Glycobiology, 2018, vol. 28, no. 9, 656–669
doi: 10.1093/glycob/cwy008
Advance Access Publication Date: 17 February 2018
Review

Review

Glycan-directed CAR-T cells

Catharina Steentoft[2], Denis Migliorini[3], Tiffany R King[3], Ulla Mandel[2], Carl H June[3], and Avery D Posey Jr.[1,3]

[2]Copenhagen Center for Glycomics, Departments of Cellular and Molecular Medicine and Odontology, Faculty of Health Sciences, University of Copenhagen, Copenhagen, Denmark and [3]Center of Cellular Immunotherapies, Abramson Cancer Center and the Department of Pathology and Laboratory Medicine, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA 19104, USA

[1]To whom correspondence should be addressed: Tel: +1 215-573-4174; Fax: +1 215-573-8590; e-mail: aposey@pennmedicine.upenn.edu

Received 6 November 2017; Revised 28 December 2017; Editorial decision 19 January 2018; Accepted 20 January 2018

Abstract

Cancer immunotherapy is rapidly advancing in the treatment of a variety of hematopoietic cancers, including pediatric acute lymphoblastic leukemia and diffuse large B cell lymphoma, with chimeric antigen receptor (CAR)-T cells. CARs are genetically encoded artificial T cell receptors that combine the antigen specificity of an antibody with the machinery of T cell activation. However, implementation of CAR technology in the treatment of solid tumors has been progressing much slower. Solid tumors are characterized by a number of challenges that need to be overcome, including cellular heterogeneity, immunosuppressive tumor microenvironment (TME), and, in particular, few known cancer-specific targets. Post-translational modifications that differentially occur in malignant cells generate valid cell surface, cancer-specific targets for CAR-T cells. We previously demonstrated that CAR-T cells targeting an aberrant O-glycosylation of MUC1, a common cancer marker associated with changes in cell adhesion, tumor growth and poor prognosis, could control malignant growth in mouse models. Here, we discuss the field of glycan-directed CAR-T cells and review the different classes of antibodies specific for glycan-targeting, including the generation of high affinity O-glycopeptide antibodies. Finally, we discuss historic and recently investigated glycan targets for CAR-T cells and provide our perspective on how targeting the tumor glycoproteome and/or glycome will improve CAR-T immunotherapy.

Key words: CAR-T cell therapy, glycome, solid tumors

Engineering an immune attack on cancer

One hallmark of cancer is the common trait of avoiding immune destruction (Hanahan and Weinberg 2011). Cancer patients are no longer able to naturally mount sufficient immune responses to tumor-associated antigens due to a multifactorial process of immune dysregulation, including upregulation of immune checkpoint molecules, loss of major histocompatibility complex (MHC) molecules and tumor-promoting inflammation.

Several interventions that promote immune proliferation and immune stimulation have demonstrated potent anti-tumor activity. Earliest of these, the delivery of high-dose interleukin-2 (IL-2), induced in vivo proliferation of polyclonal T cells and regression of metastatic melanoma and renal cancer in ~8% of patients who had failed standard therapies (Rosenberg et al. 1998). Tumor-infiltrating lymphocytes (TILs) from tumors with an elevated mutational load, like melanoma, demonstrate tumor-lysing activity and can induce durable tumor remission in patients when expanded to large quantities ex vivo and re-infused (Rosenberg et al. 2011). More recently, blocking of checkpoint molecules CTLA-4 and PD-1 (or its ligand PD-L1) reverses tumor-induced immune suppression and has led to successful remissions in patients with melanoma, lung and renal cancers, among others (Hodi et al. 2010; Topalian et al. 2012).

However, these immune interventions all rely upon prior existence of tumor-reactive, antigen-specific immune cells, and are only effective as yet in particular tumor histotypes; as such, many patients will not benefit from these therapies.

Synthetically engineering antigen-specificity into autologous or allogeneic T cells can overcome the lack of existing tumor-reactive patient T cells. Two approaches that introduce (usually through viral integration) antigen-specificity into non-reactive T cells are transgenic T cell receptors (TCRs) and chimeric antigen receptors (CARs). Introduction of a transgenic TCR involves genome integration of the Vα and Vβ chains of a previously identified TCR into polyclonal T cells, providing each cell with dual specificity, including clonality of the introduced TCR. Transgenic TCRs direct patient T cells to common mutated peptides, such as KRAS G12D and G12V mutations, which are present in the vast majority of pancreatic ductal adenocarcinomas (PDACs) (Waddell et al. 2015; Wang et al. 2016). However, transgenic TCRs hold many limitations, including restriction to MHC presentation and thus applicability to HLA-selected patients. TCRs require CD8 or CD4 co-receptors for MHC Class I and Class II engagement, respectively, and are thus only applicable in either a cytotoxic or helper T cell, but not both. The Vα and Vβ chains of the transgenic TCRs can mispair with the endogenous TCR (Shao et al. 2010), an obstacle that can now be overcome through CRISPR/Cas9 and other tools to genetically edit the endogenous TCR (Osborn et al. 2016). Lastly, tumors frequently downregulate the expression of the MHC complex on the cell surface, which prevents TCR engagement and limits additional transgenic TCR strategies after tumor escape (Tran et al. 2016).

