M urea, 1 mM CaCl₂, MgCl₂, MnCl₂ and ZnCl₂) at 1200 g for 30 s at room temperature (RT). The glycopeptides were then eluted as described above.

2.2. Synthesis of immunogenic glycochimera

To generate immunogenic glycoconjugates, CD44s-Tn and C-terminal cysteine-tagged CD44s glycopeptides were conjugated to Cross Reacting Material (CRM197; Sigma-Aldrich) and KLH (Sigma-Aldrich). Two synthesis strategies were applied. In the first strategy, CRM197 and KLH were linked to m-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS) and then conjugated with CD44s glycopeptides.

In the second strategy, the CD44s glycopeptides were first linked with MBS and then conjugated with the immunogenic proteins. To allow mild reduction of the C-terminal cysteines, tris(2-carboxyethyl) phosphine (TCEP) pH 7.4 was added to 20 µg of CD44s-Tn at a final concentration of 100 µM and incubated for 1 h at 4 °C with agitation. Posteriorly, the reduced glycopeptides were incubated with 0.015 mg/µL MBS (Sigma-Aldrich) in dimethylformamide (DMF; Sigma-Aldrich) at 4 °C overnight under agitation. Subsequently, the functionalized CD44s-Tn glycopeptides were passed through a pre-conditioned 3 kDa centrifugal filter (Amicon Ultra Centrifugal Filters, Merck) with phosphate buffer saline solution (PBS, pH 7.4) to remove the non-functionalized glycopeptides. To obtain immunogenic conjugates, CD44s-Tn-MBS glycopeptides were incubated for 30 min at RT under agitation with 50 µg of KLH (0.01 mg/µL) in PBS (pH 7.4). The solution was further passed through a pre-conditioned 50 kDa centrifugal filter (Amicon Ultra Centrifugal Filters, Merck) with PBS (pH 7.4) to remove the uncoupled glycopeptides. The endotoxin levels in KLH and KLH-CD44s-Tn were measured using the Pierce™ Chromogenic Endotoxin Quantification Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. The size and polydispersity index (Pdl) of glycoconstructs were determined by Dynamic Light Scattering (DLS), while surface charge was determined by Laser Doppler anemometry (LDA). DLS and LDA analyses were performed with a Nano ZS Zetasizer (Malvern, Worcestershire, UK) at 25 °C. Data is presented as the average of the triplicate formulations.

2.3. Synthesis of BSA-conjugates

Cysteine-tagged CD44s, CD44s-Tn, CD44s-T, CD44s-sTn, MUC16 and MUC16-Tn peptides were conjugated to bovine serum albumin (BSA; Sigma-Aldrich), to render BSA-glycoepitopes as previously described by us [20]. Briefly, 0.01 mg/µL BSA in 0.01 M phosphate buffer (pH 7; Sigma-Aldrich) were incubated with 0.015 mg/µL MBS (Sigma-Aldrich) in DMF (Sigma-Aldrich) for 30 min at room temperature under agitation. Then, BSA/MBS mixture were passed through a PD-10 desalting column (Sigma-Aldrich) conditioned with 0.05 M phosphate buffer (pH 6). Desalted BSA/MBS solutions were incubated with 1 µg of peptides or glycopeptides in 25 µL DMF pH 7.0 at 4 °C overnight under agitation, following evaporation in a SpeedVac.

2.4. NanoLC-ESI-MS/MS

Peptide products were characterized by nanoLC-ESI-MS/MS using either a LTQ-Orbitrap XL or a Q-Exactive HF. Chromatography was carried out on a nanoLC system (Dionex, 3000 Ultimate nano- LC). Eluent A was aqueous formic acid (0.2%), while eluent B was formic acid (0.2%) in acetonitrile. Samples were injected into a trapping column (C18 PepMap 100, 5 μ m particle size) and washed with an isocratic flux of 95% eluent A and 5% eluent B at a flow rate of 30 μ L/min. After 3 min, the flux was redirected to the analytical column (EASY-Spray C18 PepMap, 100 $\text{\AA}$, 150 mm x 75 μ m I.D. and 3 μ m particle size) at a flow rate of 0.3 μ L/min. The column temperature was set at 35 $^{\circ}$ C. Glycopeptide separation occurred using a linear gradient of 5–40% eluent B over 30 min. Column wash and re-equilibration were warranted before the following injection. The mass spectrometers were operated in the positive ion mode, with an m/z range from 300 to 2000, with a spray voltage of 1.9 kV and a transfer capillary temperature of 275 $^{\circ}$ C. CID and HCD fragmentation were respectively obtained in the LTQ-Orbitrap XL or Q-Exactive HF. LTQ-Orbitrap settings were tube lens voltage 100 V, full scan resolution 60 000, fragment scan resolution 7500, normalized collision energy 35%, isolation window 2 Da, activation time 30 ms and an activation Q of 0.250. Q-Exactive HF settings were full scan resolution 120000, fragment scan resolution 30000, fragment scan fixed first mass at 110 m/z, normalized collision energy 30%, isolation window 2.0 m/z. Peptides were detected in the form of multiply charged species, which were considered for downstream quantification. Individual glycopeptide relative abundance is presented in relation to the sum-total peptide abundance, calculated based on the chromatogram peak areas and considering the occurrence of triply, quadruply and quintuply charged ions. Glycosite annotation was performed manually after initial searches against the CD44 30-mer sequence with Sequest HT (Proteome Discoverer 2.5; Thermo Fisher Scientific) to identify candidate peptide spectrum matches. Variable modification of serine and threonine residues with HexNAc (+203.0794, GalNAc, Tn antigen) and peptide N-terminal by reaction with MBS linker with the possibility of multiple oxidations were considered.

2.5. Glycosylated domains in KLH glycochimera

Slot blotting was used to assess the conjugation of CD44s glycopeptides to KLH. Nitrocellulose membranes were loaded with 10 μ g of KLH, KLH-MBS, KLH-CD44s-Tn, KLH-CD44s, CD44s, CD44s-Tn, and MBS-CD44s-Tn under vacuum. The

membranes, previously blocked with Carbo-free blocking solution 1x (VectorLabs), were blotted for the Tn antigen with biotinylated VVA lectin (1:5000; VectorLabs) for 1 h at RT and incubated in the Vectastain Elite ABC reagent, Peroxidase (VectorLabs), for 15 min at RT. The Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare Life Sciences) was used as developing reagent. Data analysis was performed through Image Lab Software (Bio-Rad) in a ChemiDoc XRS (Bio-Rad).

2.6. Cell lines and culture conditions

Human BC cell line T24, and GC cell lines AGS, MKN-45 and MKN-74 were purchased from American Type Culture Collection (ATCC). Cells were maintained with RPMI 1640 GlutaMAX™ medium (Gibco) supplemented with 10% (v/v) of fetal bovine serum (FBS; Gibco) and 1% (v/v) penicillin/streptomycin (PenStrep; Thermo Fisher Scientific). Cells were kept at 37 °C in a 5% CO₂ humidified atmosphere.

2.7. Western blot

Total protein extracts from AGS, MKN-45 and MKN-74 were separated in a 4-20% precast polyacrylamide gels (Bio-Rad) and transferred to a nitrocellulose membrane (GE Healthcare Life Sciences). CD44 expression was evaluated with an anti-CD44 antibody (1:5000; 157107 Abcam) 1 h at RT. Followed by incubation with a goat anti-rabbit IgG (H+L) Horseradish peroxidase (HRP) conjugate antibody (1:60000; 656120 Thermo Fisher Scientific) 30 min at RT. Western blot was also used to assess the conjugation of CD44s glycopeptides to CRM197. Accordingly, 10 µg of CRM197, CRM197-MBS, CRM197-CD44s-Tn, CRM197-CD44s-sTn were processed as described above. The membranes, previously blocked with Carbo-free blocking solution 1x, were blotted for the Tn or sTn antigen with biotinylated VVA lectin or SNA lectin (1:5000; VectorLabs) respectively, for 1 h at RT and incubated in the Vectastain Elite ABC reagent, Peroxidase. MGL recognition was evaluated with the Recombinant Human CLEC10A/CD301 protein (1 µg/mL; R&D Systems) 1 h at RT. Then, the anti-his tag HRP conjugate antibody (1:5000; Abcam) was incubated 30 min at RT. The Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare Life Sciences) was used as developing reagent. Data analysis was performed through Image Lab Software (Bio-Rad) in a ChemiDoc XRS (Bio-Rad).

2.8. CRISPR-Cas9 glycoengineered cell models

Two guide RNAs targeting human *C1GALT1* (GTAAAGCAGGGCTACATGAG, ACAACACTTTGTTACAACGC) were cloned in a CRISPR-Cas9-containing dual gRNA expression vector (pRP[2CRISPR]-Puro-hCas; VectorBuilder, Inc). One microgram of mammalian CRISPR vector was transfected in wild-type AGS and T24 cell lines using jetPRIME® transfection reagent according to the manufacturer's instructions. Single clones were obtained by serial dilution in 96 well plates and knock-out (KO) clones were identified by Indel Detection by Amplicon Analysis (IDAA) using ABI PRISM™ 3010 Genetic Analyzer (Thermo Fisher Scientific) and Sanger sequencing. One clone with distinct out of frame indels was selected. Single clones with silent mutations provided phenotypic control cell lines. IDAA results were analysed using Peak Scanner Software V1.0 (Thermo Fisher Scientific).

2.9. Flow cytometry for glycoengineered cell models phenotype confirmation

Cells were detached using Versene solution (Thermo Fisher Scientific), fixed with 1% paraformaldehyde (PFA; Sigma-Aldrich) and screened for Tn and T antigens using fluorescein-labelled lectins (VectorLabs) as VVA (0.01 mg/mL) and Peanut Agglutinin (PNA, 0.01 mg/mL). VVA and PNA lectins were incubated for 1 h in PBS 2% FBS under mild agitation at RT. Sialylated Tn and T antigens expression was determined after treatment with 70 mU Neuraminidase from *Clostridium perfringens* (Sigma-Aldrich) in sodium acetate buffer at 37 °C overnight, under mild agitation. In parallel, unstained cells were used as negative controls. Samples were acquired in a FC500 Beckman Coulter flow cytometer and data analysis was performed using the CXP Software.

2.10. Immunofluorescence for glycoengineered cell models phenotype confirmation

To evaluate short *O*-glycans expressions, T24 and AGS glycoengineered cell models were cultured at low density and fixed with 4% PFA (Sigma-Aldrich), following immunofluorescent staining similar to the flow cytometry protocol. Sialylated glycoforms were evaluated in parallel with samples digested with 50 mU/mL α -neuraminidase from *Clostridium perfringens* for 4 h at 37 °C. After antigen staining, cells were marked with 2.3×10^{-3} $\mu\text{g}/\mu\text{L}$ 4',6- Diamidino-2-

phenylindole dihydrochloride (DAPI; Thermo Fisher Scientific) for 10 min at RT in the dark. All images were acquired on a Leica DMI6000 FFW microscope using Las X software (Leica).

2.11. Human samples and ethics statement

Human samples were obtained in agreement with the principles of the Declaration of Helsinki. Monocytes were isolated from surplus buffy coats from healthy blood donors, kindly provided by the Immunohemotherapy Department of Centro Hospitalar Universitário São João (CHUSJ), Porto, Portugal. Procedures were approved by the Hospital ethical committee (Protocol reference 90/19). Blood donors provided informed written consent that the byproducts of their blood collections could be used for research purposes. All procedures involving the manipulation of tumour tissues were performed under patient's informed consent and after approval of the IPO-Porto ethics committee (references CES-IPOFG-EPE-86R1/017 for bladder cancer and 87/2017 for gastric cancer). Healthy thyroid, liver, gallbladder, testis, lung, stomach, pancreas, colon/small intestine/appendix tissues were also included.

2.12. Human monocytes isolation and dendritic cells differentiation

Human monocytes were isolated from buffy coats from healthy donors, as previously described by Castro *et al* [24]. Briefly, buffy coats were centrifuged (30 min, 1200g, acceleration 5, RT, without brake) and the peripheral blood mononuclear cells (PBMCs) were collected and incubated with Rosette Sep human monocyte enrichment kit (StemCell Technologies), according to manufacturer's instructions. Then, the mixture was diluted 1:1 in PBS supplemented with 2% fetal bovine serum (FBS), layered over Histopaque-1077 (Sigma-Aldrich) and centrifuged (30 min, 1200g, without acceleration and brake, RT). The enriched monocyte layer was collected and washed three times with PBS. For monocyte-dendritic cell (mo-DCs) differentiation, 1×10^6 monocytes were seeded on 6-well plates and differentiated for 5 days in complete RPMI-1640 medium (supplemented with 10% FBS and 1% Pen Strep), supplemented with 50 ng/mL Interleukin 4 (IL-4; ImmunoTools) and granulocyte-macrophage colony-stimulating factor (GM-CSF;

ImmunoTools). Following this procedure, over 80% of differentiated cells were consistently found to be CD11c-positive throughout experiments.

2.13. Dendritic cells metabolic activity

The metabolic activity of mo-DCs stimulated with KLH, glycovaccine (KLH-CD44s-Tn) and monophosphoryl lipid A (MPLA) adjuvanted glycovaccine (KLH-CD44s-Tn plus MPLA) was evaluated using the MTT assay. Accordingly, mo-DCs were seeded into 96 well plates at a cell density of 1×10^4 and exposed to increasing concentrations of glycoformulations (0.1, 1, 10, 50, 100 $\mu\text{g/mL}$). After 24 h exposure, conditioned media was replaced by 100 μL fresh culture medium and 10 μL of a 12 mM 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Thermo Fisher Scientific) stock solution was added to each well, following a 4 h incubation at 37 °C in a humidified chamber. Conditioned medium was removed and 50 μL of DMSO (Sigma-Aldrich) was added to solubilize formazan crystals. Plates were incubated at 37 °C for 10 min and absorbances were read at 540 nm in a microplate spectrophotometer. Positive (untreated cells) and negative controls (cells treated with 1% Triton-X 100 [Sigma-Aldrich]) were included. Thus, the metabolic activity (%) of living cells was calculated through the following equation:

$$\text{Metabolic activity (\%)} = \frac{\text{Absorbance of treated cells}}{\text{Absorbance of positive control}} \times 100$$

2.14. Dendritic cell profile and viability evaluation

The mo-DCs were seeded into 6 well plates at a cell density of 1×10^6 cells and cultured during 24 h with the following formulations: CD44s (20 $\mu\text{g/mL}$), CD44s-Tn (20 $\mu\text{g/mL}$), KLH (50 $\mu\text{g/mL}$), KLH-CD44s-Tn (50 $\mu\text{g/mL}$), KLH-CD44s-Tn (50 $\mu\text{g/mL}$) plus MPLA (2 $\mu\text{g/mL}$), MPLA (2 $\mu\text{g/mL}$). 50 ng/mL LPS (Sigma) was used as mo-DCs activation control. After 24 h of culture, conditioned media were collected and the adhered DCs were detached with PBS on ice for 30 min. After centrifugation (5 min, 1500rpm, 4°C) DCs were resuspended in FACS buffer (PBS, 2% FBS, 0.09% sodium azide) and immune stained for flow cytometry analysis. The mo-DCs differentiation and maturation states were assessed by immunostaining in FACS buffer with anti-CD86-FITC (21480863; ImmunoTools), anti-CD11c-APC (21487116, ImmunoTools) at a dilution 1:25, anti-HLA-DR-PE (21278994; ImmunoTools) at a dilution 1:100, and PE/Cy7-anti-CD45 (368532; Biolegend) at a dilution 1:50, for 40 min at 4 °C. Cell viability was assessed through DAPI staining

(750 ng/mL) staining. Flow cytometry acquisition was performed using Beckman Coulter NAVIOS Flow Cytometer. Data files were analysed using the Infinicyt software (version 1.7, Cytognos SL) to discriminate cell populations, determine the mean fluorescence intensity (MFI) and the percentage (%) of positive cells.

2.15. Animal ethics statement

Animal experiments were carried out in strict accordance with the recommendations of the European Union Directive 2010/63/EU and the National Regulation published in 2013 (Decreto-Lei n.º 113/2013 de 7 de Agosto). *Mus musculus* C57BL/6 were housed at the I3S animal facility. The following Humane Endpoints were established: any signals of distress, suffering or pain, body weight loss greater than 20% of the body mass, dehydration, anorexia, anuria and moribund state, related or not to the experimental procedure.

2.16. Mice and immunization

Male adult 7-week-old C57BL/6 mice (Charles River Laboratories) were housed at the animal care facility for 1 week prior to the experiment to allow acclimatisation of the animals to the facility. The mice were kept 4 per cage in a Makrolon type I cage in a limited access area at a controlled RT, 12h light 12h dark cycles, with food and water *ad libitum*. Environmental enrichment was provided, consisting of corn cob bedding, nesting material, tunnel and chewing blocks. For immunization studies mice were randomly and blindly distributed in 4 groups (n=4 per group). Each group was subcutaneously injected with PBS (sham control), 50 µg of KLH, KLH-CD44s-Tn and KLH-CD44s-Tn plus 20 µg MPLA adjuvant (100 µL). Each mouse was immunized three times at one-week interval; the first immunization occurred on day 0, and the second and third occurred on day 7 and 14, respectively. Mice body weight change was monitored once a week, immediately prior to each the immunizations. One week following the last immunization, mice were euthanized to collect blood and tissues for histology and flow cytometry analysis.

2.17. Serum collection

Blood was collected from all animals prior to immunizations by the tail vein and after the experiment by cardiac puncture into vials containing heparin. Then, blood samples were centrifuged at 2500 rpm for 30 min at RT. Serum fraction was collected and further centrifuged at 1200 rpm for 5 min at 4 °C and stored at 80 °C for further use.

2.18. ELISA Protocol for IgM, IgG and IgG subclasses determination

Collected serum samples of each animal were used to quantify Immunoglobulin G (IgG) and Immunoglobulin M (IgM) antibodies concentration by Mouse IgG/IgM ELISA Antibody Pair Kit assay (STEMCELL Technologies) according to the manufacturer's instructions. Accordingly, serum was diluted to 1:50000 for IgG and 1:100000 for IgM determination, respectively. Briefly, ELISA plates were coated with Mouse IgM or IgG capture antibody overnight at 4°C. Serum was added followed by the addition of the alkaline phosphatase-conjugated detection antibody (1:1000). Plates were washed and developed using *p*-Nitrophenyl Phosphate (pNPP) substrate and the absorbance was read at 405 nm. IgG subclasses were individually determined by Mouse IgG1, IgG2b, IgG2c and IgG3 ELISA Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. Briefly, the capture antibody was dissolved in PBS (pH 7), and then was added to each well of a 96-well microtiter plate. After overnight incubation at 4 °C, the plate was washed with PBS buffer containing 0.05% Tween-20 (PBS-T) twice, and treated with a blocking buffer (1% BSA in PBST) at RT for 2 h. Afterward, the pooled or individual antiserum was added to the coated plates (100 µL per well) in serial dilutions ranging from 1:200 to 1:50000 prepared in 0.5% BSA in PBST and incubated at RT for 2h under agitation. After washing with PBS-T four times, the plates were incubated with a 1:250 diluted solution of HRP-linked goat anti-mouse at RT for 1 h under agitation. The plates were then washed with PBST four times. 100 µL 3,3',5,5'-Tetramethylbenzidine (TMB) solution was added to the plates and incubated in the dark for 15 min. Finally, 100 µL 2N H₂SO₄ solution was added, and the optical density (OD) value was detected using a microplate reader at 450 nm wavelength with 570 nm as a reference.

2.19. IgG and IgM antibodies isolation

Serum IgG and IgM were isolated with protein G-agarose and protein L-agarose, respectively. Initially, agarose beads were equilibrated with PBS 1x (pH 7) and then serums were incubated with the beads for 3h at 4 °C under agitation. Then, the beads were washed with PBS to remove the non-coupled antibodies. The antibodies were recovered in 0.2 M glycine (pH 2.0) and immediately re-equilibrated in PBS using Amicon ultra – 0.5 mL centrifugal 10 kDa filters (Merck) to avoid antibody denaturation and ensure their stability and functionality.

2.20. Histology

Mice liver, spleen, kidneys, and pancreas were collected to assess toxicity induced by the inoculation with different vaccine formulations. All fresh tissue samples were fixed and preserved overnight using a 10% buffered formaldehyde solution. After fixation, tissue specimens were manually dehydrated using a graded ethanol series and xylene, following by impregnation in paraffin at 60 °C (1 h in each step, twice). Then impregnated tissues were embedded in paraffin using a paraffin embedding system. Formalin-fixed paraffin-embedded (FFPE) tissue samples were cut in 3 µm-thick sections on a microtome with a disposable blade and transferred onto specific glass slides to perform H&E (haematoxylin and eosin; Leica) staining. Briefly, FFPE tissue sections were deparaffinized and rehydrated, incubated for 2 and 5 min with eosin and haematoxylin, respectively, followed by quick washing in distilled water. After drying at RT, slides were submerged in xylene and mounted on Entellan. Microscopical analysis was performed to detect histological variations among groups, including necrosis, inflammation, fibrosis, anisokaryosis and additional pathognomonic findings associated with toxicity.

2.21. Lymph nodes and spleen single cell suspension for flow cytometry analysis

After 21 days following the first immunization, the dorsal lymph nodes and the spleen were harvested and stored for a few hours in MACS tissue storage solution (Miltenyi Biotec) and later mechanically macerated in PBS and filtered in a 70 µm cell strainer. The cell suspension was centrifuged at 300g for 7 min at 4 °C. The cell pellet was incubated in 2 mL of Red Blood Cells Lysis Buffer for 15 min at 37 °C, then centrifuged at 300g for 7 min at 4 °C. The cells were resuspended in 500 µL FACS buffer and the cell viability and cell counting were assessed by trypan

blue staining using EVE Automated Cell Counter (NanoEnTek). Lymph nodes and splenocytes were stained with two different monoclonal antibody mixes and analysed by flow cytometry. Prior to staining, cells were counted and then 1 million cells were washed in FACS buffer and pre-treated with Fc blocking agent for 15 min, to minimise non-specific antibody binding. Then, cells were stained in the dark, for 30 min at 4°C, with the following antibodies: CD19-PB(557399; BD Bioscience), CD62L-FITC (565261; BD Bioscience), I-A/I-E-PE (557000; BD Bioscience), CD44-PB (560451; BD Bioscience), CD206-APC (141708; Biolegend) diluted 1:100; CD4-APC-H7 (560181; BD Bioscience), CD11c-PE/Cy7 (117318; Biolegend), CD8-PE-Cy7 (552877; BD Bioscience), CD45-PerCP5.5 (550994; BD Bioscience) diluted 1:50; CD3-PO (560771; BD Bioscience) and F4/80-FITC (LTI MA5-16627; Invitrogen) diluted 1:20. After additional washes, cells were acquired on a NAVIOS Flow Cytometer (Beckman Coulter). The FACS data was analysed using the Infinicyt software (version 1.7, Cytognos SL) to discriminate different cell populations, determine the MFI and the % of positive cells.

2.22. Splenocytes restimulation and flow cytometry analysis

Mice splenocytes were harvested as described above. Cells were resuspended in RPMI 1640 GlutaMAX™ medium supplemented with 10% (v/v) of FBS and 1% (v/v) penicillin/streptomycin. For *ex vivo* antigen restimulation assay, cells were seeded in 24 well plates at 5x10⁵ cells in 500 µL of RPMI. Cells were then stimulated with three concentrations of CD44s-Tn antigen (0.2, 2 and 20 µg/mL) or PBS 1x and kept in culture for 72 h at 37 °C in a 5% CO₂ humidified atmosphere. Then, Phorbol 12-myristate 13-acetate (PMA, 25 ng/mL; Sigma-Aldrich), Brefeldin A (10 µg/mL; Sigma-Aldrich) and Ionomycin (1 µg/mL; Sigma-Aldrich) were added for 4 h. Then, cells were detached and stained for flow cytometry analysis. Prior to staining, cells were pre-treated with Fc blocking agent for 15 min, to minimise non-specific antibody binding. Then, cells were stained in the dark, for 30 min at 4 °C, with the following antibodies: NK1.1-APC (561117; BD Bioscience); CD4-APC-H7 (560181; BD Bioscience); CD8-PE-Cy7 (552877; BD Bioscience), CD45-PerCP5.5 (550994; BD Bioscience) diluted 1:50; CD3-PO (560771; BD Bioscience) diluted 1:25 in FACS buffer. For intracellular staining cells were fixed with 4% PFA for 15 min and permeabilized with 0.3% Triton-X for 10 min. After washing with FACS buffer cells were stained in the dark, for 30 min at

4°C with anti-IFN- γ -PE (554412; BD Bioscience) diluted 1:50 in FACS buffer. After additional washes, cells were acquired on a NAVIOS Flow Cytometer (Beckman Coulter). The FACS data was analysed using the Infinicyt software (version 1.7, Cytognos SL) to discriminate different cell populations, determine the MFI and the % of positive cells.

2.23. IgG reactivity against synthetic chimeric structures and glycopeptides

The specificity of IgG and IgM antibodies for glycosylated domains elicited by the administration of the KLH-CD44s-Tn glycoconjugates was evaluated by slot blot. Nitrocellulose membranes (0.45 μ M) were loaded with 10 μ g of KLH, KLH-CD44s-Tn, KLH-CD44s, BSA-CD44s, BSA-CD44s-Tn, BSA-CD44s-sTn, BSA-CD44s-T, BSA-MUC16, BSA-MUC16-Tn and BSA. The membranes, previously blocked with Carbo-free blocking solution 1x (VectorLabs), were blotted for the IgG or IgM antibodies (1 μ g/mL) isolated from 3 serums derived from mice stimulated with KLH-CD44s-Tn plus MPLA glycoformulation, for 1 h at RT. Serum derived from PBS injected animals was used as control. Then, IgG anti-mouse secondary antibody (1:40000; 115-035-205 Jackson ImmunoResearch) or IgM anti-mouse secondary antibody (1:1000; 62-6820 Thermo Fisher Scientific) were added for 30 min at RT. The Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare Life Sciences) was used as developing reagent. Data analysis was performed through Image Lab Software (Bio-Rad) in a ChemiDoc XRS (Bio-Rad).

2.24. IgG reactivity against bladder and gastric cancer cell lines

IgG specificity against glycans and glycoconjugates on cancer cells was evaluated by flow cytometry using T24 wild type (T24 WT; CD44^{high}; Tn^{negative}) and T24 C1GALT1 KO (T24 C1GALT1 KO; CD44^{high}, Tn^{high}). Gastric cancer AGS wild type cells (AGS WT; CD44^{negative}; Tn^{negative}) as well as AGS C1GALT1 KO (AGS C1GALT1 KO; CD44^{negative}; Tn^{high}) developed specifically for this study were also screened. Secondary anti-mouse antibody was used as control. Briefly cells were stained with the murine IgG isolated from mice primed with the adjuvated glycovaccine for 1 h at RT (1:50) followed by the secondary antibody Alexa Fluor 488 anti-mouse IgG (A11029, Thermo Fisher Scientific) for 30 min at a 1:300 dilution in FACS buffer under mild agitation at RT. Data analysis was performed through CXP Software and results represent the standard deviation of

three independent experiments acquired in a FC500 Beckman Coulter flow cytometer.

2.25. Immunofluorescence

The specificity of isolated IgG from immunized mice was evaluated by immunofluorescence. T24 and AGS wild type cells and glycoengineered cells were cultured at 30% confluence and fixed with 4% PFA (Sigma-Aldrich) for 15 min at RT, following staining with IgG antibodies isolated from serum derived from mice stimulated with the adjuvated glycovaccine, for 1 h at RT (1:50) in PBS 2% FBS. Then, the Alexa Fluor 488 anti-mouse (1:300; A11029 Thermo Fisher Scientific) was used as secondary antibody to detect primary antibodies. Nuclear counterstain was achieved with 2.3×10^{-3} $\mu\text{g}/\mu\text{L}$ (DAPI, Thermo Fisher Scientific) for 10 min at RT in the dark. All fluorescence images were acquired on a Leica DMI6000 FFW microscope using Las X software (Leica).

2.26. IgG reactivity against bladder and gastric tumour tissues

Bladder and gastric tumours obtained from IPO Porto's biobank were screened for CD44s-Tn expression by immunohistochemistry (IHC) using IgG isolated from mice immunized with the KLH-CD44s-Tn chimera plus MPLA. Tumours negative for both CD44 and Tn antigens were used as negative controls. In parallel, a panel of relevant healthy human tissues (thyroid, liver, gallbladder, testis, lung, stomach, pancreas, colon/small intestine/appendix), were also screened. Briefly, 3 μm FFPE tissue sections were deparaffinised, hydrated, and underwent antigen retrieval using a solution of 1 mM EDTA pH8 (VWR) for 20 min in a microwave. After blocking endogenous peroxidases and nonspecific linkage, tissue sections were incubated with 3.5 $\mu\text{g}/\text{mL}$ of mice-isolated IgG at 4 °C overnight in a humid chamber. Detection of IgG binding to the tissue sections was assessed using a two-step IHC polymer-based detection system, according to manufacturer's guidelines (Novolink Polymer Detection System, Leica). All images were acquired on a Motic BA310E microscope (Motic) using the Motic Images Plus 3.0 software (Motic).

2.27. Complement-dependent cytotoxicity (CDC) assay

The functional properties of isolated IgG from immunized mice were evaluated by complement dependent cytotoxicity assay. T24 *C1GALT1* KO cells were cultured in 12-well plates at a cell density of 400,000 cells/well. After washing with PBS 1x, 0.1 µg/mL (RPMI without FBS) of IgG isolated from serum derived from mice stimulated with the adjuvated glycovaccine or PBS was added and incubated at 37 °C for 2 h. Then, cells were incubated with complement human sera in RPMI without PBS (1:10; Sigma-Aldrich) at 37 °C for 1 h. Cells incubated with 2% Triton-X were used as a positive control of cell death. Cells viability was measured using Annexin V Apoptosis Detection kit (BD Biosciences). Annexin V-FITC and propidium iodide (PI) staining was performed at 4 °C for 15 min in Annexin V binding buffer. Flow cytometry acquisition was performed using FC500 Beckman Coulter Flow cytometer and data analysis was performed using the CXP Software.

2.28. Statistical analysis

All experiments were performed at least in triplicates. The results are presented as the mean $\pm$ standard deviation. All graphs and statistical analysis were performed using the GraphPad Prism Software version 9 (GraphPad 9.0.2). One-way ANOVA test was used to compare the different groups. Statistical significance was considered when * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

3. Results

Our overall motivation is the establishment of the structural foundations for the development of a multivalent CD44 cancer glycovaccine candidate. The following sections outline a method for generating a diverse and well-characterized array of glycoepitopes that reflect the structural heterogeneity of tumours. Additionally, the molecular rationale and synthesis of immunogenic glycochimera resulting from the conjugation of CD44s-derived glycopeptides to carrier proteins, are presented. Finally, the study includes preclinical validation *in vitro* and *in vivo* that anticipates future preclinical and clinical validations.

3.1. Glycoepitope libraries for vaccine development

Aiming to create a multivalent CD44s glycovaccine candidate that mimics the heterogeneity of glycoproteoforms found in cancer, we implemented an enzymatic synthesis protocol capable of generating a multitude of glycoepitopes. To accomplish this, we used a 30mer peptide containing the ATR amino acid sequence specific to CD44s as a scaffold for installing Tn and sTn cancer-associated glycans. Inspired by the way glycosylation occurs in living cells, we simultaneously combined in a single pot reaction the sugar nucleotide UDP-GalNAc and four GalNAc-Ts (GalNAc-T1, GalNAc-T2, GalNAc-T3, GalNAc-T11) that catalyse the addition of a GalNAc to serine and threonine residues on the peptide chain, generating the Tn antigen (**Figure 1A**). We elected these enzymes out of a family of 20 ppGalNAc-Ts based on bioinformatics analysis with Isoform Specific O-Glycosylation Prediction (ISOGlyP; <https://isoglyp.utep.edu/>) [25] with the main objective of maximizing glycosite occupancy and to generate glycopeptides with glycosylation patterns frequently found in cell lines and tumours. Reaction yields, according to nanoLC-MS/MS analysis, were approximately 70% (**Figure 1B**). Moreover, we were able to glycosylate up to 6 out of 8 possible glycosites, determined by NetOGlyc (<https://services.healthtech.dtu.dk/services/NetOGlyc-4.0/>) [26] with a predominance of di-glycosylated species (36%), followed by tri- and tetra-glycosylated peptides (**Figure 1B**). Notably, hexa-glycosylated peptides were vestigial. To decrease the percentage of non-glycosylated CD44s peptides to residual amounts (<5%), while increasing the abundance of the glycopeptides, with emphasis on tri- and tetra-glycosylated species (30-35%), we introduced an enrichment step with the VVA lectin with affinity for the Tn antigen (**Figure 1A and B; Table S1**). Based on the analysis of MS/MS spectra, T9 and T15 were the main glycosites in di-glycosylated peptides; T9, T15, T20 and T6, T9, T15 in tri-glycosylated CD44s peptides and T6, T9, T15 and T20 in tetra-glycosylated peptides (**Figure 1D and Figure S1**). These glycopeptides mimic the tumour glycopatterns and serve as scaffolds for further glycan chain elongation [10]. Finally, the addition of CMP-Neu5Ac and ST6GalNAc I to the reaction media almost completely sialylated the Tn antigen, originating a complex mixture of fully sialylated glycoproteoforms (68.17% of total glycosylated species), as well as glycopeptides carrying both Tn and sTn (hybrid species; 15.07%). Predominantly, we generated glycopeptides carrying 2 sTn residues (44.73%) and, to a lesser

extent, 3 sTn residues (14.42%) (**Figure 1 A and C**). Hypersialylated glycopeptides carrying up to 5 sTn residues could also be detected, albeit in residual amounts (<5%) (**Table S2**). For hybrid species, 1 Tn/2 to 3 sTn glycopeptides were the most predominant glycoproteoforms. Enrichment with TiO₂ with affinity for vicinal hydroxyl groups on the Neu5Ac residue increased the percentage of hyperglycosylated proteoforms carrying 3, 4 and 5 sTn residues (**Table S2**). In summary, we describe a simple and easy enzymatic method to generate libraries of Tn and/or sTn glycopeptides at high yields. Although it is possible to resolve and isolate most glycoproteoforms using conventional C18 reverse phase columns, our approach provides a complex mixture of abnormally glycosylated species mimicking the heterogeneity of living systems. This constitutes a relevant milestone to ensure glycovaccine's multivalency.

