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Perspective

Tumor-Associated Glycans as Targets for Immunotherapy: The Wistar Institute Experience/Legacy

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Tumor cells are characterized by the expression of tumor-specific carbohydrate structures that differ from their normal counterparts. Carbohydrates on tumor cells have phenotypical as well as functional implications, impacting the tumor progression process, from malignant transformation to metastasis formation. Importantly, carbohydrates are structures that play a role in receptor–ligand interaction and elicit the activity of growth factor receptors, integrins, lectins, and other type 1 transmembrane proteins. They have been recognized as biomarkers for cancer diagnosis, and evidence demonstrating their relevance as targets for anticancer therapeutic strategies, including immunotherapy, continues to accumulate. Different approaches targeting carbohydrates include monoclonal antibodies (mAbs), antibody (Ab)–drug conjugates, vaccines, and adhesion antagonists. Development of bispecific antibodies and chimeric antigen receptor (CAR)-modified T cells against tumor-associated carbohydrate antigens (TACAs) as promising cancer immunotherapeutic agents is rapidly evolving. As reviewed here, there are several cancer-associated glycan features that can be leveraged to design rational drug or immune system targets, applying multiple TACA structural and functional features to be targeted as the standard treatment paradigm. Many of the underlying targets were defined by researchers at the Wistar Institute in Philadelphia, Pennsylvania, which provide basis for different immunotherapy approaches.

Keywords: monoclonal antibody, carbohydrate-targeted immunotherapy, cancer vaccines, lectins, cancer diagnostic, tumor-associated carbohydrate antigens (TACA)

Introduction

Alteration in glycosylation is a proven characteristic of cancer, and most known serological biomarkers currently used in the clinic are cancer-associated glycans. Biosynthesis of glycans is non-DNA template dependent and it is controlled by the expression of glycosyltransferases and glycosidases. Tissues of different origin and cancer cells express different carbohydrate structures due to expression of these enzymes. Aberrant glycosylation can include sialylation, fucosylation, O-glycan truncation or incomplete synthesis, and N- and O-linked glycan branching.[1] Both aberrant expression of carbohydrates and acquisition of aberrant glycosylation profiles accompany malignant transformation and progression.[2] Carbohydrates are the most diverse complex biomolecules that also play pivotal roles in many cellular physiological functions, including cell–cell interaction, cellular signaling through cell surface receptors, and immune recognition. Although tumor-specific glycans have been known for a long time (Table 1), only recently, these structures have been identified as potential targets to recruit the host immune system for cancer therapy or generate the immune response through vaccines.

Common approaches for immunotherapy include monoclonal antibodies (mAbs), vaccines, carbohydrate-specific antibody (Ab)–drug conjugates, carbohydrate mimetics, antagonists targeting selectins, and Siglec receptors. For example, several lectin families play a role in inflammatory processes and cancer, including selectins, galectins, siglecs, and macrophage galactose-type lectin (MGL). Glycan–lectin interactions are critical for cancer progression, cell proliferation, extravasation, and invasion. Targeting the glycans and interference in their interactions with specific inhibitors is currently being explored in clinical trials as a promising therapy strategy (Table 2).

Research into tumor-associated carbohydrate antigens (TACAs) has entered an exciting phase because of the recent identification of their function and implications for clinical use. These discoveries open up the possibility of using TACAs and mAbs recognizing TACAs in vaccines and immunotherapeutic strategies against cancer. Pioneering studies focusing on cancer-associated cell surface glycans have been carried out using hybridoma technology and mAbs developed at the Wistar Institute of Anatomy and Biology starting in the late 1970s.[3–5] Future studies were performed in collaboration

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Table 1. Structure of Common Tumor-Associated Carbohydrate Antigens

Tumor-associated carbohydrate antigens	*Structure*
Tn	GalNAcSer/Thr
sialyl Tn	Neu5Acα2-6GalNAcaSer/Thr
T antigen	Galβ1-3GalNAcα1-Ser/Thr
Globo-H	Fucα1-2Galβ1-3GalNAcβ1-3Galα1-4Galβ1-4Glc
LeY	Fuc(α1 → 2)Gal(β1 → 4)[Fuc(α1 → 3)]GlcNAc
SLea	Neu5Acα2,3Galβ1,3(Fucα1,4)GlcNAc
SLeX	Neu5Acα2,3Galβ1,4 (Fucα1,3)GlcNAc
GM3	Neu5Acα2,3Galβ1,4Glcβ1Cer
GD3	Neu5Acα2,8Neu5Acα2,3Galβ1, 4Glcβ1Cer
GD2	GalNAcβ1,4(Neu5Acα2,8Neu5Acα2,3)Galβ1, 4Glcβ1Cer