As an alternative to the TCR, the CAR is an artificial TCR that combines antibody-driven specificity with the activation machinery of the TCR complex to deliver antigen-specificity in a MHC-independent manner (Eshhar 1997). Structurally, CARs are hybrids of antibodies and cell surface receptors (Figure 1). The ectodomain consists of the variable domains of an antibody in a single-chain variable fragment (scFv) format. CARs are stabilized on the T cell surface through the incorporation of a transmembrane domain (usually type I) and employ the intracellular domain of the immunoreceptor tyrosine based activation motif (ITAM)-containing CD3ζ molecule required for TCR-based T cell activation. Second and third-generation adaptations of CAR molecules include the intracellular domains of T cell costimulatory molecules, such as CD28 and/or 4-1BB, which aid in enhancing CAR-T cell effector functions and survival. Unlike TCRs, which can target intracellular cancer-specific mutations, CARs are limited to targeting cell surface antigens. However, since the antigen specificity of CARs is driven through antibody-ligation of targets, CARs do not require CD4 or CD8 co-receptors, can drive antigen-specific stimulation and anti-tumor activity in both helper and cytotoxic T cells, and are unrestricted by HLA. Therefore, one CAR developed against a specific antigen or for a particular cancer histotype can theoretically treat all patients with that antigen expression or cancer histotype, assuming that the CAR targets a broadly expressed, cancer-specific, indispensable cell surface marker. As this review will discuss, that assumption is not trivial for the field.

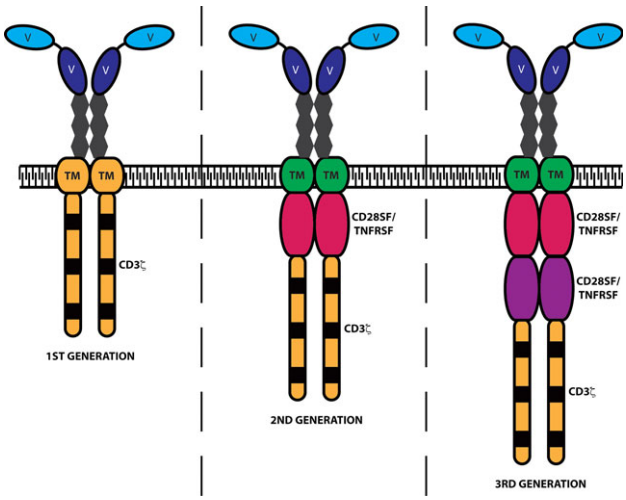

Fig. 1. CAR design by generation. First-generation CARs contain an scFv (blue and light blue), flexible spacer domain (gray), transmembrane and intracellular signaling domains of CD3ζ (gold), including the three ITAM domains of CD3ζ (black). Second-generation CARs utilize transmembrane domains of other T cell surface molecules (CD8a, CD28, etc.; green) and costimulatory domains from CD28 and TNFR superfamilies (magenta). Third-generation CARs utilize more than one costimulatory domain in cis (purple).

CD19 CAR: the gold standard

The aim of any therapeutic development should be a large index between therapeutic benefit and potential for harm. For cancer therapies, a large therapeutic index is achieved through either cancer-specific targeting that spares normal tissues or through eradication of cancer along with minimal impact on normal cell populations. For B cell malignancies, the latter strategy is employed by immune therapies, such as rituximab (anti-CD20) and anti-CD19 CAR-T cells.

B cell malignancies, such as acute lymphoblastic leukemia (ALL), chronic lymphocytic leukemia (CLL) and diffuse large B cell lymphoma (DLBCL), retain many common B cell markers, such as CD19, CD20, CD21 and CD22. CAR-T cells for B cell malignancies first targeted CD19 as CD19 is expressed from the early pro-B cell stage through at least the mature B cell stage (with some reports of expression in early plasmablasts (Arce et al. 2004)), while CD20, CD21 and CD22 have more restricted expressions in the B cell lineage.

The success

Preclinical in vitro and in vivo xenograft assessment of anti-CD19 CAR-T cells demonstrated anti-tumor efficacy and CAR-T cell survival and persistence with certain second-generation CAR molecules, which provided rationale for Phase I clinical trials. In three relapsed/refractory (r/r) CLL patients that had failed other therapies, anti-CD19 CAR-T cells (originally CTL019, now tisagenlecleucel – Kymriah™) demonstrated dramatic results – complete remissions in two patients and a partial response in the third within a month after infusion of the modified T cells. Subsequent clinical trials at multiple institutions treating pediatric and adult ALL patients with anti-CD19 CAR-T cells have been even more striking, with up to 94% of patients achieving a complete remission. After a multicenter global registration Phase II trial for r/r pediatric and young adult ALL (ELIANA), demonstrated 82% complete remissions within three months of treatment, the US Food and Drug Administration's (FDA) Oncologic Drugs Advisory Committee voted unanimously to recommend tisagenlecleucel for approval. On 30 August 2017, the FDA approved tisagenlecleucel as the first CAR-T cell therapy and the first form of therapy involving gene transfer in the US. Of note, blinatumomab is another CD19-directed immune therapy that utilizes bi-specific antibody technology to target CD19

and engage T cells without genetic modification (Newman and Benani 2016). Blinatumomab is FDA approved and a Phase III trial (TOWER; NCT02013167) of blinatumomab versus chemotherapy demonstrated a significantly longer overall survival (7.7 mo vs. 4.0 mo) and an increase in complete remission with full hematologic recover (34% vs. 16%) (Kantarjian et al. 2017).

