The sweet side of tumor immunotherapy

Carbohydrate signatures on tumor cells have functional implications in tumor growth and metastasis and constitute valuable tools in cancer diagnosis and immunotherapy. Increasing data regarding the mechanisms by which they are recognized by the immune system are facilitating the design of more efficient immunotherapeutic protocols based on cancer-associated glycan structures. Recent molecular and proteomic studies revealed that carbohydrates are recognized, not only by B cells and antibodies, but also by cells from the innate arm of immunity, as well as by T cells, and are able to induce specific T-cell immunity and cytotoxicity. In this review, we discuss and update the different strategies targeting tumor-associated carbohydrate antigens that are being evaluated for antitumor immunotherapy, an approach that will be highly relevant, especially when combined with other strategies, in the future fight against cancer.

KEYWORDS: cancer ▪ glycosylation ▪ immunotherapy ▪ tumor-associated carbohydrate antigen ▪ vaccine

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Tumors have their own glycan signatures

Cancer cells present phenotypic changes or molecular signatures that distinguish them from healthy cells. Some of these signatures are of carbohydrate nature, being the result of a series of alterations in the glycosylation pathways of proteins or lipids on these cells. Apart from representing tumor markers, these glycan motifs, often known as tumor-associated carbohydrate antigens (TACAs), have functional implications in potentiating tumor progression, spreading and invasiveness [1].

Carbohydrate signatures on tumor cells are decoded by the immune system through the interaction of several immune receptors from both the innate and adaptive arms of immunity. Receptors of innate immunity, known as pattern-recognition receptors, recognize a limited pattern of structures of both microbial and self origin, and are capable of transducing signals in a highly sensitive mode in order to activate adaptive immune responses [2]. In particular, receptors of the innate branch that recognize glycan motifs consist of soluble or membrane-associated lectins, siglecs and scavenger receptors, among others.

Receptors of adaptive immunity include B-cell receptors and T-cell receptors (TCRs). B-cell receptors recognize antigens and transduce activation signals that drive the differentiation of B cells and the production of opsonization antibodies. TCRs, contrary to what was originally predicted, can also recognize carbohydrate-containing antigens presented by CD1 or MHC molecules; although, in the latter case, short carbohydrate chains are usually attached to peptidic molecules [3]. Antigen recognition by T cells leads to signal transduction cascades, and subsequently leads to the generation of effector T cells that produce a battery of cytokines or posses cytotoxic activity.

The key mediators that bridge innate and adaptive immunity are dendritic cells (DCs). DCs acquire and process antigens, and migrate to the lymphoid organs, where they present antigens to specific T cells, thereby inducing primary T- and B-cell responses. Therefore, recognition of glycan motifs on cancer cells by immune receptors might induce immunity against tumors. However, they can also facilitate the dissemination of cancer cells [4] by different mechanisms, described below.

A diverse range of glycan motifs on cancer cells has been detected. TACAs can be found on glycolipids, *N*- and/or *O*-linked glycoproteins, and consist of unmasked carbohydrate structures that result from incomplete glycosylation [5]. In most cases, the events involved in the glycosylation process are orchestrated by glycosidases and glycosyltransferases, the levels of which have been correlated with tumor metastasis [6–8]. Common glycan alterations associated with tumor cells include an increase in β1,6-branching of N-linked chains [9], as well as sialylation, Lewis sugars (sialyl-Le^a [sLe^a], sLe^x, Le^y) [10–15] and changes in the expression

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of blood group antigens (Table 1) [16,17], that have been described on epithelial tumors, including those of the breast, colon, lung, gastric, pancreas, ovarian, cervical and prostate cancers, among others. Simpler *O*-glycan-motifs, such as the Tn, TF and the sialyl-Tn (sTn) antigens (Table 1) have been described on several tumor types such as bladder, colorectal, gastrointestinal, prostate, ovarian, breast, pancreas and lung carcinomas [18–22] while they are not found in normal tissues [23,24]. Finally, TACAs on gangliosides (glycolipids consisting of oligosaccharides chains linked to ceramide) such as GD2, GD3 and GM2 are mainly overexpressed in human melanoma, neuroblastoma and glioma [17,25–27], while the glycolipids fucosyl-GM1 and globo H have been identified in small-cell lung cancer cells [28–30] and ovarian, colon and prostate cancers [16,31], respectively (Table 1).

At first, glycan motifs on tumor cells were identified and characterized by virtue of their reactivity with tumor-specific antibodies and lectins. Their expression on cancer cells made TACAs interesting tumor markers for cancer diagnosis, and later on potential targets for immunotherapy against cancer [5]. However, the importance of these molecular signatures was highlighted with subsequent experimental demonstrations related to their (functional) role in the development and growth of cancer, revealing that most TACAs constitute essential machinery to induce metastasis and invasiveness [32].

Function of TACAs in cancer growth & antitumor immunity

The immune system attacks, but tumors escape

Pioneering work by Paul Ehrlich brought about the idea that the immune system participates in the eradication of tumors, giving rise to the 'cancer immunosurveillance' hypothesis, first postulated by Burnet and Thomas in the 1950s [33]. Later on, this hypothesis was generally accepted following a series of publications demonstrating that mice genetically deficient in key components of the immune response had higher susceptibility to tumors [34]. Additional data, however, demonstrating that the immune system is capable of not only protecting the host against tumor formation, but also of shaping tumor immunogenicity, prompted a more refined concept, leading to the formulation of the 'Cancer Immunoediting' hypothesis. This concept highlights two actions performed by the immune system: one capable of eliminating tumor cells and other capable of sculpting or editing tumors that have the capacity to 'escape' immunity [35].

