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REVIEW

CAR T cells redirected against tumor-specific antigen glycoforms: can low-sugar antigens guarantee a sweet success?

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Abstract **Immune-based therapies have experienced a pronounced breakthrough in the past decades as they acquired multiple US Food and Drug Administration (FDA) approvals for various indications. To date, six chimeric antigen receptor T cell (CAR-T) therapies have been permitted for the treatment of certain patients with relapsed/refractory hematologic malignancies. However, several clinical trials of solid tumor CAR-T therapies were prematurely terminated, or they reported life-threatening treatment-related damages to healthy tissues. The simultaneous expression of target antigens by healthy organs and tumor cells is partly responsible for such toxicities. Alongside targeting tumor-specific antigens, targeting the aberrantly glycosylated glycoforms of tumor-associated antigens can also minimize the off-tumor effects of CAR-T therapies. Tn, T, and sialyl-Tn antigens have been reported to be involved in tumor progression and metastasis, and their expression results from the dysregulation of a series of glycosyltransferases and the endoplasmic reticulum protein chaperone, Cosmc. Moreover, these glycoforms have been associated with various types of cancers, including prostate, breast, colon, gastric, and lung cancers. Here, we discuss how underglycosylated antigens emerge and then detail the latest advances in the development of CAR-T-based immunotherapies that target some of such antigens.**

Keywords cancer immunotherapy; chimeric antigen receptor; solid tumors; tumor-associated antigen; glycosylation; O-glycans; adoptive cell therapy

Introduction

Cancer immunotherapy can be presented as an example of how a journey that started with the "*magic bullet*" theory led to one of the most, if not the most, effective cancer treatment approaches [1]. Monoclonal antibodies (mAbs), antibody–drug conjugates (ADCs), T cell-redirecting bispecific antibodies (TRBAs), specific peptide enhanced affinity receptor (SPEAR®) T cells, chimeric antigen receptor (CAR) T cells (CAR-Ts), CAR natural killer cells (CAR-NKs), and CAR regulatory T cells highlight the high-level flexibility of immune-based anticancer treatment modalities. Genetic modification of T cells to express CARs specific for any given tumor-associated antigen (TAA) or tumor-specific antigen (TSA) can readily drive their cytotoxic capacity toward tumor cells of interest in a reliable fashion independent of major histocompatibility complex (MHC) [2].

As of today, four CD19-redirected and two B-cell maturation antigen-redirected CAR-T products have been granted the US Food and Drug Administration (FDA) approval based on their ability to induce high-rate disease remissions in certain patients with hematologic malignancies [2–7]. However, Yescarta™, Kymriah™, Tecartus™, Breyanzi™, Carvykti™, and Abecma™ have all been approved for the treatment of relapsed/refractory (R/R) blood-based malignancies [2–7]. In the context of solid tumors, the clinical and commercial success of CAR-T therapies have been rather bleak so far [1,8]. This issue has encouraged scientists to discover the underlying mechanisms and develop potential counterstrategies for their confrontation [2,8]. Such unwelcome clinical responses might be due to the substantial differences between solid tumors and hematologic cancers [2,9]. One

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of the main hurdles of solid tumor CAR-T therapy is the hostile tumor microenvironment (TME), which plays the role of an impenetrable obstacle toward CAR-Ts by restricting their access to the target tumor cells [2,9]. Other potential influencers might entail the type of conditioning regimens utilized before CAR-T administration, the delivery route of the adoptive cells, and even the components of the CAR construct [1,8,10]. Solid tumors are also evaders of immunosurveillance as they do so by the loss or downregulation of the target antigen recognized by a certain CAR molecule [2,9]. Moreover, unwanted adverse events that may arise from the poor cancer-specificity of the target antigens remarkably limit the applicability of CAR-T therapies in solid tumors [2,9].

Given that the discovery of favorable TSAs might be a time-consuming and difficult mission, scientists can alternatively focus on the identification of the aberrantly glycosylated forms of TAAs expressed by tumor cells (known as Tn, T, and sialyl-Tn antigens) [1]. Moreover, the development of mAbs that only target such cancer-associated glycoforms (known as cancer-specific mAbs (CasMabs)) might also be a turning point in CAR-T therapies because such CasMabs could be employed for the development of cancer-specific CAR-Ts (Cas-CAR-Ts) [1].

In this review, we briefly discuss the underlying mechanisms that give rise to cancer-specific antigen glycoforms and highlight how Cas-CAR-Ts might help in the success of solid tumor CAR-T therapies. Furthermore, we summarize the encouraging results of targeting aberrantly glycosylated antigen-expressing tumor cells with CAR-Ts, which can be a direction for scientists in this field.

CAR fundamentals

Anatomically, an FDA-approved CAR-T product expresses CARs that are composed of five components, namely, a targeting domain, a spacer region (also known as "hinge"), a transmembrane domain, a costimulatory domain, and an activation domain (Fig. 1A) [2,11]. The extracellular segment harbors the targeting domain that is often composed of a fully human, humanized, or murine single-chain variable fragment (scFv) [10]. Investigations have reported exhaustion of CAR-Ts induced by the antigen-independent aggregation of the scFv of CAR molecules [12–15]. In detail, the inherent tendency of CAR scFvs to self-aggregate leads to CAR-T exhaustion via the phosphorylation and signaling of the CD3ζ domain incorporated into the CAR construct [12–15]. Such spontaneous activation and consequent exhaustion restrict the tumoricidal efficacy of CAR-Ts; therefore, various tactics have been applied by researchers to overcome this issue [12,13]. For instance, Long *et al.* demonstrated that incorporating the 4-1BB costimulatory domain into CAR constructs reduces the level of CAR-T exhaustion induced by tonic signaling in comparison with the CD28 costimulatory domain, which increases the exhaustion level [13]. Such findings provide insights into how the choice of costimulatory domain can impact the tumoricidal efficacy of CAR-Ts and why particular CAR-Ts with different costimulatory domains are more persistent in clinical settings [13]. In 2021, Landoni *et al*. reported that particular residues within the framework regions of scFvs that contribute to their instability can be identified using *in silico* approaches [12]. Moreover, the investigators reported that substitution of the mentioned residues or scFv humanization culminated in the resolution of the tonic signaling issue without impinging on the binding specificity of the scFv and enhanced the tumoricidal functionality of the resultant CAR-Ts (regardless of CD28 or 4-1BB costimulatory domain) [12]. The abovementioned approaches alongside various other tactics, such as the introduction of disulfide bonds between the heavy chain (V_H) and light chain (V_L) of scFvs for their stabilization, can be applied as counterstrategies in this regard [12–14,16]. However, more broad investigations are required for the further elucidation of this matter. We have employed single-domain antibodies (also known as VHH), derived from camelid mAbs, in the construct of our CARs and reported encouraging results [2,10,17–24].