LeY, Lewis Y; SLea, sialyl Lewis A; SLeX, sialyl Lewis X; STn, sialyl Tn.

with the Department of Medical Biochemistry, University of Goteborg in Sweden; Department of Chemistry, University of Alberta, Edmonton, Canada; University of Pennsylvania, Philadelphia, Pennsylvania; and the National Institutes of Health (NIH), Bethesda, Maryland. These studies succeeded in identification of multiple carbohydrate tumor-associated antigens (Ags), including the first glycolipid tumor-associated antigen, sialyl Lewis A (SLea), known as CA19-9.[6,7] The SLea-specific antibody, named NS19-9, was developed at Wistar Institute by hybridoma technology using the human colorectal cancer cell line, SW1116, as an immunizing antigen.[4] The SLea antigen is widely expressed in gastrointestinal cancers and is one of the most studied serum biomarkers for diagnosis and monitoring of pancreatic and colorectal cancers. CA19-9 carbohydrate also plays a role in tumor extravasation through interaction with selectins, as discussed below. A recent study postulates that SLea promotes pancreatic cancer. This study demonstrated the possibility of CA19-9 as a therapeutic target for treatment of pancreatitis and pancreatic cancer.[8] Consequently, the CA19-9 antigen itself, its mimic, and the mAb are utilized for various immunotherapy approaches.

Examples of TACAs found by this group represent blood group-related carbohydrate series such as A, B, O, and Lewis (Le) system series, including sialyl-Lea (SLea), sialyl Lewis X (SLeX), and Lewis Y (LeY), which are widely expressed in epithelial tumors (Table 1).[9–20] SSEA-1 (CD15 or Lex) was also identified as a stage-specific embryonic antigen at the Wistar Institute using hybridoma technology.[21,22]

The ability to detect these aberrant glycans *in situ* in FFPE specimens using carbohydrate-specific mAbs has also been demonstrated by our group. These data indicate that the blood group ABO, H, Se, and Le genes are subjected to a tissue-dependent differential expression. The results of these studies laid the groundwork to evaluate blood group Ags and related glycolipids as pathological tumor markers and provide immunohistochemical evidence for a diverse repertoire of altered antigen expression in different cancers, which can be exploited for diagnosis and therapeutic intervention.[23–26]

Tumor tissues can also display gangliosides such as GD2, GD3, GM3, GM2, fucosyl GM1, and Globo-H that are sialylated glycosphingolipids found at elevated levels in tumors of neuroectodermal origin, including neuroblastomas (NBs) and melanomas. Specifically, a gradual increase in GD2, GD3, and 9-0-acetyl-GD3 ganglioside expression in subsequent stages of melanoma progression from normal melanocytes to metastatic disease, including the pivotal step of the early primary melanoma in the radial growth phase (RGP) to advanced vertical growth phase (VGP) melanoma, was characterized. The qualitative differences of gangliosides between the RPG and VGP suggest their role as prognostic indicators of risk for tumor recurrence and as a therapy target.[27–30] A phase I clinical trial has been conducted with murine mAb ME361, which recognizes GD2 and GD3 generated at the Wistar Institute.[31] The initial study, including clinical trials from this and other groups, built the foundation to further exploit the therapeutic and diagnostic potential for ganglioside-expressing tumors.

The assembly of cell surface complex carbohydrates requires the concerted action of a large number (>100) of glycosyltransferases, each of which catalyzes the transfer of a single sugar residue, usually from a sugar nucleotide, to specific hydroxyl groups on a suitable oligosaccharide acceptor. Glycosyltransferases such as sialyltransferases and fucosyltransferases involved in linking terminating residues on glycans are two of the most common glycosylation changes in carcinogenesis and progression. The increase in activity of these glycosyltransferases leads to overexpression of terminal TACA epitopes commonly found on transformed cells that include SLeX, SLea, sialyl Tn (STn), Globo H, LeY, and gangliosides.[32,33] α-2-L-Fucosyltransferase transfers L-fucose from GDP-L-fucose to the C-2 position of terminal nonreducing b-D-galactosyl residues, thus forming the H antigen from its type 1 or 2 chain precursor.