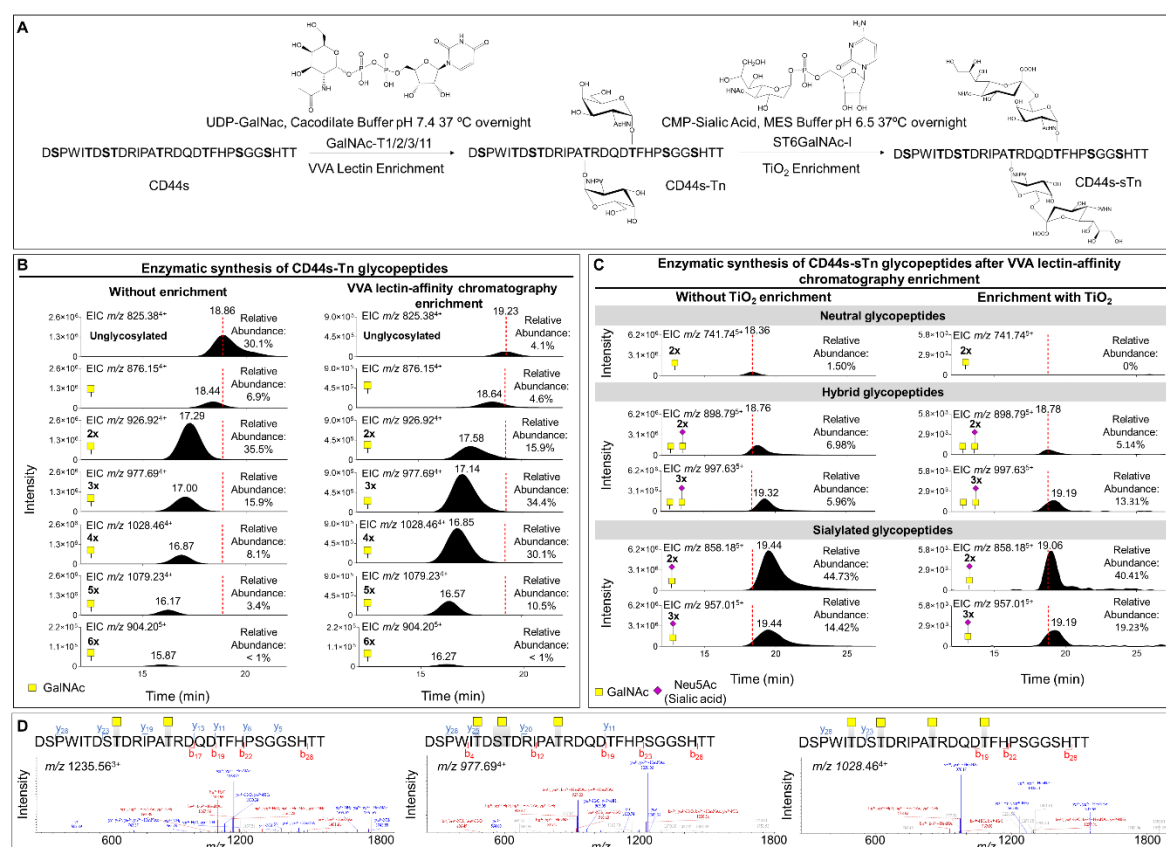


Figure 1. Enzymatic synthesis of CD44s-Tn/sTn glycopeptide libraries for cancer immunotherapy. A) Scheme for the single pot enzymatic synthesis of cancer-associated CD44s-Tn and/or sTn glycopeptides. Briefly, a 30mer CD44 derived peptide containing the ATR amino acid sequence specific of CD44s was combined with sugar nucleotide UDP-GalNAc and a mixture of GalNAc-Ts (GalNAc-T1/2/3/11) that define the peptide glycosites by generating the Tn antigen. For subsequent sialylation, CD44s-Tn

glycopeptides or VVA-enriched CD44s-Tn glycopeptides, ST6GalNAc I and CMP-Neu5Ac were added to the reaction medium, followed by TiO₂ enrichment. **B) CD44s-Tn glycopeptides library obtained before and after enrichment with the VVA lectin.** After synthesis, the products were characterized by nanoLC-MS. CD44s-Tn glycopeptide libraries obtained from the first enzymatic synthesis step with the multiple GalNAc-Ts encompass species with up to 6 occupied glycosites, being the most predominant the di-glycosylated peptides. Enrichment with the VVA lectin with affinity for the Tn antigen significantly decreased to vestigial amounts of the non-glycosylated peptide, and enriched for tri-, tetra-, and penta-glycospecies. **C) CD44s-sTn glycopeptides libraries obtained before and after TiO₂ enrichment.** Non-enriched samples present high abundance of glycopeptides carrying 2 sTn residues, even though 3 sTn glycopeptides could also be observed. Lower amounts of hybrid species carrying both Tn and sTn (>12%) were also detected. This method also suggests an enrichment for species carrying 3 sTn residues. **D) CID-M/MS spectra for the most abundant CD44s-Tn glycopeptides with precise glycosites identification.** Regarding di-glycosylated peptides the main glycosites were T9 and T15, whereas for the tri-glycosylated peptides the T6, S8/T9 and T15 appeared as the preferential glycosites. For the tetra-glycosylated peptide the T6, T9, T15 and T20 appeared as the main identified glycosites.

3.2. Synthesis of glycopeptide-protein chimeras

We focused on prototyping a glycovaccine candidate by exploiting CD44s-Tn glycopeptides. In the path towards vaccine development, we started by assessing the possibility of direct delivery of glycopeptides to immune cells by testing toxicity against mo-DCs. Strikingly, CD44s-Tn glycopeptides induced massive DCs death (**Figure S2**), impeding their direct use as immunogens. One possible explanation is that CD44s and CD44s-Tn peptides may activate apoptosis pathways through interaction with death receptors such as Fas ligand [27, 28]. We further hypothesized that linking the glycopeptide haptens to carrier proteins could hinder the glycoepitopes from being recognized by immune cells' glycan-binding receptors and/or prevent deleterious interactions with membrane components. Also, carrier proteins approved for vaccine applications such as CRM197 and KLH carry multiple T cell epitopes [29], therefore holding potential to induce both humoral and cellular immune responses. The MBS linker was selected to covalently link primary amines on the carrier protein to C-terminal cysteine-tagged CD44s-Tn and/or sTn glycopeptides via a maleimide-thiol conjugation (**Figure 2A**).

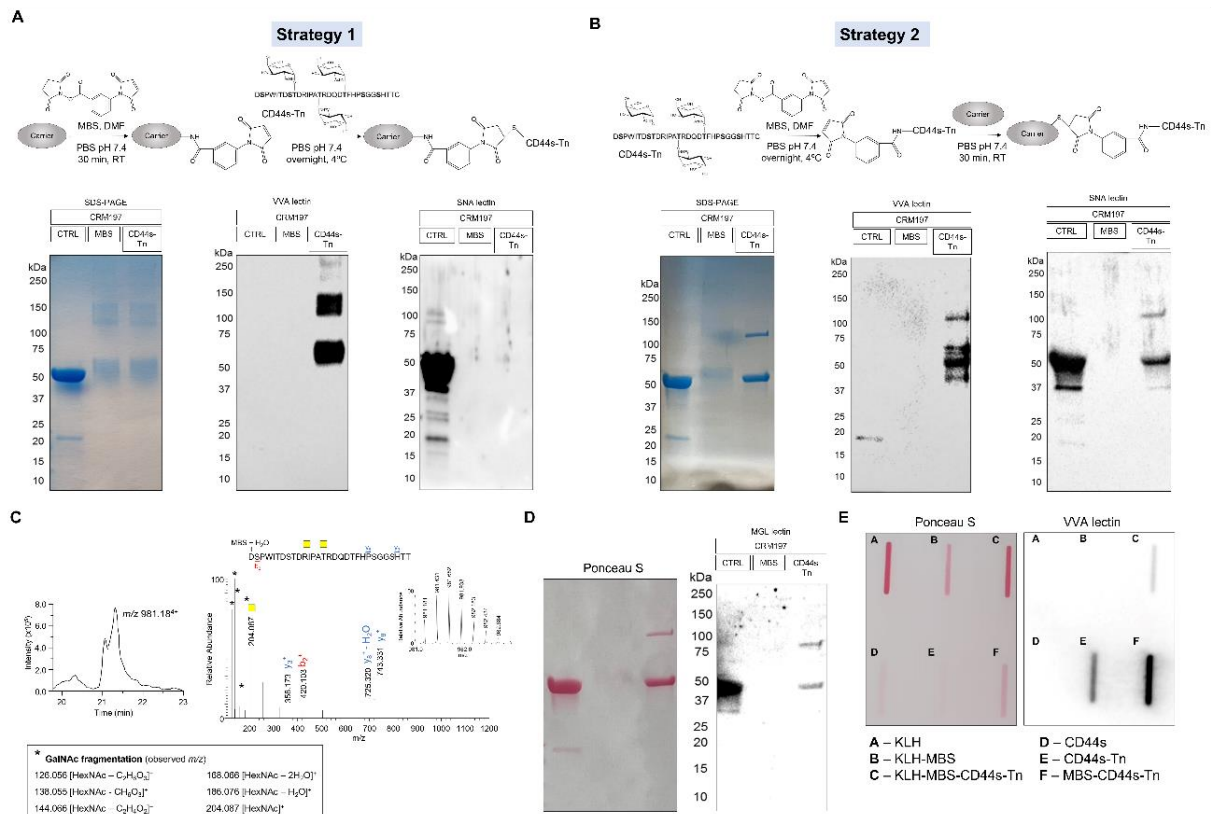


Figure 2. Synthesis of CD44s-Tn glycopeptide carrier protein chimeras exploiting MBS linker. **A) Coupling of carrier protein CRM197 with MBS prior to conjugation with CD44s-Tn glycopeptides induces protein multimerization and loss of glycosylation.** In strategy 1, carrier protein CRM197 was linked with MBS prior to CD44s-Tn conjugation. Several high molecular bands in SDS-PAGE gel suggested undesired protein-protein cross-linking induced by MBS. Nevertheless, VVA blotting confirmed the successful conjugation of the protein with the glycopeptides with the conservation of the Tn residue. In addition, SNA showed only reactivity for the non-conjugated CRM197, supporting MBS conjugation with sialic acids and undesired alterations of the protein glycosylation patterns. **B) Coupling of CD44s-Tn with MBS prior to conjugation to CRM197 prevents carrier protein multimerization while preserving glycosylation of the carrier protein.** The second conjugation strategy involved the functionalization of CD44s-Tn glycopeptides with MBS prior to CRM197 conjugation. This strategy avoided undesired CRM197 cross-linking while maintaining the availability of CD44s-Tn for VVA recognition. In addition, this strategy allowed the recognition of naturally expressed sialic acids in CRM197 by the SNA lectin. **C) NanoLC-HCD-MS/MS analysis confirming the binding of MBS to the N-terminal of CD44-Tn glycopeptide with preservation of the GalNAc residue.** **D) CRM197-CD44s-Tn glycochimerae are recognized by immune system lectin MGL.** The glycochimerae synthesized according to strategy 2 are recognizable by an immune system lectin expressed by APCs. **E) VVA blotting confirming the conjugation of CD44s-Tn to the**

carrier protein KLH. The KLH-CD44s-Tn glycochimera was synthesized following strategy 2 and VVA slot blotting confirmed the presence of the Tn antigen.

In exploratory studies, we found that MBS-functionalized CRM197 proteins generated multiple bands in SDS-PAGE more than twice its original molecular weight (**Figure 2A**). These findings strongly suggested undesired protein-protein cross-linking induced by MBS [30]. Nevertheless, VVA blotting on CRM197-MBS-CD44s-Tn glycochimeras confirmed the availability of Tn antigen, which was not observed in the controls (CRM197 and CRM197-MBS) (**Figure 2A**). This demonstrated successful carrier protein conjugation to Tn-glycopeptides with conservation of the glycan motif for recognition by targeting molecules. However, this approach had limitations associated with the use of the MBS linker, widely applied for easy protein-protein conjugation under click-chemistry settings. Furthermore, it completely changed the glycosylation pattern of the original carrier protein, namely by leading to a loss of sialic acids, as translated SNA lectin blotting (**Figure 2A**). To explore alternative synthesis routes, we assessed the possibility of activating the haptens with MBS prior to conjugation with the carrier protein (**Figure 2B**). Using nanoLC-MS/MS analysis, we first followed the reaction products for the di-glycosylated CD44s peptide, which was the most abundant glycospecies in non-VVA purified samples (**Figure 1B**). Studying non-purified samples allowed us to also follow reactions occurring at the non-glycosylated peptide, providing a relevant control. We found that the non-glycosylated peptide was preferentially conjugated to MBS via the glycopeptide N-terminal (**Figure S3**). Notably, we observed high levels of MBS hydration and, to a lesser extent, signs of oxidation, most likely of the peptide chain (**Table S3**). Similar observations were made for the di-glycosylated CD44s peptide (**Table S3** and **Figure 2C**). MS/MS spectra analysis confirmed MBS conjugation to the peptide N-terminal in both non-glycosylated (**Figure S3**) and glycosylated peptides (**Figure 2C**). These spectra also showed that the GalNAc residues in the glycopeptides were conserved, as indicated by the oxonium ion at m/z 204.087 and typical GalNAc cross-ring fragmentations [25], without any evidence of sugar derivatization. Collectively, these observations strongly suggest that MBS-linked glycopeptides may then be binding to the protein carrier via its thiol groups. Additional experiments using a CD44s cysteine-tagged peptide carrying a thiol group potentially targetable by MBS also showed the N-terminal as the main route for conjugation, irrespective of the presence of

glycosylation (**Table S3** and **Figure S3**). Interestingly, in addition to the previously identified modifications, many of the cys-tagged glycopeptides also exhibited thiol group oxidations (**Table S3**), reinforcing preferential targeting of the N-terminal by MBS. Collectively, these findings strongly support that N-terminal MBS-activated glycopeptides may than be targeting the thiol groups on the carrier protein.

In summary, we found that previous coupling of CD44s glycopeptides with MBS avoided undesired protein carrier cross-linking, while maintaining the MBS-linked glycopeptide haptens on the CRM97 available for recognition by VVA. Furthermore, the naturally occurring sialic acids on the carrier protein were preserved using this strategy (**Figure 2B**). Immunoblotting with the Macrophage Galactose-type Lectin (MGL), a well-known receptor expressed by antigen presenting cells and a known ligand for the Tn antigen showed reactivity (**Figure 2D**), suggesting that the glycosidic domains of the glycopeptide haptens could be recognized by the immune system [31]. Building on the optimized synthesis method, we have also developed a KLH-MBS-CD44s-Tn glycochimera, which due to the high immunogenicity of the protein carrier may potentially improve CD44s-Tn presentation to the immune system [32]. Successful conjugation was again confirmed by blotting for VVA (**Figure 2E**). Finally, we confirmed that these formulations presented endotoxin levels below admissible values for *in vitro* and *in vivo* testing (0.5 EU/mL) [33] (0.18 ± 0.005 EU/mL for KLH and 0.21 ± 0.003 EU/mL for KLH-CD44s-Tn) (**Figure 3A**). Moreover, administration of the glycopeptides in the form of glycoconjugates did not show any signs of toxicity against human mo-DCs (**Figure 3B**), in clear contrast with the glycopeptides (**Figure S2**).

3.3. KLH-CD44s-Tn chimera physicochemical properties

DLS measurements indicated that KLH presented an average size of 419.3 nm, a Pdl of 0.512 and a slightly negative zeta potential of -14.5 mV (**Table S1**). Notably, three distinct populations were obvious at 107.1-123.1 nm, 477.3-591.3 nm c 4690-5217 nm (**Figure S4**). This indicated some degree of self-association [34], as previously reported for this protein at physiological pH [35]. Conjugation with glycopeptides did not result in statistically significant alterations in size, polydispersion, and charge, with the KLH-CD44s-Tn measuring 327.9 nm, exhibiting a Pdl of 0.583, and a negative zeta potential of -14.0 mV (**Table S1**). Interestingly, the presence of MPLA adjuvant increased their median size to 532.9

nm, Pdl to 0.657, and negative charge to -15.6 mV (**Table S1**), possibly due to interactions between KLH and MPLA. This suggests that the glycochimera and adjuvant may be delivered together. Notably, all formulations maintained a negative charge, indicating that the conjugation process does not impact protein stability. The existence of three populations of distinct sizes was also conserved during conjugation and in the presence of MPLA. Populations around 100 nm are expected to be preferentially internalized by DCs [36], while those around 500 nm have been described to remain in circulation for longer periods and populations above 1000 nm to be rapidly eliminated [37]. Accordingly, more than 70 % of the KLH could be possibly targeting DCs (**Figure S4**) and maintained in circulation for longer periods, irrespectively of glycoconjugation and/or MPLA co-administration.

3.4. Dendritic cells activation *in vitro*

We further assessed the capacity of the KLH-MBS-CD44s-Tn chimera to activate dendritic cells *in vitro*. We found that dendritic cell activation, as indicated by an increase in CD86 and HLA-DR expression (both in terms of percentage and MFI), could only be achieved upon co-administration of the glycovaccine together with MPLA (KLH-CD44s-Tn + MPLA), a clinically approved vaccine adjuvant [38] (**Figure 3C**). Notably, neither KLH-MBS-CD44s-Tn alone nor in co-administration with MPLA induced cell death, supporting their suitability for *in vivo* applications (**Figure 3B**).

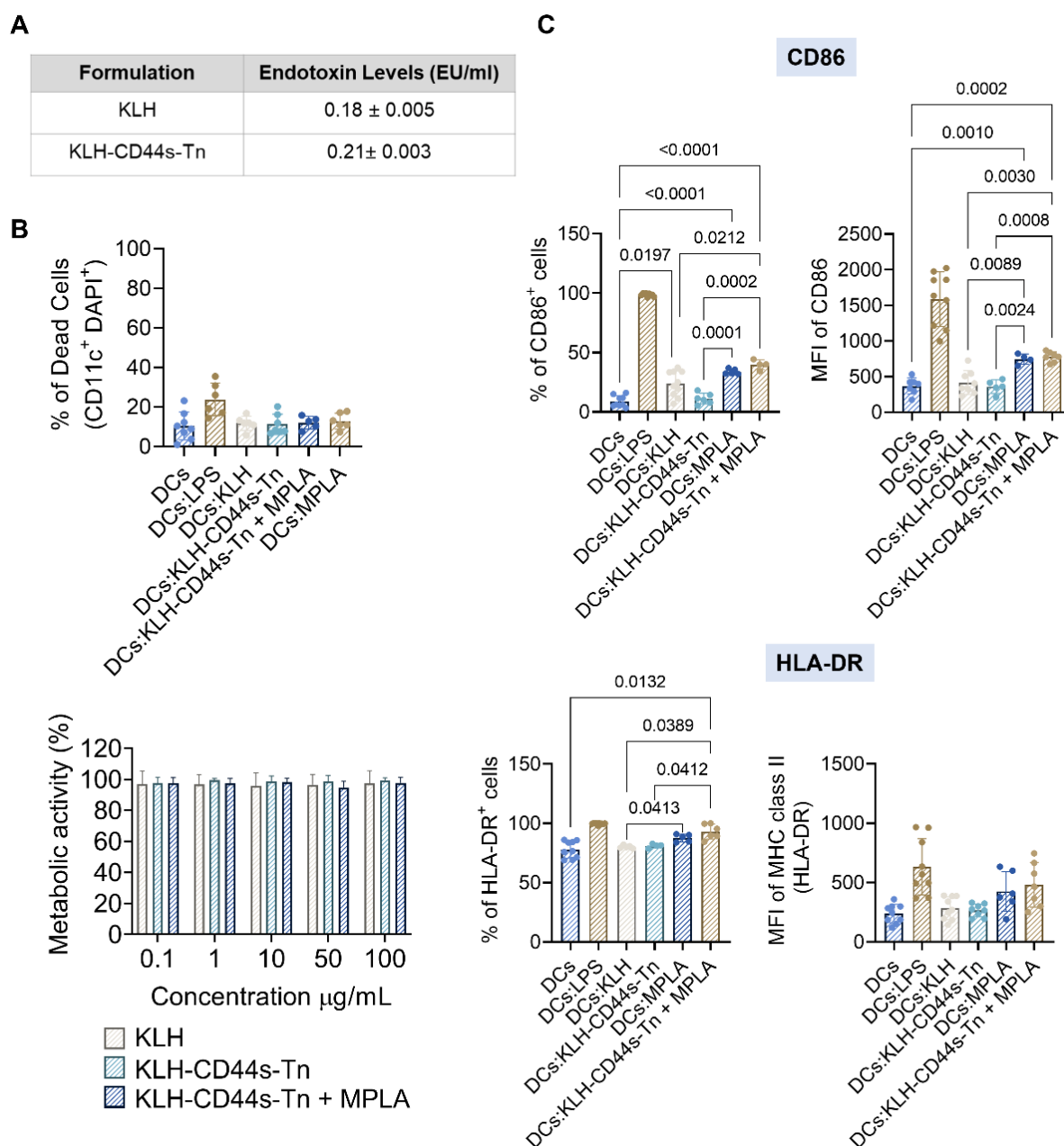


Figure 3. The KLH-CD44s-Tn glycovaccine showed no toxicity *in vitro* and was able to stimulate mo-DCs in the presence of MPLA adjuvant. A) The endotoxin levels for KLH and KLH-CD44s-Tn glycovaccine are below admissible limits (0.5 EU/ml) for *in vitro* and *in vivo* testing. B) KLH-CD44s-Tn glycovaccines are non-toxic to mo-DCs. The non-toxic nature of the glycochimera was confirmed by MTT and low DAPI incorporation assessed by flow cytometry. C) KLH-CD44s-Tn glycovaccine was able to stimulate mo-DCs in the presence of the MPLA adjuvant. Maturation markers CD86 and HLA-DR evaluation showed that KLH-CD44s-Tn alone did not stimulate mo-DCs, requiring the addition of MPLA. The results correspond to the mean and standard deviation. Data are

representative of at least 3 independent experiments. All comparisons were performed using the One-way ANOVA test.

3.5. KLH-MBS-CD44s-Tn glycovaccine does not induce signs of toxicity *in vivo*

The KLH-MBS-CD44s-Tn glycovaccine was then administered subcutaneously to immune-competent C57BL/6 mice. The mice were injected with PBS (Sham Control), KLH, KLH-MBS-CD44s-Tn with or without MPLA adjuvant three times at seven-day intervals, and finally euthanized seven days after the last immunization, as depicted in **Figure 4A**. Notably, the mice showed physiological well-being and no signs of distress over this time, as revealed by a gradual increase in weight in all groups (**Figure 4B**). Also, histological evaluation of relevant organs (spleen, lung, pancreas, kidney) at the end of the experiment revealed no signs of injury or inflammation, reinforcing the safety profile of the formulations (**Figure S5A**). Moreover, vaccinated mice did not show signs of acute inflammation, as evidenced by no changes in serum concentrations of C-reactive protein compared to the controls (**Figure S5B**). Also, these concentrations were maintained within ranges reported in the literature [39]. Following sacrifice, serum was collected to assess antibody titration and the specificity of the antibody responses against glycoconjugates, whereas dorsal lymph nodes and spleen were also collected for evaluation of cellular immune responses.

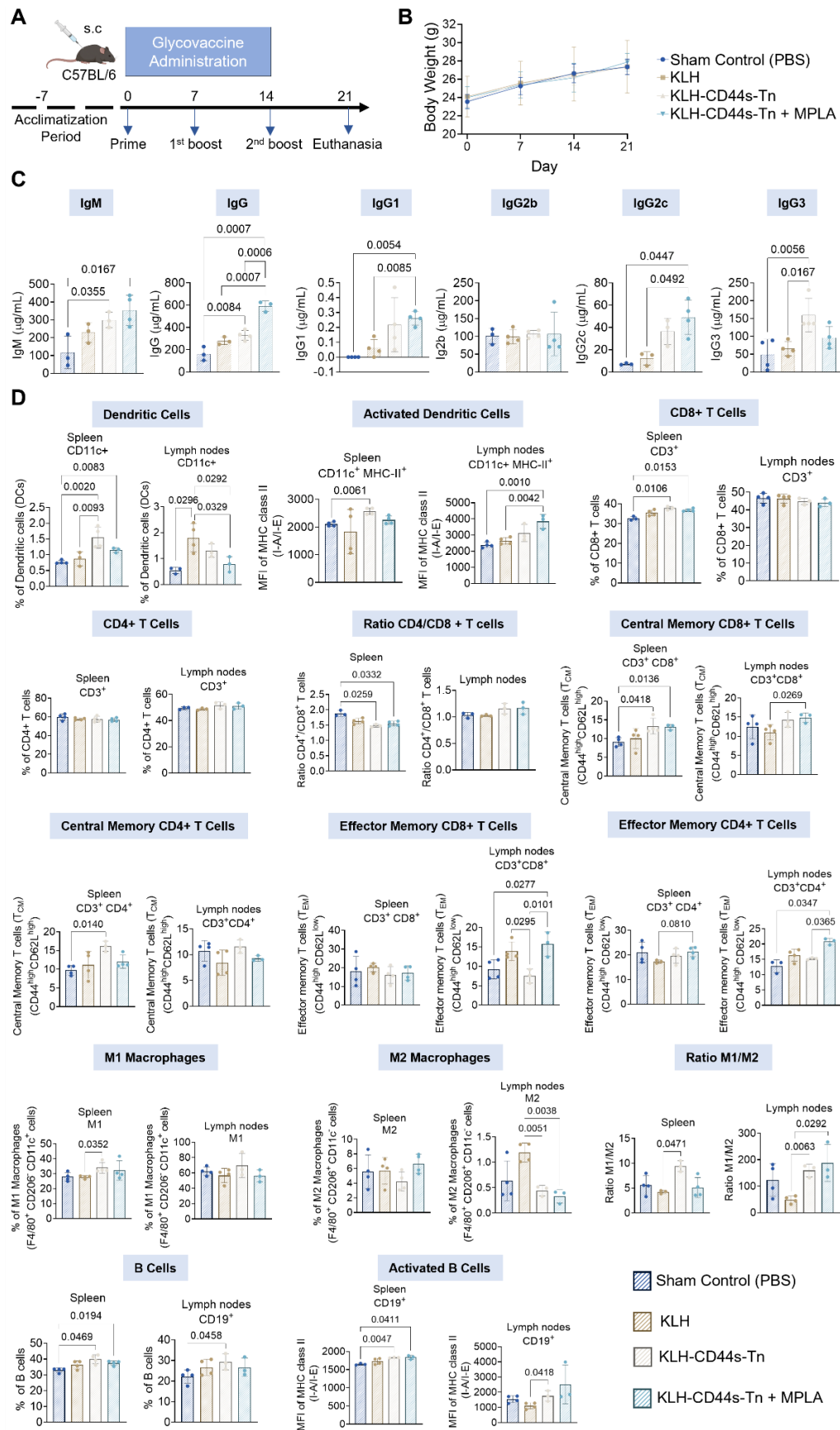


Figure 4. The glycovaccine was proven to be safe *in vivo* and elicited both humoral and cellular immune responses, including cellular memory. A) Immunization scheme of C57BL/6 mice. After 1 week of acclimatization, mice were subcutaneously administered with the formulations three times at seven-day intervals and euthanized seven days after the last immunization. **B) Body weight evaluation during the experimental period.** Mice from all groups increased in body weight during the 21 days of the evaluation, which suggests the safety of the glycovaccine. **C) Evidence of humoral response against KLH-CD44s-Tn and CD44s-Tn after glycovaccine administration.** Both adjuvanted and non-adjuvanted glycovaccine stimulated IgG and IgM production, being this effect highlighted with the adjuvanted formulation. The fact that IgG titers were higher in KLH-CD44s-Tn groups in comparison to KLH strongly suggest that specific responses are being mounted against the CD44s-Tn glycoepitope. Higher values of IgG than IgM suggest an antibody class switch. In addition, mice immunized with the glycovaccine produced IgG1, IgG2c and IgG3 antibodies. The higher values of IgG2c antibodies facing IgG1 suggest mainly Th1 immune responses and T cell-dependent response. **D) Demonstration of cellular immunity against KLH-CD44s-Tn and CD44s-Tn following glycovaccine administration.** The fact that cellular response was higher in KLH-CD44s-Tn groups in comparison to KLH strongly suggest that specific responses are being mounted against the CD44s-Tn glycoepitope. Flow cytometry analysis of cells derived from the spleen and lymph nodes showed that the glycovaccine was able to stimulate lymphoid immune cells, namely CD8⁺ T cells, and both central and effector memory T cells and myeloid immune cells namely dendritic cells, with an increase in M1 macrophages and a decrease in M2 macrophages. The results are presented as the mean and standard deviation. Data are representative of at least three independent experiments. All comparisons were performed using the One-way ANOVA test.

3.6. KLH-MBS-CD44s-Tn glycovaccine induced humoral immune responses *in vivo*

The sera of mice immunized with the glycovaccine and corresponding controls were initially screened for IgM and IgG. We observed significantly higher levels of both types of immunoglobulins following glycovaccination compared to the sham control (**Figure 4C**). Notably, the increase in IgG levels was more pronounced in the group that received MPLA as an adjuvant. Additionally, we also detected antibody responses against KLH. The evaluation of IgG antibody isotypes revealed that mice immunized with the KLH-CD44s-Tn glycovaccine produced IgG1, IgG2c, and IgG3 antibodies (**Figure 4C**). Mice immunized with the adjuvanted

glycovaccine (KLH-CD44s-Tn + MPLA) showed mainly an increase in the levels of IgG1 and IgG2c antibodies, with significantly higher concentrations when compared to the sham control and KLH formulations, indicating a glycopeptide-specific humoral response. The higher concentration of IgG2c antibodies compared to IgG1 suggests that the adjuvanted glycovaccine could stimulate mainly Th1 immune responses [40, 41]. IgG2c antibodies are associated with a stronger T cell-dependent response and Fc γ R-mediated activity [40], suggesting that our adjuvanted glycovaccine elicited antibodies capable of activating antibody-dependent cellular cytotoxicity. Interestingly, a higher concentration of carbohydrate-related IgG3 antibodies was detected in the non-adjuvanted glycovaccine, suggesting a T cell-dependent response and IgG-mediated effector functions in the early response [40, 42]. Of note, no significant alterations were observed for IgG2b upon vaccination with KLH-CD44s-Tn alone or combined with MPLA, which reinforces a T cell-dependent immune response since this class of antibodies is associated with a T cell-independent humoral response [40].

3.7. Glycovaccine induced alterations in adaptive and innate immune cell compartment *in vivo*

To investigate the impact of the KLH-CD44s-Tn glycovaccine on cellular immune response, we analysed single cells obtained from dissociated spleens and lymph nodes of mice by flow cytometry (**Figure S6 and S7**). The glycovaccine induced an immune response involving lymphoid (T-cells and B-cells) and myeloid immune cells (dendritic cells and macrophages). Effective cancer vaccines need to stimulate both humoral and cellular responses, which requires the activation of antigen-presenting cells (APCs), particularly dendritic cells, the most specialized APCs [43]. We observed an increase in the percentage of dendritic cells in the spleen following administration of the glycovaccine. Furthermore, the adjuvanted glycovaccine (KLH-CD44s-Tn + MPLA) elicited higher dendritic cell activation (CD11c⁺ MHC-II⁺) in the lymph nodes, suggesting improved presentation of CD44s-Tn to T-cells (**Figure 4D**). Both glycovaccine formulations increased the CD8⁺ T cell population in the spleen, suggesting a potential anti-tumour response resulting from CD44s-Tn presentation to T-cells via MHC class I. However, no significant changes were observed in CD4⁺ T cell frequencies, although the higher levels of IgG antibodies suggest antigen presentation to CD4⁺ helper T cells passively via

MHC class II and subsequent B cell activation (**Figure 4D**). The glycovaccine induced a higher percentage of central memory CD8⁺ T cells (CD44^{high} CD62L^{high}) in both spleen and lymph nodes, with the adjuvanted glycovaccine inducing more pronounced effects (**Figure 4D**). Additionally, the glycovaccine increased the percentage of central memory CD4⁺ T cells (CD44^{high} CD62L^{high}) in the spleen, suggesting long-term protection against CD44s-Tn. The adjuvanted glycovaccine also increased effector memory CD4⁺ T cells (CD4⁺ CD44^{high} CD62L^{low}) in both the spleen and lymph nodes and CD8⁺ T cells (CD8⁺ CD44^{high} CD62L^{low}) in lymph nodes (**Figure 4D**). In contrast, effector memory CD4⁺ T cells remained unchanged, while effector memory CD8⁺ T cells decreased in mice vaccinated with the glycovaccine alone compared to PBS and adjuvanted administrations (**Figure 4D**). These observations may be potentially attributed to the suppression of KLH T-cell immunogenic epitopes [32] responsible by triggering effector memory by conjugation with the glycopeptide, a phenomenon rescued by introducing the adjuvant. Nevertheless, the glycovaccine also increased the percentage of B cells (CD19⁺) in both spleen and lymph nodes, with higher percentages obtained with the glycovaccine alone (KLH-CD44s) in both organs (**Figure 4D**). Notably, the percentage of activated B cells (CD19⁺ I-A/I-E⁺) was higher in the lymph nodes and spleen following administration of glycovaccine alone as well as adjuvanted, indicating B cell activation and suggesting class switching resulting in increased IgG antibodies [44]. In addition, the glycovaccine increased the percentage of pro-inflammatory M1-like macrophages (F4/80⁺ CD206⁻ CD11c⁺) in the spleen, which are known to have the ability to kill tumour cells, suggesting a potential anti-tumour immune response (**Figure 4D**). Both adjuvanted and non-adjuvanted glycovaccine reduced the percentage of anti-inflammatory M2-like macrophages (F4/80⁺ CD206⁺ CD11c⁻) in the lymph nodes, indicating a rather pro-inflammatory response following vaccination (**Figure 4D**). Accordingly, a higher M1/M2 ratio was observed upon glycovaccine alone in the spleen and higher values for both glycovaccine formulations in the lymph nodes. Of note, when compared to KLH administration, the glycovaccine increased the percentage of B cells and activated B cells, while the adjuvanted glycovaccine increased frequencies of activated DCs, central memory CD8⁺ T cells, effector memory CD4⁺ T cells and increased M1/M2 macrophage ratio, indicating a KLH-independent immune response (**Figure 4D**). Taken together, our results demonstrate that the KLH-CD44s-Tn glycovaccine induced potentially long-lasting cellular immune response by eliciting

immunological memory with the additional potential anti-tumour effect by activating innate and adaptative immune cells, associated with tumour clearance. Future studies must address the durability of the immunological memory to envisage protective effects. Strikingly, we did not find significant increase in any of the evaluated cellular responses in mice administered solely with KLH, strongly supporting the induction of glycoconjugate-specific immune response.