From the early days of CAR-Ts to date, CARs can be categorized into five distinct and yet overlapping generations. First-generation CARs are merely an scFv fused to a hydrophobic membrane-spanning region and a signaling segment composed of a truncated fragment from the T-cell receptor (TCR), namely CD3ζ [2,11,25]. This construct was not a potent inducer of T-cell expansion and persistence; thus, investigators positioned a costimulatory domain (mainly CD28 or 4-1BB) between the hydrophobic region and the main signaling domain only to develop second-generation CARs [11,25]. Third-generation CARs only harbored an auxiliary costimulatory domain compared with their predecessor; thus, third-, fourth-, and fifth-generation CARs can be viewed as renovated versions of second-generation CARs [2,25]. Fourth-generation CARs (also known as "armored CARs") carry a fragment that enables the expression of cytokines of interest upon activation, whereas fifth-generation CARs contain a tailored fragment of the intracellular domain of a cytokine receptor [11,25]. However, CARs exhibit a lower level of sensitivity toward tumor cells expressing their target antigen at moderate levels (about 100–200 CAR engagements are required per CAR-T to trigger cytolytic reactions) in comparison with endogenous T cells, whose cytolytic reactions are triggered following the establishment of productive immunological synapses with a restricted number of peptide-displaying MHCs (approximately

1–10 interactions per cell) [26–28]. This phenomenon has been evident as CAR-Ts fail to cytolyze tumor cells with moderate expression levels of target antigens (rather than overexpression) [29]. The importance behind the choice of the costimulatory signaling domain is accentuated here, as CAR-Ts harboring CD28 outperform 4-1BB-based CAR-Ts in eliminating tumor cells with moderate target antigen expression [30].

The general procedure for the development of CAR-Ts includes T-cell isolation, *in vitro* expansion, genetic modification to express the desired CAR, and CAR-T administration into the desired patients. Following adoptive transfer, CAR-Ts would have to find their target cells, interact with their surface antigen by means of the CAR, and ultimately enforce tumoricidal effects against the tumor cells (Fig. 1B).

Efficacy limitations in solid tumor CAR-T therapies

Why cannot CAR-Ts eliminate solid tumor cells? It almost takes about a thousand answers to satisfy this question. One is that, in many cases, CAR-Ts do not even reach their target cells, let alone enforce cytolytic reactions against them. Now, why do CAR-Ts hardly ever reach solid tumor cells? This is due to the physical and biochemical barriers that are forced upon CAR-Ts by the tumor-associated vasculature and the harsh characteristics of the TME. After infusion into the circulation, CAR-Ts would have to migrate toward the desired tumor sites and move past the tumor vessels into the enriched stroma. However, the blood vessels exhibit morphological transformations that lead to their anergy, because of the

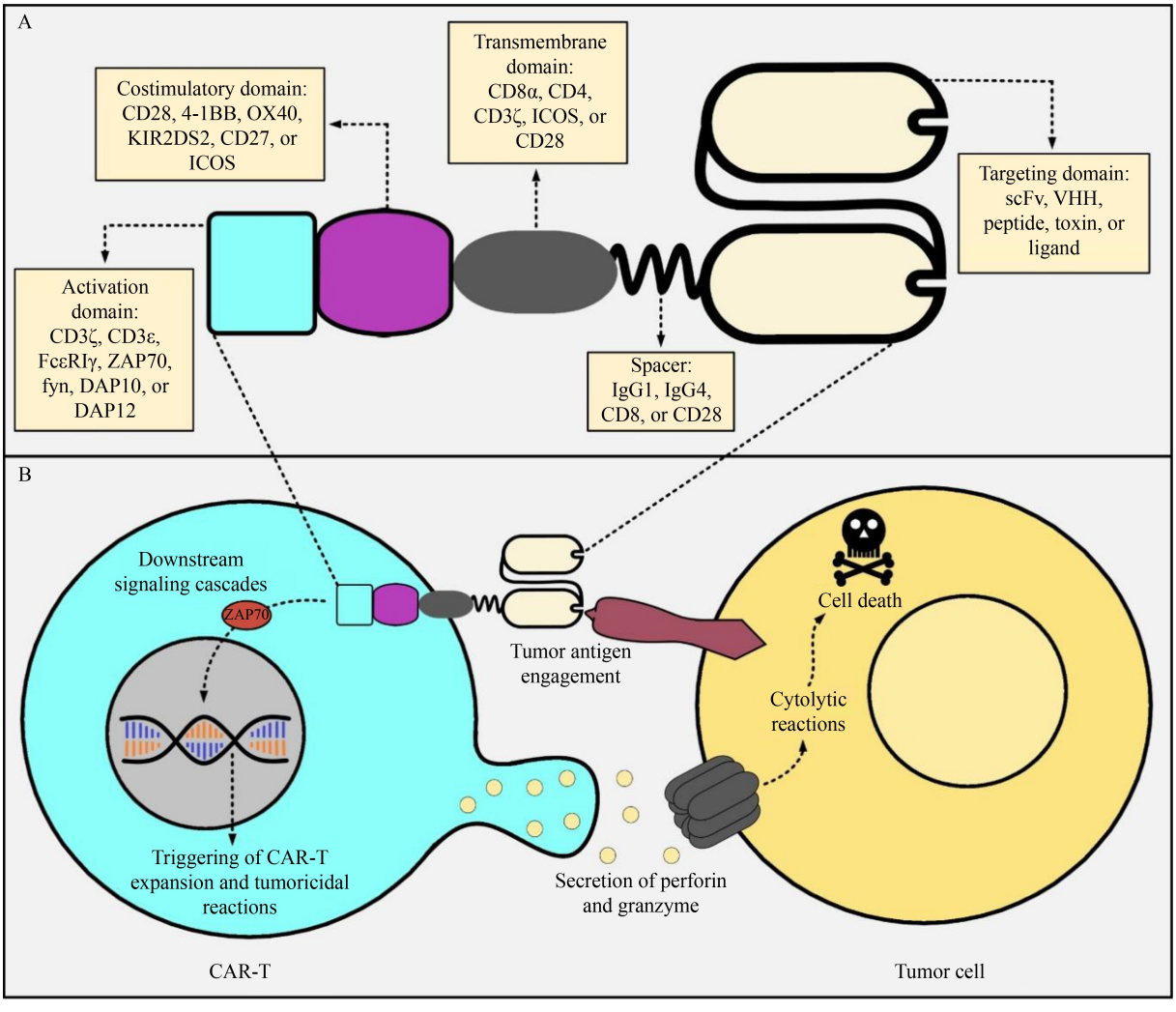

Fig. 1 Anatomy of a conventional CAR construct and action mechanism of a CAR-T. (A) Different components used in the construction of a second-generation CAR molecule. (B) The mechanism by which CAR-Ts enforce cytolytic reactions against the target tumor cells. Upon target antigen encounter, the downstream signaling cascades of the costimulatory domain(s) and the activation domain are triggered and result in the CAR-T-mediated production and secretion of granzyme and perforin. The downstream signaling of CD3ζ, 4-1BB, and CD28 is dependent on ZAP70, NF-κB, and PI3K, respectively. CAR-T, chimeric antigen receptor T-cell; ICOS, inducible T-cell costimulator; KIR2DS2, killer cell immunoglobulin-like receptor 2DS2; NF-κB, nuclear factor-κB; PI3K, phosphoinositide 3-kinase; ZAP70, the ζ-associated protein of 70 kDa.