Our group characterized kinetic and structural parameters of both Secretor (Se) and H α-2-L-fucosyltransferases that are responsible for the synthesis of H (O-type) blood group and Lewis series Ags.[34,35] We published the amino acid sequence for α-2-L-fucosyltransferase and demonstrated that the enzyme-enhanced expression correlated with colon cancer progression.[36] The elevated level of the enzyme in adenomatous polyps may represent an early event associated with tumorigenesis in colon cancer. A nucleotide sequence analysis of the protein coding region of the complementary DNAs (cDNAs) derived from adenoma, and colon adenocarcinoma revealed 100% homology, suggesting that there is no tumor-associated allelic variant within the H α-2-L-fucosyltransferase cDNA.[37]

Glycosyltransferases represent prime targets for the design of glycosylation inhibitors with the potential to specifically alter the structures of cell surface carbohydrates. The study by our group on the mechanism of glycosyl transfer demonstrated that the reactive acceptor hydroxyl groups are

Table 2. Clinical Trials Targeting Carbohydrate Antigens

Modality	*Target*	*Histology*	*Phase*	*Clinical Trial*
Vaccines				
Glycolipids/Glycoproteins	Theratope STn	Breast	3	NCT00003638
	Thomsen-Friedenreich (TF)	Prostate	1	NCT00003819
	Globo-H-GM2-sTn-TF-Tn	Ovarian	1	NCT01248273
	GD2, GD3, Globo H, Fucosyl GM1 and N-propionylated polysialic acid	Lung	1	NCT01349647
	Globo-H	Breast	2	NCT01516307
	GM2, GD2, GD3	Sarcoma	2	NCT01141491
	GD3	Melanoma	2	NCT00679289,
			1	NCT03159117
	GD3L, GD3L	Melanoma	1	NCT00597272
			1	NCT00911560
	GM2	Breast	3	NCT00003357
		Melanoma	3	NCT00005052
Anti-idiotype	NeuGcGM3	NSCLC	3	NCT01460472
		Pediatric tumors	1	NCT01598454
	ACA125	Ovarian,	2	NCT00058435
	CEA	Colorectal	2	NCT00033748
	GD2	Neuroblastoma	1	NCT00003023
Peptide Carbohydrate Mimotope	LeY, GD2	Breast	1 and 2	NCT02229084
		Lung	1/2	NCT02264236
Monoclonal Antibodies				
	GD2	Neuroblastoma	1	NCT03033303
		Neuroblastoma/	1 and 2	NCT01757626
		Osteosarcoma	2	NCT00089258
			2	NCT03363373
			2	NCT00002458
			3	NCT01704716
			1 and 2	NCT03860207
	GD3	Melanoma	2	NCT00679289
	LeY	Breast	2	NCT01370239
			2	NCT01370239
		Ovarian	2	NCT00617773
	SLea	Pancreas	1	NCT02672917
		Gastrointestinal cancers	2	NCT03801915
	MUC1	Ovarian,	2	NCT01899599
		Solid tumors	1	NCT01222624
Radiolabeled mAb	GD2	Central Nervous System, Lung, Melanoma, Neuroblastoma, Sarcoma	2	NCT00445965
Antibody drug conjugate	LeY	NSCLC	2	NCT00051571
		Prostate	2	NCT00031187
		Ovarian	2	NCT00051584
		Breast	2	NCT00028483
Lectin antagonists				
	Selectins	Acute Myeloid Leukemia	3	NCT03616470
	Galectins	Melanoma, NSCLC, H&N, Colon cancer,	1	NCT02575404,
		Solid tumors	2	NCT00110721,
		Melanoma	1	NCT00054977,
			1 and 2	NCT01723813,
			1	NCT02117362
	Siglec-15	H&N, NSCLC	1/2	NCT03665285
		Ovarian		
		TNBC		

(*continued*)

Table 2. (Continued)

Modality	*Target*	*Histology*	*Phase*	*Clinical Trial*
CAR T cells				
	LeY	Solid tumors	1	NCT03851146
		Myeloid malignancies	1 and 2	NCT02958384
	CEA	Pancreatic	2 and 3	NCT04037241
				NCT03818165
	GD2	Glioma	1	NCT04099797
			1	NCT04196413
			1	NCT04099797
		Neuroblastoma,	1 and 2	NCT04637503
		Osteosarcoma	1 and 2	NCT03373097
			1	NCT01953900
			1	NCT02107963
		B cell lymphoma	1 and 2	NCT04429438
Bispecific Antibodies	GD2	Neuroblastoma	1 and 2	NCT02173093