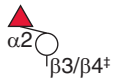
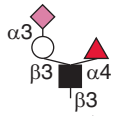
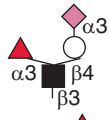
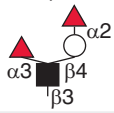
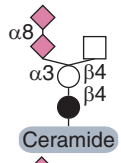
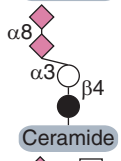
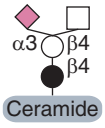
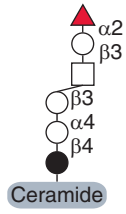
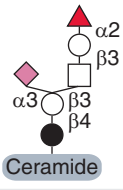
Tumor escape may be promoted by mechanisms driven by tumor cells themselves, such as loss of tumor antigens or MHC class I proteins, or developing cytotoxic resistance; all of which result in poorly immunogenic tumor cell variants. Furthermore, tumors can promote an immunosuppressive state in the tumor microenvironment by releasing molecules such as VEGF, TGF-β, galectin-1 or expressing the Trp-depleting enzyme indoleamine 2,3-dioxygenase. On the other hand, tumor cells can recruit regulatory immune cells that suppress the antitumor effector mechanisms, such as Tregs, myeloid-derived suppressor cells or M2-type macrophages [36]. Thus, the balance between immunosuppressive and antitumor effector cells (M1 macrophages, $CD8^+$ T cells and Th1 cells), can define the prognosis and the therapeutic impact of chemo- and immuno-therapy [37,38].

TACAs promote cancer development & growth

Research carried out during the past decade has provided much insight regarding the advances in our understanding of the biological function and mechanisms by which tumor-associated antigens induce cancer development and modulate immunity. It has been well demonstrated that TACAs can shape the malignant phenotype of tumor cells or suppress antitumor immunity, contributing to tumor growth. For instance, highly *O*-glycosylated mucins produced by cancer cells can provide antiadhesive properties to tumor cells by masking cell surface adhesion molecules and preventing cell–cell and cell–matrix interactions, thus contributing to the formation of metastases [39]. Indeed, some TACAs such as sTn or TF on carcinoma cells can modulate a malignant phenotype, inducing a more aggressive cell behavior, such as decreased cell–cell aggregation and increased extracellular matrix adhesion, migration and invasion [40,41]. Furthermore, sTn expressed on MUC1 affects the ability of DCs to stimulate Th1 responses, important for tumor refection [42], in a process where the sialic acid-recognizing receptors named siglecs may participate in its recognition. Finally, mucins produced by cancer cells can also favor tumor progression through the induction of COX-2 by activated monocytes, in a carbohydrate-dependent mechanism [43] that results in lower IFN-γ-producing $CD4^+$ T cells [44].

During the process of invasion of tumor cells to distant organs, tumor cells must interact with

Table 1. Structure and expression in cancer of tumor-associated glycan-motifs.

TACA	Carbohydrate nature	Structure†	Expression in cancer
Tn	*O*-glycoproteins (mucins)		Bladder, breast, colon, gastric, lung, ovarian, pancreatic, prostate
TF			
sTn			
H disaccharide	*O*-glycoproteins *N*-glycoproteins Gangliosides		Cervical, colon, gastric, lung, oral epithelial, urothelial
sLe[a]	*O*-glycoproteins *N*-glycoproteins Gangliosides		Breast, colon, gastric, lung, ovarian, pancreatic, gastric
sLe[x]			
Le[y]			
GD2	Gangliosides		Glioma, melanoma, neuroblastoma
GD3			
GM2			
Globo H	Globosides		Colon, ovarian, prostate
Fucosyl-GM1	Gangliosides		Lung (small cell)

†*Shapes represent the following monosacharides: white square:* N*-acetylgalactosamine; black square:* N*-acetylglucosamine; diamond: neuraminic acid; white circle: galactose; black circle: glucose; triangle: fucose.*
‡*β3 for type 1 H disaccharide; β4 for type 2 H disaccharide.*
TACA: Tumor-associated carbohydrate antigen.

the vascular endothelium, a process that allows extravasation of tumor cells from the blood circulation, favoring tumor spreading. During this adhesion process, TACAs are key factors that specifically interact with different lectins present on endothelial cells. Indeed, it has been proposed that sLe^x or sLe^a exposed at the cell surface of malignant cells is involved in selectin-mediated adhesion to vascular endothelium, favoring their extravasation from the blood circulation [45]. These data are supported by the fact that high amounts of sLe^x and sLe^a are present in different human adenocarcinomas [46], and that both P-selectin and E-selectin expression is significantly enhanced on the endothelial cells of cancer patients [47,48].

In a similar process, galectin-3, a member of the β-galactoside-binding animal lectin family, plays an important role in favoring adhesion and invasion of tumor cells. Indeed, the TF antigen on MUC1 produced by cancer cells interacts with galectin-3 expressed on the endothelial cell surface, promoting the adhesion of cancer cells to endothelium [40,49]. The TF–galectin-3 interactions can be efficiently disrupted using different compounds that either mimick or mask this carbohydrate structure [50]. Indeed, a modified polysaccharide fiber derived from citrus fruits that specifically inhibits the carbohydrate binding of galectin-3 [51,52] and is capable of inhibiting the *in vivo* formation of metastatic deposits [53]; this has emerged as one of the most promising antimetastatic drugs.