angiogenic factors produced by tumor cells; therefore, they cannot function normally and permit T-cell extravasation [9,24,31]. Even if CAR-Ts tackle this restriction with the aid of blood vessel disruptors, they would have to survive the tumor stroma. In sheer contrast with hematologic cancers, solid tumor cells are concentrated in so-called "tumor islets" that are surrounded by the stroma. The stroma itself is composed of cancer-associated fibroblasts (CAFs) and tumor-associated macrophages (TAMs) [9,24,31]. On the one hand, CAFs are key players in this situation as they produce and secrete molecules that make up a hyperintricate mesh of the extracellular matrix (ECM) that somehow acts as the "gravel trap" of a car racing track by slowing the CAR-Ts down until they are stuck in it [9,24,31]. On the other hand, TAMs mediate the formation of protracted contacts with CAR-Ts until they are too exhausted to execute their antitumor mission [31]. Moreover, in regard to biochemical restrictions, oxygen inadequacy and the accumulation of waste biochemicals with toxic characteristics, which are secreted by rapidly growing malignant cells, also remarkably impinge on the effector function of CAR-Ts [9]. Furthermore, CAR-Ts might be exhausted and unable to establish productive CAR–antigen interactions with tumor cells by the time they access their target cells because of antigen downregulation [9,24]. CAR-Ts might also be incapable of engaging with the respective tumor surface antigens, which might come as a result of antigen loss or the expression of alternatively spliced antigens that cannot be recognized by the current CAR [9,24]. Recent findings showed that CAR-Ts themselves might contribute to the diminished tumor antigen expression through a process known as "trogocytosis" [9,24]. In this process, CAR-Ts take up tumor antigens from tumor cells, endocytose them, and display them on their surface [9,24]. Various solid tumors have been targeted by CAR-Ts, and several other clinical trials that will start in the years to come are set to recruit patients. However, most of these trials investigate the safety and tumoricidal capacity of CAR-Ts that target TAAs. In this regard, mild to life-threatening adverse events or unwanted side effects might be expected. One of the plainest and most practical strategies to potentially minimize such off-tumor effects would entail targeting the aberrantly glycosylated forms of TAAs. In the upcoming sections, we discuss the underlying mechanisms that lead to the expression of such glycoforms by tumor cells and then focus on the CAR-Ts that have been developed for targeting these glycoforms.

Cancer-specific antigen glycoforms

The aberrant glycosylation of proteins or lipids, such as those observed in several oncological indications (including breast and colon cancers), might offer tumor cells numerous benefits, including immunosurveillance evasion, augmented angiogenesis capacity, and amplified growth-related signaling cascades [32–36]. Such benefits provide a solid ground for tumor malignancy progression and the formation of distant metastatic lesions, and a high proportion of human proteins require glycosylation for proper 3D conformation and function; hence, aberrantly glycosylated glycoproteins could be considered hallmarks for cancer diagnosis and even qualified targets for the development of anticancer therapies [32–36]. In various malignancies, certain malignant cells exhibit tremendously abnormal glycosylation patterns that lead to the production and expression of Tn antigen (short for Thomsen-nouveau), T antigen, and/or sialyl-Tn antigen (Fig. 2) [36]. Tn, T, and sialyl-Tn antigens play various distinct roles in the promotion of tumor progression and metastasis [32–36]. For instance, T antigen promotes tumor metastasis by mediating the adhesion of malignant cells to the endothelium (which is achieved through interaction with galectin-3) [33,34]. Additionally, findings have suggested that sialyl-Tn antigen might offer metastatic cells a shield against the cytotoxicity of NKs [37]. Moreover, sialyl-Tn antigen is believed to play a role in negatively impacting the cell–cell aggregation of malignant cells in primary tumor lesions and therefore mediate the release of such tumor cells [38,39]. The expression of sialyl-Tn antigen by tumor cells has been associated with the onset of malignancy-associated characteristics, including augmented ECM adhesion and increased tumor cell migration and invasiveness [40]. Furthermore, macrophage galactose-type lectin expressed by macrophages and dendritic cells enforces immunosuppressive effects by impinging on the signaling cascades of TCRs and inducing T-cell apoptosis by interacting with the N-acetylgalactosamine (GalNAc) units of Tn antigens present in the CD45 protein of effector T cells [41,42]. Such effects may result in the escape of malignant cells from immunosurveillance [36].

In 1985, the first mAb against a Tn antigen was developed, and it was reported that a high percentage of primary breast carcinomas expressed the antigen [43]. In 1988, Kjeldsen and colleagues developed the first mAbs specific for a sialyl-Tn antigen and asserted that a combination of Tn- and sialyl-Tn-targeting mAbs might offer a great diagnostic and therapeutic relevance [44]. Such early studies opened a new window for exploiting underglycosylated antigens for diagnostic and therapeutic purposes. For example, the elevated expression of Tn and sialyl-Tn antigens in ovarian, breast, colon, and pancreatic cancer patients has been linked with poor prognosis [45–48]. Moreover, Akita and coworkers demonstrated that the different glycosylation patterns of MUC16 can be exploited for differentiating endometriosis and ovarian cancer, and they can also be utilized for the classification of the type and malignancy stage of the

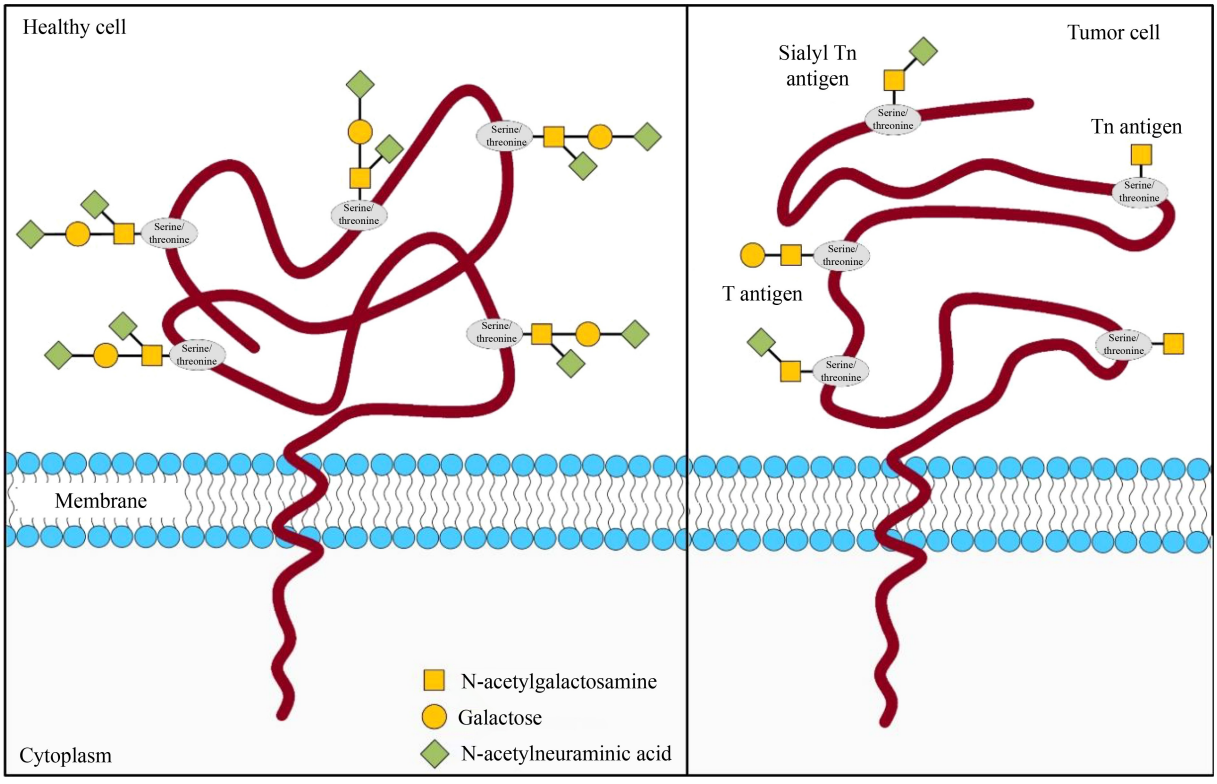

Fig. 2 Structural differences between properly glycosylated and aberrantly glycosylated forms of an antigen. The cancer-associated glycoform of the antigen exhibits a substantially different structural conformation compared with the one expressed on the surface of the normal cell. As "form is function", Tn, T, and sialyl-Tn antigens are expected to be functionally different from their normal counterparts. This altered 3D conformation might impinge on the stability and expression level of the antigen, as well as its turnover.

disease [49].