3.8. Antigen-specific cellular response

To assess the KLH-CD44s-Tn glycovaccine's capacity to elicit antigen-specific cellular responses, we examined IFN- γ production in mice splenocytes upon *ex vivo* glycoantigen restimulation, as illustrated in **Figure 5A**. Controls included mice challenged with PBS and KLH. Notably, the KLH-CD44s-Tn glycovaccine, either alone or adjuvated with MPLA, specifically induced IFN- γ (a cytokine that potentiates both innate and adaptative immune responses) production upon antigen restimulation in CD4⁺ and CD8⁺ T cells, as well as Natural Killer (NK) cells (**Figure 5B**). Significant differences in IFN- γ production were observed, particularly at the highest concentration of CD44s-Tn glycopeptide, indicating a concentration-dependent effect. This effect was absent in the control groups. The activation of T as well as NK cells further suggests stimulation of both innate and adaptive immune responses [39, 40]. Overall, our glycovaccine elicited cellular responses against the CD44s-Tn glycoantigen, as evidenced by specific IFN- γ production upon antigen restimulation. Importantly, the absence of a significant increase in IFN- γ production in mice administered with PBS and KLH strongly supports the induction of a CD44s-Tn-specific immune response.

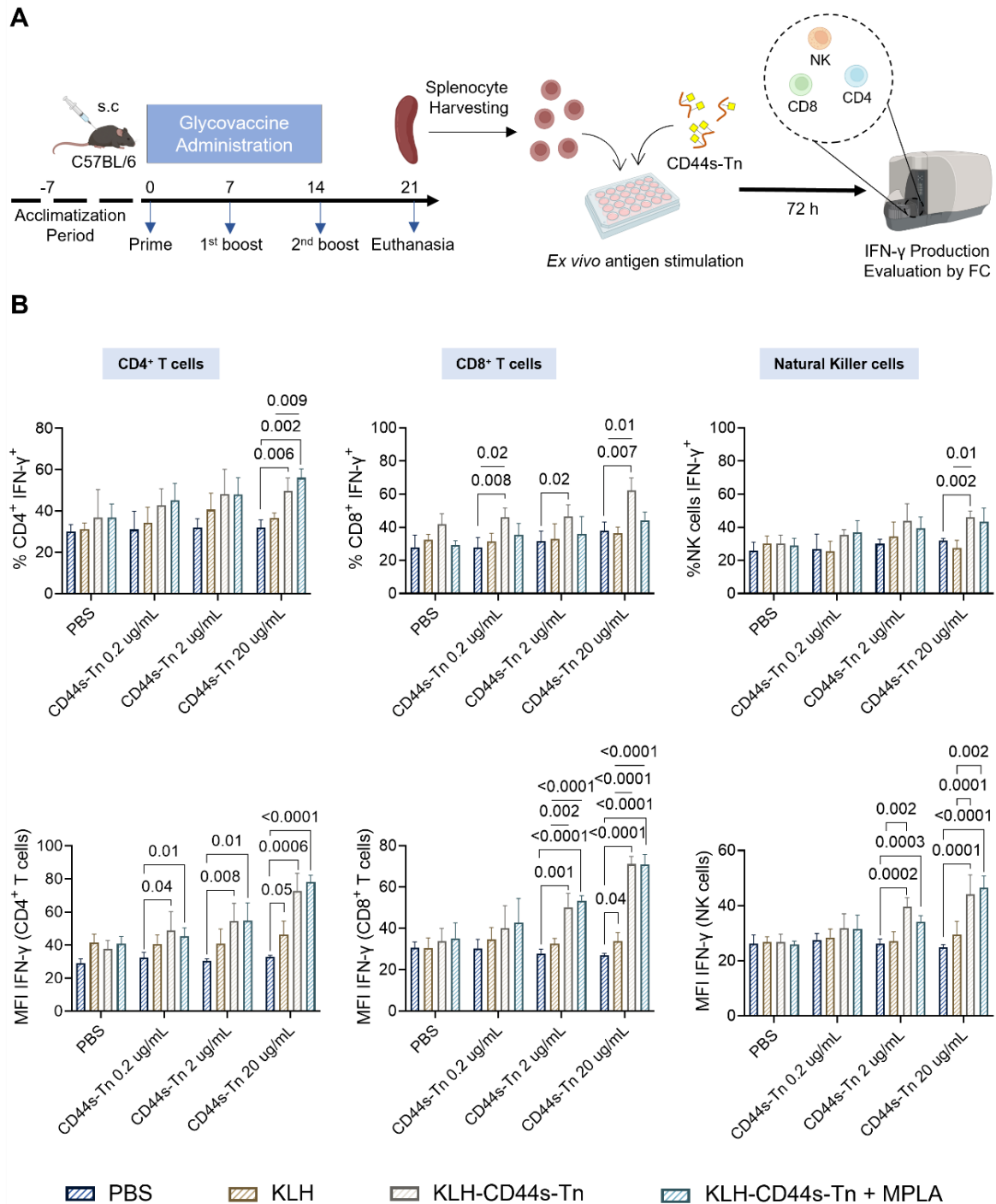


Figure 5. CD4⁺ and CD8⁺ T cells, as well as NK cells from vaccinated mice, specifically produced IFN-γ upon glycopeptide stimulation in a concentration-dependent manner. A) The immunization scheme of C57BL/6 mice and splenocyte stimulation with CD44s-Tn glycopeptides. After three glycovaccine administrations, mice splenocytes were harvested and cultured, stimulated with CD44s-Tn. After 72h, the production of IFN-γ was evaluated by flow cytometry in CD4⁺, CD8⁺ T cells, and NK cells. Splenocytes from mice stimulated with PBS and KLH were used as controls **B) Glycovaccinated mice displayed a**

cellular response against the CD44s-Tn glycoantigen. Flow cytometry analysis of mice splenocytes from glycovaccinated mice showed that CD4+, CD8+ T cells, and NK cells produced IFN- γ upon glycopeptide stimulation, with the response increasing with glycopeptide concentration. This effect was not observed in mice vaccinated with PBS and KLH. The results are presented as the mean and standard deviation of five independent replicates. All comparisons were conducted using the one-way ANOVA test.

3.9. KLH-MBS-CD44s-Tn vaccination induced the generation of specific antibodies against glycodomains

IgG were isolated from serum using protein G agarose, while IgM were recovered by affinity to protein L agarose and then screened for specificity for glycans and glycoconjugates. To achieve this objective, we constructed a slot-array that included CD44s-Tn and KLH-MBS-CD44s-Tn synthesized as described previously. Controls, such as CD44s peptides devoid of glycosylation, BSA, KLH-MBS-CD44s, KLH, MUC16-Tn, and MUC16, were also included. We found that IgM reacted more against CD44s-Tn than MUC16-Tn, but not to the corresponding glycosylated peptides, supporting peptide backbone dependent glycan recognition (**Figure 6A**). Reactivity against non-glycosylated BSA was also not observed, reinforcing these observations. In addition, we found high IgM titers against chimeric KLH constructs carrying either CD44s or CD44s-Tn, most likely linked to immunity against the carrier protein, as also suggested by observations for KLH alone. Interestingly, high IgG reactivity was found for CD44s-Tn in comparison to MUC16-Tn (**Figure 6A**), suggesting that to some extent, the generation of glycopeptide-specific responses occurred, which requires future confirmation. Again, no responses against non-glycosylated species (CD44s, MUC16, or BSA) were found (**Figure 6A**). Interestingly, IgG generation against KLH and chimeric constructs was not significant and was only observed in one mouse. Finally, we assessed IgG reactivity against other types of glycans mounted on CD44s peptides and found that the generated antibodies could also detect, although to a lesser extent, the Tn sialylated glycoform sialyl-Tn (sTn), which is also present in aggressive tumours (**Figure S8**). Notably, little reactivity was observed for CD44s carrying longer T antigens (**Figure S8**). In summary, these observations strongly suggest that IgG and IgM can specifically recognize aberrant Tn-glycosylated peptides and possibly induce specific anti-tumoral responses against CD44s-Tn expressing cells.

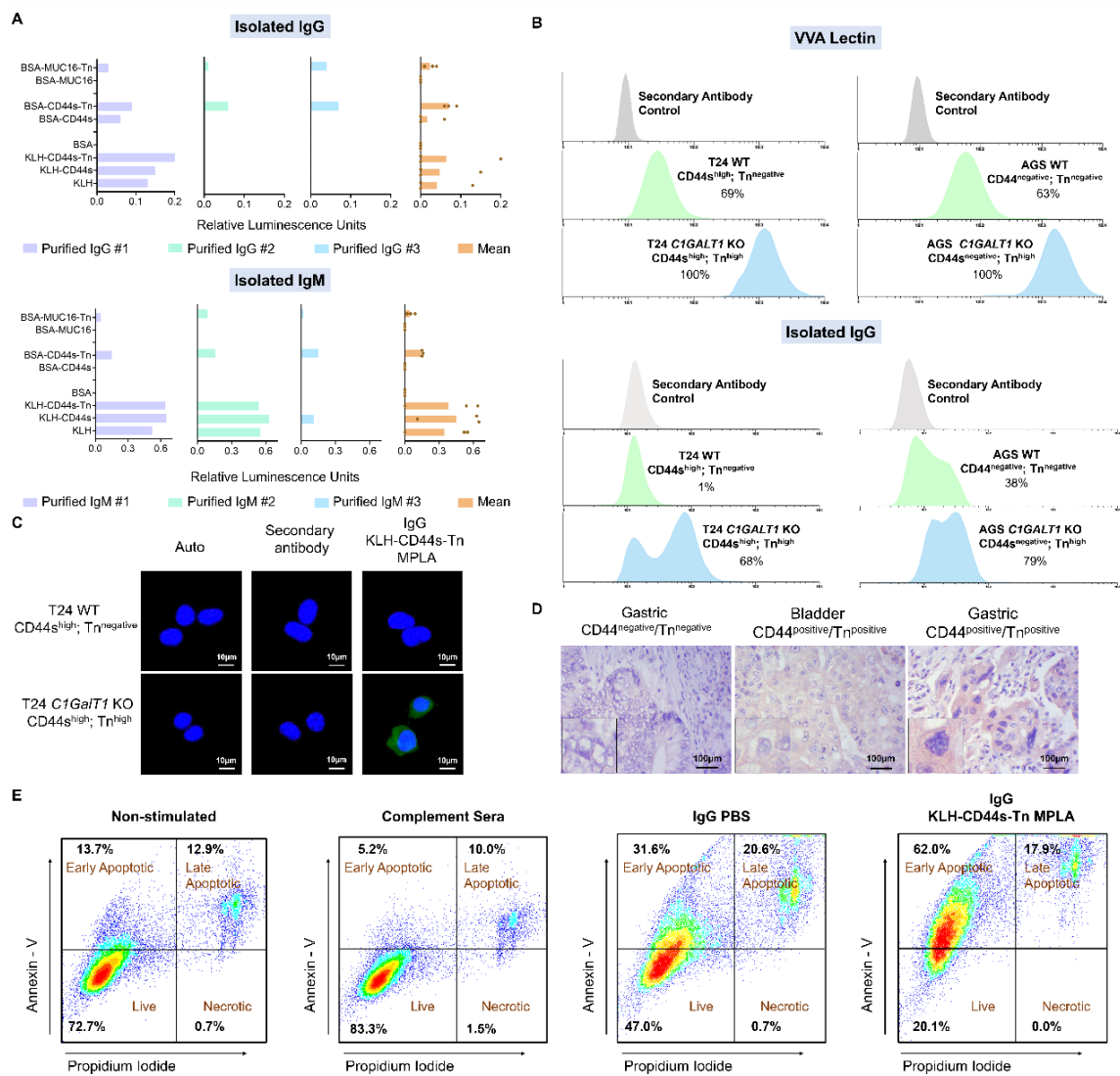


Figure 6. IgM and IgG antibodies from mice immunized with the KLH-CD44s-Tn glycovaccine recognize CD44s-Tn as well as the Tn antigen in bladder and gastric cancer cell lines and tumour tissues. A) IgM and IgG recognized CD44s-Tn as well as the Tn antigens. Slot-arrays showed IgM and IgG against CD44s-Tn and the KLH-CD44s-Tn chimera but not CD44s, demonstrating specificity for glycosylated CD44s. Antibodies recognizing the Tn antigen on a 20 mer MUC16-Tn were also observed, even though in lower abundance. B) IgG specifically recognized Tn-expressing bladder and gastric cancer cells, irrespectively of the CD44s status. IgG from KLH-CD44s-Tn plus MPLA immunized mice recognized bladder (T24 C1GALT1KO; CD44s^{high}; Tn^{high}) and gastric cancer (AGS C1GALT1KO; CD44s^{negative}; Tn^{high}) cell lines edited to express the Tn antigen (as confirmed by VVA affinity; top FACS). No reactivity was observed for corresponding control cells, expressing elongated glycosylation. C) Immunofluorescence for isolated IgG against T24 C1GALT1 KO cells overexpressing CD44s-Tn. D) IgG cross-react with CD44^{positive}/Tn^{positive} bladder and gastric tumours sections (middle and right panels) but

not CD44^{negative}/Tn^{negative} cancer tissues (left panel). E) Isolated IgG effectively activated complement system. Complement-dependent cytotoxicity assay showed that IgG from KLH-CD44s-Tn plus MPLA strongly decreased the viability of T24 C1GALT1KO tumour cell line showing their potential anti-tumoral effect. The viability of T24 C1GALT1KO tumour cell line was accessed by staining for cell surface Annexin V and Propidium Iodide (PI) followed by flow cytometry analysis. Plots comparing Annexin V to PI show the populations corresponding to live (Annexin V⁻ PI⁻), early apoptotic (Annexin V⁺ PI⁻), necrotic (Annexin V⁻ PI⁺) and late apoptotic (Annexin V⁺ PI⁺) cells. Numbers within gates indicate the percentage of cells in each of these populations.

3.11. Generated antibodies recognize cancer cell lines and human tumours

Isolated IgG from the serum of KLH-MBS-CD44s-Tn plus MPLA-immunized mice were used to screen abnormally glycosylated cell lines and human tumours. We first observed cross-reactivity against the highly aggressive bladder cancer cell line T24, which was glycoengineered to stably overexpress the Tn antigen by knocking out the *C1GALT1* gene (**Figure S9**), a gatekeeper glycosyltransferase for *O*-glycan elongation, leading to the overexpression of CD44s-Tn glycoproteoforms [10]. No reactivity was observed for control T24 cells expressing elongated *O*-glycans (**Figures 6B and C**). In parallel, we have also developed for this work a human gastric cancer AGS cell line CRISPR-Cas9 *C1GALT1* KO to overexpress the Tn antigen (**Figure S10**). AGS glycoengineered cells (**Figure 6B and C**) and corresponding controls, despite expressing high levels of Tn and no extended *O*-glycosylation (**Figure S10**), do not express CD44 (**Figure S11**). Of note, cross-reactivity against this AGS glycoengineered model was also observed. Taken together with slot-array analysis, these findings support the generation of IgG specifically against the Tn antigen expressed in different cancer cells, including those negative for CD44s. Finally, we used these IgG to screen Tn and CD44-positive and -negative bladder and gastric cancer tissue sections. In both cases, cytoplasmic and membrane staining could be observed, supporting the capacity to recognize cancer cells in human tumours (**Figure 6D**). Furthermore, we observed no cross-reactivity of the generated antibodies against a wide number of relevant human healthy tissues (thyroid, liver, gallbladder, testis, lung, stomach, pancreas, colon/small intestine/appendix; data not shown), reinforcing antibodies' specificity against cancer tissues.

3.12. Generated antibodies effectively activate the complement system

We also aimed to investigate whether the antibodies produced by vaccinated mice had the ability to induce the death of cancer cells *in vitro*. Considering its higher IgG titers, KLH-CD44s-Tn glycovaccines adjuvated with MPLA were employed for this study. The emphasis was placed on evaluating complement-dependent cytotoxicity (CDC), a mechanism extensively reported to be triggered by vaccination using carbohydrate antigens [41, 45]. Accordingly, T24 C1GALT1 KO cells were incubated with IgG from the serum of PBS- and adjuvanted KLH-MBS-CD44s-Tn-immunized mice. IgG from PBS showed a viability decrease from 72.7% to 47.0% (**Figure 6E**), suggesting some degree of unspecific reactivity. In contrast, IgG from adjuvanted glycovaccine-immunized mice exhibited a significant reduction in viable cells (20.1%) (**Figure 6E**). These changes primarily resulted from the increase in early apoptotic cells, largely attributable to the assay being halted at 1 hour due to intrinsic cell death induced by the complement alone, without the presence of antibodies (**Figure 6E**). Nevertheless, this indicates that the generated antibodies may effectively activate the complement system against cancer cells, suggesting potential to promote an anti-tumoral effect as previously observed in other models [40, 41].

4. Discussion and Concluding Remarks

Cancer immunotherapy exploiting vaccines based on cancer-associated and, ideally, neoantigens, is an emerging therapeutic and prophylactic strategy. However, its widespread application to multiple solid tumours has been limited by the number of mutations presented by many lesions [5, 6, 46]. The quest for novel sources of neoantigens has generated interest in immature glycans, such as the Tn and sTn antigens found in more advanced tumours generally associated to a worse prognosis [7, 9]. These glycans result from dysfunctional glycosylation pathways in response to microenvironmental cues that remain to be fully understood [8]. However, genetic alterations in glyco genes have also been described [9]. Notably, the adoption of immature rather than more extended glycosylation is a fundamental event driving disease progression and is rarely observed in healthy tissues, making these glycans ideal targets for cancer therapy [47]. Furthermore, there have been growing efforts supported by high throughput glycoproteomics,

to identify clinically relevant glycoproteins carrying these alterations with the goal of precise cancer targeting [6].

One of the challenges in developing glycopeptide-based immunotherapies is the identification of cancer specific glycoproteoforms. The present study builds on these efforts and presents a glycovaccine approach that targets abnormally glycosylated CD44s, a glycoprotein that is overexpressed in many solid tumours, including breast [48], liver [49], colon [50], and pancreatic [51] cancers. We recently described that CD44s is modified with immature Tn and sTn O-glycans in bladder cancer and that these specific glycoproteoforms are not present in normal tissues, making it an attractive target for cancer immunotherapy [10]. Furthermore, we found these signatures associated with more aggressive tumours enriched for epithelial-to-mesenchymal transition markers and, consequently, of higher metastatic potential [10]. To our knowledge, this is the first attempt to translate in-depth functional glycoproteomics into tangible cancer glycovaccines, materializing a relevant and needed roadmap for translational glycomedicine.

This study introduced a new enzymatic method and enrichment strategy to generate libraries of cancer-specific CD44s-Tn and/or sTn glycoproteoforms, mimicking the heterogeneity found in tumours. This approach leads to higher yields for installing glycans on peptide chains than chemical methods and provides a stable platform to generate a myriad of glycoproteoforms in a one-pot reaction [20]. Moreover, it allows to produce multivalent vaccines, unlike current single epitope strategies that display only one or two glycosites. Notably, the synthesized glycopeptide proved to be toxic for DCs, impeding their direct administration. To tackle this challenge, we conjugated CD44s-Tn derived glycopeptides to immunogenic proteins and optimized the glycopeptide-protein conjugation chemistry through a maleimide linkage to avoid unwanted cross-linking. Interestingly, despite the widespread use of this method to bind haptens to carrier proteins, the possibility of carrier protein multimerization has not been considered in the context of glycopeptide-based vaccines. We postulate that prior activation of the glycopeptide haptens at low temperature before exposure to the carrier may be key to overcome this limitation. We were able to resolve and precisely characterize the reaction products derived from the linkage of the glycopeptide with MBS using nanoLC tandem mass-spectrometry. This addressed a major milestone towards the delivery of well-characterized glycovaccines necessary to meet demanding regulatory requirements in the path towards the clinics.

Physicochemical analysis of the glycochimeras revealed three different populations regarding size, while the charge of all formulations remained slightly negative (-14.0 - -15.6 mV). The conjugation process and the addition of MPLA do not presented alterations in size and charge of the three populations, suggesting the stability of the glycoformulations upon conjugation. In addition, the slightly negative charge of our chimeras suggests reduction of the undesirable clearance by reticuloendothelial system, increasing their circulation period [37]. Furthermore, a high percentage of KLH-CD44s-Tn with and without adjuvant (>70%) presented the ideal size to remain in circulation for longer periods and to be selectively internalized by APCs [36], promoting their transport for secondary lymphatic organs to prime T cells. The glycovaccines produced in this way were well-tolerated *in vivo*. Furthermore, we provide unequivocal demonstrations that our glycovaccines is capable of eliciting both humoral and cellular responses *in vivo*. Regarding humoral responses our data indicates a class switch from IgM to IgG, showing higher affinity for glycopeptides and suggesting activation of B cells. Notably, these antibodies were reactive against tumours showing abnormal glycosylation, but not healthy tissues, supporting the cancer specificity of the immune response. Moreover, these antibodies effectively activated the complement system and decreased tumour cells viability *in vitro*, supporting potential anti-tumour effect, which warrants confirmation in future efficacy studies *in vivo*. In addition, we extensively characterized immune cell populations showing higher frequencies of T cells, B cells and M1 macrophages, which are key elements driving anti-tumour responses. We further observed both increased effector and central memory T cells, required to maintain immunological surveillance and avoid relapse [52]. Finally, we evaluated the specificity of the cellular response against the CD44s-Tn glycopeptide evaluating IFN- γ production in several immune populations. The flow cytometry evaluation allowed to specifically determine the cellular populations that produce the IFN- γ . Upon ex-vivo antigen rechallenge, we found an increase in IFN- γ production by both CD4+ and CD8+ T cells from glycovaccinated mice, indicating antigen-specific-mediated responses [53]. Moreover, an increased production of IFN- γ was also observed in NK cells, suggestive of an innate immune activation [54]. The study demonstrates the potential of glycovaccines to induce an immune response against CD44s-Tn glycoengineered cells and human tumours, indicating their potential efficacy as cancer immunotherapies. Our approach provides the first multivalent glycovaccine

candidate able to deliver a wide array of glycoepitopes to the immune system. However, before the vaccine can be used in clinical practice, several challenges need to be addressed, including determining the optimal dose and schedule and investigating potential adverse effects in relevant animal models and humans. We are presently engineering murine cells to express CD44s-Tn glycoproteoforms, envisaging syngeneic models to study the therapeutic and prophylactic efficacy of the vaccine candidate, which will be key for more in-depth pre-clinical validations. We also argue that therapeutic efficacy may require co-administration of glycovaccines with ICIs able to override the immunosuppressive cancer microenvironment [4, 21]. Our glycovaccine candidate approach targets CD44s carrying immature Tn and sTn O-glycans, which are overexpressed in many solid tumours and linked to metastasis [10, 47]. These molecular signatures are frequently found in circulating tumour cells, lymph nodes, and distant metastasis, providing an opportunity to address disease dissemination, which remains a major concern in cancer management [8, 47, 52, 55]. In conclusion, our study presents a novel approach to cancer immunotherapy using glycovaccines and warrants further investigation. Glycovaccines have the potential to revolutionize cancer therapy, and additional studies are needed to evaluate their safety and efficacy in preclinical and clinical models.

AUTHOR CONTRIBUTIONS

RF: Conceptualization, Methodology, Investigation, Formal analysis; Writing – original draft. AM: Methodology, Investigation, Formal analysis; Writing – review and editing. DF, MRS: Methodology, Investigation, Formal analysis, Writing – review and editing. FC, MPC: Methodology, Writing – review and editing. EF; CG, JS, SC, MG, ME, BS, CP: Methodology, Investigation, Writing – review and editing. MJO, BS, LLS: Conceptualization, Resources, Funding acquisition, Writing – review and editing. AP: Funding acquisition, Writing – review and editing. AMNS: Methodology, Formal analysis, Writing – review and editing. JAF: Conceptualization, Funding acquisition, Project Administration, Supervision, Resources, Writing – original draft.

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DECLARATION OF CONFLIT OF INTERESTS

José Alexandre Ferreira is the CEO of GlycoMatters Biotech and Lucio Lara Santos and André M. N. Silva are founders of the company. The authors have issued a patent on CD44 glycoepitopes and chimeric vaccine glycoconjugates for cancer therapy and synthesis methods thereof (PCT/PT2021/050025).

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Supplementary material

Table S1. Products from the single-pot enzymatic synthesis of CD44s-Tn glycopeptides, without purification and after VVA enrichment, identified by C18 reverse phase nanoLC-ESI-MS.

O-glycans	Glycopeptides	Retention time (min)	Without enrichment	Enrichment with VVA
	Monoisotopic mass [M+3H] ³⁺ / [M+4H] ⁴⁺ / [M+5H] ⁵⁺		Relative abundance (%)	Relative abundance (%)
Neutral glycopeptides				
Unglycosylated	1100.17 / 825.38 / 660.51	19.23	30.1	4.1
1 Tn	1167.86 / 876.15 / 701.12	18.64	6.9	4.6
2 Tn	1235.56 / 926.92 / 741.74	17.58	35.5	15.9
3 Tn	1303.25 / 977.69 / 782.35	17.14	15.9	34.4
4 Tn	1370.94 / 1028.46 / 822.97	16.85	8.1	30.1
5 Tn	1438.64 / 1079.23 / 863.59	16.57	3.4	10.5
6 Tn	1506.33 / 1130.00 / 904.20	16.27	0.1	0.3

Table S2. Products from the single-pot enzymatic synthesis of CD44s-STn glycopeptides after VVA affinity chromatography, without purification for sialylated glycopeptides and with TiO₂ enrichment, identified by C18 reverse phase nanoLC-ESI-MS.

O-glycans	Glycopeptides Monoisotopic mass [M+3H] ³⁺ / [M+4H] ⁴⁺ / [M+5H] ⁵⁺	Retention time (min)	Without enrichment	Enrichment with TiO ₂
			Relative abundance (%)	Relative abundance (%)
Neutral glycopeptides				
Unglycosylated	1100.17 / 825.38 / 660.51	19.43	14.67	3.52
1 Tn	701.12	----	0.16	0.00
2 Tn	926.92 / 741.74	----	1.50	0.00
3 Tn	782.35	----	0.28	0.00
4 Tn	1028.46 / 822.97	----	0.16	0.00
Hybrid glycopeptides				
1 Tn + 1 STn	999.69 / 799.96	----	1.26	0.00
1 Tn + 2 STn	1497.31 / 1123.24 / 898.79	18.78	6.98	5.14
1 Tn + 3 STn	1662.04 / 1246.78 / 997.63	19.19	5.96	13.31
1 Tn + 4 STn	1826.76 / 1370.33 / 1096.46	19.06	0.57	3.64
2 Tn + 1 STn	1050.46 / 840.57	----	0.20	0.00
2 Tn + 2 STn	939.41	----	0.10	0.00
Sialylated glycopeptides				
1 STn	1264.90 / 948.92 / 759.34	----	4.74	0.00
2 STn	1429.62 / 1072.47 / 858.18	19.06	44.73	40.41
3 STn	1594.35 / 1196.01 / 957.01	19.19	14.42	19.23
4 STn	1759.07 / 1319.56 / 1055.85	20.03	3.77	9.47
5 STn	1443.10 / 1154.68	19.89	0.51	5.27

Table S3. Products from the MBS functionalization of CD44s-Tn and CD44s(Cys)-Tn glycopeptides, identified by C18 reverse phase nanoLC-ESI-MS.

Reaction with MBS	Other peptide modifications	Chemical Formula	[M+3H] ³⁺ / [M+4H] ⁴⁺ / [M+5H] ⁵⁺	Relative abundance (%)
MBS-CD44s				
N-terminal	-	C ₁₅₀ H ₂₁₃ N ₄₃ O ₅₅	1166.51 / 875.14 / 700.31	8.88
	+ H ₂ O	C ₁₅₀ H ₂₁₅ N ₄₃ O ₅₆	1172.52 / 879.64 / 703.91	40.05
	+ H ₄ O ₂	C ₁₅₀ H ₂₁₇ N ₄₃ O ₅₇	1178.52 / 884.14 / 707.52	51.07
MBS-CD44s-2Tn				
N-terminal	+ H ₂ O	C ₁₆₆ H ₂₄₁ N ₄₅ O ₆₆	981.18 / 785.15	70.17
	+ O ₃ - H ₂	C ₁₆₆ H ₂₃₇ N ₄₅ O ₆₈	988.17	29.83
MBS-CD44s(Cys)				
N-terminal	---	C ₁₅₃ H ₂₁₈ N ₄₄ O ₅₆ S	900.89 / 720.91	17.58
	+ H ₂ O	C ₁₅₃ H ₂₂₀ N ₄₄ O ₅₇ S	905.39 / 724.52	25.02
	+ H ₂ O ₃	C ₁₅₃ H ₂₂₀ N ₄₄ O ₅₉ S	913.39 / 730.91	38.58
	+ H ₂ O ₄	C ₁₅₃ H ₂₂₀ N ₄₄ O ₆₀ S	1222.84 / 917.38	18.82
MBS-CD44s(Cys)-2Tn				
N-terminal	---	C ₁₆₉ H ₂₄₄ N ₄₆ O ₆₆ S	1002.43 / 802.14	16.73
	+ H ₂ O	C ₁₆₉ H ₂₄₆ N ₄₆ O ₆₇ S	1006.93 / 805.75	23.98
	+ H ₂ O ₃	C ₁₆₉ H ₂₄₆ N ₄₆ O ₆₉ S	1014.92 / 812.14	41.86
	+ O ₄	C ₁₆₉ H ₂₄₄ N ₄₆ O ₇₀ S	1018.42 / 814.94	4.17
	+ O - H ₂	C ₁₆₉ H ₂₄₂ N ₄₆ O ₆₇ S	1005.92	1.79
	+ O ₂	C ₁₆₉ H ₂₄₄ N ₄₆ O ₆₈ S	1010.43 / 808.54	6.19
	+ O ₃ - H ₂	C ₁₆₉ H ₂₄₂ N ₄₆ O ₆₉ S	1013.92 / 811.34	5.28

Table S4. Physicochemical properties of KLH, KLH-CD44s-Tn and KLH-CD44s-Tn plus MPLA, including mean size, polydispersity index (Pdl) and zeta potential. The values are represented as mean values ± SD (n=3).