CAR, chimeric antigen receptor; CEA, carcinoembryonic antigen; H&N, head and neck cancer; mAbs, monoclonal antibodies; NB, neuroblastoma; NSCLC, non small cell lung cancer; sTn, sialyl Tn; TNBC, triple negative breast cancer.

involved in a critical hydrogen bond donor interaction with a basic group on the enzyme, which removes the developing proton during the glycosyl transfer reaction. The resulting deoxygenated acceptor analogs can no longer be substrates for the corresponding glycosyltransferases, which should act as competitive inhibitors. Alternatively, basic groups would be logical targets for irreversible covalent inactivation of the enzymes. Inhibitors of glycosylation can be invaluable tools in deciphering both the biosynthetic pathways for the assembly of active cell surface oligosaccharides, as well as tools for drug discovery.[38]

Defining the epitopes for antibodies and T cell receptors (TCRs) is of great importance for optimization of antigenic and immunogenic properties of effective vaccines and other immunotherapeutic approaches based on the Ab or TCR antigen recognition. To understand the basic principles of antibody-targeting TACAs and their binding specificity, structural studies, including biochemical methods, mass spectrometry, and proton nuclear magnetic resonance (NMR) spectroscopy, as well as conformations established by computer modeling, were undertaken by our group.[39–44]

While missing detailed crystallographic information, topographical features of antibody recognition and the conformational properties of a series of related tissue blood group (Lewis) carbohydrates were established. For example, using two-dimensional NMR in combination with hard-sphere energy calculation, it was established that the NS-19-9 antibody does not cross-react with Lea antigen and the presence of Neu5Ac residue can cause conformational alteration, which are crucial to the formation of the antigenic determinant.[39] Combining molecular modeling and experimental structural information may be possible to rationally modify Lewis antigen-binding antibodies by fine-tuning specificities and affinities to optimize their *in vivo* functionalities.[45,46] Importantly, these studies were conducted at the time when the methods of cocrystallizing antibody Fab fragments and carbohydrate antigens had not yet been used for antibody recognition and the conformational properties of blood group (Lewis) carbohydrates.[47,48]

The efficacy of the antibody can be further improved by increasing the specificity for the carbohydrate antigen that may be important for antibody-based approaches, including chimeric antigen receptor (CAR)-modified T cells, as even minor alterations in binding of the carbohydrate structure may have an impact on the bound conformations and affinity of the antigen important for recognition of the tumor antigen.

Recent efforts on carbohydrate-based cancer immunotherapies, including bispecific antibodies (BsAbs) and CAR T cells against TACAs, are rapidly evolving. This perspective discusses the role of carbohydrates for the current applications in oncology, including diagnostics and immunotherapy approaches. We also would like to acknowledge that many of today's modern cancer drugs may owe their conceptual basis to the pioneering work by Koprowski's team at the Wistar Institute decades ago, which can inform current and future developments in the field, including carbohydrate Ags as diagnostics, mAbs as drugs, vaccines, adhesion antagonists, and even BsAbs and CARs.

Carbohydrates as Diagnostics

Current clinically approved serological biomarkers for cancer diagnosis and biomarkers of disease recurrence in different cancers are glycosylated biomarkers. Most of the clinically relevant glycoprotein biomarkers in cancer patients include the prostate-specific antigen (PSA); carcinoembryonic antigen (CEA); ovarian carcinoma antigen CA125 also known as MUC16; breast cancer CA15-3, that is, aberrantly glycosylated MUC1; CA72-4 antigen in gastric cancer alpha fetoprotein (AFP) in liver cancer; and breast cancer antigen CA27-29.[49] Early studies from our group introduced mAbs for serological detection of TACAs.[50–55] The SLea antigen expressed in epithelial tumors is detected by the serological assay NS19-9 used for patients with an established diagnosis of pancreatic, colorectal, gastric, or biliary cancer and is used to monitor clinical response to therapy. It is the most clinically validated serum biomarker used for the management of pancreatic cancer patients to date.[56,57] One of the most important limitations of SLea as a tumor marker is that 5%–10% of the population lacks the ability to synthesize

the SLea precursor due to failure of the Lewis α-4-L-fucosyltransferase expression and, as a result, cannot produce CA19-9, as noted in our report.[58]

Carbohydrate Targets and mAbs for Immunotherapy

Multiple approaches targeting carbohydrates have been investigated, and multiple clinical trials support the potential of targeting glycosylation in cancer immunotherapy. The comprehensive list of clinical trials applying different approaches targeting cancer-specific glycans has been listed in Table 2.[59,60] Considering the therapeutic approaches in which TACAs are targeted, their expression should be required as an eligibility criterion for patient stratification or selection to personalize patients' clinical outcomes.