O-glycosylation is dependent on a series of glycosyltransferases, including polypeptide N-acetyl-α-galactosaminyltransferases (ppGalNAcTs), T-synthase (core 1 β1,3-galactosyltransferase), and sialyltransferase ST6GalNAc-I [36,50,51]. In detail, a GalNAc unit is transferred from a UDP-GalNAc to the serine/threonine residue(s) of a protein by N-acetyl-α-galactosaminyltransferases, which results in the formation of Tn antigen [36,50,51]. Further on, T antigen is biosynthesized as core 1 β1,3-galactosyltransferase transfers a galactose (Gal) unit from UDP-Gal to a Tn antigen [36,50,51]. In the absence of functional T-synthase, Tn antigen can also be modified to sialyl-Tn antigen as ST6GalNAc-I transfers an N-acetylneuraminic acid (Neu5Ac) unit (derived from CMP-Neu5Ac) to Tn antigen [36,50–52]. To this date, various underlying mechanisms have been linked to the emergence of Tn, T, and sialyl-Tn antigens. The most outstanding scenario entails substantial changes in the expression of the chaperone Cosmc and/or T-synthase (Fig. 3) [53]. Moreover, several factors, including mutations, epigenetic silencing, or attenuated signaling pathways, might contribute to the altered expression of Cosmc and/or T-synthase [53–55]. Other potential scenarios have also been proposed regarding the expression of such underglycosylated antigens, which include the mislocalization or impinged expression of ppGalNAcTs or their glycosylated substrates, the highly basic environment of the Golgi apparatus leading to the mislocalization of its glycosyltransferases, the abnormal expression of ST6GalNAc-I, the mislocalization of nucleotide-sugar transporters caused by certain mutations, and aberrations in the formation of the heterodimeric complexes of Golgi glycosyltransferases [56–60]. Regardless of what mechanism (or mechanisms) leads to the emergence and expression of Tn, T, and/or sialyl-Tn antigens, the potential applicability of such glycoforms in cancer diagnostics and therapy cannot be ignored. In the upcoming section, we will briefly summarize the tumoricidal capacity of CAR-Ts that target such antigens in different oncological indications.

Antigen examples

Tn, T, and sialyl-Tn antigens are exclusively expressed by tumor cells; hence, their targeting via CasMabs considerably diminishes the existing adverse events of mAb-based therapies that arise from the poor cancer-specificity of the targeted antigens. The development of such CasMabs might consequently accelerate the development of other mAb-based treatment modalities, such TRBA, ADC, CAR-T, and CAR-NK therapies. Fig. 4 represents how the Cas-CAR-T platform can maximize

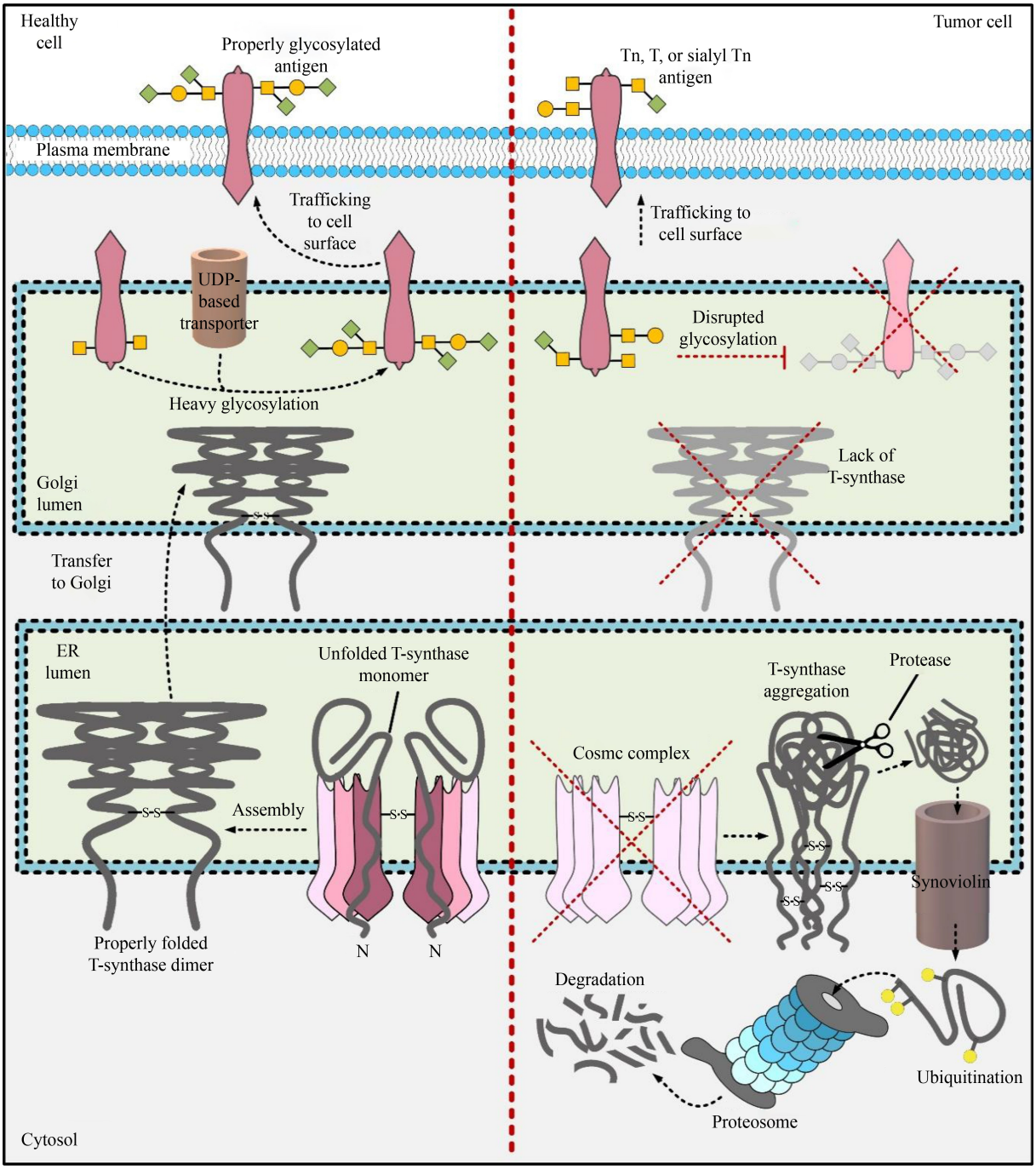

Fig. 3 Underlying mechanism of the emergence of Tn, T, and/or sialyl-Tn antigens. In normal situations (left panel), the Cosmc complex helps the unfolded T-synthase fold and assemble properly by serving as an endoplasmic reticulum chaperone. Once properly folded, the active T-synthase dimer exits the endoplasmic reticulum and enters the Golgi where it plays a critical role in the O-glycosylation of glycoproteins. In some tumor cells (right panel), somatic mutations or epigenetic gene silencing, such as those induced by hypoxia, can lead to Cosmc deficiency, which results in the aggregation of unfolded T-synthase, its proteolytic cleavage and retrotranslocation into the cytosol, and its polyubiquitination and consequent degradation. The loss of functional T-synthase, which might also be caused by some undeciphered mechanisms as detailed before, gives rise to the aberrant O-glycosylation of proteins and the surface expression of Tn, T, and sialyl-Tn antigens. In the case of sialyl-Tn antigen, a Neu5Ac may also be added to Tn antigen by ST6GalNAc-I, which results in the formation of sialyl-Tn antigen [36]. The aberrant expression of sialyl-Tn antigen is also speculated to be a result of the suppressive effects of ST6GalNAc-I on the functionality of T-synthase because of the high-level expression of ST6GalNAc-I [36]. ER, endoplasmic reticulum.