Material	Construct	Size (nm)	Pdl	Zeta Potential (mV)
KLH	Control	419.3±83.5	0.512±0.017	-14.50±1.62
	KLH-CD44s-Tn	327.9±52.3	0.583±0.138	-14.00±0.45
	KLH-CD44s-Tn MPLA	532.9±86.7	0.657±0.099	-15.60±0.76

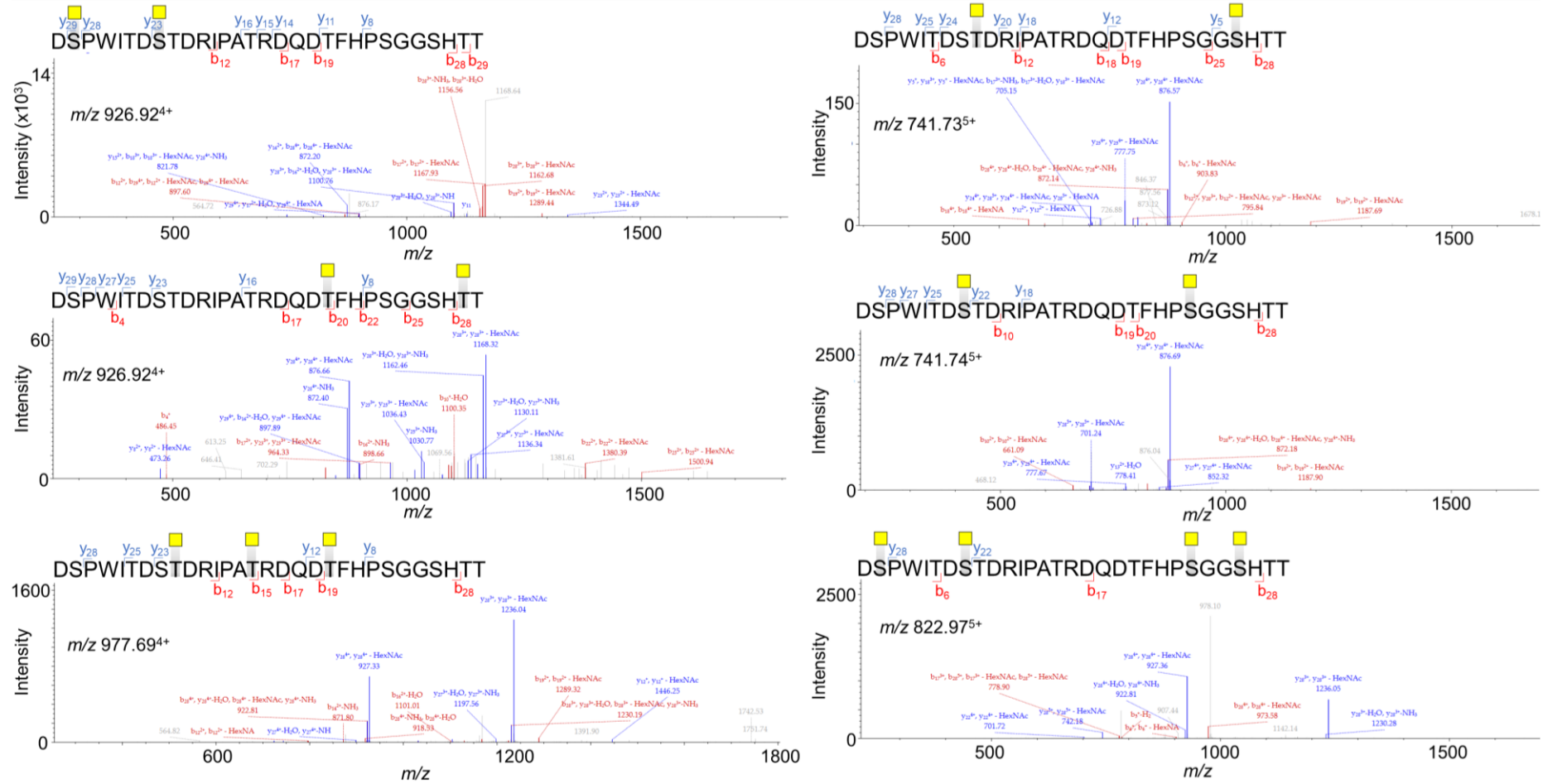


Figure S1. Glycosite identifications for 30-mer CD44s peptides after glycosylation by a combination of GalNAc-T1, GalNAc-T2, GalNAc-T3 and GalNAc-T11. Regarding CD44s-2Tn glycopeptides, S2/S8, T6/S27, T20/T29 and S8/S23 were identified as glycosites. Identification of T9, T15 and T20 as modified glycosites in CD44s-3Tn glycopeptides. Identification of S2, S8, S24 and S27 as modified glycosites in CD44s-4Tn glycopeptides

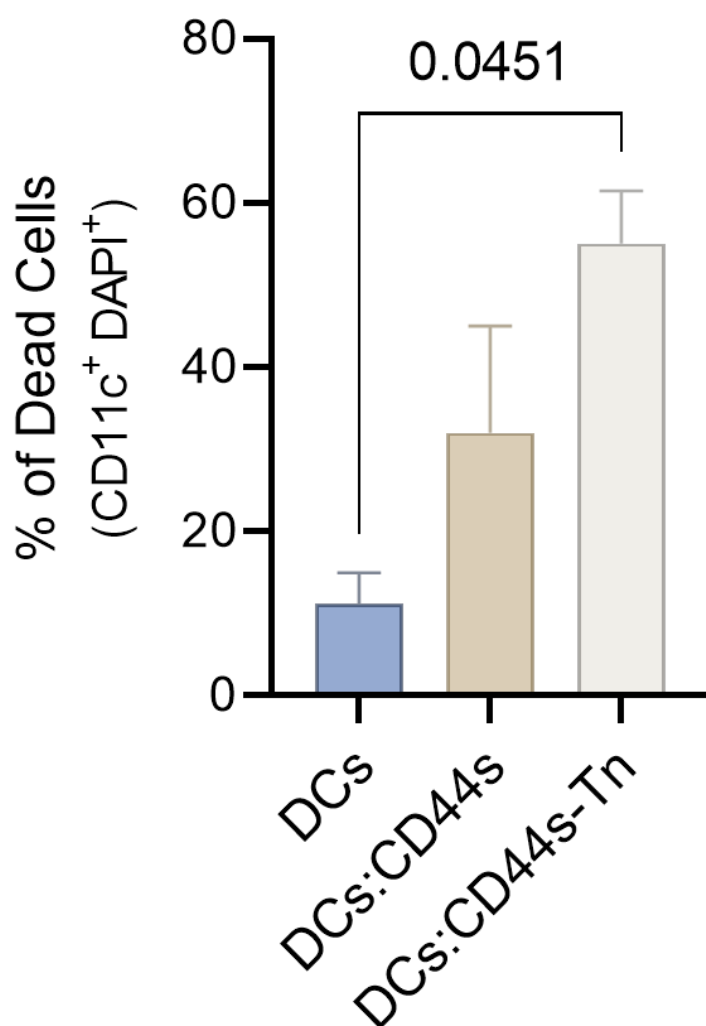


Figure S2. CD44s-Tn induced dendritic cells death *in vitro*. Flow cytometry analysis using the cell death marker DAPI showed that the CD44s-Tn was toxic for the dendritic cells, with a percentage of cell death near 60%. All comparisons were performed using the One-way ANOVA test.

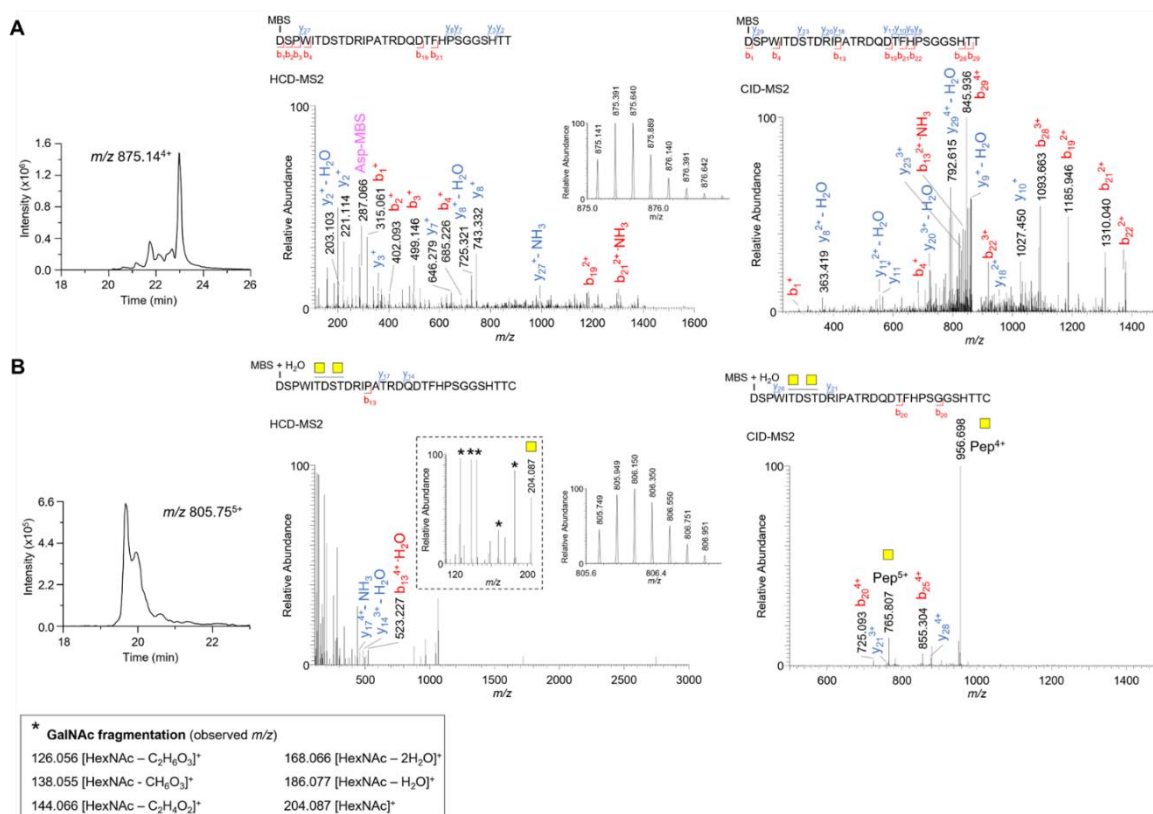


Figure S3. Nano-LC-MS and HCD and CID-MS/MS spectra for CD44s and CD44s-Tn with a cysteine tag. For reporter ions we show the MS isotopic envelop and, the corresponding HCD and CID products ion spectra highlighting GalNAc oxonium ions. GalNAc cross-ring fragments and y- and b-series peptide backbone ions that support protein annotation. The full MS showed that for both CD44s and CD44s-Tn cysteine tag the MBS preferentially binds through the terminal amine.

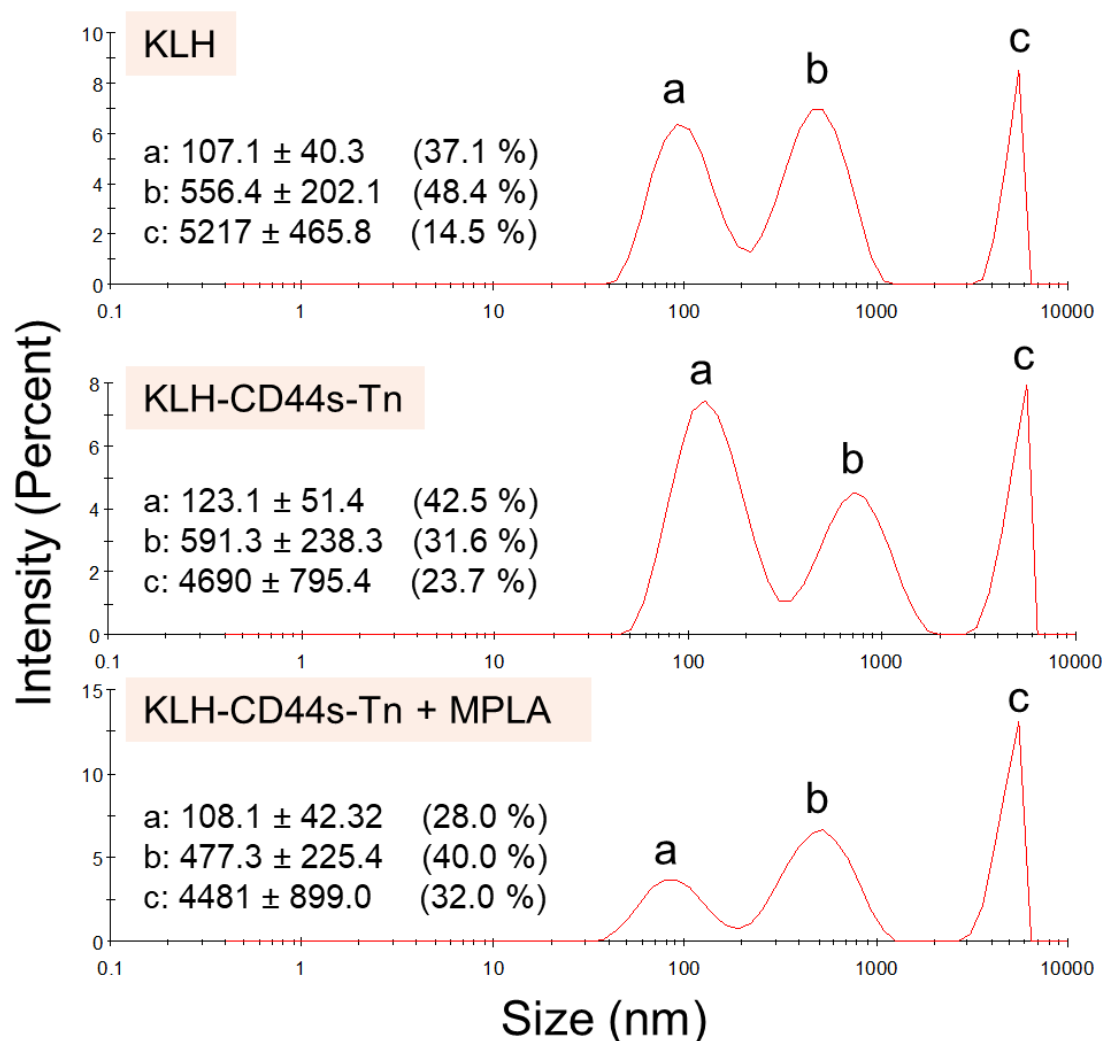


Figure S4. Dynamic Light Scattering analysis of KLH-based chimeras identified three different populations that showed no significant differences after conjugation with CD44s-Tn and addition of MPLA. DLS was able to distinguish three different populations: small, medium, and large, represented by letters a (107.1-123.1 nm), b (477.3-591.3 nm), and c (4690-5217 nm), respectively. The conjugation of CD44s-Tn to KLH and the addition of MPLA did not significantly change the size of the three populations, suggesting glycoformulation stability. CD44s-Tn conjugation to KLH slightly increased the percentage of small and large populations while decreasing the percentage of the medium-sized population. Adjuvated glycochimera presented a reduction in the percentage of the smaller population while increasing the percentage of the medium and large populations. Data are representative of at least 3 independent experiments.

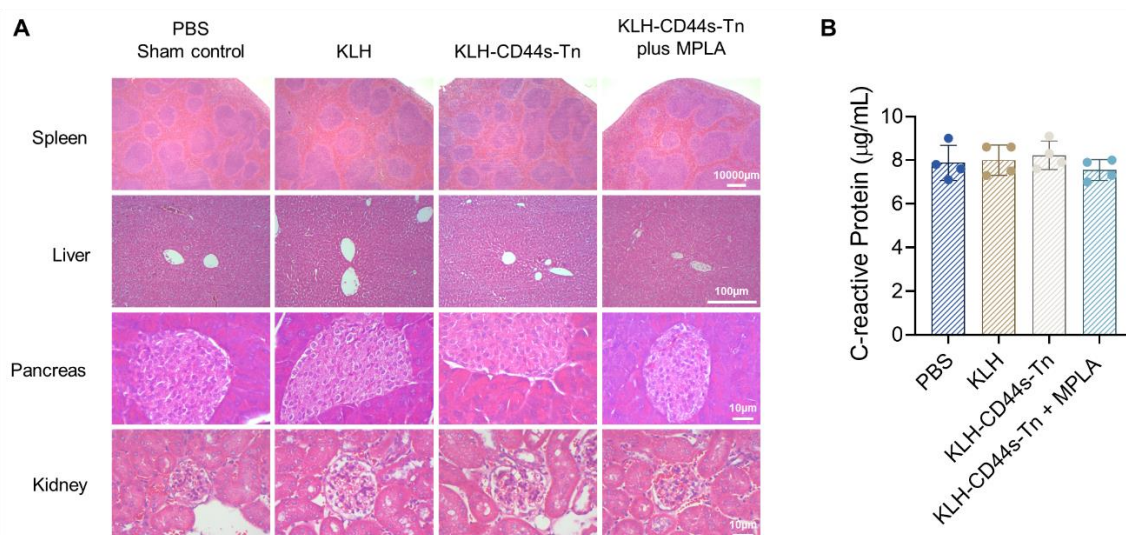


Figure S5. All tested formulations in mice were proven to be non-toxic *in vivo*. A) Histological evaluation of several vital organs from immunized mice. Histological analysis of relevant organs from mice after glycovaccine administration (spleen, liver, pancreas, kidney) revealed no signs of injury or inflammation, suggesting the safety profile of all tested formulations. **B) Mice did not present signs of an acute inflammatory condition.** C-reactive protein levels are within the range for healthy mice and no differences between experimental groups were founded.

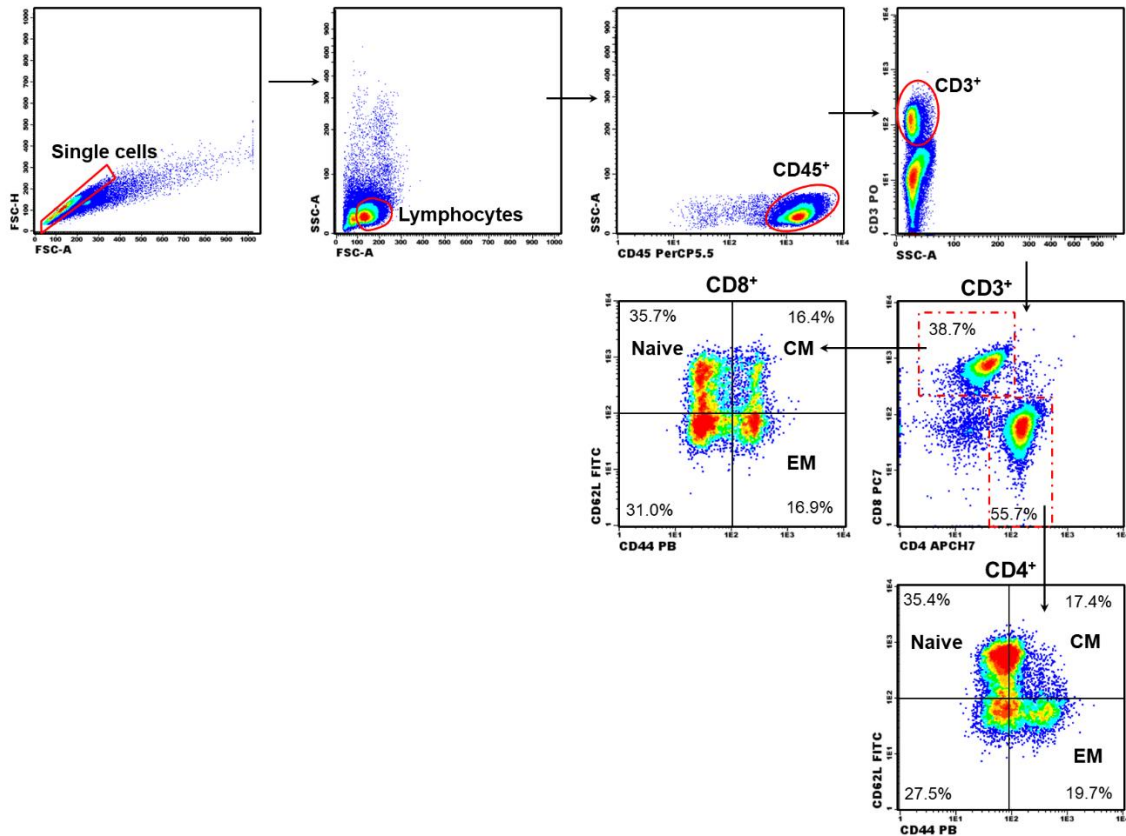


Figure S6. Flow cytometric gating strategy to identify T cells. Aggregates and cellular debris were excluded based on forward scatter (FSC). Lymphocytes were gated on single cells (FSC-H vs FSC-A). CD3⁺ cells were gated on CD45⁺. CD8⁺ and CD4⁺ cells were gated on CD3⁺ cells. Naïve, central and effector memory T cells were gated on CD3⁺CD4⁺ or CD3⁺CD8⁺ cells.

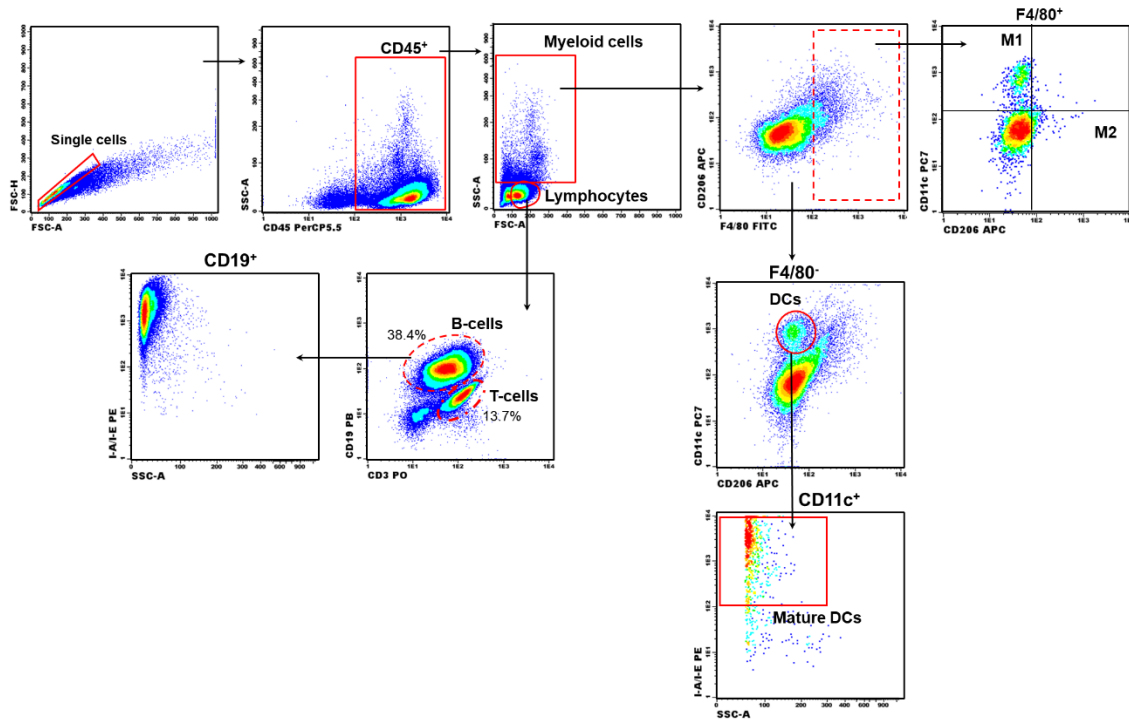


Figure S7. Flow cytometric gating strategy to identify lymphoid and myeloid populations. Aggregates and cellular debris were excluded based on forward scatter (FSC). CD45⁺ cells were gated on single cells (FSC-H vs FSC-A). Lymphocytes and Myeloid cells were gated on CD45⁺ cells. B-cells were gated on lymphocytes (SSC-A vs FSC-A) and CD19⁺IA/IE⁺ cells were gated on B-cells. M1 and M2 macrophages were gated on F4/80⁺ cells. Dendritic cells were gated on F4/80⁻ cells and mature dendritic cells were gated on dendritic cells (CD11c⁺) cells.

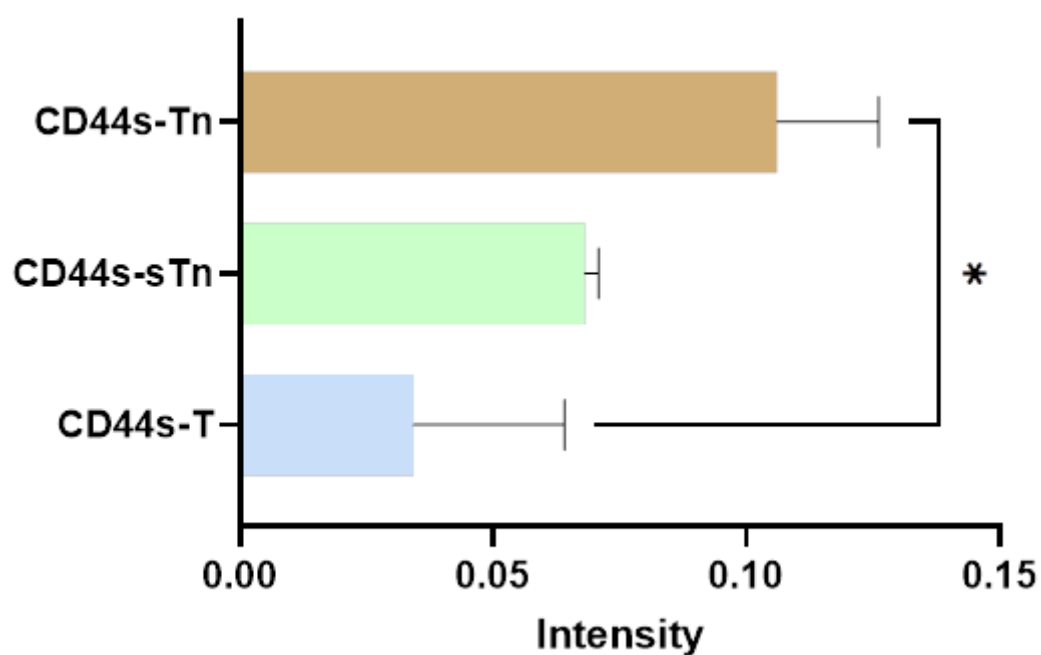


Figure S8. Isolated IgG antibodies in serum of mice immunized with KLH-CD44s-Tn glycovaccine specifically recognized synthetic CD44s-Tn antigen. IgG antibodies showed low reactivity for synthetic CD44s-sTn while presented residual recognition of CD44s-T antigen.

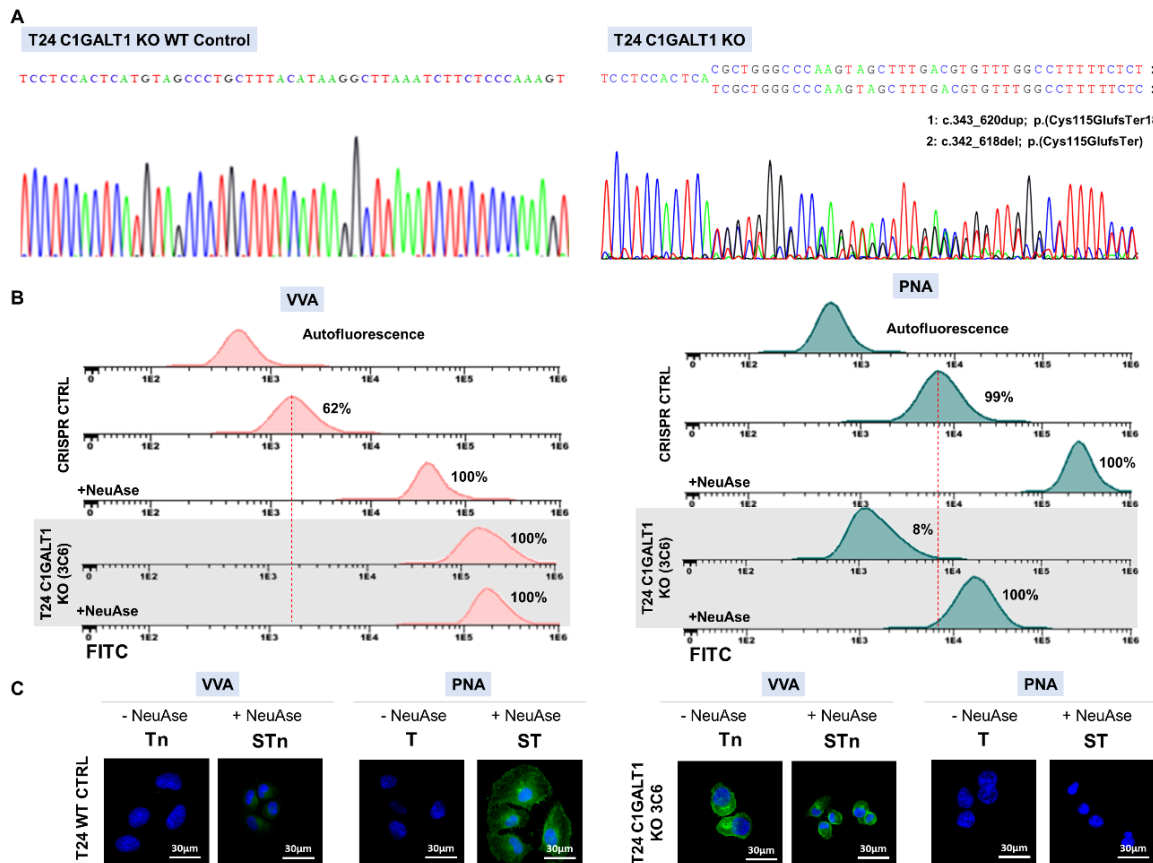


Figure S9. Glycoengineered T24 bladder cancer cell line was successfully knocked-out for the *C1GALT1* and overexpress short-chain *O*-glycans such as Tn antigen while expression of elongated *O*-glycans is not observed. A) T24 bladder cancer cell line was successful knocked-out for the *C1GALT1*. Sanger sequencing showed that T24 *C1GALT1* KO clone is characterized by two different DNA sequences, with the following observed variants and predicted consequences: 1: c.343_620dup; p.(Cys115GlufsTer18), leading to a 278bp deletion and the replacement of Cys 115 by Glu, resulting in a frameshift introducing a stop codon 18 a.a ahead; 2: a [c.342_618del; p.(Cys115Ter)], leading to a 277bp deletion and introducing of a stop codon at Cys 115.; B) Glycoengineered T24 cell line overexpress short-chain *O*-glycans and the expression of matured glycans is absent. Flow cytometry using VVA lectin confirmed the overexpression of the Tn antigen (left panel) while PNA lectin confirmed the decrease of more elongated *O*-glycans (right panel). C) Immunofluorescence studies on glycoengineered T24 bladder cancer cell line confirming the overexpression of short-chain *O*-glycans and the absence of elongated *O*-glycans.

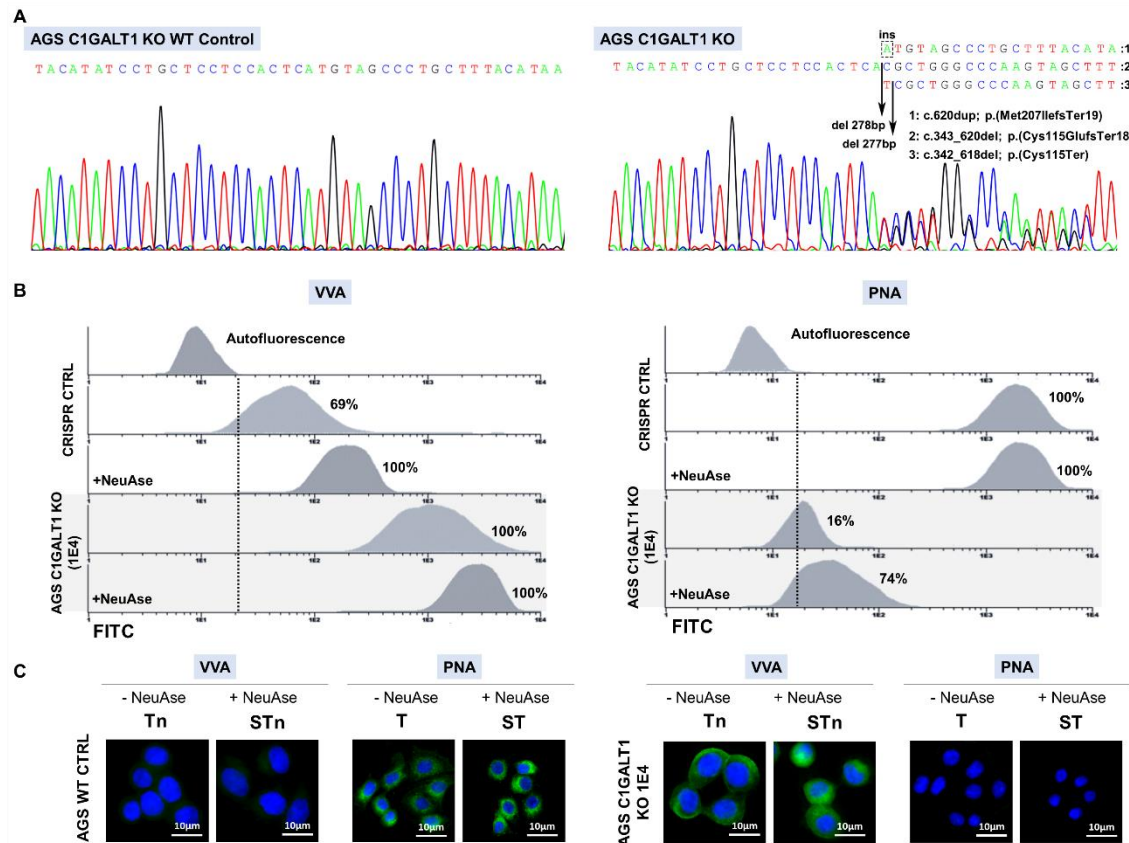


Figure S10. Glycoengineered AGS gastric cancer cell line was successfully knocked-out for the *C1GALT1* and overexpress short-chain *O*-glycans such as Tn antigen while expression of elongated *O*-glycans is not observed. A) AGS gastric cancer cell line was successful knocked-out for the *C1GALT1*. Sanger sequencing showed that AGS *C1GALT1* KO clone is characterized by three different DNA sequences, with the following observed variants and predicted consequences: 1: c.620dup [p.(Met207IlefsTer19)], where the duplication of nucleotide 620 results in the replacement of Met 207 by Ile, leading to a frame-shift and the introduction of a stop codon 19 a.a ahead; 2: c.343_620del [p.(Cys115GluTer18)], resulting in a 277bp deletion and the replacement of Cys 115 by Glu, leading to a frame-shift and the introduction of a stop codon 18 a.a ahead; 3: c.342_618del [p.(Cys115Ter)], where a 278bp deletion results in the replacement of Cys 115 by a stop codon. B) Glycoengineered AGS cell line overexpresses short-chain *O*-glycans and the expression of matured glycans is absent. Flow cytometry using VVA lectin confirmed the overexpression of the Tn antigen (left panel) while PNA lectin confirmed the decrease of more elongated *O*-glycans (right panel). C) Immunofluorescence studies on glycoengineered AGS gastric cancer cell line confirming the overexpression of short-chain *O*-glycans and the absence of elongated *O*-glycans.

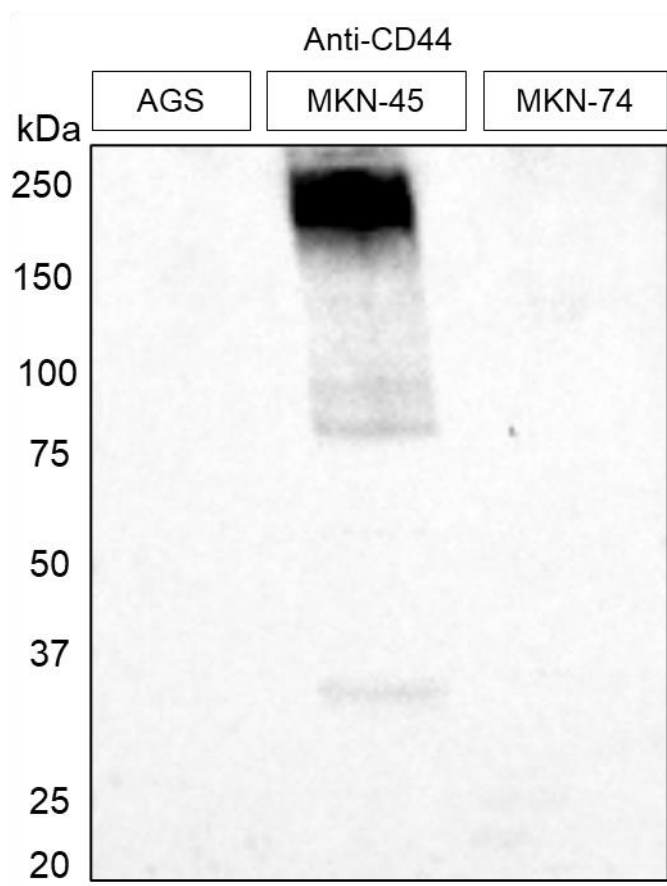


Figure S11. AGS gastric cancer cell line does not express CD44. Western blot analysis using an anti-CD44 antibody showed no reactivity against AGS cell line. The gastric cancer cell lines MKN-45 and MKN-74 were used as positive and negative controls, respectively.