TACAs modulate antitumor immune responses

In addition to the promoting effects of TACAs to tumor development, carbohydrates attached to proteins or lipids may also modulate the induction of an effective antitumor immune response. For instance, carbohydrates can affect B- or T-cell immunogenicity, by hampering antigen processing or presentation [54], T-cell proliferation [55,56] or skewing T-cell immune responses [57,58].

Although antigen-presenting cells (APCs) are able to process glycoproteins to glycopeptides and present them in association with both MHC class I [59] and class II [60] molecules, both antigen processing and presentation of glycopeptides is highly dependent on the complexity and size of carbohydrate chains, as well as the site where they are attached [61]. In particular, short-linked glycans on the tumor-associated mucin MUC1 control both the extent and site specificity of the proteolysis of tumor-associated MUC1 glycopeptides [61,62], impairing processing and presentation through the MHC class II pathway [63].

Apart from their participation in antigen processing and presentation processes, tumor-associated glycan motifs may impair the development of stimulatory immune responses by inducing tolerance. For instance, glycosylated MUC1-loaded DCs increase Treg production, which suppresses the induction of MUC1 B- and T-cell responses *in vivo* [64]. In a similar way, gangliosides can impair the differentiation and function of DCs and even induce DC or T-cell death by apoptosis [65,66].

In spite of their role in cancer and in the modulation of immune responses, TACAs constitute potential tools for immunotherapy. Indeed, carefully designed glycoproteins or glycopeptides incapable of impairing tumor immunity can be effectively used for immunotherapy in experimental models and, in adequate immunostimulating conditions, they induce effective immune responses that mediate cancer regression. Of great importance in the design of these molecules is the comprehension and study of the mechanisms by which TACAs modulate the immune system.

Carbohydrate-based immunotherapy: success or failure in cancer treatment?

Induction of antitumor carbohydrate-specific immunity

The induction of both innate and adaptive immune responses is necessary for efficient antitumor immunity. The innate immune response makes a crucial contribution to the activation of adaptive immunity, through the recognition of nonself determinants using pattern-recognition receptors. In addition, lymphoid cells of the acquired immunity play a critical and defined role in the host's defense against cancer. In fact, B and T cells are critical in cancer surveillance, since mice deficient in RAG-2 develop spontaneous malignancies [67].

The expression of carbohydrate signatures by tumors makes them interesting targets for cancer diagnosis or immunotherapy. Hence, of particular importance is how carbohydrate-reactive lymphocytes and cells of the innate immunity mediate cellular immune responses.

Recognition of tumor glycan signatures by innate immune cells

It is now well established that peptide modifications such as glycosylation, phosphorylation or ubiquitination can be recognized by both

branches of the immune system: the innate and the adaptive immunities [68,69]. For instance, γδ T cells comprise innate-like lymphocytes capable of recognizing low-molecular-weight, nonpeptidic, phosphate-containing molecules termed phosphoantigens without conventional MHC restriction [70]. These cells may participate in tumor surveillance, since they posses cytotoxic activity [70]. On the other hand, invariant NK T cells (iNKT cells) are glycolipid antigen-reactive cells, that express a limited array of αβ TCRs. Unlike classical T cells, which recognize peptides presented by highly polymorphic MHC molecules, iNKT cells recognize (glyco)lipids via MHC-like, nonpolymorphic, CD1d molecules. They produce both Th1 and Th2 cytokines in response to glycosphingolipid stimulation [71,72], and much accumulated evidence support the idea that iNKT cells have effector roles in the regulation of both innate and adaptive immunity involved in cancer [73].

Carbohydrate motifs expressed on cancer cells may be also recognized as a type of danger signal to the innate immune system [74]. Indeed, several carbohydrate-binding lectin receptors participate in the recognition of glycan structures derived from tumor cells [75]. Thus, cells expressing these receptors may play a role in bridging innate and adaptive immune responses to cancer cells [76]. However, as described for several glycans from pathogens, TACAs on tumor cells might target lectin receptors in order to escape immune surveillance, either by inducing tolerance or skewing the protective immune responses [75].

Carbohydrate-binding receptors, such as C-type lectin receptors (CLRs) or sialic-acid-binding immunoglobulin-like lectins (siglecs), are associated with the cell surface of APCs, where they can modulate T-cell signaling and survival through glycan recognition on the tumor cell surface. Siglecs comprise a sialic acid-dependent cell adhesion molecule family of receptors that exhibits distinct and varied specificity for sialoside sequences on glycoconjugates [77,78]. Some siglecs have been identified as inhibitory receptors that signal via their proximal immunoreceptor tyrosine-based inhibition motif, or immunoreceptor tyrosine-based inhibition motif-like motifs. Ligand binding results, in this case, in potent inhibition of NK cell and T-cell antitumor functions [79]. Sialylated TACAs on cancer-associated mucins, such as MUC1 or MUC16 (CA125), have previously been shown to be ligands for siglecs, promoting invasion of cancer cells [80] or by immunomodulating the antitumor immune response, allowing tumor cells to escape recognition by NK cells [81].