the tumoricidal capability of effector cells while abrogating their cytolytic reactions against healthy cells. In this section, we will briefly discuss some of the most outstanding antigens in this field against which Cas-CAR-

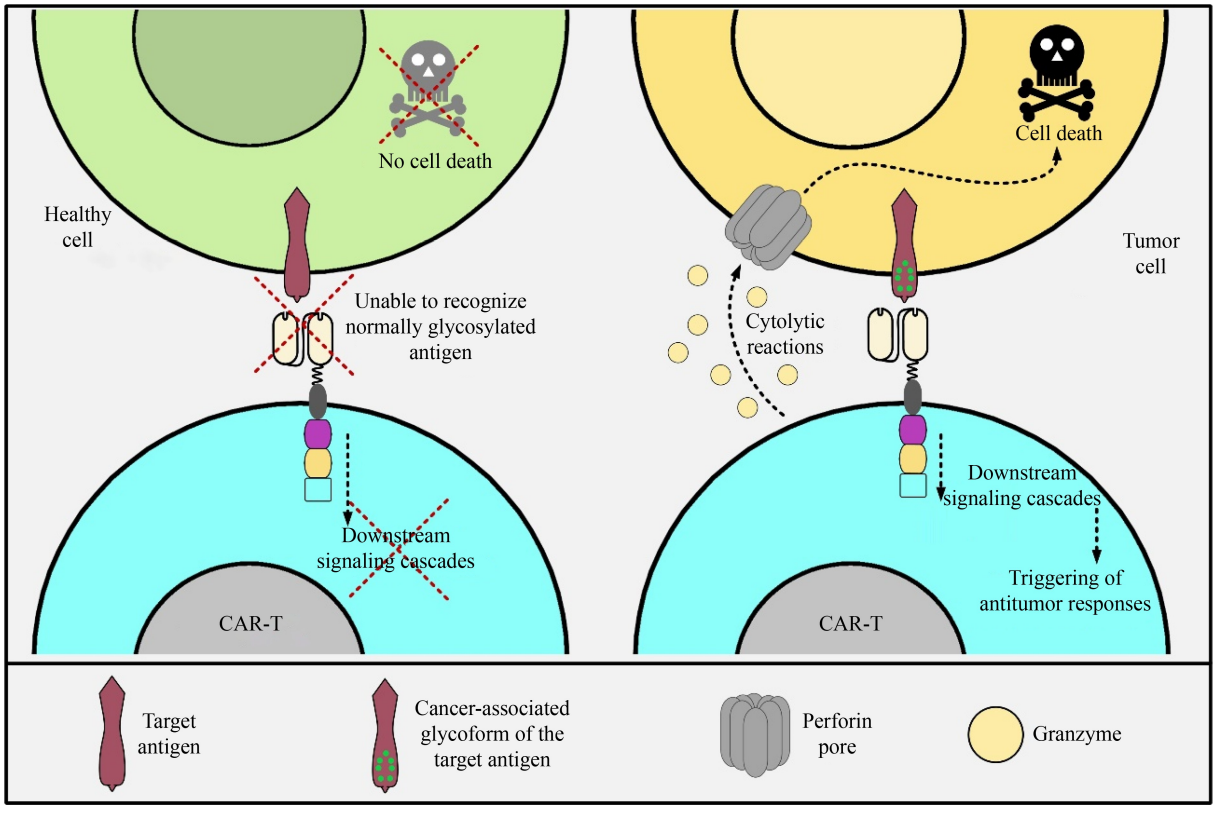

Fig. 4 Underlying mechanism of how incorporating a targeting domain that only binds the tumor-specific glycoform of an antigen into the CAR construct considerably diminishes the "on-target, off-tumor" toxicities of CAR-Ts. The CAR represented here only recognizes the Tn, T, or sialyl-Tn glycoform of the target antigen, which is restricted to tumor cells. Such CAR-Ts are unable to enforce tumoricidal reactions toward healthy cells expressing the properly glycosylated form of the antigen because they cannot engage with them. CAR-T, chimeric antigen receptor T cell.

Ts have been developed so far.

Dysadherin

In 2019, Steentoft and collaborators introduced a highly feasible platform for the development of desired CasMabs, which entails the application of genetically manipulated cancer cells that express cancer-specific antigen glycoforms [61]. Using this platform, these researchers developed a CasMab (named 6C5) against the Tn glycoform of dysadherin [61]. In detail, this technique is based on the application of different cancer cell lines of "SimpleCell" with pancreas, ovary, and breast origin whose *COSMC* gene is knocked out for the meticulous screening and isolation of potent high-affinity CasMabs [61]. Dysadherin (also called FXYD5) is a malignancy-associated cell membrane glycoprotein that plays critical roles in the formation of metastatic tumor lesions by disrupting cell adhesion through different mechanisms, including E-cadherin downregulation [62]. Dysadherin may play roles in the regulation of blood pressure, and its reduced expression is linked to hypertension [63]. The elevated expression of dysadherin has been linked to poor cancer prognosis, as well as the augmented aggressiveness and metastatic capacity of different malignancies (including colorectal carcinoma, testicular tumors, breast cancer, lung cancer, tongue cancer, melanoma, hepatocellular carcinoma, thyroid carcinoma, esophageal squamous cell carcinoma, gastric cancer, pancreatic ductal adenocarcinoma, and cervical squamous cell carcinoma) [61]. This phenomenon makes dysadherin an interesting target for the development of immune-based therapies. Despite the broad expression of the normally glycosylated form of dysadherin in healthy tissues, targeting its Tn glycoform using 6C5 tremendously minimizes the unfavorable side effects of dysadherin targeting because of the high tumor-specificity of the targeted epitope [61]. Even though CAR-Ts against the aberrant glycoform of this antigen have not yet been developed, incorporating 6C5 into the CAR construct of future CAR-Ts might result in therapeutically favorable outcomes in a broad range of solid tumors. Additionally, 6C5 could also be recombinantly linked to a CD3-specific moiety for the development of a dysadherin-specific TRBA that can itself be applied for cancer therapy as a monotherapy or in combination with CAR-Ts or even other types of anticancer treatment modalities as a part of combination

therapy.

CEACAM5

Carcinoembryonic antigen-related cell adhesion molecule 5 (also known as CEACAM5 or CD66e) has been recognized as an ideal immunotherapy target in numerous solid tumors, such as gastric, lung, pancreas, ovary, colon, and breast cancers [64]. CEACAM5 is a member of the gene family of carcinoembryonic antigen (CEA) and it plays roles in cell–cell contact, as well as in cell adhesion and migration [65,66]. Additionally, CEACAM5 prevents the occurrence of anoikis (which is a form of programmed cell death) [66]. In this regard, resistance to anoikis is an intrinsic feature of malignant cells; hence, this characteristic of CEACAM5 reveals its involvement in tumorigenesis and metastasis [66]. In 2010, Hawkins and colleagues conducted a dose-escalation phase I clinical trial (NCT01212887) to evaluate the tumoricidal efficacy and safety of CEACAM5-redirected first-generation CAR-Ts (in combination with aldesleukin, fludarabine, and cyclophosphamide) in 14 patients with advanced solid tumors [64]. This CAR-T product was equipped with an scFv (named MFE23) that was previously proven to be safe when applied as an antibody conjugate or imaging probe [64]. However, without therapeutically valuable clinical responses, this trial was prematurely terminated because of the poor persistence of the adoptively transferred effector cells and the emergence of life-threatening toxicities, including acute respiratory side effects (attributed to the expression of the targeted antigen by lung epithelial cells) and cytokine release syndrome (characterized by the augmented levels of serum IFN-γ and IL-6) [64]. The issue of poor persistence could be simply resolved by the development of second- or third-generation CAR-Ts; however, in such cases, the severity of the mentioned off-tumor toxicities could even be greater. MFE23 could be substituted with a CEACAM-specific CasMab, such as 5G2, to increase the safety index of CEACAM-redirected CAR-Ts. This mAb has been developed using tumor tissue-derived spheroids as an approach to overcome the limitations of mimicking tumor-specific glycosylation patterns *in vitro* using common cell lines [67]. Moreover, 5G2 specifically recognizes the aberrantly glycosylated form of CEACAM5, which is upregulated in colorectal cancer, and disrupts the adhesion of the mentioned spheroids to the ECM [67].