CHAPTER III

**Targeted and Self-Adjuvated Nanoglycovaccine Candidate for
Cancer Immunotherapy**

The information provided in this chapter is based in the following publication:

Rui Freitas, Eduardo Ferreira, Andreia Miranda, Dylan Ferreira, Marta Relvas-Santos, Flávia Castro, Beatriz Santos, Martina Gonçalves, Sofia Quintas, Andreia Peixoto, Carlos Palmeira, André M. N. Silva, Lúcio Lara Santos, Maria José Oliveira, Bruno Sarmiento, José Alexandre Ferreira. Targeted and Self-Adjuvated Nanoglycovaccine Candidate for Cancer Immunotherapy. ACS Nano (Impact Factor in 2024: 17.1, Q1). 2024. 10.1021/acsnano.3c12487.

Targeted and Self-Adjuvated Nanoglycovaccine Candidate for Cancer Immunotherapy

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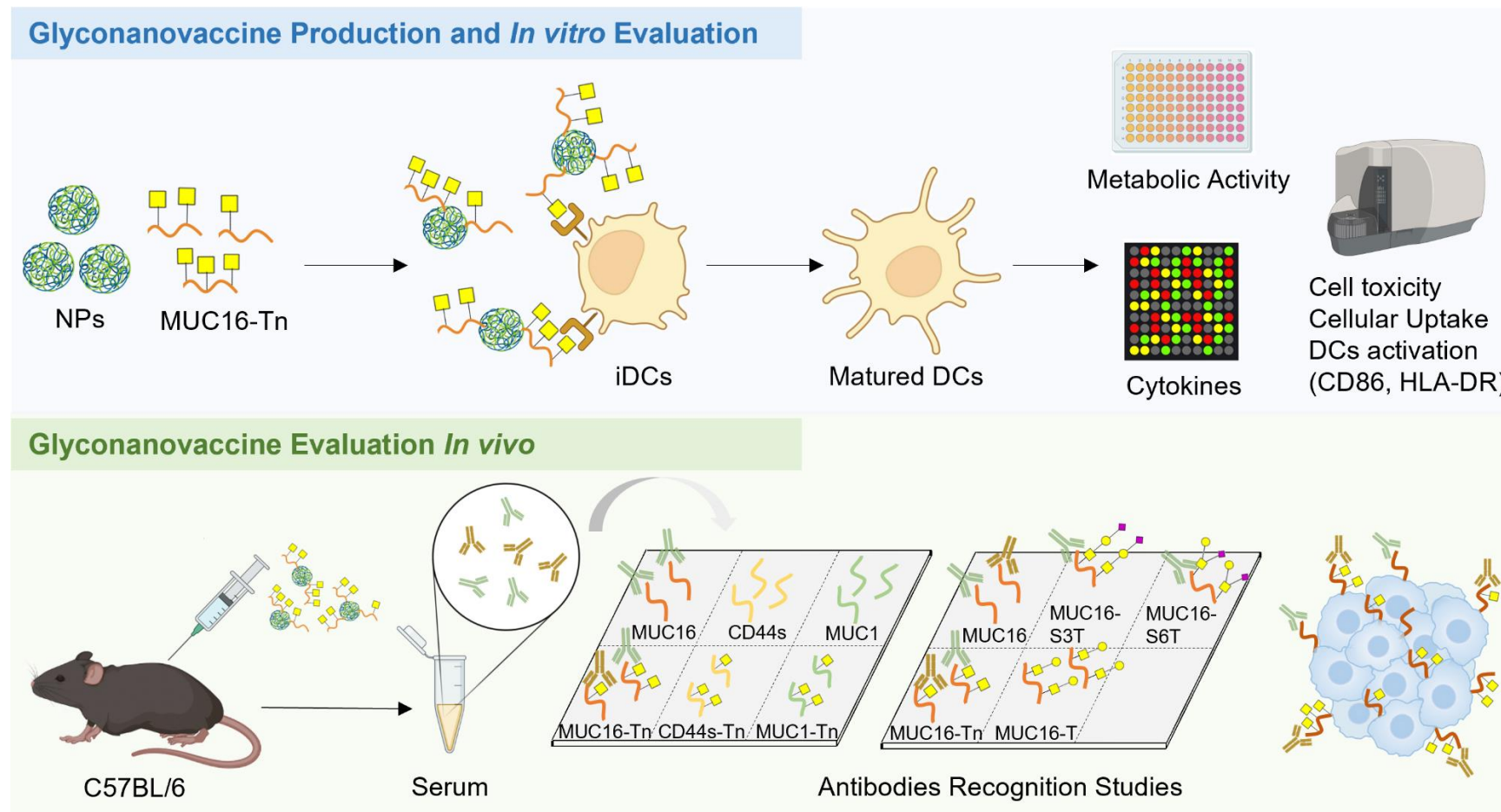
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Abstract

Advanced-stage solid primary tumours and metastases often express mucin 16 (MUC16), carrying immature glycans such as the Tn antigen, resulting in specific glycoproteoforms not found in healthy human tissues. This presents a valuable approach for designing targeted therapeutics, including cancer glycovaccines, which could potentially promote antigen recognition and foster the immune response to control disease spread and prevent relapse. In this study, we describe an adjuvant-free PLGA-based nanoglycoantigen delivery approach that outperforms conventional methods by eliminating the need for protein carriers, while exhibiting targeted and adjuvant properties. To achieve this, we synthesized a library of MUC16-Tn glycoepitopes through single-pot enzymatic glycosylation, which were then stably engrafted onto the surface of PLGA nanoparticles, generating multivalent constructs that better represent cancer molecular heterogeneity. These glycoconstructs demonstrated affinity for Macrophage Galactose-type Lectin (MGL) receptor, known to be highly expressed by immature antigen-presenting cells, enabling precise targeting of immune cells. Moreover, the glycopeptide-grafted nanovaccine candidate displayed minimal cytotoxicity and induced the activation of dendritic cells *in vitro*, even in the absence of an adjuvant. *In vivo*, the formulated nanovaccine candidate was also non-toxic and elicited the production of IgG specifically targeting MUC16 and MUC16-Tn glycoproteoforms in cancer cells and tumours, offering potential for precise cancer targeting, including targeted immunotherapies.

Keywords: glycovaccines; nanovaccines; cancer immunotherapy, cancer glycosylation; glycoantigens; glycoconjugates

Graphical Abstract



1. Introduction

Immunotherapy, particularly cancer vaccines, has emerged as a valuable tool to control disease dissemination and relapse [1]. In recent years, the design and development of personalized nanoglycovaccines have raised significant attention due to their ability to exploit tumour-specific antigens, thus enhancing the immune response while minimizing off-target effects [2]. Among the diverse array of potential tumour-associated antigens, the Mucin 16 (MUC16) glycoprotein, overexpressed in numerous cancer types³, has emerged as a potential target for immunotherapeutic interventions. MUC16 carries the truncated carbohydrate antigen Tn (GalNAc- α -O-Ser/Thr), which is minimally expressed in healthy tissues but highly abundant at the cell-surface of more aggressive primary tumours and metastases, playing key functional roles in disease progression [4]. These features make MUC16-Tn glycoproteoforms a potential signature for selectively targeting by nanoglycovaccines for precise cancer immunotherapy.

Classical cancer glycovaccine designs comprise the coupling of a glycopeptide or glycosidic chain to a protein carrier able to elicit T-cell engagement and antigen presentation [5]. These approaches have been shown to induce humoral immunity with high specificity for mucin cancer-specific truncated glycoforms, such as the Tn antigen, in relevant animal models, holding an opportunity for cancer vaccine development [5, 6]. However, this strategy raises immunogenicity concerns linked to unspecific responses against the protein carrier and epitope suppression [7], also known as antigenic competition or antigenic interference, occurring when multiple antigens are presented together on the same protein carrier. This may be particularly critical for poor immunogens such as short glycoepitopes carrying immature glycosylation. Employing advanced antigen delivery platforms like poly(lactic-co-glycolic acid) (PLGA) nanoparticles (NPs) mitigates epitope suppression through NPs uptake and antigen presentation [8,9]. PLGA's versatility to carry several molecules, biocompatibility and biodegradability make it an ideal antigen carrier, allowing controlled and sustained release of antigens for prolonged immune stimulation and enhanced vaccine efficacy [10]. By engineering PLGA NPs to carry individual antigens it is possible to promote a balanced and robust immune response against glycopeptides, thus enhancing cancer glycovaccine efficacy [11]. In addition, the possibility to tailor the physicochemical properties of PLGA NPs to target antigen presenting cells (APCs)

makes it an attractive carrier for use in vaccination [12]. Furthermore, the shielded antigens within PLGA NPs ensure effective immune presentation, maximizing the vaccine's overall efficacy in cancer immunotherapy and other diseases [13, 14]. Grafting the surface of PLGA NPs with relevant ligands facilitates specific recognition by immune or tumour cells, while additional adjuvants enhance immunogenicity [15]. Furthermore, certain types of PLGA have been proven a potent adjuvant through the activation of the non-canonical inflammasome mechanisms leading to production of pro-inflammatory cytokines, inducing cell-mediated immunity and humoral immunity [16]. Precise tailoring of particle size to 50-60 nm has been proven key towards this objective [16]. Moreover, the addition of polyethylene glycol (PEG) to PLGA NPs (PLGA-PEG NPs) impede their rapid elimination, thus increasing their blood circulation half-life which is essential for selective immune cells targeting [17]. This study capitalizes on the versatility of PLGA as a scaffold to induce humoral responses against MUC16-Tn glycopeptides. Through surface grafting of the immunogen, we aimed to confer targetability to the nanovaccine, leveraging the strong affinity of Tn antigens for calcium dependent C-type lectin receptor Macrophage Galactose-type Lectin (MGL), highly expressed by immature dendritic cells (DCs) and macrophages [18]. The primary goal was to create innovative targeted and self-adjuvated nanovaccine candidates, utilizing glycans as both antigens and targeting molecules for APCs, aiming for highly effective and personalized cancer treatment.

2. Material and Methods

2.1. Enzymatic synthesis of MUC16, MUC1 and CD44s-glycopeptides epitopes

A MUC16 20-mer peptide (VDVGTSGTPSSSPSPTTAGP) and its cysteine-tagged form (at C-terminal) were purchased from GenScript Biotech. Synthesis of glycopeptides carrying the Tn-epitope (GalNAc- α -O-Ser/Thr) was performed using polypeptide N-acetilgalactosamine transferases (GalNAc-Ts) individually or mixed (0.31 μ g GalNAc-T1, 0.62 μ g GalNAc-T2, 0.62 μ g GalNAc-T3; R&D Systems) in a 100 μ L reaction mixture containing glycosylation buffer (125 mM sodium cacodylate, 50 mM MnCl₂, pH 7.4; Sigma-Aldrich), 50 μ g UDP-GalNAc (Sigma-Aldrich) and 50 μ g of MUC16 20-mer peptide by overnight incubation at 37 °C. Similarly, a cysteine-tagged MUC1 20-mer peptide (PDTRPAPGSTAPPAHGV TSA) and CD44s-Tn 30-mer

peptide (DSPWITDSTDRIPATRDQDTFHPSSGSHHT) were produced using the same procedure. Accordingly, a mix of 0.12 μg GalNAc-T1, 0.24 μg GalNAc-T2, 0.24 μg GalNAc-T3 was used to glycosylate MUC1 20-mer peptide and 0.18 μg GalNAc-T1, 0.36 μg GalNAc-T2, 0.36 μg GalNAc-T3, 1.69 μg GalNAc-T11 was used to glycosylate CD44s 30-mer peptide. MUC16-Tn, MUC1-Tn and CD44s-Tn glycopeptides were loaded on 80 μL of agarose-bound *Vicia villosa* agglutinin (VVA) column (ThermoFisher Scientific), for enrichment. The column was washed with 10 column volumes of LAC A buffer (20 mM Tris/HCl pH 7.4, 150 mM NaCl, 1 M urea, 1 mM CaCl_2 , MgCl_2 , MnCl_2 and ZnCl_2) at 1200 g for 30 seconds at room temperature (RT). The glycopeptides were then eluted with 3x100 μL 3% acetic acid. Synthesis of MUC16-T, MUC16-S3T and MUC16-S6T was performed, as previously described by us [23].

2.2. nanoLC-ESI-LTQ-Orbitrap mass spectrometry

Synthesized glycopeptides were characterized by nanoLC-ESI-MS, as previously described by us [23].

2.3. BSA and KLH-MUC16-Tn glycoconjugates

Cysteine-tagged MUC16-Tn was conjugated to keyhole limpet hemocyanin (KLH, Sigma-Aldrich) and cysteine-tagged MUC16-Tn, MUC16-T, MUC16-S3T, MUC16-S6T, MUC1-Tn and CD44s-Tn glycopeptides and their non-glycosylated counterparts were conjugated to bovine serum albumin (BSA, Sigma-Aldrich), as previously described by us [23].

2.4. Production of PLGA-PEG-MAL NPs

The PLGA-PEG-MAL NPs were produced by a water-in-oil-in-water (w/o/w) double emulsion-solvent evaporation technique modified from Fernandes and Ferreira *et al* [48]. Briefly, 95% (w/w) core PLGA (50:50 LA:GA; 44,000 Da; 5004) and 5% (w/w) 30,000 Da polyethylene glycol (5000 Da)-maleimide (PLGA-PEG-MAL; Polysciotech/Akina) were dissolved in 1 mL ethyl acetate, following the addition of 100 μL ultrapure water to form the primary (w/o) emulsion and homogenization in a Vibra-Cell™ ultrasonic processor for 30 seconds. The second (w/o/w) emulsion was achieved by adding 4 mL of 2% Pluronic F-127 (Sigma-Aldrich) and

homogenization for 1 min. The final mixture was added to another solution of 15 mL of 2% Pluronic while stirring at 300 rpm. The mixture was kept stirring for 3 h for solvent evaporation. NPs were washed three times with ultrapure water using 100 kDa centrifuge filters (Amicon Ultra Centrifugal Filters, Merck) at 2500 g for 10 min at 4 °C, followed by resuspension in 1 mL of ultrapure water. Fluorescent NPs were obtained as described above using 85% core PLGA, 5% PLGA-PEG-MAL and 10% PLGA-FKR648 (20,000-30,000 Da; Polysciotech/Akina).

2.5. MUC16 peptide and MUC16-Tn glycopeptides conjugation to NPs

MUC16 and MUC16-Tn peptides were diluted to a final concentration of 30 µg/µL in 1x PBS pH 7.4. To allow mild reduction of C-terminal cysteines in the glycopeptide, tris(2-carboxyethyl) phosphine hydrochloride (TCEP) was added to peptides at a final concentration of 100 µM and incubated for 1 h at 4 °C under agitation. Then, 500 µg of PLGA-PEG-MAL NPs was added to the solution and incubated overnight at 4°C under agitation. NPs without peptides conjugation were handled similarly but with PBS 1x only (Control NPs). NPs were washed three times with ultrapure water using 0.5 mL 100kDa centrifuge filters (Amicon Ultra Centrifugal Filters, Merck) at 2500 g for 10 min at 4°C, followed by resuspension in 200 µL of ultrapure water. All NPs were maintained in low light conditions. In parallel, MUC16 and MUC16-Tn without cysteine tag were used to determine peptide adsorption to NPs. The endotoxin levels of MUC16-Tn and PLGA-PEG-MAL-MUC16-Tn (MUC16-Tn NPs) were measured using the Pierce™ Chromogenic Endotoxin Quantification Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. LPS and MPLA adjuvant were used as positive controls.

2.6. Peptides conjugation efficiency (CE) determination

The conjugation efficiency of MUC16 and MUC16-Tn peptides was determined by quantifying the amount of free peptide in the supernatants collected after NPs washing, through fluorescence measurement using fluorescamine (Em/Ex = 460/400; Sigma-Aldrich). The calibration curve was made using supernatants of the corresponding control NPs. Fluorescence acquisition was made on a Synergy MX microplate reader (BioTek). The conjugation efficiency was determined according to the following equation:

$$\text{Conjugation efficiency (\%)} = \frac{\text{amount of glycopeptide} - \text{amount of glycopeptide in supernatant}}{\text{total amount of glycopeptide}} \times 100$$

In parallel, we validate our conjugation efficiency determination by HPLC-UV on a Hitachi 7000 series instrument (Chiyoda, Tokyo, Japan). Samples were run through a LiChrospher 100 RP-18 (5 µm) 125-4 column (Merck), 5 µm (particle size) x 4 mm (internal diameter) x 125 mm (length). The mobile phase A consisted in water with 0.065% trifluoroacetic acid and the mobile phase B consisted in acetonitrile with 0.05% trifluoroacetic acid. The following gradients were used: 95% phase A and 5% phase B for 9 min, 5% phase A and 95% phase B for 2 min and 95% phase A and 5% phase B for 5 min. Flow was set to 1 mL/min. UV detector wavelength was set to 220 nm and the injection volume was established to 20 µL.

2.7. NPs Physicochemical and Morphological Characterization

The size and polydispersity index (Pdl) of formulations were determined by Dynamic Light Scattering (DLS), while surface charge of NPs was determined by Laser Doppler anemometry (LDA). After dispersion, NPs were diluted in 10 mM sodium chloride solution (pH 7.0) and DLS and LDA analyses were performed with a Nano ZS Zetasizer (Malvern, Worcestershire, UK) at 25 °C using the Smoluchowski model. Data is presented as the average of the triplicate NP formulations. Surface morphology and NPs size confirmation were performed by transmission electron microscopy (TEM) using a JEM-1400 microscope (JEOL, Tokyo, Japan) at an acceleration voltage of 80 kV.

2.8. Nuclear Magnetic Resonance Analysis (NMR)

PLGA NPs were washed with ultrapure water and poured into semi-stoppered glass vials with slotted rubber closures at a maximal formulation volume of 2 mL. Samples were frozen by ramped cooling at -20 °C for 24 h followed by lyophilization using a VirTis Advantage Plus Benchtop lyophilizer from SP Scientific (Warminster, PA, USA). The condenser surface temperature was maintained at -60 ± 5°C. The primary drying occurred at -32 °C and 150 mtorr during 24 h, while secondary drying occurred at 20 °C and 50 mtorr during 6 h. ¹H NMR spectra of MUC16, MUC16-Tn, PLGA-PEG-MAL NPs and MUC16-Tn covalently linked to PLGA-PEG-MAL NPs were acquired, at RT, on a Brüker AMX 300 spectrometer operating at 400.13 MHz. The samples were analysed at the final concentration of 2 mg/mL

in 1:3 H₂O-d₆:DMSO-d₆. The chemical shifts are expressed in δ (ppm) values relative to tetramethylsilane (TMS) as internal reference and coupling constants (J) are given in Hz. Data analysis was performed through MestReNova Software version 14.2.0 (MestreLab Research, Spain).

2.9. NPs affinity to VVA lectin and MGL

NPs affinity for VVA lectin was indirectly evaluated through fluorescence measurement of the supernatant of VVA lectin affinity chromatography. Briefly, 500 μ g in 500 μ L of fluorescent NPs were loaded on 100 μ L of agarose bound VVA column and lectin affinity chromatography was performed according to the described for glycopeptide synthesis. The column was washed with 10 column volumes of LAC A buffer at 1200 g for 30 seconds at RT. The flow-through was collected, and the fluorescence was measured ($E_m/E_x = 460/400$) on a Synergy MX microplate reader (BioTek).

NPs affinity for VVA lectin and MGL was directly evaluated through slot blot. Nitrocellulose membranes (0.45 μ M) were loaded with the equivalent 10 μ g of MUC16 and MUC16-Tn conjugated to NPs. Control NPs and MUC16-NPs were used as negative controls. The membranes, previously blocked with Carbo-free blocking solution 1x (VectorLabs), were blotted for histidine-tagged MGL (10 μ g/mL, 4888-CL-050; R&D Systems) in MGL buffer or biotinylated VVA (1:5000, VectorLabs) for 1h at RT. Then, the anti-his tag horseradish peroxidase (HPR) conjugate antibody (1:5000, Abcam) or Vectastain Elite ABC reagent were added for 30 min at RT, for MGL and VVA detection, respectively. The Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare Life Sciences) was used as developing reagent. Data analysis was performed through Image Lab Software (Bio-Rad) in a ChemiDoc XRS (Bio-Rad).

2.10. MUC16 and MUC16-Tn peptide stability in PLGA-PEG-MAL NPs

The release profiles were determined using 500 μ g of NPs in PBS pH 7.4 under agitation at 37 °C. Briefly, 50 μ L of NPs suspensions supernatant were collected at 0.5, 1, 2, 4, 8, 12, 24, 48, 72, 96 and 168 h and replaced with the same amount of respective buffer. The supernatants were centrifuged at 10 000 g

for 20 min and quantified using the Pierce™ Quantitative Peptide Assay (ThermoFisher Scientific) according to manufacturer's instructions.

2.11. NPs Stability Study

Before performing the *in vivo* experiments, we evaluated the stability of the MUC16-Tn NPs throughout 21 days. NPs formulations were maintained at 4 °C under low light conditions. At days 0, 7, 14 and 21 the size, Pdl and charge of formulations were determined as described above.

2.12. Cell lines and culture conditions

Human bladder cancer cell line T24 and kidney epithelial cell line HEK293 were purchased from American Type Culture Collection (ATCC). Cells were maintained with RPMI 1640 GlutaMAX™ medium (Gibco) supplemented with 10% (v/v) of fetal bovine serum (FBS; Gibco) and 1% (v/v) penicillin/streptomycin (PenStrep; Thermo Fisher Scientific). Cells were kept at 37 °C in a 5% CO₂ humidified atmosphere.

2.13. Immunofluorescence for Tn and MUC16-Tn expression detection

To evaluate Tn antigen and MUC16 expressions, T24 wild-type and T24 C1GALT1 KO cell lines were cultured at low density and fixed with 4% paraformaldehyde (PFA; Sigma-Aldrich), following immunofluorescent staining. Cells were stained with FITC – label VVA (0.01 µg/µL, FL-1231; Vector Laboratories) in PBS 2% FBS or anti-MUC16 antibody (1:20; HPA065600; Sigma-Aldrich) for 1 hour at RT. The goat anti-rabbit IgG (H+L), Alexa Fluor 594 (1:300; A11012; Thermo Fisher Scientific) was used as a secondary antibody for MUC16 detection and incubated 30 min at RT in obscurity. After antigen staining, cells were marked with 2.3x10⁻³ µg/µL 4',6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI, Thermo Fisher Scientific) for 10 min at RT in the dark. All images were acquired on a Leica DMI6000 FFW microscope using Las X software (Leica).

2.15. Glycoproteomics for MUC16

Plasma membrane protein from T24 cell line was extracted and enriched for MUC16 using the anti-CA125 (MUC16) monoclonal antibody (sc-365002; Santa Cruz Biotechnology), as previously described by us [49]. Glycoprotein digestion and analysis were performed according to Peixoto *et al* [50].

2.16. Human samples and Ethics statement

Human samples were obtained in agreement with the principles of the Declaration of Helsinki. Monocytes were isolated from surplus buffy coats from healthy blood donors, kindly provided by the Immunohemotherapy Department of Centro Hospitalar Universitário São João (CHUSJ), Porto, Portugal. Procedures were approved by the Hospital ethical committee (Protocol reference 90/19). Blood donors provided informed written consent that the byproducts of their blood collections could be used for research purposes. All procedures involving the manipulation of tumour tissues sections were performed under patient's informed consent and after approval of the IPO-Porto ethics committee (references CES-IPOFG-EPE-86R1/017 for bladder cancer).

2.17. Human monocyte isolation and differentiation in dendritic cells

Human monocytes were isolated from buffy coats from healthy blood donors, as previously outlined by Castro *et al* [51]. Briefly, buffy coats were subjected to centrifugation (30 min, 1200 g, acceleration 5, RT, without breaking), and the peripheral blood mononuclear cells (PBMCs) were collected and treated with the Rosette Sep human monocyte enrichment kit (StemCell Technologies), following the instructions provided by the manufacturer. Subsequently, the resultant mixture was combined 1:1 with PBS containing 2% FBS and layered over Histopaque-1077 (Sigma-Aldrich), followed by centrifugation (30 min, 1200 g, no acceleration or braking, RT). The enriched monocyte layer was retrieved and washed thrice using PBS. For the differentiation of monocyte-dendritic cell (mo-DCs), a total of 1×10^6 monocytes were seeded on 6-well plates or 5×10^5 monocytes on 24-well plates and allowed to differentiate over a span of 5 days in complete RPMI-1640 medium (with supplementation of 10% FBS and 1% Pen Strep), along with the addition of 50 ng/mL Interleukin 4 (IL-4; ImmunoTools) and granulocyte-macrophage colony-stimulating factor (GM-CSF; ImmunoTools). This

procedure consistently yielded over 80% of differentiated cells exhibiting CD11c-positive lineage marker throughout the course of the experiments.

2.18. Mo-DCs metabolic activity

The metabolic activity of mo-DCs stimulated with of Control NPs, MUC16 functionalized nanoparticles (MUC16 NPs), glyconanovaccine (MUC16-Tn NPs), monophosphoryl lipid A (MPLA) adjuvated glycovaccine (MUC16-Tn NPs plus MPLA) and free MUC16-Tn was evaluated using the MTT assay as previously described by us [52]. Accordingly, mo-DCs were seeded into 96 well plates at a cell density of 1×10^4 and exposed to increasing concentrations of nanoformulations (0.1, 1, 10, 50 $\mu\text{g/mL}$). After 24 h exposure, conditioned medium was replaced by 100 μL fresh culture medium and 10 μL of a 12 mM 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Thermo Fisher Scientific) stock solution was added to each well, following a 4 h incubation at 37 °C in a humidified chamber. Conditioned medium was removed and 50 μL of DMSO (Sigma-Aldrich) was added to solubilize formazan crystals. Plates were incubated at 37 °C for 10 min and absorbances were read at 540 nm in a microplate spectrophotometer. Positive (untreated cells) and negative controls (cells treated with 1% triton-X 100) were included. Thus, the metabolic activity (%) of living cells was calculated through the following equation:

$$\text{Metabolic activity (\%)} = \frac{\text{Absorbance of treated cells}}{\text{Absorbance of untreated cells}} \times 100$$

2.19. Mo-DCs viability evaluation

A preliminary viability study using increasing concentrations of glyconanovaccine (0.1, 1, 10 and 50 $\mu\text{g/mL}$) and adjuvated glyconanovaccine was performed. Briefly, 1×10^6 cells were cultured into 6 well plates and stimulated during 24 h with the NPs. After 24 h of NPs contact, the adhered mo-DCs were detached with PBS on ice for 30 min. Following centrifugation (5 min, 1500 rpm, 4 °C), DCs were resuspended in FACS buffer (PBS, 2% FBS, 0.09% sodium azide) stained with DAPI (750 ng/mL) for flow cytometry analysis. After selecting the concentration for downstream assays (1 $\mu\text{g/mL}$), mo-DCs viability was measured using Annexin V Apoptosis Detection kit (BD Biosciences). Briefly, Annexin V-FITC

and propidium iodide (PI) staining was performed for 15 min in a Binding buffer at 4 °C. Flow cytometry acquisition was performed using Beckman Coulter Navios Flow Cytometer. Data files were analysed using the Infinicyt software (version 1.7, Cytognos SL).

2.20. *In vitro* uptake studies

For NPs uptake evaluation, T24 and HEK293 were seeded in a 12-well plate with a density of 5×10^5 cells in complete RPMI medium. DCs were seeded in a 24-well plate with a density of 5×10^5 cells in complete RPMI medium. Cells were incubated with 50 µg/mL of fluorescent NPs formulations for 4 h at 37 °C. Cells were then detached and fixed with 2% PFA for 15 min at RT. The cellular uptake was evaluated by flow cytometry. Data analysis was performed through CXP Software in a FC500 Beckman Coulter flow cytometer.

2.21. Mo-DCs profile evaluation

Mo-DCs profile evaluation was performed as previously described by us [52]. The mo-DCs were seeded into 6 well plates at a cell density of 1×10^6 cells and cultured during 24 h with the following formulations (1 µg/mL): Control NPs, MUC16 NPs, glyconanovaccine, MPLA (2 µg/mL) adjuvated glyconanovaccine (MUC16-Tn NPs plus MPLA), free MUC16-Tn and KLH-MUC16-Tn. Unstimulated DCs were used as negative control and 50 ng/mL LPS (Sigma-Aldrich) was used as mo-DCs activation positive control. Previously, a preliminary study using increasing concentrations of glyconanovaccine (0.1, 1, 10 and 50 µg/mL) and adjuvated glyconanovaccine was performed. After 24 h of culture, conditioned media were collected and adhered DCs were detached with PBS on ice for 30 min. After centrifugation (5 min, 1500 rpm, 4°C) mo-DCs were resuspended in FACS buffer and immune stained for flow cytometry analysis. The mo-DCs differentiation and maturation states were assessed by immunostaining in FACS buffer with FITC-anti-CD86 (21480863, ImmunoTools), APC-anti-CD11c (21487116, ImmunoTools) at a dilution 1:25 and PE-anti-HLA-DR (21819984, ImmunoTools) at a dilution 1:100, for 40 min at 4 °C. Cell viability was assessed through DAPI staining (750 ng/mL). To define background staining, negative controls were employed using isotype-matched antibodies. Flow cytometry acquisition was performed using Beckman Coulter Navios Flow Cytometer. Data files were analysed using the Infinicyt

software (version 1.7, Cytognos SL) to discriminate cell populations, determine the mean fluorescence intensity (MFI) and the percentage (%) of positive cells.

2.22. Mo-DCs cytokine profile evaluation

Mo-DCs supernatants were assayed after 24 h of nanoformulations stimulation. IL-1 β , IL-1RA, IL-6, IL-8, IL-10, IL-12(p70), IL-12(p40), IFN- γ and TNF- α were outsource determined by Eve Technologies (Calgary, AB) using Luminex human plex-discovery assay. IL-23 was measured by Legend Max ELISA (Biolegend) according to manufacturer's instructions.

2.23. Animal Ethics Statement

Animal research followed strictly to the guidelines outlined in the European Union Directive 2010/63/EU and the corresponding National Regulation released in 2013 (Decreto-Lei n.º 113/2013 de 7 de Agosto). Accordingly, *Mus musculus* C57BL/6 specimens were housed at the i3S animal facility. Humane endpoints were defined, encompassing indications of distress, suffering, or pain, body weight reduction exceeding 20% of the total mass, dehydration, anorexia, anuria, and a moribund condition, irrespective of its association with the experimental intervention.

2.24. Mice and immunization

Seven-week-old female adult C57BL/6 mice (from Charles River Laboratories) were housed at the animal facility for one week before the beginning of the experiment to facilitate their acclimatization to the environment. The mice were housed in groups of three per cage, utilizing Makrolon type I cages placed in a restricted access area with controlled room temperature and a 12-hour light-dark cycle, with water and food ad libitum. Environmental enrichment was provided, including corn cob bedding, nesting material, tunnels, and chewing blocks. For immunization studies mice were randomly and blindly distributed in 2 groups (n=3 per group) and the study was performed in triplicate with animals from different litters. Each group was subcutaneously injected with PBS (sham control) and 3 μ g of MUC16-Tn functionalized to PLGA-PEG-MAL NPs (100 μ L). Each mouse was immunized three times; the first immunization occurred on day 0, the second

occurred on day 14, and the third occurred on day 21, respectively. Mice body weight change was monitored once a week, immediately prior to each immunization. One week following the last immunization, mice were euthanized to collect blood and tissues for histology analysis.

2.25. Serum collection

Blood samples were obtained from all animals before immunization via the tail vein and post-experiment through cardiac puncture into heparin-containing vials. Subsequently, the samples were centrifuged at 2500 rpm for 30 minutes at RT. The serum fraction was isolated, followed by additional centrifugation at 1200 rpm for 5 minutes at 4 °C, and then stored at -80 °C for future applications.

2.26. C-reactive Protein Evaluation by ELISA

C-reactive protein levels from mice serum were determined by ELISA using Mouse C-Reactive Protein/CRP Quantikine ELISA Kit (R&D Systems) according to manufacturer's instructions. All serum samples were diluted 2000-fold.

2.27. Histology

Mice liver, spleen, kidneys, heart, brain, and pancreas were gathered to evaluate the toxic effects resulting from the administration of various nanovaccine formulations and processed as previously described by us [52]. Accordingly, freshly collected tissue samples were fixed and preserved in a 10% buffered formaldehyde solution overnight. Following fixation, the tissue specimens underwent manual dehydration using a series of ethanol concentrations and xylene. Subsequently, the tissues were impregnated with paraffin at 60 °C, repeating the process twice for one hour each time. The impregnated tissues were then embedded in paraffin using a specialized embedding system. Sections, measuring 3 µm in thickness, were cut from the formalin-fixed paraffin-embedded (FFPE) tissue samples using a microtome equipped with a disposable blade. These sections were then transferred onto designated glass slides for haematoxylin and eosin staining using a Leica staining protocol. In brief, the FFPE tissue sections underwent deparaffinization and rehydration, followed by a 2-min incubation with eosin and a 5-min incubation with haematoxylin. This was followed by a quick rinse with distilled water. After

air-drying, the slides were immersed in xylene, mounted with Entellan, and subjected to microscopic analysis. The purpose of this analysis was to identify histological variations among the different groups, encompassing features such as necrosis, inflammation, fibrosis, anisokaryosis, and other distinctive findings associated with toxicity.

2.28. IgG and IgM determination by ELISA

Serum samples from each animal were utilized to measure the concentration of Immunoglobulin G (IgG) or Immunoglobulin M (IgM) antibodies using the Mouse IgG or IgM ELISA Antibody Pair Kit assay from STEMCELL Technologies, following the manufacturer's guidelines and previously described by us [52]. Serum was appropriately diluted to 1:50000 for IgG and 1:100000 for IgM analysis. Briefly, ELISA plates were coated overnight at 4 °C with Mouse IgG or IgM capture antibody. Subsequently, serum was added, followed by alkaline phosphatase-conjugated detection antibody (at a 1:1000 dilution). After washing, plates were developed using p-Nitrophenyl Phosphate substrate, and the absorbance was measured at 405 nm.