CLRs, on the other hand, comprise a calcium-dependent carbohydrate-binding protein family of receptors that, through glycan interactions, contribute to carbohydrate-mediated endocytosis and presentation by APCs [82,83]. Some CLRs can also induce signaling pathways directly, whereas other CLRs affect or modulate signaling by Toll-like receptors [84], inducing specific T-cell polarization. Accumulating evidence has shown that CLRs on DCs, such as DC-SIGN or macrophage Gal/GalNAc lectin (MGL), recognize TACAs on the tumor associated mucins CEA or MUC1, probably by interacting with Le^y and Tn antigens, respectively [85].

However, the recognition of tumor cells by glycan interactions mediated by CLR may induce antigen-specific tolerance, enabling the tumor to escape immunosurveillance. In fact, MGL has been identified on tolerogenic DCs and was proposed as an immunomodulatory receptor [86], although no strict evidence demonstrating immunosuppressive effects has been reported [85]. A better understanding of the mechanisms that govern glycan recognition on tumor cells should be of great importance in the design of immunotherapies against cancer that target glycan receptors.

Carbohydrate recognition by T lymphocytes

The evidence regarding nonpeptide antigen recognition by immune cells not only focuses on cells from the innate branch or innate-like lymphocytes. Indeed, T cells reactive to glycopeptides as well as glycolipids presenting different degrees of specificity have been described [87]. Also, some purely carbohydrate antigens capable of mimicking helical peptides may directly bind to MHC class II molecules [88], indicating that carbohydrate antigens devoid of lipids or protein can directly influence T-cell proliferation. Altogether, these advances illustrate the modern and accepted fact that carbohydrate structures are recognized, not only by innate-, but also by acquired-immune T cells, questioning the accuracy of the classical T-cell-dependent versus T-cell-independent antigen perspective, where carbohydrates are seen as T-cell-independent antigens. In this context, we will discuss how carbohydrates are recognized by T lymphocytes and, of particular importance in tumor immunity, the generation of cellular cytotoxicity mediated by $CD8^+$ T cells specific for carbohydrate antigens, an expanding new field of

research for the development of TACA-based immunotherapy.

The capacity of T cells to control tumors has been widely documented, and the importance of both antitumor CD4+ (cytokinic) and CD8+ (cytotoxic) T-cell responses has been evidenced in many models as well as in clinical settings. In fact, CD4+ T helper cells are essential in promoting antitumor immunity, effective B-cell priming, antibody isotype switching, cytotoxic T lymphocyte (CTL) expansion and CD8+ T memory cell responses, through the production of different sets of cytokines [89].

There has been evidence that the TCR of CD4+ T cells is able to recognize TACA-containing glycopeptides that are presented in the context of MHC class II molecules on APCs [60]. This fact is of great importance considering that some mucin-derived peptides posses a low immunostimulatory capacity, and they become highly immunogenic when attached to short carbohydrate chains [56]. In some cases, the TCR cross-reacts with both the glycopeptide and the peptide, while in other situations, it is specific of the TACA-containing glycopeptide. For instance, the introduction of the Tn antigen within a hemoglobin-derived peptide was shown to induce Tn-specific CD4+ T-cell responses [90,91]. Furthermore, a TF antigen-specific MHC class II restricted T-cell hybridoma, recognizing a MUC1 peptide glycosylated in two independent sites, has been described, indicating a certain degree of permissiveness regarding the carrier peptide sequence for the recognition of nonbulky carbohydrate moieties [92]. Moreover, MUC1 peptide and glycopeptide-specific TCR transgenic mice have recently been generated, and it was elegantly demonstrated that glycopeptide-specific CD4+ T cells were able to activate enhanced CTL responses, while peptide-specific CD4+ T cells did not [56].

TACA-specific CD8+ CTLs have also been described. Abdel-Motal and colleagues demonstrated that a vesicular stomatitis virus-derived peptide substituted with galabiose was able to induce CTLs that recognize target cells carrying the carbohydrate moiety as part of a glycolipid [93]. In this study, carbohydrate-specific CTLs were able to kill carbohydrate-bearing target cells independently of any MHC presentation. In another study, Xu *et al.* have also documented the induction of a carbohydrate-specific CTL response by the TF antigen linked to a Sendai virus-derived peptide. These cells were able to kill, although with poor efficiency, B16 melanoma cells transfected with MUC1, independently of the peptide backbone carrying the TF antigen, but in a MHC class I-restricted manner [94].

In conclusion, these data demonstrate that CD4+ T helper and CD8+ cytotoxic T cells are able to accommodate small carbohydrate molecules, but not more complex carbohydrate structures, within their α/β TCR. Furthermore, in some cases, the immunogenicity of a peptide can be enhanced by the addition of one carbohydrate residue. Indeed, a mucin peptide containing the Tn antigen was shown to be more immunogenic in MUC1-transgenic mice compared with the nonglycosylated peptide [56]. In addition, a CD8+ T cell epitope of the MUC1 human mucin carrying the Tn antigen was much more immunogenic than the nonglycosylated peptide, as shown by the increased MHC affinity of the glycopeptides [95]. Thus, apart from their role in T-cell recognition, some TACAs are able to modulate the affinity of the peptide for the MHC, increasing immunogenicity.

Antitumor carbohydrate-specific antibody responses

It has long been known that cancer patients produce antibodies directed to certain TACAs. For instance, high levels of anti-Tn- [96], anti-TF- [97] or anti-GM2- [98,99] -specific IgG antibodies have been detected in the blood sera of patients with cancer, suggesting an ongoing tumor immune response in these patients, indicated by the switching of antibodies to the IgG class.