Podocalyxin

Podocalyxin is a heavily glycosylated membrane-spanning protein expressed by a variety of healthy cells, including podocytes, hematopoietic stem cells, and the endothelium of blood vessels [68]. Podocalyxin deficiency has been linked to a series of disorders, such as exomphalos and kidney failure, whereas its overexpression has often been correlated with augmented tumor malignancy, accelerated tumor cell proliferation, and amplified tumor motility capability in a broad spectrum of hard-to-treat cancers (including colorectal cancer, oral squamous cell carcinoma, liver cancer, astrocytic gliomas, and other epithelial cancers) [69–73]. A research group from Tohoku University recently generated two distinct mAbs against podocalyxin named "PcMab-60" and "PcMab-47" and set out to test their specificity on podocalyxin$^+$ cancer cell lines (of pancreas and brain origins) and healthy cells of the blood vessel endothelium [74,75]. PcMab-60 managed to only react with the podocalyxin on the surface of the cancer cell lines (hence a CasMab), whereas PcMab-47 recognized the podocalyxin expressed by all three cell types (hence, it is not a CasMab). The researchers further engineered PcMab-60 to augment its antitumor impact and reported encouraging findings after administering it into preclinical mouse models of pancreatic cancer [74]. Such *in vivo* outcomes could be the infrastructure for the development of CasMab-based treatment modalities, such as CAR-Ts, which could enforce tumoricidal reactions without negative impacts on podocalyxin$^+$ healthy tissues.

Podoplanin

Podoplanin is a highly glycosylated cell surface protein typically expressed by the endothelial cells of lymphatic vessels, osteocytes, and ependymal cells [76]. However, its elevated expression has been observed in germ cell tumors, malignant mesothelioma, angiosarcomas, and astrocytic tumors [76,77]. Besides its speculated roles in thrombogenesis, podoplanin is believed to be engaged in the elevation of tumor aggressiveness and motility, which lead to the formation of distant tumor clumps [78]. NZ-1 is a podoplanin-specific mAb (isolated from rat) that has shown considerable antitumor capacity against podoplanin$^+$ aggressive tumor cell lines of pleural cancer and has induced tumor regression in preclinical mouse models [79]. In 2016, Shiina and colleagues incorporated the scFv of NZ-1 into a third-generation CAR construct to generate podoplanin-redirected CAR-Ts and reported acceptable tumoricidal efficacy directed against the podoplanin-overexpressing cells of glioblastoma *in vitro* [77]. Additionally, the *in vivo* findings following the systemic administration of the mentioned CAR-Ts were also encouraging, as the CAR-Ts induced glioma tumor rejection in preclinical mouse models [77]. Moreover, Abe and co-researchers also tailored NZ-1 to develop potent human chimeric mAbs (named NZ-8 and NZ-12), which mediated pronounced tumoricidal responses in mesothelioma xenograft mouse models (even though in combination therapy with NKs) [79]. In 2019, He and collaborators developed podoplanin-redirected CAR-Ts

equipped with a murine anti-podoplanin CasMab (named 237Ab) that only recognizes the Tn glycoform of the antigen [80]. Surprisingly, the CAR-Ts also reacted with a spectrum of distinct tumor cell types that expressed different sets of Tn antigens [80]. All the antigens that engaged in immunological synapses with the targeting domains of the CAR-Ts were aberrantly glycosylated glycoforms restricted only to tumor cells; therefore, the presence of such antigens on the surface of a particular tumor cell considerably increases the possibility of the Cas-CAR-T-meditated elimination of that cell, which minimizes the risks of tumor relapse [80]. The researchers attributed this phenomenon to the slightly altered specificity of the scFv, which might be a result of its structural variation following its fusion to the hinge fragment of the CAR molecule [80]. Furthermore, the mentioned occurrence was also suggested to be a result of the participation of a population of CARs in the formation of immunological synapses with the targeted antigens (overcoming the hindrance of weak antigen engagement), which can lead to the amplification of the signaling cascades driving the cytolytic reactions of the effector cells [80]. Another well-known human podoplanin-specific CasMab is LpMab-2, which has considerable specificity toward tumor cells expressing the abnormally glycosylated form of podoplanin [81]. Even though the capacity of LpMab-2 as the targeting fragment of CAR-Ts has not yet been evaluated, such CAR-Ts might also be safe therapeutic options for the treatment of podoplanin$^+$ malignancies after careful preclinical and clinical assessments [81]. Additionally, T cells can also be engineered to co-express two distinct CAR constructs (one based on LpMab-2, and the other based on 237Ab) or simply express a bivalent CAR based on a bivalent scFv derived from the mentioned CasMabs [10]. Apart from these, two different populations of CAR-Ts (237Ab- and LpMab-2-based CAR-Ts) can also be utilized concurrently in animal models and clinical trials as a proposed pooled CAR-T therapy that might increase the rate of disease remission and overall survival [10].