The side effects

Treatment with anti-CD19 CAR-T cells, when successful, induces a deep molecular remission and aplasia of B cells (Porter et al. 2011). Patients are treated with intravenous immunoglobulin (IVIG) in the absence of a B cell repertoire; however, long-lived plasma cells and humoral immunity remain in these patients, suggesting IVIG may not be required (Bhoj et al. 2016). Other obstacles and some disappointments have accompanied the success of tisagenlecleucel and other anti-CD19 CAR-T cell therapies. The first is the identification of a therapy-induced cytokine release syndrome (CRS), characterized by an intense fever and high serum concentrations of pro-inflammatory cytokines, including a spike in serum IL-6 and IFN-γ. The initial CRS observation was made in the first pediatric ALL patient infused with tisagenlecleucel, who was subsequently treated with the anti-IL6R antagonistic antibody tocilizumab (Grupp et al. 2013). Anti-IL6R blockage is now recommended for management of CAR-T cell-induced CRS and tocilizumab gained expanded FDA approval for CRS on 30 August 2017, coinciding with tisagenlecleucel approval. The severity of CRS is positively correlated with patient tumor burden (reviewed in Frey and Porter (2016)).

The second obstacle presented in CAR-T cell therapy is tumor relapse. Approximately 10–20% of anti-CD19 CAR-treated ALL patients experience relapses after complete remission, either due to expansion of a CD19-negative leukemia or loss of CAR-T cell persistence. Genetic analysis of CD19-negative leukemias from CAR-T cell treated patients revealed the outgrowth of CD19 splice variants that exclude the epitope for which the CAR's antibody is specific (Sotillo et al. 2015); these isoforms of CD19 exist in leukemic blasts at the time of diagnosis (Fischer et al. 2017). Combinatorial CAR targeting strategies, such as dual CARs targeting both CD19 and CD20 or CD22 (NCT03241940), are currently under development and in clinical trials in order to reduce tumor escape. Loss of CAR-T cell persistence is less understood; however, some suggested mechanisms for depletion of the engineered T cells are humoral responses against the CAR scFv, phagocytosis of the T cells due to Fc binding to incorporated portions of IgG1 or IgG4 constant domains of some CAR ectodomains, or inherent features of the intracellular costimulatory signaling domains (Beatty et al. 2014; Hudecek et al. 2015; Long et al. 2015).

The third obstacle of anti-CD19 CAR therapies are neurological and cerebral toxicities, including transient confusion and aphasia, and more gravely, cerebral edema and death. One anti-CD19 CAR, which is no longer under clinical investigation, JCAR015, reported four cases of severe neurotoxicities, which included cerebral edema (Gust et al. 2017). Patients with evidence of endothelial activation prior to lymphodepletion demonstrated increased risk for severe neurotoxicity. One patient experienced fatal cerebral edema after treatment with axicabtagene ciloleucel (Yescarta^{TM}), the second FDA-approved CAR-T therapy (Locke et al. 2017). There are several unknowns related to these neurological and cerebral side effects of anti-CD19 CAR-T cells; dissecting the differences in effector functions of each CAR molecule as well as any potential roles that preconditioning regimens may play in increasing toxicities need to be investigated. Although not directly compared, CD19 CAR-T with CD28 signaling domains appear to elicit more neurotoxicity than CAR-T with 4-1BB domains.

Other CAR-T therapies for hematopoietic cancers

CD19 represents a gold standard in CAR-T therapy, but it is not the only antigen targeted. Other CAR-T therapies for hematopoietic malignancies include targeting of CD22 in B cell malignancies, either as a single agent (Haso et al. 2013), or in combination with CD19 to prevent antigen escape, as mentioned above. Another preclinical model of dual CAR-T targeting to prevent antigen escape demonstrated xenograft control of CD19^{-} relapse by targeting CD19 and CD123, the α-subunit of the interleukin-3 receptor (Ruella et al. 2016). However, CD123 expression on various hematopoietic progenitor and endothelial cells make it a potential toxicity concern for targeting with CAR-T cells. Strategies to fine-tune the affinity of CARs targeting antigens expressed on normal tissues, including CD123, in order to prevent therapy-induced toxicity are currently in preclinical development.

Another developmentally restricted marker targeted by CAR-T cells is B-cell maturation antigen (BCMA), a marker expressed on some B cells, normal plasma cells, and multiple myeloma. In a first-in-human clinical trial with anti-BCMA CAR-T cells, two patients achieved very good partial responses and one patient achieved a complete remission, with all three patients demonstrating substantial decreases in serum BCMA (Ali et al. 2016). Other CAR-T targets for multiple myeloma include CS1, which is also expressed at low levels on natural killer cells, normal B cells, and some T cell subsets (Chu et al. 2014); kappa-light chain (Ramos et al. 2016); and CD138 (Tian et al. 2017).

Steering cars to solid tumors

Translation of CAR-T cell therapies from hematologic malignancies into solid tumors comes with challenges. For one, T cells share lymphoid-rich milieu with B cell malignancies, including spleen, bone marrow, lymph nodes and peripheral blood vessels, that are not shared with malignancies in solid organs. This co-localization allows for interaction and exertion of anti-tumor effects in the absence of enhanced CAR-T cell homing mechanisms. By contrast, T cell enhancements are likely necessary to surmount the solid tumor microenvironment, which can limit T cell immune responses through a variety of cellular and physical immunosuppressive measures (Figure 2). In solid tumors, homing and resilience in anti-inflammatory environments is of concern for adoptive immunotherapies. CAR-T cells modified to ectopically express chemokine receptors have demonstrated enhanced migration properties (Moon et al. 2011), which may mediate improved solid tumor targeting. Remediation of tumor-induced exhaustion can be achieved through combination of CAR-T cells with checkpoint blockade (e.g., anti-PD1), which can modulate the tumor microenvironment, including decrease infiltrating MDSCs, and restore CAR-T effector functions in vivo (John et al. 2013; Moon et al. 2014; Cherkassky et al. 2016).