2.29. IgG antibodies specificity evaluation

The specificity of IgG antibodies for glycosylated domains elicited by the administration of the MUC16-Tn NPs glycoconjugates was evaluated by slot blot. Nitrocellulose membranes (0.45 µM) were loaded with 10 µg of BSA-MUC16, BSA-MUC16-Tn, BSA-MUC1, BSA-MUC1-Tn, BSA-CD44s, BSA-CD44s-Tn, BSA-MUC16-T, BSA-MUC16-S3T and BSA-MUC16-S6T. The membranes, previously blocked with Carbo-free blocking solution 1x (VectorLabs), were blotted for the serums (10 µg/mL) derived from mice stimulated with the glyconanovaccine, for 1 h at RT. Serum derived from PBS injected animals was used as control. Then, IgG anti-mouse secondary antibody (1:70000; 115-035-205 Jackson ImmunoResearch) was added for 30 min at RT. The Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare Life Sciences) was used as developing reagent. Data analysis was performed through Image Lab Software (Bio-Rad) in a ChemiDoc XRS (Bio-Rad).

2.30. IgG reactivity against bladder cancer cell lines

IgG specificity against glycans and glycoconjugates on cancer cells was evaluated by flow cytometry using T24 wild type (T24 WT; MUC16positive; Tnnegative) and T24 C1GALT1 KO (T24 C1GALT1 KO; MUC16positive, Tnhigh) as previously described us [52]. Briefly cells were stained with the murine IgG isolated from mice primed with the 3 µg of MUC16-Tn glyconanovaccine for 1 h at RT (1:50), followed by the secondary antibody Alexa Fluor 488 anti-mouse IgG (A11029, Thermo Fisher Scientific) for 30 min at a 1:300 dilution in FACS buffer under mild agitation at RT. A secondary anti-mouse antibody was used as control. Data analysis was performed through CXP Software and results represent the standard deviation of three independent experiments conducted in a FC500 Beckman Coulter flow cytometer.

2.31. IgG reactivity against bladder cancer tissues

Bladder tumours obtained from IPO Porto's biobank were screened for MUC16 and Tn antigens expression by immunohistochemistry (IHC). The reactivity of IgG isolated from mice immunized with the glyconanovaccine was also accessed. Negative controls included tumours lacking both MUC16 and Tn antigens. Briefly, 3 µm FFPE tissue sections were deparaffinised, hydrated, and endured antigen retrieval using 1 mM EDTA pH 8 (VWR) for 20 min in a microwave. After blocking endogenous peroxidases and nonspecific linkage, tissue sections were incubated with biotinylated VVA (10 µg/mL, VectorLabs) for 1 h at 37 °C for Tn screening or 1:200 dilution of mice-isolated IgG at 4 °C overnight in a humidified chamber. MUC16 evaluation was performed as previously described by us [4]. Detection of lectin and IgG binding to the tissues was assessed through a two-step IHC polymer-based detection system, according to manufacturer's guidelines (Novolink Polymer Detection System, Leica). All images were obtained on a Motic BA310E microscope (Motic) using the Motic Images Plus 3.0 software (Motic).

2.32. Statistical analysis

All experiments were performed at least in triplicates. The results are presented as the mean $\pm$ standard deviation. All graphs and statistical analysis were performed using the GraphPad Prism Software version 9 (GraphPad 9.0.2). Shapiro-Wilk test was used to verify if the obtained values were normally distributed. One-way ANOVA test was used to compare the different groups. Two-way ANOVA

followed by Tukey's multiple comparisons test was used in evaluation of cellular uptake. Welch's t test was used to compare the two groups in the IgG, IgM and C-reactive protein evaluation. Statistical significance was considered when * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

3. Results and Discussion

This work explores the potential of PLGA-PEG NPs as an innovative and effective delivery system for glycovaccines, utilizing cancer-associated MUC16-Tn glycoproteoforms as both neoantigens and targeting molecules for APC (**Figure 1**). The following sections describe the synthesis and physicochemical characterization of MUC16-Tn functionalized PLGA-PEG NPs through maleimide (MAL) thiol chemistry. Furthermore, it assesses the capacity of this molecular construct to activate human monocyte-derived dendritic cells (mo-DCs) *in vitro*. Finally, it evaluates the safety profile of these NPs *in vivo*, using immune competent C57BL/6 mice, and their ability to induce MUC16-Tn targeting IgG. We anticipate that this may mark a significant milestone in the development of effective anti-cancer vaccines against MUC16-Tn, which is prevalent in aggressive solid tumours such as bladder [4], ovarian [21], and pancreatic [22] tumours. Furthermore, it may contribute to the rationale of utilizing other relevant glycoepitopes for cancer immunotherapy.

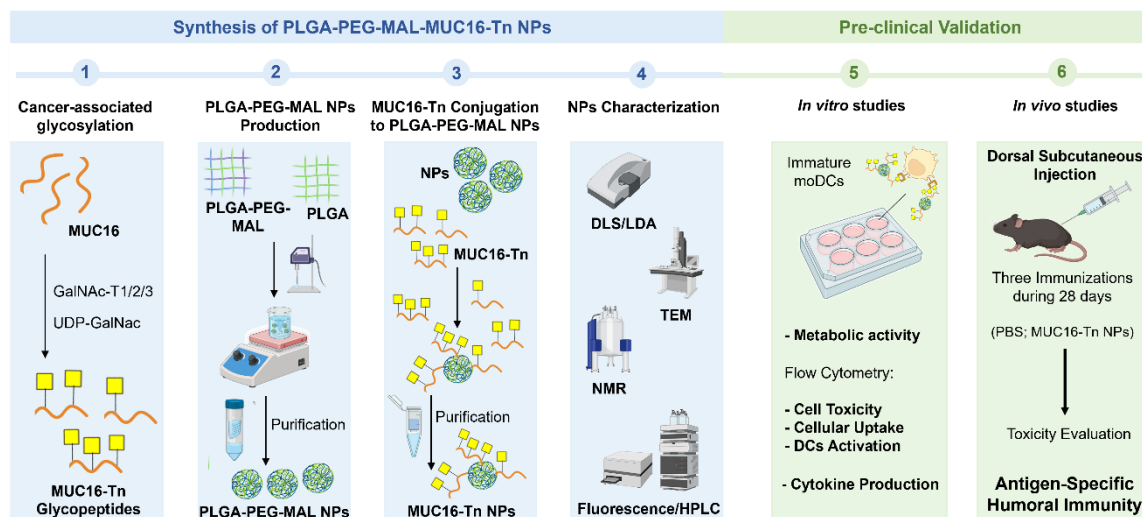


Figure 1. Production of a multivalent PLGA MUC16-Tn Nanoglycovaccine Candidate workflow. MUC16-Tn glycopeptides were obtained by enzymatic synthesis combining three GalNAc-Ts and the UDP-GalNac sugar donor (step 1). PLGA-PEG-MAL NPs were produced through a water-in-oil-in-water (w/o/w) double emulsion-solvent evaporation technique

(step 2). Subsequently, the maleimide-thiol conjugation chemistry was employed to functionalize the PLGA-PEG-MAL nanoparticles with MUC16-Tn glycopeptides, resulting in the creation of a PLGA MUC16-Tn nanoconstruct (step 3). The physicochemical properties and glycopeptide conjugation efficiency was accessed by DLS/LDA, TEM, NMR and Fluorescence/HPLC (step 4). Studies *in vitro* were then conducted to assess the pre-clinical potential of the PLGA MUC16-Tn nanoglycovaccine candidate (step 5). These included the evaluation of metabolic activity, toxicity, cellular uptake, activation, and cytokine production using moDCs. Finally, *in vivo* studies were conducted to determine the toxicity of the glyconanovaccine candidate and to determine antigen-specific humoral immunity (step 6). Created with Biorender.com.

3.1. Synthesis of MUC16-Tn glycopeptide libraries

We began by introducing Tn antigens onto a 20 mer MUC16 variable tandem repeat peptide, with 7 putative glycosylation sites, as previously described by us [23]. This process involved combining multiple recombinant ppGalNAcTs (GalNAc-T1, GalNAc-T2, GalNAc-T3) and uridine 5'-diphospho-N-acetylgalactosamine (UDP-GalNAc) in a single pot reaction, aiming to maximize glycosite occupancy and foreseeing multivalent vaccines that accurately represent the molecular heterogeneity observed in tumours. We specifically selected these glycosyltransferases from a family of 20 ppGalNAc-Ts based on Isoform Specific O-Glycosylation Prediction software (ISOGlyP; <https://isoglyp.utep.edu/>) [24]. Our primary goal was to maximize glycosite occupancy, while minimizing non-glycosylated products. According to nanoLC-MS analysis, glycosylation yields for GalNAc-T1 and T3 exceeded 50% and 70%, respectively (**Figures S1-S3 and Tables S1-S3**). Consequently, glycopeptides with up to 4 occupied glycosites for GalNAc-T1 and 5 occupied glycosites for GalNAc-T3 were obtained. When utilizing GalNAc-T1, a predominance of unglycosylated and mono-glycosylated species was observed (**Figure S1 and Table S1**). This pattern was also noted when employing GalNAc-T3 (**Figure S3 and Table S3**). In contrast, GalNAc-T2 was significantly less efficient, generating only 13 % of mono-glycosylated MUC16-Tn and no more than two occupied glycosites (**Figure S2 and Table S2**). The introduction of an additional enrichment step by Vicia villosa lectin (VVA) affinity chromatography, targeting the Tn antigen, proved crucial for significantly enhancing the yield of glycosylated products (resulting in a 1.5 to 3 times enrichment for glycopeptides; **Figures S1-S3 and Tables S1-S3**). Building on these preliminary findings, further

synthesis explored the combination of the three glycosyltransferases to improve the synthesis. This significantly increased the density of glycosite occupancy, generating predominantly di- and tri-glycosylated peptides (50 % of total glycopeptides), followed by, mono- and tetra-glycosylated species (**Figure 2 and Table S4**). VVA lectin enrichment resulted in significant changes in glycopeptide abundance, namely by the reduction of unglycosylated and mono-glycosylated species and the consequent increase in highly glycosylated species, highlighting the importance of including this enrichment step. Of note, we consistently observed low variability between glycosylated species obtained by three different experiments (**Table S4**), demonstrating the reproducibility of the enzymatic and enrichment strategies. These glycopeptides were further explored as APC-targeting agents and antigenic molecules in subsequent nanovaccine candidate development.

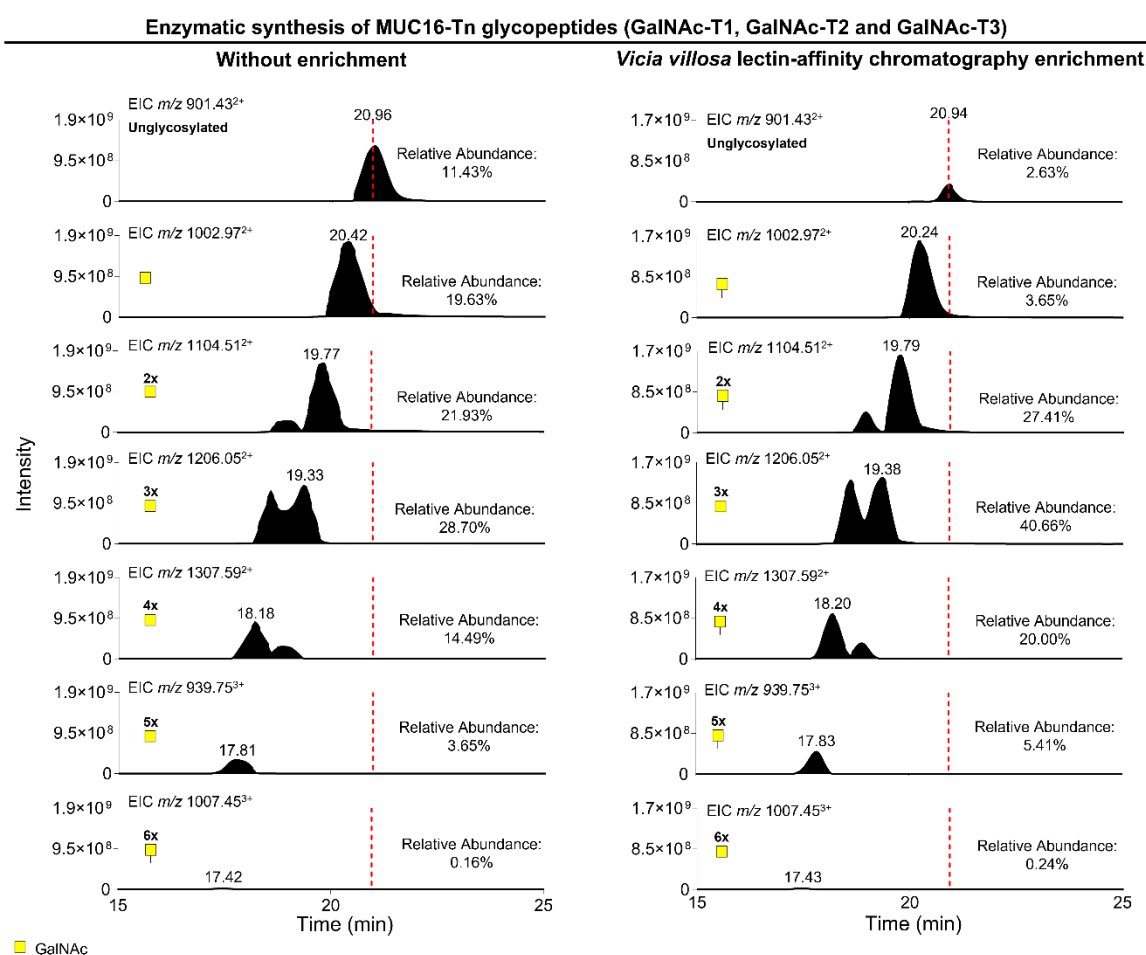


Figure 2. MUC16-Tn glycopeptide libraries obtained by enzymatic synthesis for cancer nanovaccines production. After enrichment with VVA lectin or the absence of enrichment, the products were characterized by C18 reverse phase nanoLC-MS. MUC16-Tn glycopeptide

libraries obtained from the enzymatic synthesis with multiple GalNAc-Ts contain species with up to 6 occupied glycosites, being the most predominant the tetra-glycosylated peptides. Vestigial amounts of unglycosylated peptides were detected, confirming high yields of glycopeptide synthesis. Enrichment with the VVA lectin, with affinity for the Tn antigen, slightly reduced di-glycosylated peptides, while increasing the amount of tetra-glycosylated peptides. The abundance of tri- and penta-glycosylated peptides were reasonably maintained.

3.2. Production of PLGA-PEG-MAL-MUC16-Tn nanoparticles

We generated Poly(lactide-co-glycolide)-b-poly(ethylene glycol)-maleimide (PLGA-PEG-MAL) NPs using the double emulsion-solvent evaporation method. Nanoparticles were then functionalized with MUC16-Tn glycopeptides, resulting in the formation of PLGA-PEG-MAL-MUC16-Tn constructs. The choice for the double emulsion-solvent approach was primarily related to its versatility for encapsulating various biomolecules and/or drugs [15, 25], enabling the future development of vaccine delivery systems with improved pharmacological properties. The inclusion of PEG envisaged to increase NPs stability and preventing early *in vivo* elimination by the immune system [26]. To conjugate MUC16-Tn, we introduced a cysteine tag at the glycopeptides' reducing end, and this tag was then linked to the maleimide group anchored to the NPs surface. This approach allowed precise control of the conjugation reaction stoichiometry after reduction with Tris(2-carboxyethyl)phosphine (TCEP). NPs functionalized with non-glycosylated MUC16 (MUC16 NPs) and non-functionalized NPs (Control NPs) were also produced as control systems. Simultaneously, unconjugated and conjugated NPs labelled with the FKR648 fluorophore were produced for fluorescent assays. Dynamic light scattering (DLS) measurements indicated that the size of PLGA-PEG-MAL NPs ranged from 104.8 to 137.4 nm (**Table 1**). Functionalization resulted in minimal alterations in size and heterogeneity, with the particles measuring 137 nm and exhibiting a polydispersity index (Pdl) of 0.240 (**Table 1**). All formulations maintained a negative zeta potential, indicating stability, low aggregation tendency, and reduced risk of repulsion by negatively charged cell surfaces (**Table 1**) [27]. Interestingly, the presence of MUC16-Tn glycopeptides on the NPs surface increased their negative potential, possibly due to the negative charge presented by the peptides/glycopeptides under the reaction's pH conditions. Fluorescent NPs were slightly larger, ranging from 110.8 to 219 nm (**Table 1**), with a Pdl within the range

of 0.098 to 0.289, confirming the low heterogeneity of these NPs (**Table 1**). Like non-fluorescent NPs, the increase in negative charge observed in fluorescent functionalized NPs supports the presence of functionalized glycopeptides (**Table 1**). Additionally, transmission electron microscopy (TEM) revealed consistent spherical shape, size range, and polydispersion of the NPs, supporting the findings of DLS analysis (**Figure 3A**).

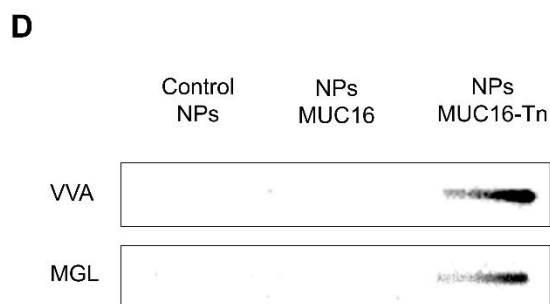
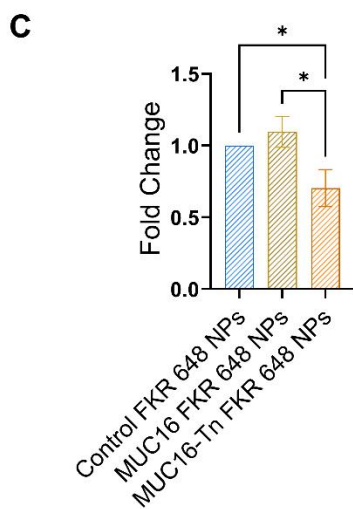
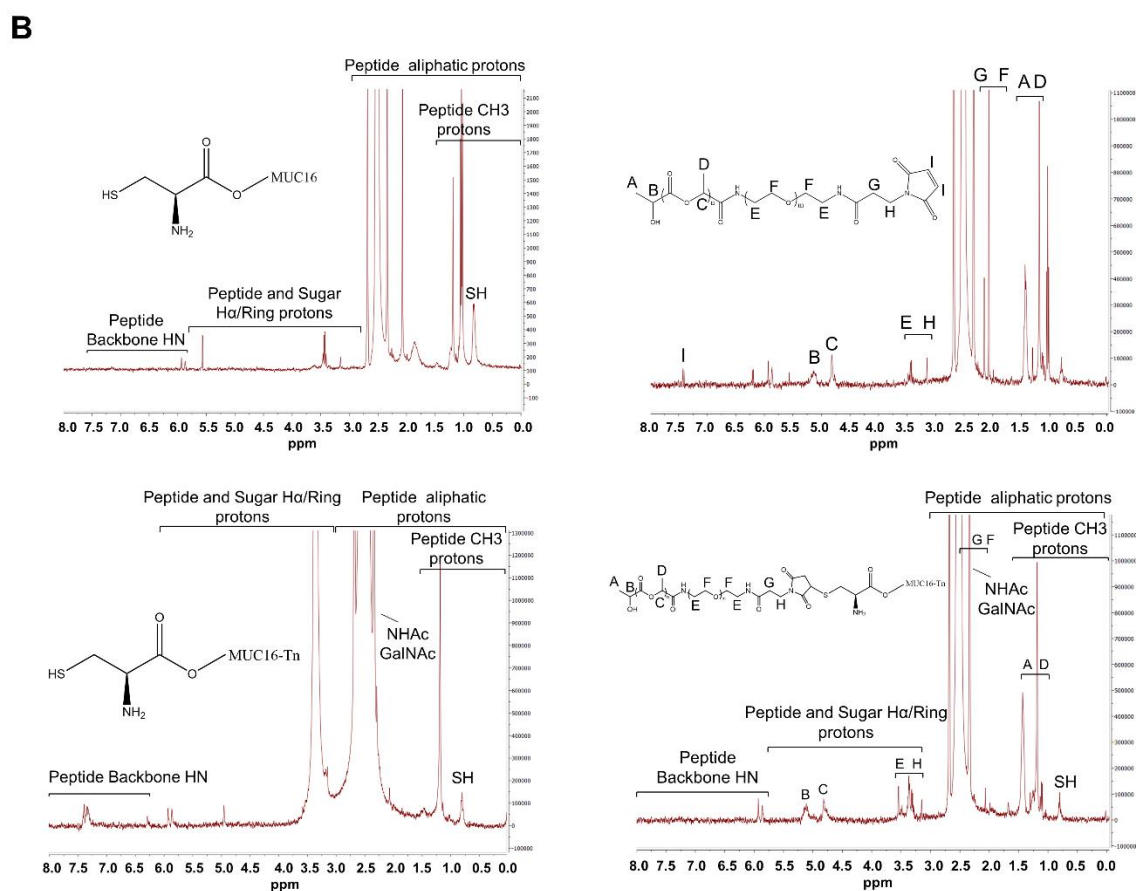
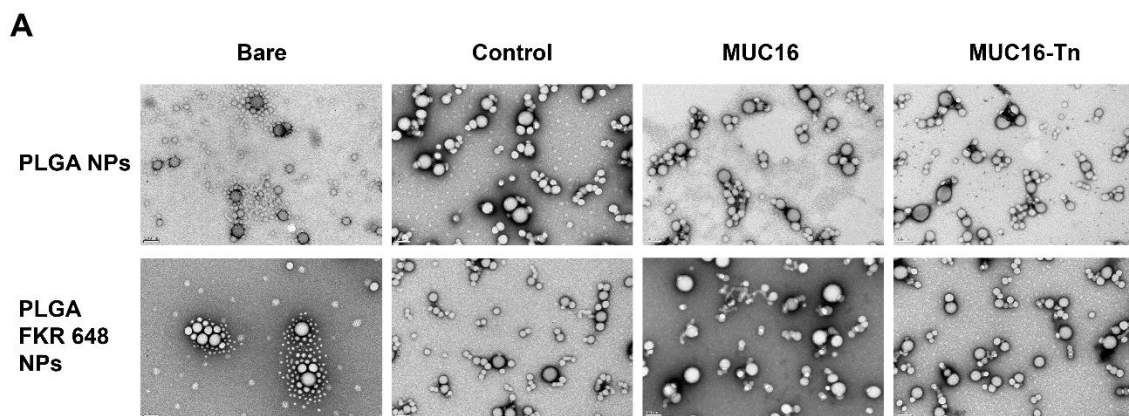


Figure 3. Morphological characterization of nanoconstructs and evaluation of Tn recognition by VVA/MGL lectins. A) Nanoparticles presented spherical shape and low intrinsic aggregation tendency. TEM analysis of all produced nanoconstructs. Scale bar represents 100 nm. Images were obtained with 100.000x magnification. B) ^1H NMR spectra confirms the successful functionalization of MUC16-Tn to PLGA-PEG-MAL NPs. The spectra presented characteristic signals corresponding to peptide, Tn-containing glycopeptide, and PLGA-PEG-MAL NPs, highlighting the successful functionalization of MUC16-Tn glycopeptides to NPs. Some degree of glycopeptide adsorption is also suggested. C) Fluorescent MUC16-Tn NPs presented high affinity for VVA lectin. Lectin affinity chromatography selectively retained MUC16-Tn NPs, as highlighted by the decrease in fluorescence of the supernatant, indicating more NPs retention in the agarose bound VVA lectin. Data is representative of at least 3 independent experiments. Comparisons were performed using One-way ANOVA. Statistical significance was achieved when $*p < 0.05$. D) VVA and MGL selectively recognized MUC16-Tn NPs. Lectin immunoblotting showed specific reactivity of VVA/MGL for MUC16-Tn NPs, while failing to recognize MUC16-NPs or control NPs.

To indirectly assess the conjugation efficiency of MUC16 and MUC16-Tn, we quantified the unbound peptides in the resulting supernatants of NPs washing fractions. This quantification was performed through fluorescence measurement following fluorescamine labelling. The conjugation efficiency was further validated by HPLC analysis to confirm the results obtained by fluorescence measurement. The conjugation efficiency for NPs functionalized with MUC16-Tn exceeded 96%, while it was lower than 65% for MUC16 (Table 1).

Table 2. Physicochemical properties of PLGA-PEG-MAL based nanoconstructs, including mean size, polydispersity index (Pdl), zeta potential, and conjugation efficiency (CE). The values are represented as mean values $\pm$ SD (n=3).

Material	Nanoconstruct	Functionalized Agent	Size (nm)	PdI	Zeta Potential (mV)	CE (%)
PLGA-PEG-MAL	-	Bare	104.8 $\pm$ 3.0	0.084 $\pm$ 0.004	-5.16 $\pm$ 0.80	-
	Functionalized PBS 1x	Control	111.1 $\pm$ 0.3	0.204 $\pm$ 0.007	-5.52 $\pm$ 1.93	-
	Functionalized	MUC16	137.4 $\pm$ 1.0	0.235 $\pm$ 0.011	-7.79 $\pm$ 1.35	29.7 $\pm$ 6.1
	Functionalized	MUC16-Tn	136.7 $\pm$ 1.0	0.240 $\pm$ 0.007	-7.25 $\pm$ 1.17	96.7 $\pm$ 1.0
PLGA-PEG-MAL-FKR648	-	Bare	110.8 $\pm$ 2.0	0.098 $\pm$ 0.014	-4.37 $\pm$ 0.453	-
	Functionalized PBS 1x	Control	219 $\pm$ 4.5	0.158 $\pm$ 0.018	-5.39 $\pm$ 1.32	-
	Functionalized	MUC16	197 $\pm$ 4.5	0.194 $\pm$ 0.009	-8.22 $\pm$ 1.28	64.3 $\pm$ 5.4
	Functionalized	MUC16-Tn	202.9 $\pm$ 3.7	0.289 $\pm$ 0.021	-8.76 $\pm$ 0.711	98.3 $\pm$ 0.5

These differences suggest that the presence of GalNAc likely plays a significant role in augmenting the binding capacity of MUC16, though the specific molecular mechanism remains to be fully elucidated. Moreover, to determine peptide adsorption to NPs, we incubated the NPs with MUC16/MUC16-Tn peptides lacking the cysteine tag. Forty-two percent of MUC16-Tn was adsorbed onto the NPs, whereas no MUC16 adsorption was observed (data not shown), reinforcing the notion of successful functionalization. Finally, controlled release experiment at pH 7.4 was performed to estimate the stability of the covalently linked MUC16 and MUC-16-Tn PLGA NPs. No glycopeptide was released from both constructs over a 168-hour period, suggesting efficient covalent immobilization and high affinity of the glycopeptide to the NPs. In summary, after functionalization, the NPs displayed the appropriate size (20-200 nm), spherical shape, and homogeneity required for preferential targeting of DCs and efficient transport to lymph nodes for antigen presentation [28]. Additionally, we verified that over 95% of the produced glycopeptide was functionalized to NPs, indicating successful conjugation, although we cannot entirely rule out some degree of adsorption. Moreover, these NPs exhibited excellent stability at physiological pH for an extended period.

3.3. Nuclear Magnetic Resonance (NMR) and Nanoparticles affinity for Tn binding lectins

Complementary studies by proton NMR were performed to confirm the presence of MUC16-Tn linked to the NPs (**Figure 3B**). The MUC16 peptide used as control exhibited signals in the range of 0.5-1.5 ppm, corresponding to CH₃ protons. Additionally, signals in the range of 3.0-5.5 ppm corresponded to peptide H α protons, while signals in the range of 6.0-7.5 ppm resulted from NH protons in the peptide backbone. Additionally, the signal corresponding to the SH group from the cysteine tag used for conjugation was detected between 0.5-1.0 ppm [29] (**Figure 3B**). The MUC16-Tn spectrum presented additional signals between 2.0-3.0 ppm, corresponding to NHAc groups, and 3.0-5.5 ppm, corresponding to H α and H₂-H₆ ring protons of the GalNAc residues [30] (**Figure 3B**). Control PLGA-PEG-MAL NPs presented a typical spectral fingerprint, in accordance with previous publications [31, 32]. Intense signals at 1.0-1.5 ppm, characteristic of CH₃ from PLGA [31], 2.0 ppm and 3.5 ppm, corresponding to PEG [31], and 7.0-7.5 ppm, corresponding to MAL [32], were detected (**Figure 3B**). Finally, the spectrum corresponding to MUC16-Tn NPs was markedly different from the PLGA-PEG-MAL NPs, exhibiting signals corresponding to both peptide and Tn protons. Notably, resonances at 7.0-7.5 ppm, corresponding to maleimide, were missing, suggesting conjugation through maleimide group (**Figure 3B**). However, the signal corresponding to SH could still be observed, indicating some degree of glycopeptide adsorption.

Subsequently, we investigated the recognition of MUC16-Tn glycopeptides on the NPs by VVA and MGL, two lectins known for their strong affinity for the Tn antigen [33, 34] (**Figure 3C and D**). First, NPs' binding capacity to VVA was assessed exploiting lectin chromatography and MUC16-Tn functionalized PLGA-FKR648 NPs. The significant reduction in fluorescence intensity observed in the flow-through of MUC16-Tn NPs suspensions compared to controls strongly suggests enhanced retention of MUC16-Tn NPs by the lectin (**Figure 3C**). Orthogonal validation by lectin blotting confirmed that glycan motifs on the MUC16-Tn glycopeptides remained conserved following conjugation to the NPs and could be specifically targeted by VVA and MGL. Collectively, these findings support effective functionalization of PLGA-PEG-MAL NPs with MUC16-Tn glycopeptides and

underscore their potential as vehicles for targeting GalNAc-binding receptors such as MGL in antigen presenting cells.

3.4. Mo-DCs metabolic activity, NPs toxicity and uptake *in vitro*

Before proceeding for *in vitro* and *in vivo* assays, the endotoxin levels of formulations were determined. Accordingly, formulations presented endotoxin levels below admissible values for *in vitro* and *in vivo* testing (0.5 EU/mL) [35, 36] (0.09 ± 0.003 EU/mL for MUC16-Tn and 0.42 ± 0.014 EU/mL for MUC16-Tn NPs) (**Figure 4A**) demonstrating their ability to be used in further assays. The toxicity of MUC16-Tn NPs on mo-DCs was assessed through 4',6-diamidino-2-phenylindole (DAPI) incorporation by apoptotic cells (**Figure S4**). Toxicity studies were also conducted in the presence of the vaccine adjuvant monophosphoryl lipid A (MPLA) with the goal of facilitating future clinical translation. This Food and Drug Administration (FDA)-approved MPLA adjuvant is a non-toxic derivative of lipopolysaccharide lipid A, enhancing immune response by activating APCs and promoting immune memory [37]. Based on this assay, NPs toxicity increased with concentration; however, with acceptable levels within the range of 0.1-1.0 $\mu\text{g/mL}$ (**Figure S4**; acceptable toxicity < 30% cell death according to ISO 10993-5 [38]). The presence of the adjuvant resulted in significant toxicity, regardless of the NPs concentration. Interestingly, MPLA alone exhibited no toxicity, in perfect agreement with its well-established pharmacological safety profile [37]. The MTT assay was employed to assess the metabolic activity of mo-DCs when exposed to various formulations, revealing a significant increase in metabolic activity upon stimulation with 1 and 10 $\mu\text{g/mL}$ of MUC16-Tn NPs (**Figure 4B**), suggesting cell activation. A preliminary flow cytometry activation assay using CD86, a TCR co-stimulatory receptor, and HLA-DR, also known as MHC-II, markers was performed to assess mo-DCs maturation profiles (**Figure S5 and S6**). Cell activation, translated by increased expression of CD86-positive mo-DCs, was observed for MUC16-Tn NPs concentrations beyond 0.1 $\mu\text{g/mL}$, while no differences were observed for HLA-DR expression. This effect was consistent whether the NPs were administered individually or in combination with MPLA, clearly demonstrating that mo-DCs activation is independent of NPs concentration even in the presence of MPLA adjuvant (**Figure S6**). In summary, MUC16-Tn functionalized NPs evidenced a notable ability to induce activation of mo-DCs, akin to the activation achieved with

lipopolysaccharides (LPS) positive controls, even at low NPs concentrations and in the absence of an adjuvant. Considering the NPs toxicity profile and active concentrations, a concentration of 1 $\mu\text{g/mL}$ of MUC16-Tn NPs without MPLA was elected for subsequent experiments.