MUC1

Mucin 1 (MUC1, or alternatively known as CA15-3 or Episialin) is a highly glycosylated membrane-bound glycoprotein normally expressed in the lung, pancreas, esophagus, small intestine, and prostate, as well as by hematopoietic stem cells [82]. Normally, MUC1 protects the epithelium from infectious, physical, and biochemical elements and therefore majorly contributes to cell survival [82]. The underglycosylated forms of MUC1 have consistently been linked to advanced oncological indications, including head and neck squamous cell carcinoma (HNSCC), pancreatic cancer, breast cancer, and various other adenocarcinomas [82–84]. This makes MUC1 one of the most suitable antigens for the development of targeted immunotherapies. In 2004, our research group generated MUC1-specific VHHs with a high affinity range (0.2×10^{-9}–0.6×10^{-9} mol/L) and then incorporated them into CAR constructs [85,86]. Later on, we developed the first VHH-based CAR-Ts against MUC1 and reported encouraging antitumor responses against the MUC1$^+$ cell lines T47D and MCF-7, which was also accompanied by elevated levels of IFN-γ, TNF-α, and IL-2 [21]. However, MUC1 is abundantly expressed by healthy tissues; thus, minimizing off-tumor toxicities requires the development of MUC1-specific CasMabs. In 2008, Wilkie and colleagues developed the first Cas-CAR-Ts against the tumor-related glycoform of MUC1 that harbored the HMFG2 scFv for the redirection of T-cell cytotoxicity against MUC1$^+$ cancer cells [87]. Alongside favorable *in vitro* outcomes, the study was expanded into the *in vivo* phase where a single round of CAR-T administration led to considerable tumor growth inhibition in preclinical mouse models [87]. According to the first case report regarding the treatment of a patient with seminal vesicle cancer with MUC1-redirected CAR-Ts, You and collaborators reported considerable tumor elimination following the localized administration of pSM3-based CAR-Ts into the metastatic tumor lesions without mediating adverse events [88]. Of note, pSM3 is an scFv specific for the sialyl-T glycoform of MUC1 and is a slightly modified version of SM3 with a stronger antigen binding capacity [88]. The V_L of SM3 and the V_H of HMFG2, which exhibits more than a 7-fold stronger binding capacity toward unglycosylated MUC1 compared with SM3, were tailored into a single construct to build the targeting domains of the CAR-Ts developed and evaluated by Mei and colleagues against HNSCC cell lines [83]. IL-22 could positively impact the expression level of the targeted antigen by tumor cells; thus, the investigators further engineered their CAR construct (developed a fourth-generation CAR) to enable it to secrete transgenic IL-22 following CAR-T activation [83]. The findings indicated promising outcomes, as the CAR-Ts enforced pronounced cytolytic reactions against their targets and sufficiently eliminated MUC1$^+$ cells [83]. According to a study by Zhou and colleagues, MUC1-redirected Cas-CAR-Ts might also be considered potential options for the treatment of patients with triple-negative breast cancer [89]. Their MUC1-redirected CAR-Ts harbored TAB004 (which binds the cancer-specific glycoform of MUC1) as the targeting domain, and these CAR-Ts secreted substantial amounts of IFN-γ and granzyme B upon engagement with the surface antigen of the corresponding cells and enforced tumor-specific cytolytic reactions [89]. Furthermore, a single round of the mentioned MUC1-redirected CAR-T administration (1×10^7 cells) into preclinical animal models mediated considerable tumor rejection without the onset of severe toxicities toward healthy tissues [89]. The therapeutic benefits of MUC1-redirected Cas-CAR-Ts have also been evident in xenograft mouse models of

pancreatic cancer and T-cell leukemia [90]. In detail, Posey and colleagues employed the scFv "5E5" for the construction of a CAR molecule that only recognizes the Tn glycoform of MUC1 [90]. Maher and collaborators conducted the first dose-escalation phase I clinical trial (NCT01818323) in this matter in 2015 with 30 patients with head and neck cancer [91]. This investigation is expected to be completed in April 2022 [91]. Fingers are crossed until then.

Aside from the application of HMFG2, TAB004, and 5E5 as the targeting domains of Cas-CAR-Ts, these mAbs have also been applied in various other fields. For instance, Berry *et al*. evaluated HMFG2 mAb in the immunohistochemical staining of the breast cancer, benign lesion, and normal tissue samples of human subjects [92]. These researchers reported that HMFG2 staining was mostly extracellular in the benign lesions and normal tissue samples but was intracellular in the malignant tissue samples [92]. Berry *et al*. also indicated that HMFG2 is not suitable for determining tumor differentiation degree or prognosis in breast cancer [92]. Years later, Athanassiou *et al*. demonstrated that HMGF2 can be used as a noninvasive tool for the precise detection of axillary lymph node metastases in patients with breast cancer [93]. Moreover, Bamias *et al*. reported that HMFG2 labeled with radioactive agents can be administered via the intravesical route to patients with bladder carcinomas [94]. According to the findings, tumors showed an increased uptake of this mAb compared with normal samples [94]. However, the researchers stated that there is no relationship between the uptake level of this mAb and the tumor grade [94].

In 2019, Bose and Mukherjee reported that TAB004 inhibits the growth of the human pancreatic ductal adenocarcinoma cell line, Capan2, in a dose-dependent fashion [95]. In detail, TAB004 triggers apoptosis in the mentioned cell line following internalization, and the combination therapy of TAB004 with certain chemotherapeutics increased the susceptibility of pancreatic ductal adenocarcinoma cells to conventional chemotherapy [95]. Additionally, Curry and colleagues investigated the potential of TAB004 for detecting circulating MUC1 and cancer stem cells in human and mouse subjects with pancreatic cancer [96]. According to the researchers, ~80% of cancer stem cells expressed MUC1 in patients with cancer as determined by TAB004 [96]. Moreover, circulating MUC1 was only identified in the sera of mouse tumor models established using the HPAF-II cell line (which highly expresses MUC1) [96]. Curry *et al*. also reported that TAB004 could be employed as a pancreatic cancer stage diagnostic tool because it was capable of precisely distinguishing the stage progression of the disease [96]. Additionally, in 2018, Wu *et al*. conjugated TAB004 to a fluorescent probe and evaluated its ability to detect pancreatic ductal adenocarcinoma in preclinical mouse models [97]. The researchers indicated that TAB004 is beneficial for the early detection of pancreatic cancer and is a potent antibody for the delivery of imaging probes to the TME of pancreatic cancer [97]. In another study, Moore *et al*. conjugated TAB004 to indocyanine green and set out to monitor tumor progression in mouse models [98]. The results of *in vivo* imaging indicated that the conjugate enabled early tumor detection following administration as compared with physical approaches [98]. Researchers were able to detect lung metastatic lesions via this approach without the detection of the healthy epithelium [98]. Additionally, in 2017, Roy and collaborators demonstrated that TAB004 could be beneficial for breast cancer screening in patients with dense breast tissues [99].

Lavrsen and colleagues investigated the potential of 5E5 for the development of tumor-specific immune-based therapies by evaluating its ability to induce antibody-dependent cellular cytotoxicity (ADCC) [100]. The researchers reported that this mAb induced ADCC in two particular breast cancer cell lines (T47D and MCF7), and indicated that antibodies that target aberrantly glycosylated mucins are solid options for developing precise immune-based therapies [100].

TAG72

Tumor-associated glycoprotein 72 (TAG72) is the truncated sialyl-Tn O-glycan hapten highly expressed in glycoproteins and mucins on the surface of multiple solid tumors, such as ovarian cancer, lung adenocarcinoma, and colorectal cancer [44,101–104]. TAG72 can be targeted using mAbs, such as B72.3 and CC49 [44,101]. In 1998, Hombach *et al*. generated CAR-Ts against TAG72 and reported that these cells demonstrated remarakble targeted cytotoxicity toward gastrointestinal tumor cell lines [101,105]. In 2017, Hege *et al*. reported the results of a phase I clinical trial that investigated the safety, tumor site migration, and immunogenicity of first-generation TAG72-redirected CAR-Ts delivered through direct hepatic artery administration to patients with metastatic colorectal cancer [106]. According to this report, a proportion of patients experienced reductions in their serum levels of CA125 and TAG72; however, these findings were not accompanied by substantial clinical responses most possibly because of the immunogenicity of the scFv incorporated into the targeting domain of these CAR-Ts and the poor persistence of the CAR-Ts due to insufficient effector cell costimulation signaling [106].

In 2018, Murad *et al*. generated humanized second-generation TAG72-redirected CAR-Ts and reported that these cells demonstrated target antigen-specific antitumor

activity and cytokine secretion toward TAG72-expressing ovarian cancer cell lines and ascites derived from patients with ovarian cancer according to *in vitro* assessments [107]. Moreover, the regional intraperitoneal delivery of these CAR-Ts in preclinical xenograft models of peritoneal ovarian cancer resulted in meaningful suppressed tumor progression and prolonged overall survival [107]. The researchers also indicated that these results were even more enhanced when CAR-T administration was repeated sequentially [107]. In comparison, the level of TAG72 expression declined in cases of disease relapse and was associated with poor CAR-T persistence *in vivo* [107]. Conclusively, such data might pave the way for further clinical investigations in this field.