In addition to overcoming the tumor microenvironment, the first barrier for CAR-T cells in solid tumors is antigen targeting. Lethal and severe toxicities have been observed through targeting of solid tumor antigens by CAR and TCR-engineered T cells, which distinguishes CAR-T targeting from that of therapeutic antibodies or from the targeting of CAR-T cells against the dispensable B cell compartment. For example, trastuzumab-based (anti-HER2) CAR-T

therapy caused lethal cardiopulmonary toxicity in a patient with metastatic colon cancer that was attributed to low-level antigen expression in normal lung tissues (Morgan et al. 2010). Cardiotoxicity is observed in cancer patients treated with trastuzumab, but instances of toxicity are related to long-term usage (Soo Park et al. 2017); in the case of the trastuzumab-CAR-T cells, lethality occurred within five days post treatment and was most likely due to CRS (Morgan et al. 2010). Another CAR-T therapy targeting carbonic anhydrase IX caused Grade 4 liver toxicity in patients with metastatic renal cell carcinoma due to antigen expression on epithelial cells lining the bile ducts (Lamers et al. 2006). These examples illustrate the necessity of defining tumor-specific antigens, which are distinct from tumor-associated antigens that share normal tissue expression, in order to maintain safety while also generating anti-tumor efficacy for CAR-T cell therapy in solid tumors. We propose that this barrier can be overcome through the targeting of tumor-associated epitopes arising through aberrant glycosylation.

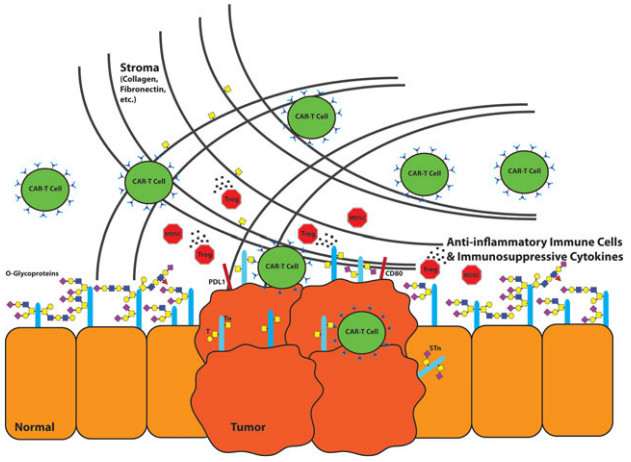

Fig. 2. Barriers for CAR-T in the tumor microenvironment. The tumor microenvironment suppresses anti-tumor immune responses through the formation of dense stroma, which limits the trafficking of effector T cells, and recruitment of anti-inflammatory cells, which secrete immunosuppressive cytokines. The figure displays an example of O-glycopeptide targeting CAR-T cells against a specific Tn-mucin epitope on the tumor cell surface.

Monoclonal antibodies targeting tumor-associated glycan related epitopes

Thus far, the majority of antigens targeted by CARs have been tumor-associated protein epitopes overexpressed on cancer cells. As presented above, this may represent a major hazard when targeting solid tumors. Proteins similar to CD19, those that are uniquely restricted to a dispensable cell-type, and also essential for cancer cell growth, have currently not been identified on cells giving rise to solid tumors. Tumor-specific antigens caused by mutations and/or alternative splicing may provide safer targets, but these are often personal or limited to a subset of cancer cells, and therefore difficult to utilize for general immunotherapy. However, recent advances in neoantigen vaccines and personalized tumor RNA vaccines demonstrate that personal mutations can be used to develop a feasible, yet personal, immunotherapy (Linette and Carreno 2017; Sahin et al. 2017).

Aberrant glycosylation may offer targetable epitopes

One of the most characteristic features of cancer cells is altered glycosylation capacities that result in exposure of aberrant glycans (Hakomori 2002). Changes in glycosylation occur in all classes of glycoconjugates including glycolipids, N- and O-linked glycoproteins, and glycosaminoglycans (GAGs), and they may induce a range of different cancer-associated epitopes (Dube and Bertozzi 2005; Stowell et al. 2015). These epitopes can be divided into three classes (Figure 3). First, there are glycan hapten epitopes, which include the immature truncated O-glycans designated T, Tn and STn antigens (Springer 1984), as well as terminal structures shared by glycoproteins and lipids including the histo-blood group Lewis-related