The presence of TACA-specific autoantibodies in cancer patients may be a useful tool to predict cancer progression. In keeping with this, a recent study demonstrated that autoantibodies to aberrant *O*-glycopeptide epitopes represent a fruitful source of sensitive biomarkers for the early detection of cancer. Cancer-associated IgG autoantibodies to Tn-MUC1 and sTn-MUC1 were identified in sera from newly diagnosed breast, ovarian and prostate cancer patients [100].

On the other hand, such antibodies may act as effectors of the immune system and mediate tumor cell killing. Interestingly, the presence of naturally occurring antibodies to the ganglioside GM2 in melanoma patients has been correlated with improved survival of stage III melanoma patients [99]. These data may indicate that the immune system of cancer patients is capable of developing TACA-specific antibodies that suppress melanoma growth in patients. Also, a protective cancer-specific autoantibody recognizing the Tn antigen was identified in a spontaneous mouse cancer model [101].

The biological importance of TACA-specific antibodies in eliminating tumor cells has been demonstrated by different mechanisms. TACA-specific antibodies may enhance the uptake and presentation of tumor antigens by APCs to induce higher immune responses [102]. They can also mediate the killing of tumor cells by complement-dependent cytotoxicity (CDC), as well as by antibody-dependent cellular cytotoxicity (ADCC), performed by NK cells or macrophages [103,104]. These two mechanisms are strongly dependent on the antibody class and on the carbohydrate target type. Indeed, targeting gangliosides would preferentially allow killing through CDC, whereas this mechanism is poorly efficient for TACAs expressed on mucins (TF, Tn or sTn) [103,105].

Based on these data, antibodies directed to specific TACA structures on peptides or proteins become interesting and may be potential tools to be used in immunotherapy against cancer. Also, the presence of such autoantibodies in cancer patients demonstrates the capacity of the immune system in these patients to develop IgG-class tumor-specific antibodies. In this regard, the design of molecules containing TACAs may represent a promising strategy to enhance the anti-tumor immune response in cancer patients.

Carbohydrate-targeting immunotherapy: an update

Over the last decades, anticancer immunotherapy has emerged as a new exciting area for controlling tumors. In this context, glycan motifs present on the cell surface of human cancer cells may be potent targets for immunotherapy against tumors.

On the one hand, monoclonal antibodies (mAbs) have been studied for its use in passive immunotherapy protocols. Although several mAbs for the treatment of cancer are now available to the market [106], so far, none of them are directed to carbohydrate structures. Nevertheless, some progress has been made in experimental models regarding the capacity of TACA-specific mAbs in mediating tumor regression. Indeed, we have recently reported that a chimeric antibody recognizing the Tn antigen is able to induce killing of tumors cells by ADCC mediated by macrophages [104]. In addition, a large number of mAbs targeting tumor-associated glycolipids, such as GD2, GD3, GM2 or sLe^a, has the ability to induce direct cell death in target cells [107,108], as well as mediating potent ADCC and CDC [109–111]. In particular, anti-GD2 and anti-GD3 mAbs are being evaluated to target neuroblastoma, melanoma and non-small-cell lung cancer [112]. Indeed, a mAb against the GD2 structure fused to IL-2 is capable of mediating the killing of $GD2^+$ neuroblastoma and melanoma cancer cells by increasing activating immune synapse formation between tumor cells and NK cells expressing high-affinity IL-2 receptors [113].

Antibody and cell-based immunotherapy of cancer has converged with the development of chimeric antigen receptor (CAR) technology [114]. In this approach, T cells are engineered to express the antigen binding site of a mAb (commonly an antibody-derived single-chain fragment) coupled via hinge and transmembrane elements to a signaling domain. A key advantage is that CARs are targeted against native rather than processed antigens; consequently, their function is not hampered by the frequent occurrence of HLA downregulation in human cancer [115]. CAR-T cells targeting cancer-associated carbohydrate antigens such as GD2 [116] or GD3 [117] on gangliosides have been developed. A chimeric GD2-specific receptor on T lymphocytes exhibited antimelanoma activity *in vitro* and increased the survival of mice xenografted with a human melanoma cell line [118]. Interestingly, the coexpression of a CD28 costimulation domain on CAR-T cells targeting GD3 improved tumor-induced proliferation *in vitro* and suppression of tumors *in vivo* [119]. It was also found that CAR-T cells targeting the Le^y antigen significantly inhibit established human ovarian tumors in mice [120]. More recently, MUC1-specific CAR-T lymphocytes were developed [121,122]. In order to improve the possibilities of using these CAR-T cells in immunotherapeutic protocols, coexpression of a chimeric IL-4 receptor was evaluated. These cells exhibited an IL-4-selective proliferation and a very high capacity to elicit repeated destruction of MUC1-expressing tumor cells [123], providing proof of principle for the development of IL-4-enhanced T-cell immunotherapy of cancer. Whether this kind of approach will be efficient for clinical settings rests to be explored; although, promising results have already been obtained [124].