Monoclonal Antibodies

Developing antibodies against TACAs for clinical use has been challenging and few tumor-targeting antibodies have reached clinical trials due to low affinity and toxicity. Nevertheless, several glycan-specific mAbs and their chimeric and/or humanized versions showed promise in *in vitro* and *in vivo* models and have been used for passive immunotherapy in clinical protocols. Several mAbs targeting glycolipids, such as GD2, GD3, GM2, LeY, or SLea, have demonstrated the ability to mediate potent antibody-dependent cellular cytotoxicity (ADCC) and complement-mediated cytotoxicity (CMC). The efficacy of mAb treatment was often enhanced with combination therapies or antibody-mediated delivery of cytotoxic payloads such as radioisotope and antibody–drug/toxin conjugates or cytokines such as granulocyte–macrophage colony-stimulating factor (GM-CSF) and interleukin-2 (IL-2).

As protein engineering technology has become more accessible, newer antibody constructs are being tested in clinical trials. Fully human antibodies were derived from lymphocytes of individuals immunized with the SLea-KLH vaccine demonstrated to be potent in CMC and ADCC.[61,62] SLea-targeting antibody MVT-5873 is currently in phase I clinical trial (NCT02672917) and have been reported to be well tolerated. A phase II trial was designed to determine the efficacy and safety of perioperative Ab use in patients with gastrointestinal cancers (NCT03801915). Promising antibodies with high affinity for SLea (5B1 and 7E3) were also expressed as recombinant antibodies and were potent in CDC ADCC assays.[63] The most recent example demonstrates that reengineering the Fc portion of SLea-targeting antibodies generated in response to the SLea/KLH vaccine enhanced their affinity for activating human FcγRs and led to an enhanced therapeutic effect suggesting that an Fc-optimized variant could potentially be translated to the clinic.[64] Potentially natural class-switch variants can also show enhanced therapeutic potential. Spontaneously occurring mAb specific for SLea and LeY heavy chain variants, IgG1, IgG2b, and IgG2a, identified in our study had the same binding specificity and were active in ADCC; however, IgG2a gave the highest percentage of lysis and inhibited growth of human colorectal carcinoma, while IgGl and IgG2b were ineffective.[65–67] The results demonstrate the potential usefulness of the subclass switching antibody as a therapeutic agent.

Although GD2 and GD3-specific mAbs demonstrated *in vivo* and *in vitro* activity against melanoma and NB, the concerns with toxicity and low efficacy of mAbs prompted new advances in genetic engineering and development of newer generation chimeric and humanized anti-GD2 mAbs. The human–murine chimeric Ab ch14.18, subsequently renamed dinutuximab, was generated by combining the variable regions of the original murine IgG3 anti-GD2 mAb 14.18 and the constant regions of human IgG1.[68,69] Dinutuximab was the first antibody to be approved by the US FDA and the European Medicines Agency (EMA) for NB and other pediatric solid tumors and was established as the new standard of care for maintenance therapy in patients with high-risk NB. Phase 2 and 3 studies are underway for NB NCT03363373 NCT00002458 NCT01704716.

The LeY antigen is also a member of a family of blood group antigens expressed in human epithelial cancers, which makes it an attractive target for treatment with mAbs. Humanized or chimeric forms of LeY-specific mAbs have demonstrated signal of efficacy in preclinical models and have been tested in clinical trials.[70] The humanized mAb against the LeY antigen (Hu3S193) has been demonstrated to be safe in previous studies and has also been indicated as a potential intervention in solid tumors.[71–73] Hu3S193 is a humanized anti-LeY IgG1 mAb with strong complement and antibody-dependent cytotoxicity with clinical benefit shown in a phase II study (NCT00617773).