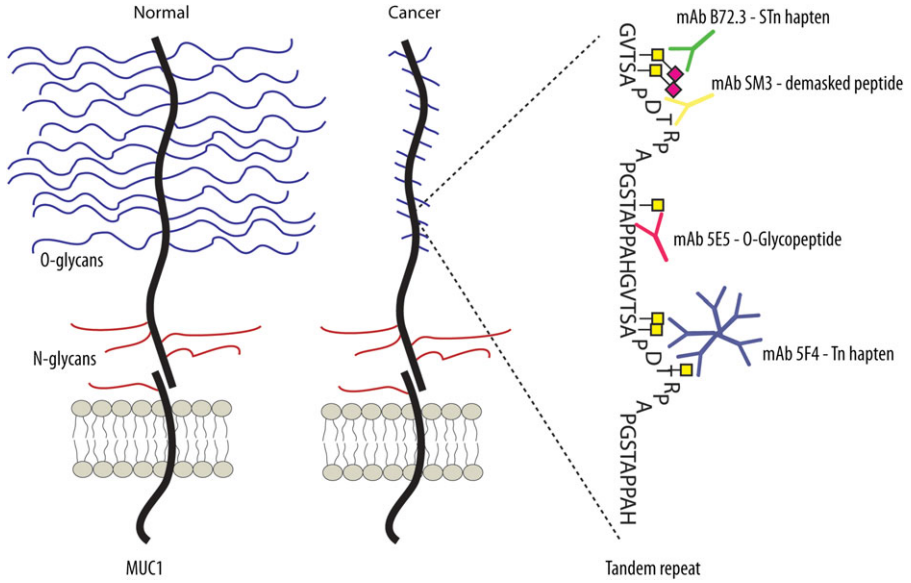

Fig. 3. Classes of anti-glycan mAbs. Glycan-dependent mAbs can contact the peptide backbone in the absence of glycosylation (SM3, anti-MUC1), bind to glycan haptens in the absence of peptide recognition (5F4, anti-Tn), (B72.3, anti-TAG-72 or STn), or combination O-glycopeptides that require glycan and peptide recognition (5E5, anti-Tn-MUC1).

structures (SLe^a, SLe^x) (reviewed in Fuster and Esko (2005)). Of note, T antigen is found on the ductal cells of normal pancreas and may not be as cancer-specific as Tn and STn antigens (Remmers et al. 2013). Second, there are epitopes comprised by an altered context of glycans and the carrier protein, e.g., truncated O-glycans at specific peptide sequence motifs in the large mucin protein MUC1 (Sorensen et al. 2006). Glycolipids may also generate antibody epitopes comprised of the glycan moiety and the carrier lipid (Nudelman et al. 1982), and several cancer-associated glycolipids have been recognized (Cheung et al. 1985; Tai et al. 1988). Third, there are protein epitopes that are exposed due to changes in glycosylation sites and/or glycan structures, e.g., the cancer-associated MUC1 epitope targeted by monoclonal antibody (mAb) SM3 (Burchell et al. 1987). Epitopes induced by changes in glycosylation may be more or less unique to cancer cells, and a multitude of mAbs to these epitopes have been reported in the last 3–4 decades (Sell 1990; Kannagi 2000; Hendriks et al. 2017).

The majority of cancer-associated glycan-targeting mAbs have been raised by classical hybridoma technology using cancer cells or tissue, purified glycoprotein/lipids as well as synthetic conjugates as immunogens (Kannagi 2000). However, there are also examples of antibodies generated by phage display (Lee et al. 2002) or derived from cancer patients (Hirohashi et al. 1986; Yamaguchi et al. 1987). Table I lists representative examples of mAbs targeting all three classes of glycosylation-related tumor-associated epitopes.

MAbs to tumor-associated glycan hapten epitopes

Many of the first mAbs to tumor-associated glycans bind to glycolipids (Hakomori 2001). Some of these mAbs are specific for glycolipids or the unique glycan structures only found on glycolipids, such as gangliosides GM2, GM3, GD2 and GD3, while others bind carbohydrate haptens that are found on both glycolipids and glycoproteins, including Le^x/Le^y and SLe^x/SLe^a glycan hapten structures. For example, the antibody F77 recognizes a core 2 branched O-linked glycan with α-2-fucose and exclusively reacts with malignant prostate tissue and inhibits growth of aggressive prostate cancer tumors in xenografts (Zhang et al. 2010; Nonaka et al. 2014). Other antibodies generated by immunization with cancer cell lines and bovine or ovine mucins target truncated O-glycans found on mucins. The truncated T, Tn and STn O-glycans, normally only found as biosynthetic intermediates in the secretory pathway, are often presented on the cancer cell surface, and were recognized decades ago as pancarcinoma antigens (Springer 1984) (Figure 2). While natural antibodies to these truncated O-glycans are found in all individuals causing polyagglutinability (Moreau et al. 1957; Berger 1999), antibody titers appear to be elevated in cancer patients (Springer et al. 1979; Springer 1984). A number of mAbs targeting T, Tn and STn O-glycans have been raised (see examples in Table I and Figure 3); however, these mAbs exhibit low affinity (Haji-Ghassemi et al. 2015) and are mainly IgM, although anti-STn mAbs are mostly IgG1 (Table I). Low affinity mAbs targeting truncated O-glycan haptens for passive immunotherapy have failed to produce effective and long-term protections in a Phase III trials of a STn-KLH vaccine to treat metastatic breast cancer (Theratope) (Miles et al. 2011). Although patients developed significant anti-STn titers and high seroconversion from IgM to IgG, there was no benefit to disease progression or overall survival. Antibodies targeting O-glycan haptens may exhibit reduced activity due to the repetitive and non-uniform nature of O-glycan haptens in addition to aggregation of the antibodies (Whitlow et al. 1993). Improvements to low-affinity anti-glycan antibodies to prevent aggregation may still yield them amenable as cancer therapeutics with potent effector strategies, such as CAR-T cells (see next section).

High affinity mAbs to O-glycopeptide epitopes

The characteristic truncation of O-glycosylation in cancer also offers another group of interesting epitopes that consists of a small, truncated O-glycan and part of the peptide backbone. mAbs targeting these O-glycopeptide epitopes are characterized by relatively high affinity (~10^{-9} M range) and restricted reactivity against the glycopeptide epitope with little or no reactivity against the peptide with elongated O-glycans or the unglycosylated peptide (Schietinger et al. 2006; Tarp et al. 2007; Steentoft et al. 2010; Lavrsen et al. 2013). Although these antibodies do not react with the O-glycan hapten structure *per se*, they may show binding to high concentrations of the glycan (Tarp et al. 2007). O-glycopeptide mAbs exhibit rather restricted reactivity with cancer cells; although if reactivity is found with normal cells, this is generally limited to Golgi-like or perinuclear localization, suggesting detection of accumulated biosynthetic intermediates (Sorensen et al. 2006; Posey et al. 2016).