Contrary to passive immunotherapy, the field of vaccinology targeting TACAs has been highly developed. In fact, several cancer vaccine strategies based on TACAs have been produced, and some of these have been evaluated in clinical trials. Nevertheless, the design of carbohydrate-based vaccines against cancer still remains a challenge. As with most vaccines, they have to

elicit specific humoral and/or cellular immune responses against cell surface antigens capable of eliminating tumor cells. In addition, in order to be efficient, these vaccines have to break the immunotolerance to TACAs, which are usually self-antigens. Moreover, most isolated TACAs are generally poor immunogens, and they require an appropriate immunogenic carrier or a favorable molecular context (adjuvancy) in order to elicit a therapeutically efficient immunological response. Regardless of these apparent drawbacks, the surprising and growing body of knowledge concerning the induction of carbohydrate-based immunity holds promise for the future success of these vaccines.

TACA-based vaccines can be classified in hemisynthetic or totally synthetic vaccines, depending on the nature of their synthesis. The former encompass mainly synthetic carbohydrate moieties covalently coupled to a carrier protein. In the majority of cases, covalent conjugation to the keyhole limpet hemocyanin (KLH) has proven to be the most effective strategy to enhance TACA immunogenicity [125–127]. However, the latter kind of vaccines refers to compounds fully synthesized by chemical methods, representing homogenous and pure chemically defined structures that override certain limitations of hemisynthetic conjugates [5]. These vaccines can also be designed to contain unnatural TACA analogs, an adjuvant (i.e., TLR agonists, such as palmitoyl) or other immunological $CD4^+$ or $CD8^+$ T cell epitopes to guarantee the induction of strong cytotoxic immunity [5,128,129].

During the last decade, results from a considerable number of vaccination experiments performed in cancer animal models have been made available. Vaccination of mice with TACA-based vaccines has been demonstrated to be effective in eliciting TACA-specific IgM or IgG antibody responses against Tn, *s*Tn, TF, Le^y, fucosyl-GM1, GD3 and globo H antigens [126,127,130–138], which, in some cases, recognize the native forms of TACAs produced by human cancer cells [127,137], mediate ADCC [134] and prolong survival of vaccinated tumor-bearing mice [130,133,134]. However, when tested in clinical settings, specific humoral immune responses did not always correlate with a good clinical response [139,140], in spite of the presence of high antibody titers against the tumor carbohydrate. The only two carbohydrate–KLH glycoconjugates (GM2–KLH and *s*Tn–KLH) that reached Phase III clinical trials so far, unfortunately, failed to meet clinical end points.

Vaccines based on carbohydrate antigens can also be divided into those containing a single copy of a given TACA or those that contain multiple copies of TACAs arranged in clusters (Figure 1). Indeed, carbohydrate antigens produced by tumor cells tend to cluster on their surface [141]. A clear example is constituted by cancer mucins that contain series of multiple and consecutive serine and threonine residues that are aberrantly glycosylated. Since both the clustering and presentation of some TACAs have shown to be important parameters for eliciting adequate antitumor immunity [126,132,134], vaccines exposing TACAs organized in artificial or natural (i.e., mucins) clusters have been evaluated in the past years. Indeed, a linear glycopeptide carrying a cluster of six Tn was shown to induce higher levels of anti-Tn antibodies than a glycopeptide based on clusters of three Tn [142]. Immunization of breast cancer patients with clustered *s*Tn–KLH conjugate confirmed that TACAs arranged in clusters more closely resemble carbohydrate antigens expressed at the tumor cells surface [141]. Thus, TACAs arranged in clusters seem to be more relevant targets to induce an antitumor immune response than single epitopes. In this line, Tn-specific mAbs recognizing tumor cells bind this antigen better when arranged in clusters of three, while nonclustered Tn structures are not recognized [143].

Furthermore, glycosylated tumor-associated mucin peptides might constitute better vaccines than conjugates presenting only the carbohydrate structure. Indeed, a 32-mer MUC1 peptide glycosylated at three sites per tandem repeat produced significantly higher anti-Tn antibody titers than a Tn–KLH conjugate exposing clusters of three Tn [126]. Interestingly, Karsten and colleagues have shown that glycosylation with *N*-acetylgalactosamine enhances binding of a series of MUC1-specific mAbs. The fact that two of these mAbs were developed against nonglycosylated peptides suggests that glycosylation induced a change in the peptide conformation that resulted in enhanced binding to the glycosylated antigen [144,145]. On the other hand, natural MUC1 antibodies of patients with breast cancer recognized higher affinity glycosylated MUC1 peptides as compared with unglycosylated MUC1 [146]. Additional structural and immunological studies have shown that the carbohydrate structures on mucins may be essential for inducing strong immune responses [147–149]. Altogether, these data suggest that vaccines presenting TACAs arranged in clusters on mucins may develop tumor-specific immune responses

with a higher affinity to tumor cells. The mucin-based epitopes are included to act, potentially, as T-cell epitopes in order to provoke a strong immune response. Current studies are on their way to determine the efficacy of this type of vaccines [150,151], although much attention has to be paid since the amino acid sequence on a peptide can have critical influence on the conformation of TACA clusters [152].

Attempts to improve B- and especially T-cell immunogenicity of TACA-based vaccines included the preparation of modified forms of TACAs, resulting in unnatural analogues of these tumor antigens [153,154]. In order to ensure cross-reactivity between antibodies induced by unnatural TACA analogs and TACAs expressed on tumor cells, Guo and co-workers proposed to induce expression of an artificial form of TACA by supplying tumors with an unnatural monosaccharide precursor, in a strategy they named 'metabolic cell engineering' [128]. Promising *in vitro* results demonstrated that a modified form of GM3 was expressed by different tumor cell lines that were killed by CDC mediated by specific antibodies against the GM3 analog [155,156]. However, a critical point of this strategy is to provide the unnatural saccharide precursor the correct specificity for tumor cells. More results in experimental models *in vivo* might validate the potential of this strategy in patients with melanoma.