Other antigens

Alongside the mentioned antigens, several other antigens also undergo deregulated glycosylation in tumor cells against which CasMabs have been developed (Table 1). Reis *et al*. developed a mouse mAb (named PMH1) against the Tn glycoform of MUC2 by immunizing experimental animals with a peptide based on the tandem repeat of the intestinal MUC2 (amino acid sequence of the GalNAc glycosylated peptide: PTTTPISTTTMVTP-TPTPTC) [108]. According to the results of Western blot analysis, the investigators reported that PMH1 also reacted with colonic mucin, which led to the conclusion that this mAb may be reactive toward some MUC2 glycoforms [108]. According to another investigation, Pedersen and collaborators developed a large library of aberrantly glycosylated tumor-specific human MUC1-2, MUC4, MUC5AC, and MUC6-7 and reported that the sera of patients with colorectal cancer contain autoantibodies reactive to aberrantly glycosylated MUC1 and MUC4 peptides [109]. In particular, mAb 6E3 specifically recognized the Tn glycoform of recombinant MUC4, whereas no reactivity to the unglycosylated fusion protein of MUC4 or Tn glycoforms of other unrelated peptides was reported [109]. Moreover, Pedersen *et al*. demonstrated that screening the sera of patients with cancer might culminate in the identification of qualified autoantibodies against the tumor-specific glycoforms of glycopeptides, which could be suitable for therapeutic and/or diagnostic purposes [109]. In 1994, Tassone and colleagues developed a mouse mAb named UN1 and reported that this mAb specifically bound an unknown ~120 kDa transmembrane protein surface-expressed by thymocytes and a particular subtype of peripheral blood lymphocytes [110]. In 2011, de Laurentiis and co-investigators employed mass spectrometry along with other confirmatory experiments to identify the specific antigen recognized by UN1 [111]. CD43 was identified as the antigen, and UN1 specifically recognized an epitope that is glycosylated with a GalNAc as elucidated using glycosidase digestion; therefore, the Tn glycoform of CD43 was reported as the antigen recognized by CasMab UN1 [111]. Of note, CD43 plays roles in cell adhesion, programmed cell death, and differentiation [111]. Moreover, the Tn glycoform of CD43 was reported to be present in patients with certain carcinomas (including breast and colon cancers) and absent from the healthy tissues of the same individuals; hence, de Laurentiis *et al*. further corroborated the tumor association of the CD43 epitope recognized by UN1 [111]. According to another study, Matsuura and Hakomori reported the development of an IgG1 CasMab called "FDC-6," which only recognizes a fibronectin domain specific to liver and colon cancers, amniotic fluid, and fetal connective tissues and is unable to react with plasma and healthy adult tissue fibronectin [112]. Moreover, the investigators reported that oncogenic transformation is correlated with the presence of the FDC-6-recognized domain in fibronectin, whereas the development of fetal fibronectin to its adult form coincides with the loss of the mentioned domain as demonstrated by the CasMab [112].

Conclusions

Immune-based therapies have proven to be one of the most prosperous choices for the treatment of a wide spectrum of immunological and oncological indications. CAR-T therapies have entered the market for the treatment of certain leukemias and lymphomas. However, CAR-T therapies have not yet achieved applaudable success in the fight against solid tumors because of their inefficiency and nonnegligible toxicities toward the vital organs of recipients. In the past decade, scientists assiduously tried to discover TSAs that could be targeted to expand the success zone of CAR-T therapies in the context of solid tumors. In recent years, several research groups alternatively leveraged aberrantly glycosylated antigens for the development of safer CAR-Ts and reported outcomes that might come as clinically favorable. Moreover, targeting the Tn, T, and sialyl-Tn glycoforms of non-TSAs via Cas-CAR-Ts holds considerable therapeutic promise; hence, it might highlight the importance of discovering more aberrantly glycosylated glycoforms of common TAAs. Substantial differences in the glycosylation pattern of cancer-associated antigen glycoforms and the practical caveats in the characterization of CasMabs act as the main limitations that have hindered the generation and broader application of such mAbs [61]. Additionally, scientists can focus on the biochemical characteristics of tumor cells and the cancer-specific glycosylation patterns of currently known TAAs. CasMabs that are generated against such antigens can be multidimensionally applied for the treatment of solid tumors, whether in the form of

Table 1 Summary of various CasMabs specific for tumor-specific antigen glycoforms

Monoclonal antibody name	Target (notes)	Involved indications	Reference(s)
6C5	Tn glycoform of dysadherin	Various solid tumors	[61]
5G2	CEACAM5 and CEACAM6	Various solid tumors	[67]
PcMab-60	Podocalyxin (tumor-specific form)	Epithelial cancers and astrocytic gliomas	[74]
237Ab	Tn glycoform of podoplanin	Astrocytic tumors, malignant mesothelioma, and germ cell tumors	[113,114]
LpMab-2	Podoplanin (with aberrant O-glycosylation or sialylation)		[81]
HMFG2	MUC1 (glycosylation dependent)	HNSCC, pancreatic cancer, breast cancer, and various other adenocarcinomas	[87,92]
SM3	MUC1 (glycosylation dependent)		[115]
pSM3	Sialyl-T of MUC1		[88]
TAB004	The tumor form of MUC1 (tMUC1)		[89,99]
PankoMAb	Tn glycoform of MUC1		[116]
2D9	Tn glycoform of MUC1		[117]
MY.1E12	Sialylated MUC1		[118,119]
VU-2-G7	Tn glycoform of MUC1		[120]
1B9	T glycoform of MUC1		[117]
5E5	Tn glycoform of MUC1		[121]
PMH1	Tn glycoform of MUC2	Mucinous adenocarcinoma	[108]
6E3	Tn glycoform of MUC4	Epithelial carcinomas	[109]
UN1	Tn glycoform of CD43	Breast cancer, colon cancer, and T-cell leukemia	[110,111]
FDC-6	Tn glycoform of fibronectin	Colon and liver cancers	[112]
B72.3	Sialyl-Tn epitope (expressed on glycoproteins and mucins)	Ovarian cancer, lung adenocarcinoma, and colorectal cancer	[44,101]
CC49	Sialyl-Tn epitope (expressed on glycoproteins and mucins)		[122]

CAR-Ts, immunotoxins, TRBAs, or even naked mAbs, to produce more improvement. Moreover, tumor cells will do whatever it takes to be invisible to the eyes of the immune system. In this regard, the combination of CAR-T therapies with other conventional or innovative treatment methods, including surgery, radiotherapy, chemotherapy, immune checkpoint inhibitors, mAbs, and anticancer vaccines, might come as the future face of a successful solid tumor CAR-T therapy. Additionally, numerous parameters might need to be taken into consideration from case to case only to devise a highly personalized treatment suitable for a given patient's condition because of the complex nature of solid tumors. Such parameters include the patient's disease burden, tumor malignancy rate and stage, age, sex, and history of prior treatment (the pressure of a prior treatment has sometimes been correlated with the emergence of resistance against that particular type of treatment) [123]. As discussed in this review, most of the developed Cas-CAR-Ts are still under experimental or early clinical investigations; therefore, how successful each CAR-T product is in a particular oncological indication or how suitable their target antigens are will be determined in the upcoming years as more comprehensive clinical investigations are conducted. In conclusion, an ideal treatment modality requires to be precise and effective because of the aggressiveness of solid tumors.

Compliance with ethics guidelines

Pooria Safarzadeh Kozani, Pouya Safarzadeh Kozani, and Fatemeh Rahbarizadeh declare that they have no conflict of interest. This manuscript is a review article and does not involve a research protocol that requires the approval of the relevant institutional review board or ethics committee.

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