Other applications of TACA-specific mAbs include drugs delivered through antibody–drug conjugates (ADCs). LeY-specific BR96 mAbs conjugated to doxorubicin and docetaxel were tested in phase II trials for advanced non small cell lung cancer (NSCLC) and breast cancer.[74,75] Multiple ADC approaches targeting STn antigens by conjugating them to monomethyl auristatin E (MMAE) showed promising anti-tumor activity.[76,77] Immunotoxins such as diphtheria toxin fused to the single-chain variable fragment (scFv) 5F11 and pseudomonas toxin fused to scFv14.18—demonstrated specific killing of $GD2^+$ tumor cells. However, immunogenicity of foreign toxin proteins has remained a major concern, and no GD2-directed immunotoxins are currently in human trials.[78] Regression of lung, breast, and bladder carcinomas in patient derived xenograph (PDX) models was demonstrated upon administration of BR96 sFv-PE40 Pseudomonas exotoxin A.[79] The first human study to obtain data on the safety and feasibility of ^{89}Zr-DFO-HumAb-5B1 to image pancreatic tumors and other SLea-positive malignancies is ongoing (NCT02687230).

Vaccines

Targeting TACAs as an immunotherapeutic strategy with anticancer vaccines provides an appealing option for cancer treatment. Examples include vaccines targeting the mucin-related Tn, STn, and T antigens, the gangliosides GM2 and GD3, and the glycosphingolipid Globo-H.[80–82] Several carbohydrate-based vaccines have shown some promise and are presently undergoing clinical evaluation (Table 2). Strategies to overcome poor immunogenicity of glycan-based vaccines by displaying vaccine glycans in a multivalent context are currently being pursued. For example, a triantigenic vaccine containing Globo H, Ley, and Tn has been shown in animal models to elicit an immune response against

each oligosaccharide antigen and it may result in recruiting both humoral and T cell-mediated immune responses against tumors in human patients.[83] Similarly, vaccination of small cell lung carcinoma patients with polysialic acid (PSA)-KLH or N-propionylated PSA-KLH (PrPSA-KLH) conjugates was tested in a clinical trial.[84]

Successful tumor immunotherapy might require the induction of cytotoxic T lymphocytes (CTLs) in addition to antibodies, which explains early attempts of developing a carbohydrate-based vaccine such as Theratope that elicits a B cell-mediated immune response, but does not seem to trigger a T cell-mediated immune response.[85] Another approach to overcome poor immunogenicity and lack of T cell engagement has been developed using anti-idiotype antibodies (Ab2) as surrogate antigens. Ab2 vaccines induce anti-GD2 immune responses and have several advantages over native gangliosides; as proteins, they induce T cell responses. Anti-idiotypic GD2 mAbs were tested in phase I trials.[86]

An alternative approach to develop T-dependent responses to carbohydrate Ags is the use of peptide or polypeptide surrogates of carbohydrates, which has also been carried out by our group in collaboration with the University of Pennsylvania. Peptides may functionally mimic carbohydrates and induce IgG and cellular immune responses, which the carbohydrate itself is usually unable to induce.[87–90] Carbohydrate-mimetic peptides (CMPs), unlike carbohydrate antigens, can prime for memory responses to TACAs, suggesting that CMPs facilitate cognate interactions between B cells and T cells. Antibodies induced by a CMP to the meningococcal group C capsular polysaccharide[91] were shown to be reactive with the LeY antigen and activated peptide-specific T helper type 1 (Th1) and type 2 (Th2) responses.[92,93] To induce sustained immunity against both LeY and GD2, CMP as a surrogate pan-immunogen that mimics both was developed. To test the feasibility of inducing proapoptotic antibodies reactive with LeY and GD2, advanced breast cancer subjects were immunized with the P10s-PADRE vaccine and limited clinical benefit was observed following vaccine treatment, which was not only caspase 3 dependent but also demonstrated synergy with chemotherapeutics[94] (NCT02229084 and NCT 02264236).

Similarly, a DNA vaccine encoding a peptide isolated with the GD2-specific mAb 14G2a GD2, resulted in the induction of antibodies that exhibited CDC against $GD2^+$ melanoma cells and inhibited growth of human melanoma cell xenografts. A study suggests that peptides mimicking the GD2 ganglioside inhibit tumor growth through antibody and/or $CD4^+$ T cell-mediated mechanisms. DNA vaccination studies in mice showed that plasmids encoding peptides mimicking LeY induced LeY cross-reactive IgG2a Abs and mediated CMC.[95] Peptide mimetic of carbohydrate Ags encoded as DNA plasmids are novel immunogens providing a means to manipulate carbohydrate cross-reactive Th1 responses. This approach provides a way for development of messenger RNA (mRNA) vaccines targeting the immune response to glycans.