Another novel approach having a great impact in the design of glycosidic vaccines during the last years focuses on the efficient delivery of carbohydrates to specialized immune cells (i.e., DCs). DCs recognize carbohydrate antigens to induce primary T- and B-cell responses through various pattern-recognition receptors, mainly CLRs. In addition, CLRs are differentially expressed in DC subsets, which may be specialized in particular functions. For instance, mouse CD8α$^+$ DCs are believed to play a dominant role in cross-presentation [157], while human CD14$^+$ DCs from the dermis would be specialized in the control of mature B-cell differentiation [158]. Furthermore, CLRs may trigger innate signals on DCs, which can skew T-cell polarization [159]. Thus, one could theoretically target one CLR on a determined DC subset, according to the type of carbohydrate on the vaccine, to induce the immune response of choice. Using this kind of approach, we have recently demonstrated that intradermal immunization with Tn-vaccines resulted in an efficient and selective delivery of the antigen to dermal DCs expressing the *N*-acetylgalactosamine-specific lectin receptor MGL. This delivery, in turn, led to strong T-cell activation and expansion of the germinal center B-cell population, together with the induction of high levels of specific anti-Tn antibodies [82]. Thus, *in vivo* DC-targeting strategies based on carbohydrate–lectin interactions, such as Tn–MGL, would therefore constitute a promising approach for enhancing antigen presentation and Tn-specific immunity.

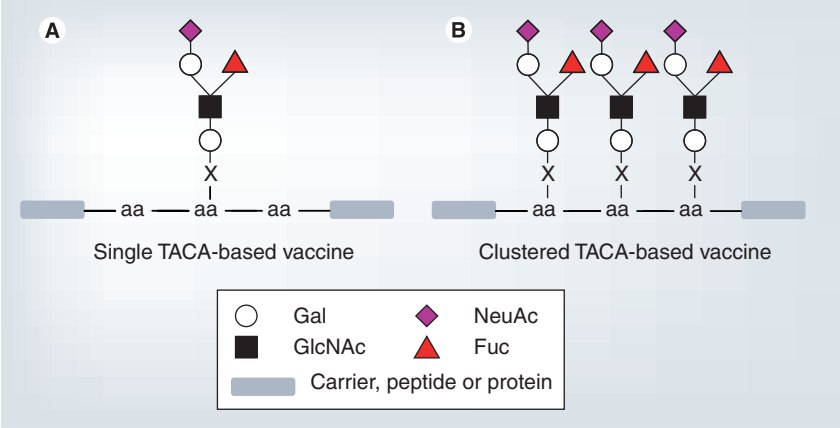


Figure 1. Single-copy and clustered tumor-associated carbohydrate antigen-based vaccines. Vaccines based on carbohydrate antigens can present a single copy of a given TACA **(A)** or multiple copies of TACAs arranged in clusters **(B)**. 'X' represents O (if aa is Ser/Thr), N (if aa is Asn in the Asn-X-ThrSer sequence) or an artifical linker (non-natural aa).
aa: Amino acid; Fuc: Fucose; Gal: Galactose; GlcNAc: *N*-acetylglucosamine; NeuAc: Neuraminic acid; TACA: Tumor-associated carbohydrate antigen.

Taking this strategy as a basis, several laboratories have also tried to improve cytotoxic immune responses by decorating protein antigens with carbohydrates. Indeed, when modified with glycans, the model antigen ovalbumin showed enhanced cross-presentation and increase generation of IFN-γ-producing T cells [160]. In another study, it was shown that MUC1 conjugated to oxidized mannan induced a high frequency of cytotoxic T lymphocytes and antitumor responses [161]. In both cases, glycosylated antigens targeted the mannose receptor. These results emphasize the importance of glycans on antigens and raise the question if a general glycosylation procedure will be available in the future for antigens targeting CLRs on DCs.

Conclusion & future perspective: what is missing in carbohydrate-based immunotherapy against cancer?

Pioneering work by Springer and coworkers showed that immunization with desialylated red blood cells expressing the Tn and TF antigens increased survival of breast cancer patients and prevented recurrences [162,163] and constituted one of the initial promising results that TACA-based vaccines would bring success in

the treatment of cancer. However, and despite the achievements obtained with some vaccines in preclinical models, no carbohydrate-containing cancer vaccine has so far produced any significant improvement in clinically relevant end points when evaluated in Phase III clinical trials. Several factors might constitute the causes of this failure. First, it is noteworthy that most of the Phase I and II studies have been carried out in patients with late-stage disease who have already experienced unsuccessful treatments. Furthermore, in some cases, patients presented

Executive summary

Glycan signatures on tumor cells: tumor-associated carbohydrate antigens

- Tumor-associated carbohydrate antigens (TACAs) carbohydrate motifs on proteins or lipids are expressed by tumor cells resulting from alterations in the glycosylation pathways.
- Tn, TF and sialyl-Tn antigens are found in most epithelial carcinomas.
- Sialyl-Le^a, sialyl-Le^x and sialyl-Le^y antigens are also found in epithelial tumors.
- GD2, GD3 and GM2 structures are overexpressed in melanoma, neuroblastoma and glioma.
- Fucosyl-GM1 antigen is identified in small-cell lung cancer cells.
- Globo H is detected in ovarian, colon and prostate cancers.
- TACAs are useful for cancer diagnosis since they are absent in healthy tissues.