To our knowledge, the first mAb identified and characterized with O-glycopeptide specific reactivity was FDC-6, developed in 1985 by immunization with fibronectin from hepatoma cells with an aim to identify cancer-associated forms of fibronectin (Matsuura and Hakomori 1985). While this mAb reacted specifically with fibronectin isolated from hepatoma, fibrosarcoma and fetal fibroblasts, it showed no reactivity with what was considered normal fibronectin from plasma at the time. Considerable efforts to clone a gene variant for the proposed fibronectin variant failed, and it was shown that the epitope was comprised of a short peptide sequence motif located in the IIICS variable region of fibronectin (VTHPGY). Reactivity of FDC-6 mAb was entirely dependent on a truncated O-glycan at the Thr residue in this motif, and the minimum epitope was in fact a Tn-hexapeptide (Matsuura et al. 1988). Moreover, it was found that the site-specific O-glycosylation of this motif was directed by two polypeptide GalNAc-transferase isoforms, GalNAc-T3 and T6 (Wandall et al. 1997; Bennett et al. 1999; Freire-de-Lima et al. 2011), known to be upregulated or de novo expressed in many cancers (reviewed in Bennett et al. (2012)). O-GalNAc glycosylation is directed by up to 20 GalNAc-transferases providing a highly complex and differentially regulated process that if perturbed in cancer can lead to differential decoration of O-glycans on proteins. The FDC-6 antibody, and other second-generation mAbs have been widely studied and exhibit a high-degree of cancer-specific reactivity with tumor stroma in several different cancer forms (Loridon-Rosa et al. 1990; Mandel et al. 1992; Inufusa et al. 1995).

Generation of mAbs to combination O-glycopeptide epitopes

A small number of mAbs targeting different O-glycopeptide epitopes have subsequently been generated and characterized (Table I), and, while similar to the FDC-6 mAb, their discoveries have largely been coincidental (Schietinger et al. 2006). It is therefore worthwhile to review the experimental strategies that have led to the development of existing O-glycopeptide mAbs.

It is well established that immunization with bovine and ovine mucins with high densities of T and Tn/STn O-glycans, respectively, induce potent antibodies directed to O-glycan haptens without recognizable protein backbone specificity (Kurosaka et al. 1988;

Table I. Representative examples of mAbs targeting all three classes of glycosylation-related tumor-associated epitopes (glycan mAbs)

mAb name	Antigen	Isotype	Immunogen	Reference
Hapten mAbs				
O-glycans				
1E3 (BM8)	Tn (GalNAc)α1-O-Ser/Thr)	IgG2a	AOSM	Takahashi et al. (1988), Mandel et al. (1991)
5F4	Tn	IgM	AOSM	Thurnher et al. (1993)
B72.3	STn (NeuAcα2-6GalNAcα1-O-Ser/Thr)	IgG1	Membrane enriched extract of human metastatic carcinoma	Colcher et al. (1981), Thor et al. (1986)
TKH2	STn	IgG1	OSM	Kjeldsen et al. (1988)
3F1	STn	IgG1	OSM	Mandel et al. (1991), Steentoft et al. (2013)
3C9	T (Galβ1-3GalNAcα1-O-Ser/Thr)	IgM	Gal-A^b glycolipids absorbed to *Salmonella minnesota*	Bohm et al. (1997)
HH8	T	IgM	Gal-A^b glycolipids absorbed to *Salmonella minnesota*	Clausen et al. (1988)
Shared structures				
3S193	Le^Y (Fucα1-2Galβ1-4[Fucα1-3]GlcNAc)	IgG3	Le$_y$-expressing cells from the MCF-7 breast carcinoma cell	Scott et al. (2000)
FH6	SLe^x (NeuAcα2-3Galβ1-4[Fucα1-3] GlcNAc)	IgM	6B fucoganglioside absorbed to *Salmonella minnesota*	Fukushi et al. (1984), Fukushi et al. (1985)
N-19-9	Sle^a(NeuAcα2-3Galβ1-3[Fucα1-4) GlcNAc)	IgG1	Colorectal carcinoma cell line	Magnani et al. (1982)
Glycolipids				
F3, F12, F14	FucGM1(Fucα1-2Galβ1-3GalNAcβ1-4 [NeuAcα2-3]Galβ1-4Glcβ1-Cer)	IgG	Fuc-GM1 adsorbed to *Salmonella minnesota*	Nilsson et al. (1986)
F1, F2, F4	FucGM1	IgM	Fuc-GM1 adsorbed to *Salmonella minnesota*	Nilsson et al. (1986)
FCM1	GM3(NeuAcα2-3Galβ1-4Glcβ1-Cer)	hIgM	Melanoma patient	Yamaguchi et al. (1987), Furukawa et al. (1989)
14.18	GD2(GalNAcβ1-4[NeuAcα2-8NeuAcα2-3]Galβ1-4Glcβ1-Cer)	IgG3	Human neuroblastoma cell line LAN-1	Mujoo et al. (1987)
R24	GD3(NeuAcα2-8NeuAcα2-3Galβ1-4Glcβ1-Cer)	IgG3	Human melanoma	Pukel et al. (1982)
O-Glycopeptide mAbs				
FDC-6	Tn-FN1	IgG1	FN1 isolated from hepatoma	Matsuura and Hakomori (1985)
5E5	Tn-MUC1	IgG1	Tn-MUC1-KLH	Sorensen et al. (2006), Tarp et al. (2007)
2D9	Tn-MUC1	IgG1	Tn-MUC1-KLH	Tarp et al. (2007)
1B9	T/ST-MUC1	IgG1	MUC1-T from CHO ldlD cells (mAb 1B9)	Tarp et al. (2007)
6E3	Tn-MUC4	IgG1	Tn-MUC4-KLH	Pedersen et al. (2011)
PMH1	Tn-MUC2	IgM	Tn-MUC2-KLH	Reis et al. (1998)
MY.1E12	ST-MUC1	IgG2a	Human milk fat globule	Yamamoto et al. (1996), Takeuchi et al. (2002)
PankoMAb	Tn/TMUC1	IgG1	MUC1 from a desialylated human breast cancer	Danielczyk et al. (2006)
mAb237	Tn-OTS8	IgG2a	Mouse spontaneous fibrosarcoma with *Cosmc* mutation	Schietinger et al. (2006)
LpMab-21	Sialyl-PDPN	IgG2a	LN229 expressing PDPN	Kaneko et al. (2017)
UN1	Tn/T-CD43	IgG1	Human thymocytes	Tassone et al. (1994); de Laurentiis et al. (2011)
Glycosylation-dependent protein mAbs				
SM3	MUC1	IgG1	Partially deglycosylated MUC1 purified from human milk using mAb HMFG1	Burchell et al. (1987)
HMFG2	MUC1	IgG1	Human milk fat globule	Taylor-Papadimitriou et al. (1981), Burchell et al. (1983)
AR20.5	MUC1	IgG1	Mixture of MUC1 from three different sources	Qi et al. (2001), Movahedin et al. (2017)