Adhesion Antagonists

Lectins such as C-type, Galectins, and Siglecs are important for adaptive immune responses. Examples of a lectin–ligand interaction (important for the homing and tissue recruitment of leukocytes and tumor cells) involve the C-type lectins, E-selectin, P-selectin, and L-selectin (CD62E, CD62P, and CD62L), and LeX or SLea that are the major ligands expressed on the surface of tumor cells.[96,97] During inflammation, selectins mediate the initial attachment of leukocytes to the endothelium during the process of leukocyte extravasation. In cancer, SLeX and SLea interactions with selectins regulate the metastatic cascade by forming emboli of cancer cells and platelets and favoring their arrest on endothelia.

We hypothesized that the tumor cell transmigration from the bloodstream to metastatic sites in analogy with lymphocyte rolling is mediated by interaction of selectins on endothelial cells and TACAs on the surface of tumor cells. Applying the concept of the functional equivalence of chemically dissimilar molecules such as carbohydrates and proteins sharing common surface topology, our study of administration of a monovalent peptide mimetic of SLea showed reduced neutrophil recruitment *in vivo*.[98–100] In a subsequent study, colonization of tumor cells expressing SLea was blocked by the peptide mimetic of this antigen and was completely abolished in E-selectin knockout mice.[101,102] Using combinatorial synthetic chemistry technology, this study allowed for delineating the positions of amino acids containing carboxyl groups that improved upon peptide mimicry and increased mAb binding. Developing reagents that are stereochemically equivalent to carbohydrate ligands for E-selectin that can effectively block lymphocyte rolling and tumor colonization *in vivo* might provide an effective treatment of the metastatic process and inflammatory conditions. Consequently, interference with selectin functions has become a potential therapeutic strategy, and these compounds are currently in clinical development. Glycomimetics' E-selectin inhibitor Uproleselan in combination with chemotherapy has been shown to improve survival in patients with acute myeloid leukemia (NCT03616470).[103]

Galectins are endogenous lectins recognizing galactose that allows for specific binding to carbohydrate epitopes, which can be shared by several T cell surface proteins. Galectins are expressed in cancer and stromal cells and mediate interactions between tumor cells and innate and adaptive immune cells. Galectin-3 binds to the cytotoxic T lymphocyte antigen 4 (CTLA-4) and lymphocyte activation gene 3 (LAG-3), while galectin-9 binds to T cell immunoglobulin and mucin-domain containing-3 (TIM-3).[104,105] Galectin-1 binding induces partial phosphorylation of TCRz and induces partial z-chain phosphorylation (pp21z) that cannot initiate downstream protein tyrosine phosphorylation, IL-2 production, or T cell proliferation.[106] Because of their immunosuppressive role, targeting galectins represents a potential therapeutic approach to restore antitumor immunity. Galectin antagonists in combination with chemotherapy, peptide vaccinations, or immune checkpoint inhibitors are currently in clinical trials for treatment of different cancers (Table 2).

The Siglec family of lectins comprises immunoglobulin-type lectins, which recognize predominantly sialic acid-containing glycans that are expressed in most white blood cells of the immune system and play critical roles in immune cell signaling.[107] For example, Siglec-9 plays a critical role

in suppressing antigen-specific T cell responses *in vitro* and *in vivo*. Siglec-9 is coexpressed with several known inhibitory T cell receptors, for example, PD-1, CTLA-4, and TIM-3, in healthy individuals and melanoma patients.

A subset of Siglec-9 CD8 T effector cell engagement was associated with phosphorylation of the inhibitory protein tyrosine phosphatase SHP-1, but not SHP-2, resulting in suppressed TCR signaling and effector functions.[108] Siglec-15 is not expressed in normal tissue, but is upregulated in tumor cells and tumor-associated myeloid cells as well as M2 macrophages, leading to profound immunosuppression in the tumor microenvironment (TME). Siglec-15 expression is mutually exclusive with that of PD-L1, suggesting that a drug that alleviates Siglec-15–driven immunosuppression could be viable in patients with low PD-L1 expression to benefit from checkpoint blockade.[109] Many avenues to exploit sialic acid–Siglec interactions to advance cancer therapy are under investigation for advanced solid tumors (NCT 03665285).