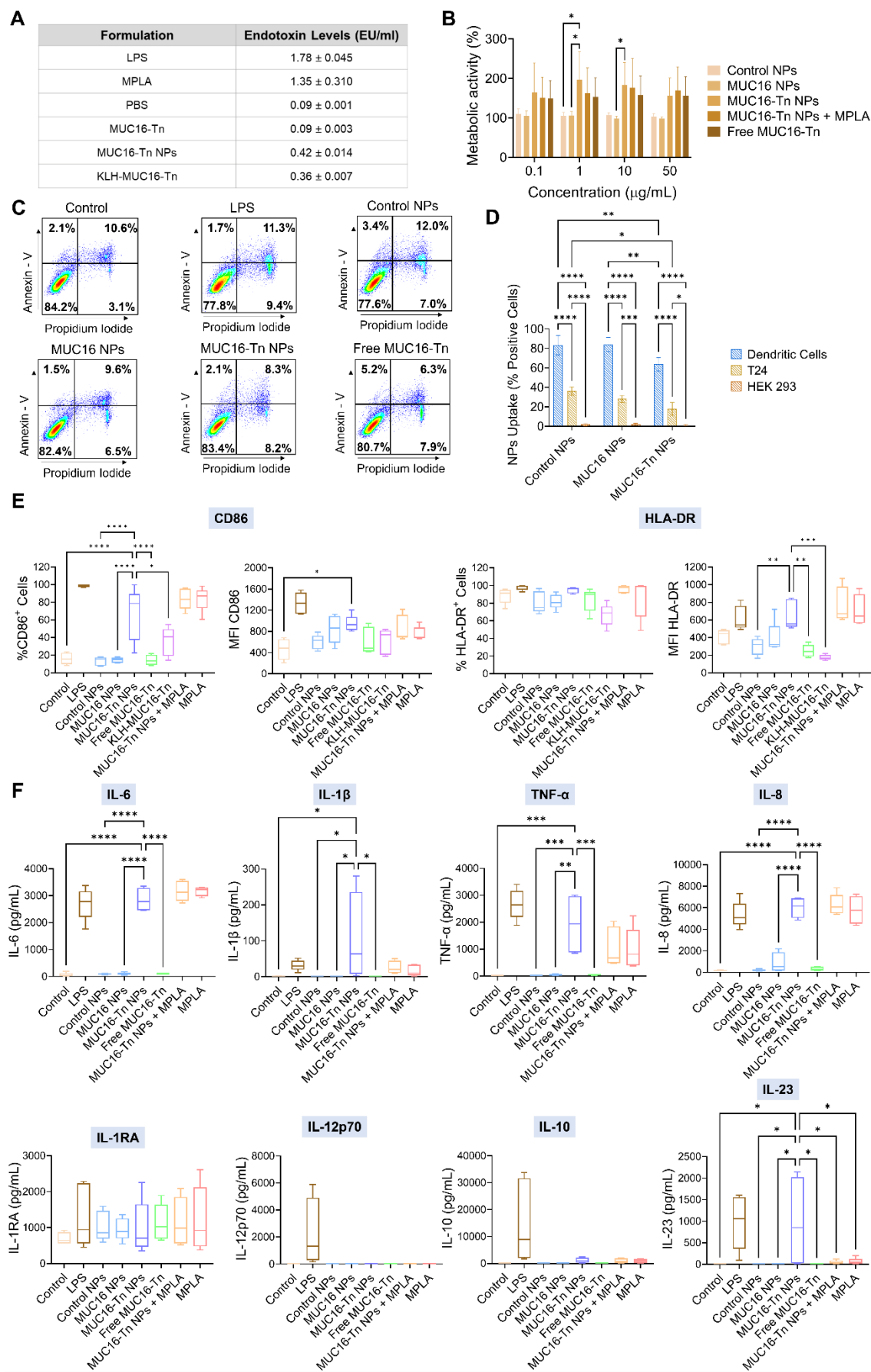


Figure 4. MUC16-Tn NPs showed no toxicity *in vitro* and showed preferential internalization by mo-DCs, increasing its metabolic activity and stimulating a pro-inflammatory profile in the absence of an adjuvant. A) The endotoxin levels for MUC16-Tn, MUC16-Tn NPs and KLH-MUC16-Tn formulations were found below admissible limits (0.5 EU/mL) for *in vitro* and *in vivo* applications. B) MUC16-Tn NPs increased metabolic activity of mo-DCs. The MTT assay showed an increase in metabolic activity of mo-DCs in the presence of 1 µg/mL of glyconanovaccine. C) MUC16-Tn NPs did not affect the viability of mo-DCs. The viability of mo-DCs was assessed by staining for cell surface Annexin V and Propidium Iodide (PI), followed by flow cytometry analysis. Plots comparing Annexin V to PI from gated single cells show the populations corresponding to viable (Annexin V- PI-), early apoptotic (Annexin V+ PI-), necrotic (Annexin V- PI+) and late apoptotic (Annexin V+ PI+) cells. Numbers within gates indicate the percentage of cells in each of these populations. D) MUC16-Tn NPs are preferentially internalized by mo-DCs. The uptake of the glyconanovaccine was evaluated by flow cytometry using fluorescent PLGA NPs. Both bladder cancer cell line T24 and normal tissue cell HEK293 were less susceptible to NPs internalization than mo-DCs. Comparisons were performed using Two-way ANOVA. E) MUC16-Tn NPs were able to stimulate mo-DCs in the absence of MPLA adjuvant. Maturation markers CD86 and HLA-DR evaluation showed that the glycovaccine alone stimulated mo-DCs. F) MUC16-Tn NPs induced the production of several pro-inflammatory cytokines by mo-DCs. Multiplex and ELISA studies showed that mo-DCs stimulated with the glyconanovaccine presented increased levels of pro-inflammatory cytokines as IL-6, IL-1β, TNF-α, IL-8, and IL-23. In addition, low levels of IL-12(p70) were detected, while differences in anti-inflammatory cytokines IL-10 and IL-1RA were not found. The results correspond to the mean and standard deviation. Data is representative of at least 5 independent experiments. For MTT and activation/cytokine evaluation, all comparisons were performed using the One-way ANOVA test. Statistical significance for comparisons with LPS positive control was omitted for better graph interpretation. Statistical significance was achieved when * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

The viability of mo-DCs stimulated with MUC16-Tn NPs and respective controls was assessed by Annexin V Apoptosis assay. Both MUC16-Tn NPs and controls preserved mo-DCs viability, suggesting biocompatibility of the nanoformulations (**Figure 4C**). Selective uptake of control NPs, MUC16 NPs, and MUC16-Tn NPs by mo-DCs, by the non-cancer human embryonic kidney HEK293 cells, and by the aggressive T24 bladder cancer cells was also explored (**Figure 4D**). Mo-DCs exhibited the highest uptake, while uptake in non-cancerous HEK293 cells was nearly negligible. This suggests the selective internalization of the nanoformulations by mo-DCs and, to less extent, cancer cells. Notably, the

percentage of mo-DCs uptake was 20% lower for MUC16-Tn in comparison to MUC16-functionalized and non-functionalized NPs, indicating distinct uptake mechanisms, most likely mediated by the glycosidic moiety.

3.5. Mo-DCs activation and cytokine profile *in vitro*

The capacity of the nanovaccine candidate to activate mo-DCs *in vitro* was also pursued (**Figures 4E-F and S7**). Of note, a classical glycovaccine construct composed by the immunogenic protein keyhole limpet hemocyanin (KLH) conjugated to MUC16-Tn (KLH-MUC16-Tn) was also tested. Importantly, MUC16-Tn functionalized NPs effectively stimulated mo-DCs, as evidenced by increased CD86 (both percentage of positive cells and MFI) and HLA-DR (MFI) expression, when compared to free MUC16-Tn, KLH-MUC16-Tn, and Control/MUC16 NPs (**Figure 4E**). Moreover, the level of stimulation was comparable to that observed in experiments with the MPLA adjuvant. Consistent with these observations, distinct mo-DCs aggregates were detected following exposure to MUC16-Tn NPs alone or in combination with MPLA, which were not apparent in the case of non-functionalized and MUC16-functionalized NPs, and free MUC16-Tn glycopeptide stimulation (**Figure S7**). To further confirm the activation and maturation status of mo-DCs, the profile of secreted cytokines was investigated (**Figure 4F**). MUC16-Tn NPs strongly induced pro-inflammatory cytokines, including IL-1 β (mediator of T cell immunity [39]), IL-6 (responsible for proliferation of T lymphocytes and differentiation in cytotoxic T cells [40]), IL-8 (involved in neutrophils recruitment [41]), IL-23 (induces memory T cell proliferation [42]) and TNF- α (enhances phagocytosis and cytotoxicity of granulocytes; promotes T cells activation and differentiation [43]), with no changes in anti-inflammatory cytokines such as IL-1RA and IL-10 (**Figure 4E**). Furthermore, low levels of the pro-inflammatory cytokine IL-12(p70) (**Figure 4E**) were detected, while its antagonist, IL-12(p40), was overexpressed (**Figure S8**), suggesting that IL-12 is not a driver of the pro-inflammatory response promoted by the glyconanovaccine candidate. A trend towards increased secretion of INF- γ (**Figure S8**) was also observed. Collectively, these findings support that MUC16-Tn NPs induce mo-DC activation and their presumed capacity to contribute to both innate and adaptive immune responses [40]. In addition, these results suggest that the self-adjuvating function of the glyconanovaccine candidate is dependent of the synergistic effect of MUC16-Tn glycopeptide conjugated to PLGA-PEG-MAL NPs, as

no immunostimulatory effect has been observed for non-functionalized NPs or the glycopeptides. Moreover, the mo-DCs activation occurs probably due to MGL receptor targeting by the Tn glycan [34, 44], following activation of NF- κ B pathway and the consequent production of pro-inflammatory cytokines TNF- α , IL-8, IL-6, IL-1 β and IL-23 similarly to what have been previously observed by targeting of other C-type lectin receptors (CLRs) [45], which warrants future validation.

3.6. Glyconanovaccine candidate humoral immune response evaluation *in vivo*

Finally, the toxicity of MUC16-Tn NPs, as well as their ability to elicit antibody responses against the glycoimmunogens *in vivo* was investigated. Prior to *in vivo* studies, the stability of the glyconanovaccine candidate at physiological pH was studied over a 21-day period, matching the duration of the predicted *in vivo* immunization protocol (**Figure 5A**). Of note, NPs size, Pdl, and zeta potential suffered only minor variations throughout the stability test (**Figure S9**), indicating the vaccine's suitability for further *in vivo* studies. The PLGA-PEG-MAL-MUC16-Tn glyconanovaccine candidate was then administered subcutaneously to immunocompetent C57BL/6 mice and PBS was used as Sham Control. Mice were immunized three times, at day 1, 14 and 21, and euthanized seven days after the last immunization (**Figure 5A**). Notably, mice showed no signs of distress over the experiment course, demonstrating signs of physiological well-being by gradually increasing body weight in all experimental groups (**Figure 5B**). Also, histological evaluation of relevant organs (spleen, liver, pancreas, kidney, brain, and heart) at the end of the experiment revealed no signs of injury or inflammation (**Figure S10**), reinforcing the safety profile of the administered formulations. In addition, no evidence of systemic inflammation after inoculation was found, as indicated by normal C-reactive protein levels in mice serum (5-9 μ g/mL) [46] (**Figure 5C**), reinforcing the biocompatibility and no acute inflammatory profile of the nanovaccine candidate.

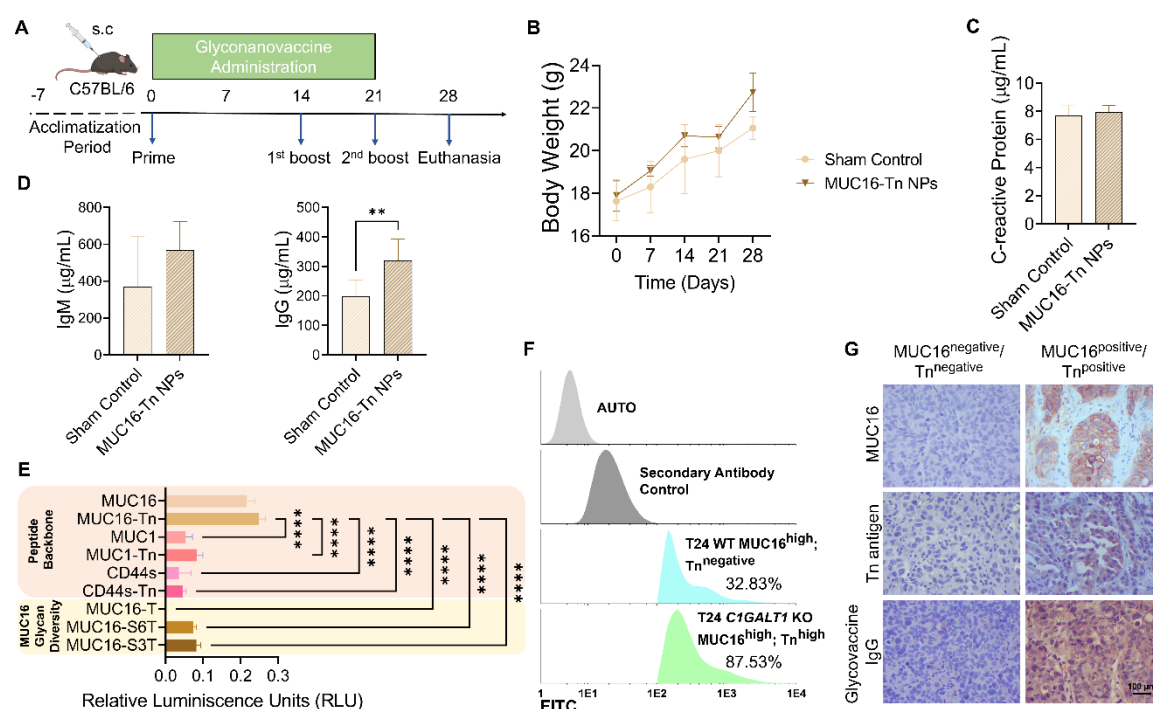


Figure 5. The glyconanovaccine candidate showed no toxicity *in vivo* and generated antibodies showing specific recognition of synthetic MUC16/MUC16-Tn and a cancer cell line and tumour tissues overexpressing MUC16-Tn and Tn antigens. A) Immunization scheme of C57BL/6 mice. After 1 week of acclimatization, mice were subcutaneously (s.c) primed with the nanoformulations, following a boost injection 14 day later and a second boost 7 days after. Seven days after the last immunization, mice were euthanized. Created with Biorender.com. B) Body weight evaluation during the experimental period. Mice from both groups increased their body weight during the 28 days of experimental procedures, which suggests a clearly safe profile of the glyconanovaccine. C) Mice presented no signs of acute inflammatory conditions. C-reactive protein levels were found within the normal range for healthy mice, and no differences were detected between experimental groups. D) Humoral responses generated after glyconanovaccine administration. The glyconanovaccine stimulated IgG production, while no effect on IgM levels were found. Welch's t test was used to compare the two different groups. E) IgG antibodies in serum of mice immunized with the glyconanovaccine specifically recognized synthetic MUC16 and MUC16-Tn. IgG antibodies showed low reactivity for synthetic CD44s/CD44s-Tn and MUC1/MUC1-Tn antigens, failing to recognize MUC16-T antigens and demonstrating high reactivity against MUC16/MUC16-Tn compared to sialylated glycoforms. Comparisons were performed using the One-way ANOVA test. Statistical significance was achieved when * $p < 0.05$, ** $p < 0.01$, * $p < 0.001$, **** $p < 0.0001$. F) IgG specifically recognized MUC16-Tn-expressing bladder cancer cells. PLGA-PEG-MAL-MUC16-Tn glyconanovaccine immunized mice IgG recognized**

bladder (T24 C1GALT1 KO; MUC16^{high}; Tn^{high}) cancer cell lines edited to express the Tn antigen. **G) IgG cross-reacted with MUC16^{positive}/Tn^{positive} bladder tumour sections (right panel), but not with MUC16^{negative}/Tn^{negative} cancer tissues (left panel).**

3.7. IgG antibodies production and reactivity against glycodomains, cancer cell lines and tumour tissues

Serum samples of immunized mice were used to assess antibody titration and the specificity of antibody responses against glycoconjugates. Both IgG and IgM antibody production following stimulation were determined by ELISA (**Figure 5D**). A significant increase in IgG antibody production upon glyconanovaccine administration was found, with no discernible differences in IgM production (**Figure 5D**). To assess IgG antibody specificity for glycans and glycoconjugates, a slot-array composed by MUC16, MUC16-Tn, as well as cancer-associated (glyco)peptides CD44s, CD44s-Tn, MUC1, and MUC1-Tn was explored. The array also included MUC16 carrying other types of typical and more extended O-glycans expressed by both normal and cancer cells, namely the T (Gal- β 1-3-GalNAc- α 1-O-Ser/Thr), sialyl-3-T (S3T; Neu5Ac- α 2-3-Gal- β 1-3-GalNAc- α 1-O-Ser/Thr), and sialyl-6-T (S6T; Gal β 1-3[Neu5Ac α 2-6]GalNAc- α 1-O-Ser/Thr) antigens. IgG from immunized mice exhibited significantly higher reactivity toward MUC16 and MUC16-Tn, with negligible recognition of Tn antigens on other peptide backbones and MUC16 carrying more extended glycans (**Figure 5E**), indicating specificity for the vaccine immunogen. Additionally, IgG recovered from immunized mice recognized T24 cells glycoengineered to stably and homogeneously express the Tn antigen through the knockout of C1GALT1 (T24 C1GALT1KO; **Figure 5F**), a key glycosyltransferase for O-glycan extension. Notably, dedicated glycoproteomics and immunoassays confirmed the expression of MUC16-Tn glycoepitopes in this cell line (**Figure S11**). As expected, and consistent with slot-array analysis, lower levels of antibody binding to the wild-type T24 cell line were observed, which is most likely due to cross reactivity of the antibodies generated against the MUC16 peptide backbone. Finally, serum IgG were used to screen Tn and MUC16-positive and -negative bladder cancer tissues. Purified IgG showed reactivity against bladder tissues, supporting the capacity to recognize cancer cells in human tumours (**Figure 5G**). Furthermore, no cross-reactivity was observed against tissues that were negative for both MUC16 and Tn antigens, supporting antibodies' specificity. Collectively, these results show that the produced IgG exhibited specific

recognition for MUC16-Tn, as well as for cell lines and tumour tissues overexpressing MUC16-Tn and Tn antigens. This finding underscores the potential of our glyconanovaccine candidate to elicit an anti-tumour response. Moreover, slot-array analysis revealed minimal reactivity towards Tn antigens on other peptide scaffolds, indicating that the generated antibodies primarily targeted the glycopeptide domains rather than the glycan itself. This observation is particularly distinctive since most glycan-binding antibodies developed thus far often lack the essential glycopeptide specificity needed for precise cancer targeting [47].

4. Concluding Remarks

Cancer vaccines are an emerging approach in the field of anti-tumour immunotherapy, given their potential to eradicate tumours and prevent relapse [1]. Moreover, cancer-specific glycoproteoforms, arising from immature O-glycosylation due to premature termination of protein glycosylation, are potential candidates for vaccine design [5]. Their cancer-associated nature and cell surface localization facilitates cancer cells recognition by the immune system, providing a solid foundation for vaccine development. Classical anti-tumour glycovaccines involve conjugating antigens to immunogenic proteins, with the goal of inducing humoral and cellular immune responses [5]. However, despite increasing the immunogenicity of the targeted antigen, this approach can potentially lead to non-specific immune responses against the carrier protein and subsequent epitope suppression [5]. Here, we demonstrate that PLGA functionalization with glycoepitopes may provide means to overcome this limitation, enabling effective glycoantigens delivery to immune cells and triggering of immune responses without the need of a carrier protein.

In this study, we further hypothesized that pancreatic carcinoma MUC16-Tn glycoepitopes could serve a dual role as neoantigens and targeting molecules for immature APCs, which express MGL receptor targeting the Tn antigen. The potential of PLGA-PEG-NPs to stimulate immune responses was also explored. Of note, these NPs bring considerable innovation to glycovaccine design by eliminating the requirement for additional adjuvant co-administration that characterized most glycovaccine immunization protocols [37]. As such, this work offers a multifaceted PLGA-based multivalent glyconanovaccine candidate potentially translatable to multiple solid tumours associated with a poor prognosis,

where the prevalence of MUC16-Tn expression is well-documented [4, 21]. We started by implementing an enzymatic strategy to create a library of densely glycosylated MUC16-Tn glycopeptides, mimicking the molecular heterogeneity observed in tumours. The combined employment of three GalNAc-Ts led to increased glycosylation yields and the introduction of multiple glycosites, rendering it the most suitable approach for producing MUC16-Tn glycopeptides essential for the development of a multivalent nanoglycovaccine. Well-established maleimide-thiol click chemistry conjugation was used to functionalize PLGA-PEG nanoparticles with MUC16-Tn glycopeptides.

In vitro studies demonstrated the low toxicity profile functionalized NPs, as well as their preferential uptake by DCs, especially when compared to healthy cells. Furthermore, it became evident that co-administration with MPLA significantly amplified the toxicity of the formulations, underscoring the importance of a meticulous assessment of approved adjuvants to combine with PLGA nanoparticles. In addition, the proposed glyconanovaccine candidate was capable of significantly stimulate DCs in the absence of MPLA adjuvant. Leveraging the translational potential of the proposed glyconanoconstruct, an *in vivo* study with the primary objective of assessing the tolerability and the production of specific IgG against MUC16-Tn glycopeptides was conducted. Our findings clearly demonstrated that the glycovaccine candidate was well-tolerated, as indicated by the absence of distress signals, organ injury, and biochemical signs of inflammation. Furthermore, generated IgG showed specific recognition of MUC16 and MUC16-Tn, as well as cell lines and tumours overexpressing MUC16-Tn and Tn antigens, which supports the potential anti-tumour response of our glyconanovaccine.

In summary, our glyconanovaccine candidate has been designed to target APCs expressing Tn binding lectin receptors, serving as a carrier for antigen presentation and as an adjuvant to enhance anti-tumour immune responses. Our research lays the essential molecular and pre-clinical foreground for an innovative, self-adjuvated cancer glycovaccine. To our knowledge, this represents a pioneering effort to harness glycan-associated molecular signatures in the creation of a polymeric glyconanovaccine. Future studies should involve a comprehensive assessment of humoral and cellular responses to vaccination, as well as the exploration of prophylactic and therapeutic potentials. This includes integration with existing therapeutic approaches such as immune checkpoint inhibitors, with the aim of achieving effective clinical translation.

The assessment of immunological memory will also be paramount for the effective deployment of a glycovaccine. However, we acknowledge significant challenges on the path to clinical translation. A pivotal milestone involves establishing a humanized murine cancer model that expresses MUC16-Tn, a crucial step in advancing pre-clinical development. Notably, this entails achieving both the expression of the targeted protein epitopes and precise fine-tuning of glycosylation pathways to ensure the expression of immature cancer-associated glycans. We are currently pursuing this objective with the vision of laying the groundwork for a paradigm shift in cancer immunotherapy. Our goal is to introduce cancer-protective approaches through active immune surveillance by specifically targeting these metastasis-associated glycosignatures, which, to date, remain largely unexplored.

AUTHOR CONTRIBUTIONS

RF: conceptualization; data acquisition; data analysis; writing; illustration; **EF, AM:** data acquisition; data analysis; illustration; **DF:** data acquisition; data analysis; **MRS:** data analysis; illustration; **FC, BS, MG, SQ:** data acquisition; review and editing; **CP:** data acquisition; data analysis; **MJO, BS, LLS:** conceptualization, funding acquisition, review and editing; **AP:** funding acquisition: review and editing; **AMNS:** data acquisition; data analysis; review and editing; **JAF:** conceptualization; coordination; funding acquisition; writing; review and editing.

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DECLARATION OF CONFLIT OF INTERESTS

JAF is the CEO and scientific founder of GlycoMatters Biotech, AMNS and LLS are scientific founders of GlycoMatters Biotech. A provisional patent request has been filled for this technology by JAF, RF, EF, AM, DF, FC, LLS, MJO, and BS.

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Supplementary material

Table S1. Products from the single-pot enzymatic synthesis of MUC16-Tn glycopeptides using GalNAc-T1, without purification and after VVA enrichment, identified by C18 reverse phase nanoLC-ESI-MS.

<i>O</i> -glycans	Glycopeptides Monoisotopic mass [M+2H] ²⁺ / [M+3H] ³⁺	Retention time (min)	Without enrichment	Enrichment with VVA
			Relative abundance (%)	Relative abundance (%)
Unglycosylated	901.43 / 601.29	20.99	46.97	32.10
1 Tn	1002.97 / 668.98	20.38	32.45	42.61
2 Tn	1104.51 / 736.67	19.92	17.89	21.19
3 Tn	1206.05 / 804.37	19.38	2.65	4.05
4 Tn	1307.58 / 872.06	0.05	0.04	0.05

Table S2. Products from the single-pot enzymatic synthesis of MUC16-Tn glycopeptides using GalNAc-T2, without purification and after VVA enrichment, identified by C18 reverse phase nanoLC-ESI-MS.

<i>O</i> -glycans	Glycopeptides Monoisotopic mass [M+2H] ²⁺ / [M+3H] ³⁺	Retention time (min)	Without enrichment	Enrichment with VVA
			Relative abundance (%)	Relative abundance (%)
Unglycosylated	901.43 / 601.29	16.93	86.72	30.74
1 Tn	1002.97 / 668.98	16.18	12.65	67.02
2 Tn	1104.51 / 736.67	15.64	0.63	2.23

Table S3. Products from the single-pot enzymatic synthesis of MUC16-Tn glycopeptides using GalNAc-T3 without purification and after VVA enrichment, identified by C18 reverse phase nanoLC-ESI-MS.

<i>O</i> -glycans	Glycopeptides Monoisotopic mass [M+2H] ²⁺ / [M+3H] ³⁺	Retention time (min)	Without enrichment	Enrichment with VVA
			Relative abundance (%)	Relative abundance (%)
Unglycosylated	901.43 / 601.29	21.00	30.31	9.13
1 Tn	1002.97 / 668.98	20.36	42.23	56.70
2 Tn	1104.51 / 736.67	19.81	20.43	21.79
3 Tn	1206.05 / 804.37	18.66	5.52	9.87
4 Tn	1307.58 / 872.06	18.24	1.40	2.33
5 Tn	1409.12 / 939.75	17.93	0.12	0.17

Table S4. Products from the single-pot enzymatic synthesis of MUC16-Tn glycopeptides using GalNAc-T1, GalNAc-T2 and GalNAc-T3 without purification and after VVA enrichment, identified by C18 reverse phase nanoLC-ESI-MS. The values are represented as mean values $\pm$ SD and the coefficient of variation (CV) (n=3).

<i>O</i> -glycans	Glycopeptides Monoisotopic mass [M+2H] ²⁺ / [M+3H] ³⁺	Retention time (min)	Without enrichment	CV	Enrichment with VVA	CV
			Relative abundance (%)		Relative abundance (%)	
Unglycosylated	901.43 / 601.29	20.71	12.17 $\pm$ 0.81	0.07	2.09 $\pm$ 0.58	0.28
1 Tn	1002.97 / 668.98	19.98	19.75 $\pm$ 0.64	0.03	14.25 $\pm$ 9.18	0.64
2 Tn	1104.51 / 736.67	19.55	21.78 $\pm$ 0.36	0.02	24.75 $\pm$ 2.30	0.09
3 Tn	1206.05 / 804.37	18.40	28.75 $\pm$ 0.54	0.02	36.64 $\pm$ 3.52	0.10
4 Tn	1307.58 / 872.06	18.07	13.95 $\pm$ 0.47	0.03	17.67 $\pm$ 2.03	0.11
5 Tn	1409.12 / 939.75	17.63	3.39 $\pm$ 0.51	0.15	4.40 $\pm$ 0.97	0.22
6 Tn	1510.66 / 1007.45	17.31	0.15 $\pm$ 0.05	0.35	0.19 $\pm$ 0.07	0.37

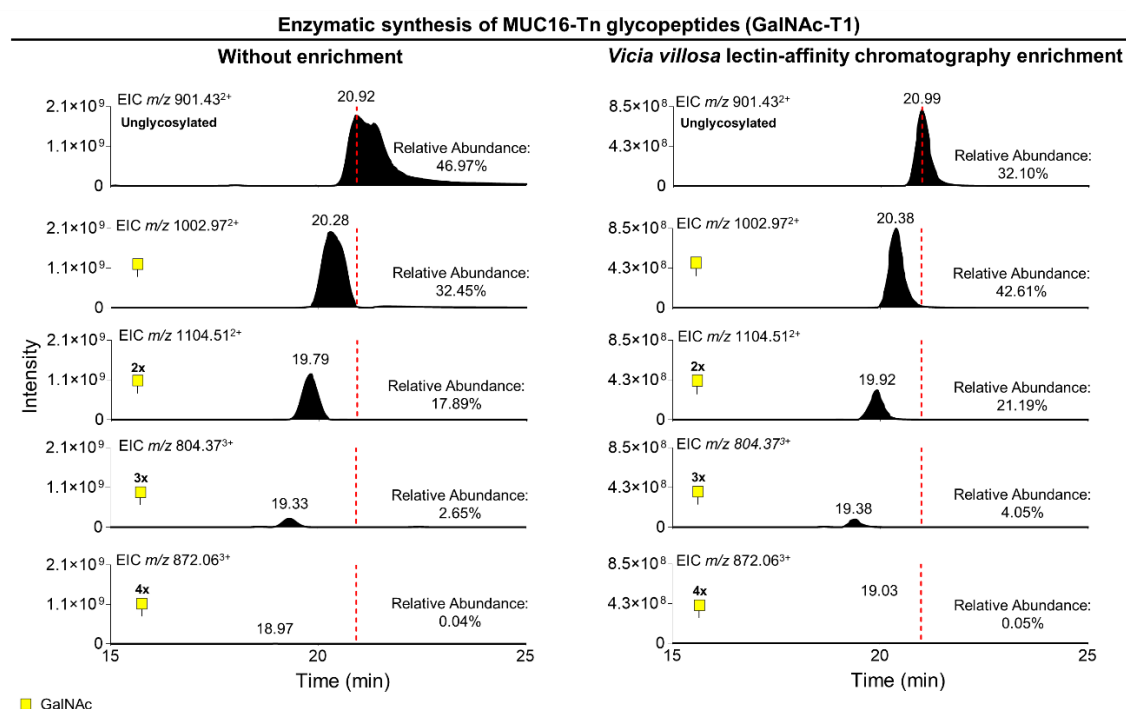


Figure S1. Enzymatic synthesis of MUC16-Tn glycopeptide libraries using GalNAc-T1, obtained before and after enrichment with the VVA lectin. After synthesis, the products were characterized by C18 reverse phase nanoLC-MS. MUC16-Tn glycopeptide libraries obtained from the enzymatic synthesis with GalNAc-T1 contains species with up to 4 occupied glycosites, being the most predominant the mono-glycosylated peptides. Enrichment with the VVA lectin with affinity for the Tn antigen slightly increased the amount of mono-, di- and tri-glycosylated peptides.

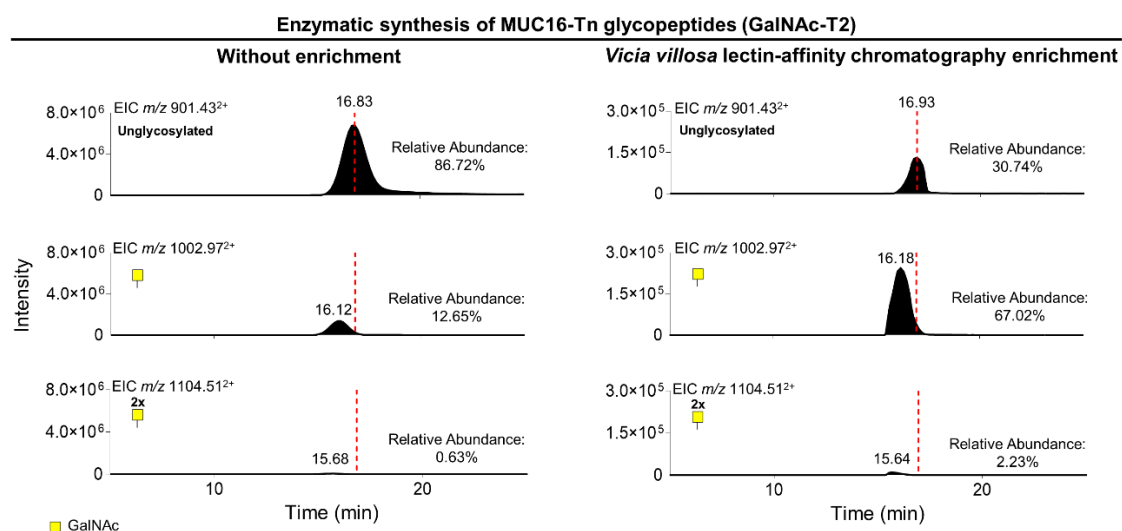


Figure S2. Enzymatic synthesis of MUC16-Tn glycopeptide libraries using GalNAc-T2, obtained before and after enrichment with the VVA lectin. After synthesis, the products were characterized by C18 reverse phase nanoLC-MS. MUC16-Tn glycopeptide libraries obtained from the enzymatic synthesis with GalNAc-T2 contains species with up to 2 occupied glycosites, being the most predominant the unglycosylated peptides, demonstrating low glycosylation yield. Enrichment with the VVA lectin with affinity for the Tn antigen significantly reduced unglycosylated peptides while increasing the amount of mono- and di-glycosylated peptides.

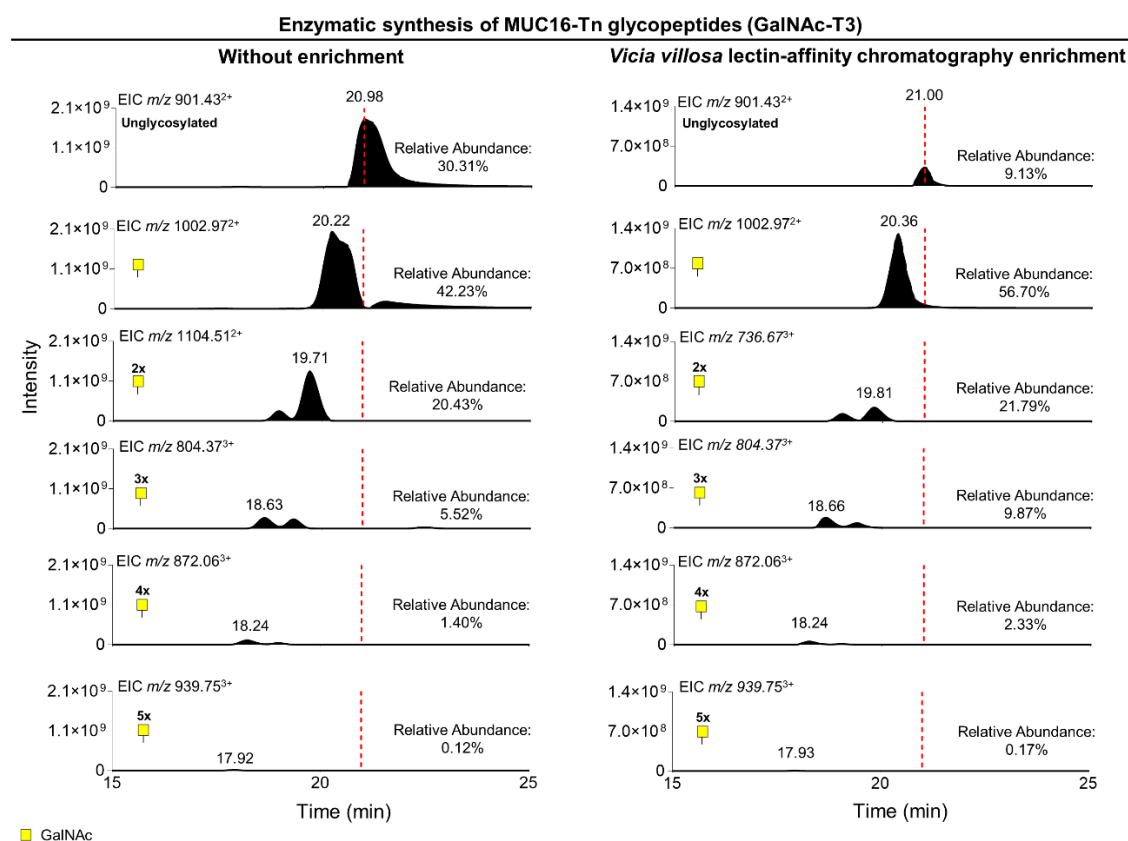


Figure S3. Enzymatic synthesis of MUC16-Tn glycopeptide libraries using GalNAc-T1, obtained before and after enrichment with the VVA lectin. After synthesis, the products were characterized by C18 reverse phase nanoLC-MS. MUC16-Tn glycopeptide libraries obtained from the enzymatic synthesis using GalNAc-T3 contains species with up to 5 occupied glycosites, being the most predominant the mono and di-glycosylated peptides. Enrichment with the VVA lectin with affinity for the Tn antigen significantly decreased the amount of mono-glycosylated peptides and increased the amount of mono-, tri- and tetra-glycosylated peptides.