Ragupathi et al. 1999; Blixt et al. 2012); although, one may question if these studies indeed could have detected antibodies to combination epitopes. An attempt to develop a synthetic Tn-hapten vaccine, using a highly clustered Tn-glycopeptide based on the MUC2 tandem repeat sequence, was unable to induce antibodies to the Tn O-glycan hapten. Unlike immunization with animal mucins, this approach instead exclusively found antibodies directed to Tn-MUC2 glycopeptide, as exemplified by the PMH1 mAb (Reis et al. 1998). This led to immunization with the MUC1 tandem repeat, which could be synthesized as extended peptides (60–100mers) but

with less dense O-glycan decoration. Immunization with defined O-glycopeptides carrying 5 O-glycans per 20 amino acid repeat coupled to KLH resulted in almost exclusive reactivity with a small peptide epitope (GSTAP) with one or two Tn glycans attached, and a panel of mAbs to Tn-MUC1 were generated (Sorensen et al. 2006; Tarp et al. 2007). These anti-Tn-MUC1 mAbs include the murine IgG1 mAb 5E5 (Figure 3), which exhibits remarkable affinity for Tn-MUC1, as well as cancer-specific reactivity (Cloosen et al. 2006; Sorensen et al. 2006; Andrulis et al. 2014; Posey et al. 2016; Tarp et al. 2007; Van Elssen et al. 2010). The epitope recognized is immunodominant in mice as well as in humans, as demonstrated in a Phase I pilot trial using a Tn-MUC1 glycopeptide-based vaccine that resulted in a strong specific IgG responses (Sabbatini et al. 2007). Moreover, circulating autoantibodies to the Tn-MUC1 epitope may be found in cancer patients (Wandall et al. 2010). Altogether, these findings suggest that the Tn-MUC1 epitope may represent a safe immune target.

Interestingly, decades of work generating MUC1 antibodies using many different strategies, including isolated mucins and cancer cells, almost exclusively resulted in mAbs to a different peptide epitope (PDTR), which also carry O-glycans. Immunization with desialylated MUC1 from a human breast cancer source and selective hybridoma screening generated PankoMAb, an antibody that reacts against a glycopeptide epitope within the PDTR motif (Danielczyk et al. 2006).

There are only a few other well-documented mAbs with truncated O-glycopeptide specificities (Table I). These include the mAb 237 directed to a Tn-glycopeptide in murine podoplanin that was discovered in a mouse spontaneous fibrosarcoma model as a result of a mutation in *Cosmc* (Schietinger et al. 2006). COSMC is a chaperone protein required for T synthase activity and loss of COSMC leads to expression of the Tn (and in some cases STn) antigen on O-glycosylated proteins (Ju et al. 2008). The Tn-glycopeptide epitope recognized by mAb 237 was also shown to be immunodominant in mice eliciting a high-affinity IgG response specific to the Tn-glycopeptide (Steentoft et al. 2010). mAb 237 is the only O-glycopeptide mAb that has been crystalized with the glycopeptide bound, and the structure shows that the mAb envelopes the carbohydrate while interacting with the peptide backbone (Brooks et al. 2010). This is in contrast to conformational peptide mAbs, such as SM3 (Figure 3), that recognize a peptide conformation formed upon glycan truncation (Burchell et al. 1989; Dokurno et al. 1998).

mAbs targeting O-glycopeptide epitopes, in contrast to mAbs targeting O-glycan hapten epitopes, while demonstrating increased antibody affinity, may exhibit reduced tumor and patient applicability due to heterogeneous expression of the carrier protein. For example, Tn and STn antigens are expressed in nearly all pancreatic cancers (Julien et al. 2012; Radhakrishnan et al. 2014), yet CAR-T cells targeting Tn-MUC1 are reactive against ~50% of tested pancreatic cancer cell lines (Posey et al. 2016). This difference may be likely accounted for by differences in MUC1 expression. In other O-glycoproteins where there may only be a single O-glycopeptide epitope present, mutations in the binding epitope may also account for loss of reactivity. In contrast, the epitope for the 5E5 CAR-T cells is within the variable tandem repeat of MUC1 and must be mutated >-40–200 times to lose reactivity but retain MUC1 expression.