CAR-Modified T Cells and BsAbs

CARs and BsAbs are exciting developments for TACA-based immunotherapy. Adoptive transfer of CAR T cells is a promising immunotherapy strategy to treat cancer in an MHC-independent manner. CARs are genetically encoded artificial TCRs that combine the antigen specificity of an antibody with the machinery of T cell activation. CARs are generated by linking the scFv of an mAb with the TCRζ-chain transmembrane and cytoplasmic regions. The second- and third-generation CARs include additional signaling domains (CD27, 4-1BB, or OX40) aimed at improving proliferation, survival, and cytokine release from the cells. CAR immunotherapies have been shown to be highly effective in hematological malignancies (KYMRIAH and CARTA) and have been approved by the FDA. However, translation of CAR-T cell therapies from hematologic malignancies into solid tumors comes with challenges, including immunosuppressive TME.

CAR T cells based on mAbs targeting GD2 and LeY were demonstrated to be effective in eradicating leukemia and pancreatic cancer in mice[110] and delayed growth of myeloma xenografts.[111] Humanized Ab-based second generation of CARs targeting LeY coupled to the cytoplasmic domains of CD28 and the TCRz chain showed durable persistence.[112] An ongoing phase I clinical trial is now testing the safety and tolerability of using these CAR T cells in patients with advanced solid tumors presenting Ley surface expression (NCT03851146). A third generation of GD2-specific CAR-T cells has been developed and tested in a phase I clinical trial.[113] Built-in costimulatory domains such as CD28 and OX40 in T cells help to maintain the ability of cells to proliferate as well as to reduce T cell exhaustion and activation-induced cell death.[114] Other stimulatory molecules have been incorporated into GD2 CAR T cells, which enabled long-term persistence of the cells in human patients and led to improved clinical outcome.[115]

As an alternative to CAR T cells, BsAbs have shown great promise in anticancer therapy. BiAbs, similar to CAR T cells, do not require HLA for antigen presentation. The most common TACA-based BsAbs target GD2. BsAbs produced by conjugating anti-CD3 (OKT3) and anti-GD2 (3F8) antibodies recognize the tumor-associated ganglioside GD2, and the T cell receptor antigen CD3 can activate and redirect non-MHC-restricted cytotoxic activity.[116] More recent studies have substituted the 5F11-scFv with the higher-affinity hu3F8-scFv to form hu3F8-scBA. These BsAb-redirected T cells induced stronger T cell activation *in vitro* and more effectively suppressed NB xenograft growth *in vivo* compared with 5F11-scBA.[117] Bispecific anti-CD3 × anti-GD2 is tested in children with NBs and other GD2-expressing tumors (NCT02173093).

A direct comparison of GD2 BsAbs and CAR T cells found that BsAbs lead to longer survival of activated T cells. Furthermore, BsAb T cells provided more effective tumor protection in tumor models. The superiority of BsAb T cells could be partly attributed to the presence of $CD4^+$ helper T cells, while the infused CAR T cells were almost exclusively $CD8^+$ T cells. This incomplete benefit may reflect the difficulties of sustaining human T cell function and trafficking in a xenogeneic environment, which may represent the limitations of even the third-generation constructs that cannot completely recapitulate the temporo-spatial features of costimulatory events required to physiologically sustain T cell activation.[118]

Concluding Remarks

Advances in molecular targeted therapy and immune checkpoint inhibition have led to unprecedented improvement in overall survival of patients with cancer. Despite these improvements, there is an unmet need for novel approaches for next-generation, antitumor immune modulatory drugs. There is increasing evidence that altered glycans that are active players throughout cancer development and progression can be targeted for effective therapies.

While the results from the studies conducted at the Wistar Institute decades ago using carbohydrate-binding mAbs have not been recognized for their clinical potential, these early studies provided the conceptual framework for the current advances that will likely further progress in development of agents based on recognition of TACAs.

Currently, diverse and innovative approaches targeting cancer-associated glycans, such as mAbs, BsAbs, and glycan-specific CAR-T cells; carbohydrates and carbohydrate analog-based vaccines; adhesion antagonists; and small molecules with potential clinical application are tested. A structure-based design of mAbs with improved potency and selectivity for tumors over normal tissues may now be a tangible option, especially in systems where detailed, three-dimensional structural information is available, such as the Lewis histo-blood group and related TACAs. Such innovative strategies are likely to overcome many current limitations in the diagnosis and treatment of cancer patients. As discussed in this review, the strategies that exploit aberrant glycosylation of cancer cells can provide therapeutic options and act synergistically with current targeted approaches and immunotherapy approaches, improving their specificity and efficacy.

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