Role of tumor-associated carbohydrate antigens in cancer & antitumor immunity

- They contribute to cell growth, migration and invasiveness of tumor cells.
- TACAs potentiate metastasis by interacting with lectins on endothelial cells.
- They can suppress antitumor immunity by: inducing expression of immunosuppressive mediators, hampering antigen processing or presentation or increasing Treg production.
- They can also impair differentiation and function of dendritic cells (DCs) and induce apoptosis on DCs or T cells.
- Carbohydrates on tumor cells that are recognized by receptors on cells from the innate immune arm (C-type lectin receptors and siglecs) can modulate T-cell signaling and survival and abrogate NK cell function.
- TACAs constitute potential tools for immunotherapy against cancer as long as they are utilized in adequate immunostimulating conditions.

Why use TACAs for immunotherapy against cancer?

- TACAs are, in principle, present on tumor cells but absent on healthy cells.
- The detection of TACA-specific autoantibodies in patients with cancer may predict cancer progression.
- TACA-specific antibodies can mediate tumor cell killing by complement-dependent cytotoxicity or antibody-dependent cellular cytotoxicity.
- CD4$^+$ and CD8$^+$ T cells reactive with glycosylated antigens are able to accommodate small carbohydrate molecules.
- In appropriate stimulating conditions TACAs can induce specific cytotoxic T-cell responses that recognize and kill tumor cells.
- Data regarding the recognition of TACAs by immune cells, as well as the mechanisms by which they modulate the immune system, are essential in the design of effective immunotherapeutic strategies against cancer.

Progress made on antitumor immunotherapy targeting TACAs

- TACA-specific monoclonal antibodies can mediate tumor regression by cytotoxic processes including complement-dependent cytotoxicity or antibody-dependent cellular cytotoxicity, and are gaining importance in antitumor passive immunotherapeutic approaches.
- Only a small number of antibodies elicited against hemisynthetic or completely synthetic TACA-based vaccines can recognize the native forms of TACAs produced by human cancer cells and mediate tumor cell killing.
- Specific antibody immune responses do not always correlate with a good clinical response.
- In this context, glycosylated mucins, especially those that present cluster of glycan motifs and artificial forms of TACAs, appear to be an interesting option since they are more immunogenic.
- Delivery of carbohydrates to DCs is also a very promising alternative strategy since it may enhance antigen uptake and presentation and induce specific polarized T-cell immune responses.

Future perspective

- Prophylactic vaccination with TACAs of individuals who are at high risk of developing cancer may constitute an attractive and promising strategy to fight against cancer.
- Chimeric antigen receptors targeting TACAs on T cells obviate antigen presentation on MHC class I molecules by tumors cells, and might be key tool in future immunotherapeutic protocols, especially when coexpressed with costimulatory molecules or cytokine receptors.
- Combining anti-TACA immunotherapy with some chemo- or radio-therapeutic regimens that rely on the induction of immunogenic tumor cell death may improve the induction of an anticancer immune response.

a relatively large tumor burden, which is usually characterized by its biologically complex and heterogeneous properties, as opposed to experimental animal models. In this context, prophylactic vaccination with TACAs of individuals who are at high risk of developing cancer may constitute an attractive and promising strategy to fight against cancer, since the primed immune system would destroy the tumor before it becomes heterogeneous and capable of evading the immune response [164]. In this sense, the US FDA has recently approved two vaccines that protect against cervical cancer induced by HPV [201]. Some types of nonvirally induced cancers may be ideal to test prophylactic TACA-based vaccines, such as breast cancer (especially those with mutations in the genes encoding BRCA1 or BRCA2), colon cancer (15% of colon cancer corresponds to familial adenomatous polyposis syndrome), or pancreatic cancer (i.e., patients with familial pancreatic cancer or hereditary pancreatitis). Patients exhibiting some of these characteristics have an increased risk of developing these types of cancer and could strongly benefit from such preventive approaches.

Another parameter to be taken into account is the age of the patients that receive vaccine treatment, since the function of the immune system might be hampered in elderly patients. It is known that immune system functions decrease with age, and that adaptive immune responses seem to be the main affected with aging. For instance, a diminished potential of the bone marrow to generate new DCs in aged individuals as well as DC maturation has been documented [165].

Last but not the least, there is a need to combine cancer immunotherapeutic treatments with other interventions, such as effective adjuvants or traditional treatments, such as surgery, radiotherapy or chemotherapy. Other innovative strategies might also be combined with carbohydrate-based passive or active immunotherapy. In fact, the possibility of modifying the cancer cell with the introduction of genetic material opens the way to a new approach based on gene therapy. Recent advances in the molecular and cellular biology of gene transfer may allow activation of the patient's own immune cells to eliminate cancer cells by expression of molecules that enhance immune responses, silencing genes related to the development of drug resistance in patients or inhibition of angiogenesis of solid tumors [166]. Thus, gene therapy could rapidly play an important role in clinical practice. In this context, a combination of immunotherapy-targeting carbohydrates with expression or silencing of certain glycosyltransferase genes by the tumor, which will enhance the expression of a determined carbohydrate antigen, could be considered in the future.

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