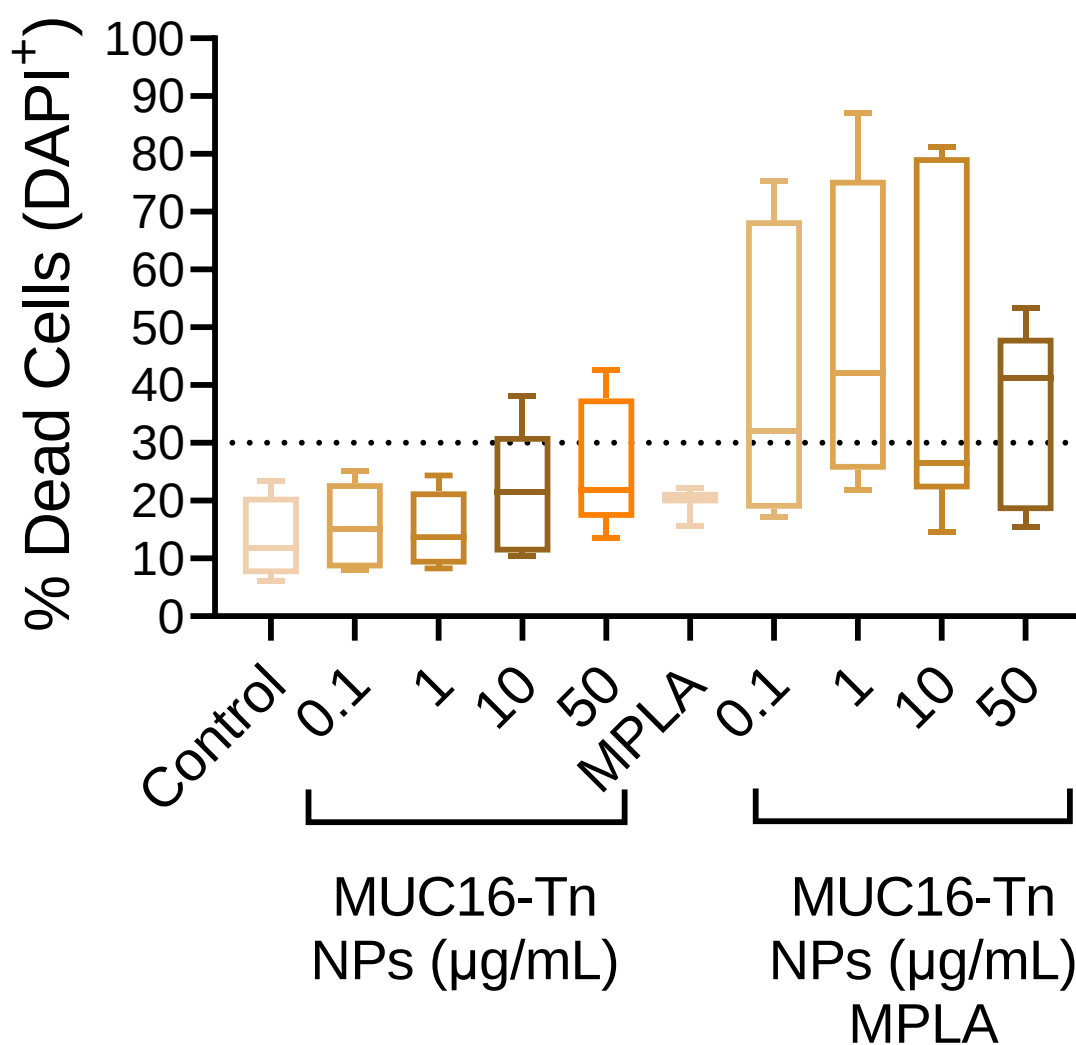


Figure S4. MUC16-Tn NPs are non-toxic to mo-DCs for concentrations below 1 µg/mL. The non-toxic nature of the glyconanovaccine candidate below 1 µg/mL and in the absence of MPLA adjuvant was confirmed by low DAPI incorporation assessed by flow cytometry. Data is representative of at least 5 independent experiments.

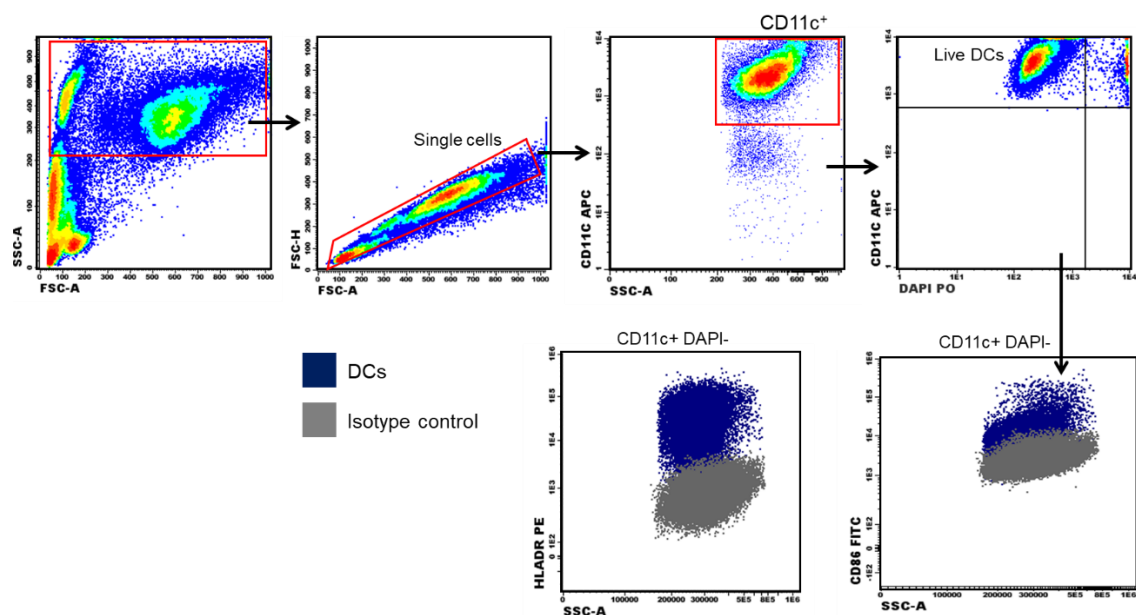


Figure S5. Flow cytometric gating strategy used to identify mo-DCs and evaluate CD66 and HLA-DR MFI and % of positive cells. Aggregates and cellular debris were excluded based on forward scatter (FSC) and side scatter (SSC). CD11c⁺ cells were gated to identify differentiated mo-DCs. Live mo-DCs were gated on CD11c⁺ DAPI⁻. Within the CD11c⁺ cells gate, CD86 and HLA-DR positive cells were determined to infer the maturation state of mo-DCs. Isotype-matched antibodies were used as negative controls.

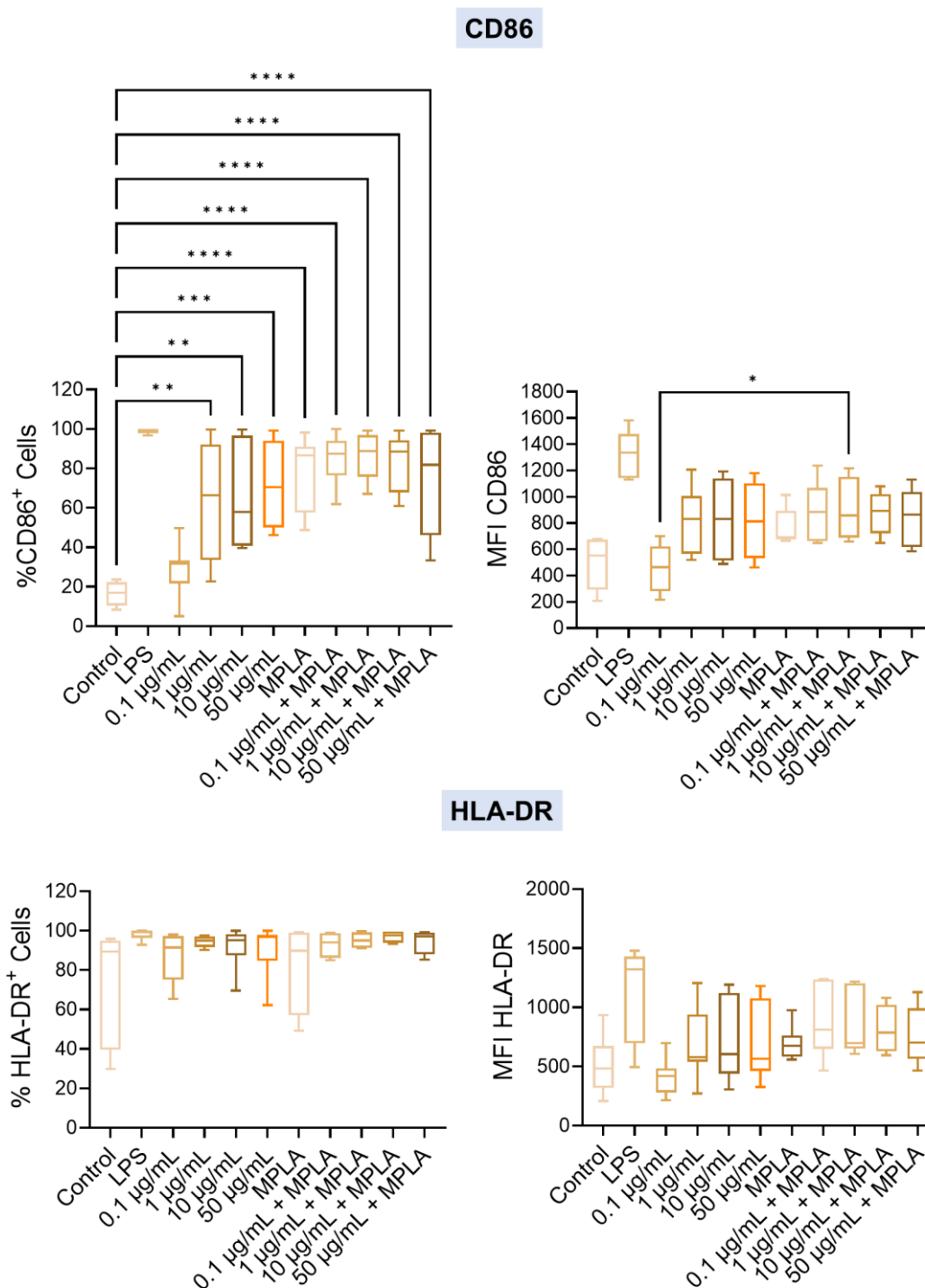


Figure S6. MUC16-Tn NPs suggested activation of mo-DCs from 1 µg/mL alone or adjuvated with MPLA. Preliminary flow cytometry analysis evaluating maturation markers CD86 and HLA-DR suggested that the glyconanovaccine candidate was able to mature mo-DCs from 1 µg/mL alone or in the presence of MPLA (indicated by % CD86). Data is representative of at least 5 independent experiments. Comparisons were performed using the One-way ANOVA test. Statistical significance was achieved when * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

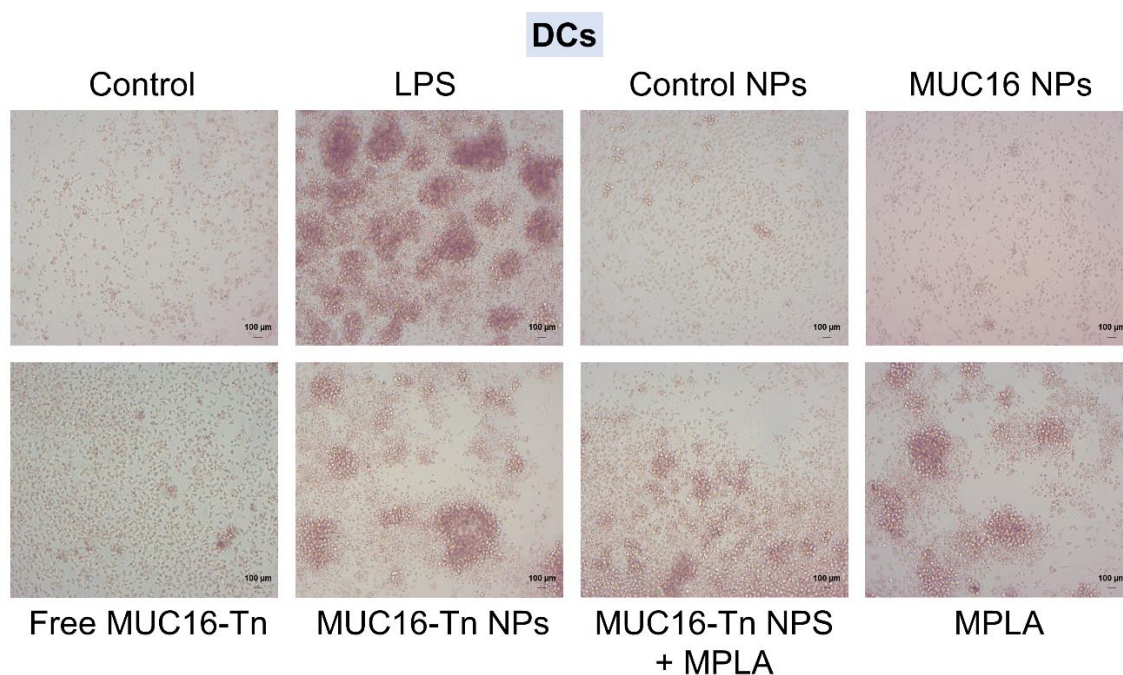


Figure S7. Cell morphology of mo-DCs confirmed the activation upon stimulation with positive control LPS and MUC16-Tn NPs alone or adjuvated with MPLA for 24 h. Brightfield microscopic images of mo-DCs stimulated with the nanoconstructs and controls for 24h. Activated mo-DCs are represented by the presence of cellular clusters. Scale bar represents 100 μm in all images.

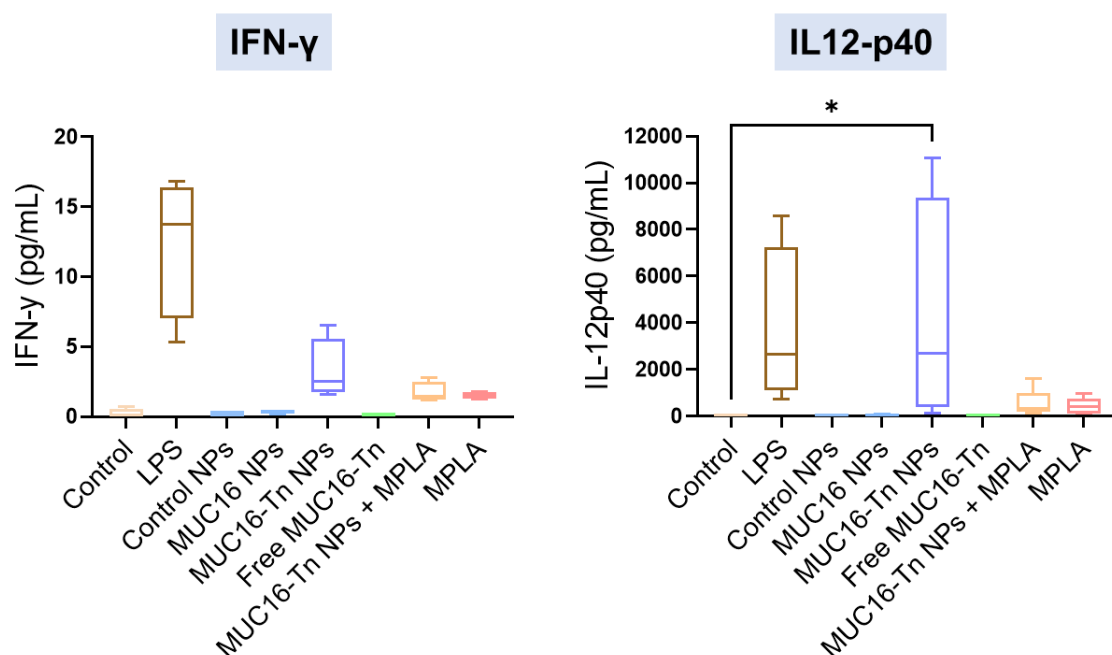


Figure S8. MUC16-Tn NPs slightly induced the release of IFN- γ and increased the production of IL-12(p70) antagonist: IL-12(p40). Multiplex showed that mo-DCs stimulated with the glyconanovaccine candidate presented slightly increased levels of pro-inflammatory cytokine IFN- γ . On contrary, high levels of the antagonist IL-12(p40) were detected suggesting that IL12 is not involved in mo-DCs immunogenic phenotype. The results correspond to the mean and standard deviation. Data is representative of at least 5 independent experiments. Comparisons were performed using the One-way ANOVA test. Statistical significance for comparisons with LPS positive control was omitted for better graph interpretation. Statistical significance was achieved when * $p < 0.05$.

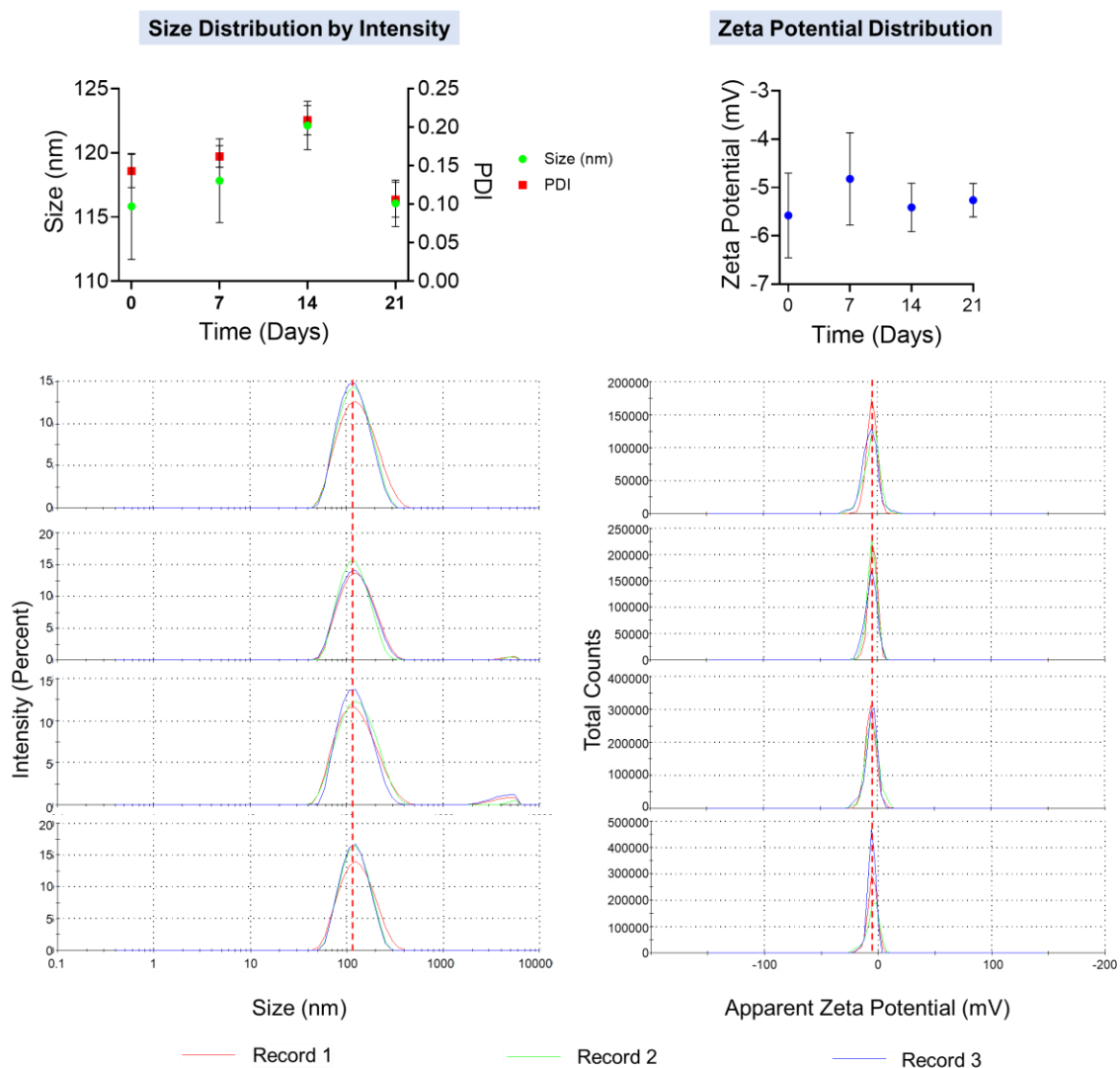


Figure S9. MUC16-Tn NPs showed stability in size, Pdl and zeta potential throughout 21 days. Prior to *in vivo* studies, DLS and LDA analysis throughout 21 days showed no alterations in NPs size, Pdl and zeta potential suggesting NPs stability during this period. Data is representative of at least 3 independent experiments.

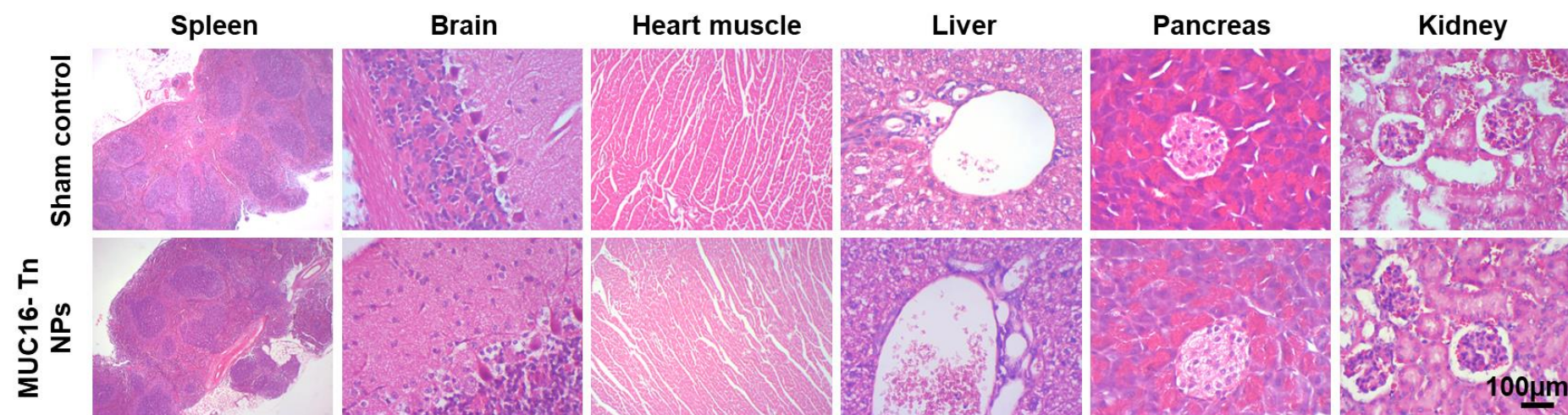
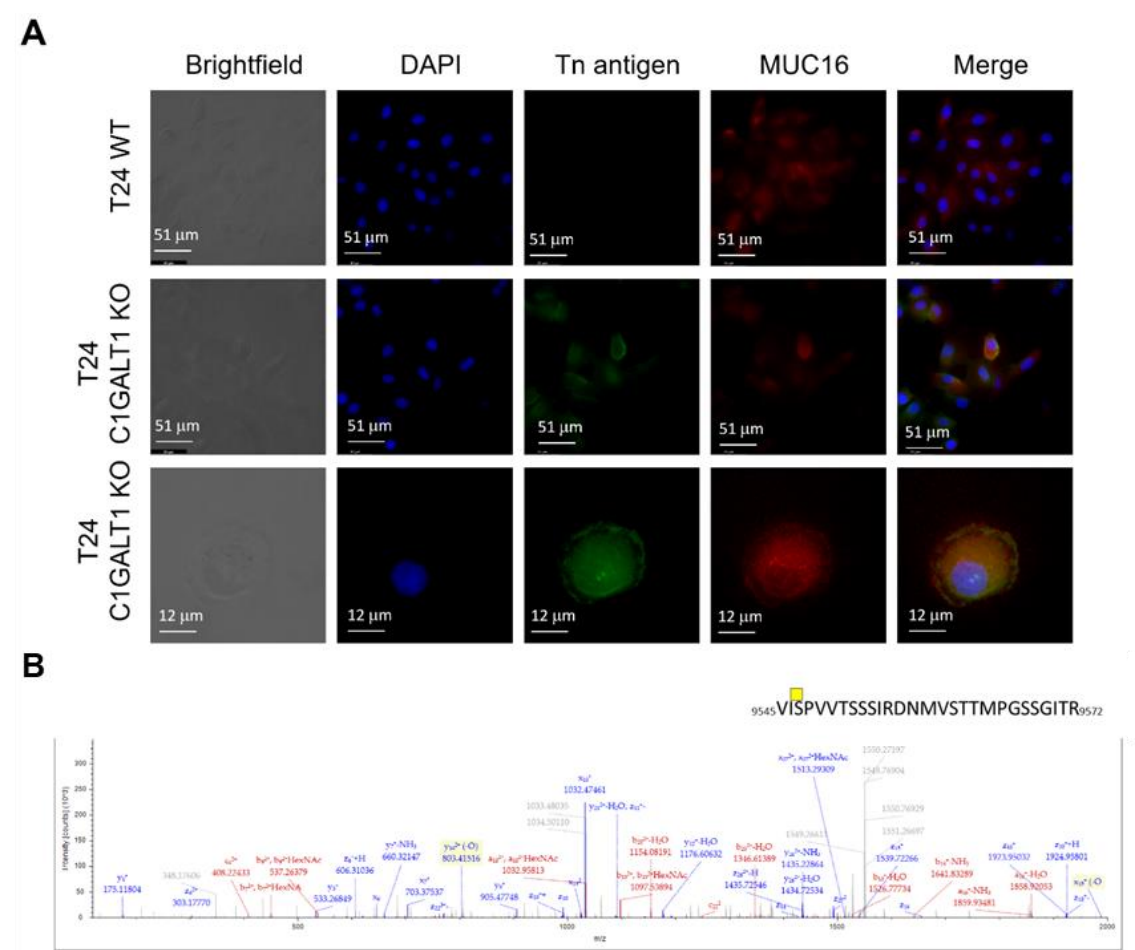


Figure S10. Histological evaluation of vital organs collected from mice after nanoglycovaccine candidate administration. No signals of injury were observed in the histological sections, such as fibrosis, inflammation, vascular ectasia, necrosis, rearrangement of chromatin, karyorrhexis or anisokaryosis.



CONCLUDING REMARKS & FUTURE PERSPECTIVES

Concluding Remarks and Future Perspectives

Bladder Cancer is one of the most frequently diagnosed malignancies and the second deadliest cancer of the genitourinary tract [1]. Disease management is challenged by the absence of predictive and patient stratification biomarkers and the need for safer and more effective therapeutics. Current therapeutic modalities, such as chemotherapy and immune checkpoint inhibitors, are often hurred by deleterious side effects and frequently fail to prevent tumour relapse, urging novel therapies. Cancer vaccines hold tremendous potential for eradicating tumours and prevent recurrence due to induced immunological memory [2]. However, this approach is challenged for lack of cancer-specific molecular signatures [3, 4]. Exploiting aberrant glycosylation, a common feature of malignant cells, together with alterations in protein abundance and composition, could be a valuable approach to identify cancer-specific molecular glycosignatures and boost the development of successful cancer vaccines [5-8]. Taking these insights into consideration, this thesis focused on the development of two cancer glycovaccine candidates based on two BLCA specific/associated glycoantigens. It started by developing an enzymatic method to produce synthetic glycopeptides, using MUC16 as a scaffold, as well as glycosyltransferases and sugar nucleotide combinations *in vitro*. Several glycospecies were synthesized, including MUC16-Tn, MUC16-STn, MUC16-T, MUC16-S3T and MUC16-S6T. To increase the ratio of sialylated glycopeptides, a TiO₂ enrichment protocol was employed. All synthetic products were characterized by mass spectrometry. Subsequently, Tn/STn glycopeptides were conjugated with two protein carriers through maleimide-thiol conjugation chemistry to produce immunogenic glycoconjugates. Overall, the first chapter established the biotechnological ground to produce cancer glycopeptides and protein glycoconjugates to be employed as potential cancer vaccine candidates.

Chapter 2 progresses to develop a multivalent anti-tumour cancer vaccine candidate based on the conjugation of the BLCA-specific glycosignature CD44s-Tn with the immunogenic protein Keyhole Limpet Hemocyanin (KLH). CD44s-Tn glycopeptides were produced by employing the single pot enzymatic strategy described in Chapter 1. Several optimizations were implemented, including the combination of glycosyltransferases and an enrichment step with *Vicia villosa* Lectin (VVA) to remove unglycosylated peptides. Moreover, glycosite annotation of glycopeptides identified CD44s-Tn species resembling the glycopatterns observed

in bladder tumours [5]. Then, CD44s was conjugated with two immunogenic proteins, and the conjugation process was optimized to avoid conjugate aggregation. In line with this, KLH-CD44s glycoconjugates were selected as vaccine candidates and pre-clinical characterization *in vitro* and *in vivo* was pursued. Namely, the safety profile of the glycovaccine candidate was evaluated *in vitro* using monocyte-derived dendritic cells (mo-DCs). The glycovaccine candidate showed no toxicity to moDCs, inducing mo-DCs maturation when combined with the adjuvant monophosphoryl lipid A (MPLA). The glycovaccine candidate was also well tolerated *in vivo*. It induced cellular specific immune responses through the production of interferon gamma cytokine (IFN- γ) by natural killer cells (NKs), CD4⁺ and CD8⁺ T cells, together with increased frequencies of mature DCs, B cells and immune memory cells. Finally, the glycovaccine candidate promoted specific humoral responses, inducing the production of IgG antibodies capable of recognizing synthetic CD44s-Tn as well as CD44s-Tn expressing cancer cells and tumour tissues. Vaccine-induced antibodies were also capable of inducing tumour cell cytotoxicity *in vitro* through complement system activation. Notwithstanding, the therapeutic efficacy of the glycovaccine candidate remains to be addressed. In line with this, future studies will test the prophylactic and therapeutic efficacy of the glycovaccine candidate *in vivo*. This will ultimately complete pre-clinical validation and open a path for clinical trials.

Chapter 3 challenged the classical conjugation of tumour antigens with protein carriers in an attempt to avoid commonly described strong immune responses against the carrier [9]. This effect often culminates in epitope suppression, hampering tumour-specific immune responses and leading to vaccines failure. To this end, the final chapter describes the construction of a multivalent glycovaccine combining the tumour associated antigen MUC16-Tn with poly(lactide-co-glycolide)-b-poly-(ethylene glycol)-maleimide (PLGA-PEG-MAL) nanoparticles. Firstly, MUC16-Tn glycopeptides were obtained by employing the enzymatic strategy described in chapters 1 and 2, followed by VVA enrichment. Then, PLGA-PEG-MAL were produced using the double emulsion-solvent evaporation method and further conjugated with glycopeptides through maleimide-thiol chemistry. The physicochemical and morphological characterizations showed the suitability of the nanoformulations for specific DCs targeting. MUC16-Tn NPs were found to be specifically recognized by macrophage galactose-type lectin (MGL), a receptor expressed in immature DCs with high

affinity for the Tn antigen [10]. Moreover, the glyconanovaccine candidate was specifically internalized by mo-DCs and showed no toxicity *in vitro*. The glyconanovaccine candidate alone stimulated mo-DCs *in vitro* through the induction of the maturation markers CD86 and HLA-DR as well as the production of several pro-inflammatory cytokines, demonstrating targeted and self-adjuvated properties. The glyconanovaccine candidate was demonstrated safe *in vivo*, inducing IgG antibodies-mediated tumour-specific humoral responses against synthetic MUC16-Tn, MUC16-Tn-expressing cell lines and tumour tissues. Despite promising results, cellular-specific immune responses should be further evaluated, given they are required for effective anti-tumoural responses. In addition, to complete pre-clinical validation, the nanovaccine anti-tumoural effect should be addressed *in vivo*, fostering future inclusion in clinical trials.

Overall, this work fully integrates for the first time previously identified cancer-associated/specific molecular glycosignatures into multivalent anti-bladder cancer vaccines. It established an optimized enzymatic strategy to produce glycopeptides and decrease the amount of unglycosylated species in the final product through chromatography. These optimizations consist in a significant improvement to mainstay single pot synthesis methods by avoiding immune responses against unglycosylated molecular targets. Moreover, glycopeptides were employed in multivalent glycovaccine construction, bringing the formulation one step closer to human tumour heterogeneity, and promoting optimized anti-tumour specific immune responses. This work also developed a multivalent glyconanovaccine candidate based on MUC16-Tn glycopeptides, which acted simultaneously as antigens and guiding molecules to dendritic cells. Of note, this approach dismissed the use of additional carrier proteins or adjuvants, constituting a significant improvement toward the generation of specific immune responses.

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