Glycan-targeting CARS

In an effort to combine targeting the promising epitopes exposed in cancer due to aberrant glycosylation with the anti-tumor efficacy of CAR-T cells, a number of CARs have been developed from mAbs specific for abnormal glycans and some tested in the clinic. In the following, we review the state of glycan-directed CAR-T cells.

TAG72 – the STn O-glycan epitope

The classic tumor-associated glycoprotein 72 (TAG72), recognized by antibodies B72.3 and CC49, is the truncated STn O-glycan hapten (Kjeldsen et al. 1988), which is widely expressed on glycoproteins and mucins on the surface of a variety of solid tumors, such as squamous cell lung carcinoma, lung adenocarcinoma, ovarian carcinoma, endometrial cancer and colorectal cancer (Qi et al. 1995; Metcalf et al. 1998; Myriokefalitaki et al. 2015). CARs specific for TAG72 were first reported in the nineties (Hombach et al. 1997; McGuinness et al. 1999) and gastrointestinal tumor cell lines were efficiently targeted by anti-TAG72 CAR-T cells (Hombach et al. 1997, 1998). Recently, long-term results of a clinical trial investigating a retrovirally transduced, first-generation CAR-T cell therapy targeting TAG72 in patients with metastatic colorectal cancer were reported (Hege et al. 2017). T cell products were administered either intravenously (IV) or via direct hepatic artery infusion. Despite encouraging drops in serum CA125 and TAG72 in some patients, clinical responses were not observed, potentially due to rejection of the CAR-T cells due to CAR antigenicity (the scFv was of humanized but of murine origins (Kim and Hong 2007)), lack of T cell co-stimulation (T cells require a secondary signal for persistence and survival), or the affinity of the CC49 anti-STn mAb (K_a ~16 nM (Muraro et al. 1988)). A concern when targeting STn with CAR T cells is potential side effects due to reactivity towards inflamed tissue known to upregulate STn (Itzkowitz et al. 1996; Ishino et al. 2010). Although vaccination and generation of circulating STn antibodies have proven safe (O'Boyle et al. 1992; Sandmaier et al. 1999), a more potent therapy with CAR-T might react differently.

Lewis y

Le^y is a difucosylated carbohydrate antigen found on different glycoconjugates (or glycolipids and glycoproteins) that is expressed in normal tissues, including renewing skin, and also expressed by myeloid cell malignancies, such as AML, and 70% of epithelial-derived tumors, such as colorectal, lung and ovarian cancer (Sakamoto et al. 1986; Yin et al. 1996; Zhang et al. 1997). The exact function of Le^y is unclear, but the expression correlates with poor prognosis (Miyake et al. 1992). Le^y-specific, second-generation (CD28-ζ) CAR-T cells were generated from a humanized 3S193 mAb (Scott et al. 2000), which has a K_d of 11.5 μM (Burvenich et al. 2016). The Le^y CAR-T cells demonstrated reactivity against tumor cell lines with high or medium Le^y expression, but no reactivity against tumor cell lines with low or no Le^y expression or neutrophils, which also express low levels of Le^y (Westwood et al. 2005). Cytotoxicity was observed against MCF7 breast cancer cell line and against OVCAR3 ovarian cancer cell line in xenograft models. In a Phase I trial, Le^y CAR-T cells were infused in four patients with relapsed AML after fludarabine preconditioning (Ritchie et al. 2013). One patient experienced a cytogenetic remission, one had a long-term complete response, and one had a partial response with 10-month persistence of the adoptively transferred cells. The study demonstrated efficient bone marrow trafficking in the patients with the best clinical response. No patients experienced Grade 3 or 4 toxicity, although one patient had a Grade 2 neutropenia that resolved spontaneously. In a patient with a history of leukemia cutis, transient

skin rashes with accumulation of AML and T cell infiltrate were observed.

GD2

The disialoganglioside GD2 is a glycoconjugate expressed on neural crest derived tumors, such as melanoma, desmoplastic small round cell tumor and neuroblastoma (Modak et al. 2002). As previously mentioned, monoclonal antibodies targeting GD2 were among the earliest glycan-specific mAbs (Wu et al. 1986), and dinutuximab (anti-GD2, also called ch14.18) is an FDA-approved drug for the treatment of high-risk neuroblastoma (Yu et al. 2010). GD2-specific CAR-T cells have been developed using an scFv sequence derived from the 14G2a mAb (~49 nM), which is an isotype switch variant of 14.18 (Mujoo et al. 1989; Alvarez-Rueda et al. 2011). The first trial investigated infusions of Epstein Barr Virus (EBV)-specific T cells engineered with first-generation CAR for added specificity to GD2 in patients with high-risk neuroblastoma and demonstrated that GD2-specific T cells were safe, virus specificity enhanced T cell persistence, and anti-GD2 CAR-T cells induced tumor necrosis (Pule et al. 2008). Long-term analysis of 19 patients receiving GD2 CAR-T cells showed that three patients (out of 11 total with active disease) achieved complete responses and two continued to have long-term remissions with 24 and 48 months of T cell persistence respectively (Louis et al. 2011). Grade 1 to 3 localized pain was experienced in three patients approximately 2 weeks post T cell infusion but no dose-limiting toxicities were observed. More recently, evaluation of a third-generation GD2 CAR, in addition to a lymphodepleting preconditioning regimen and an anti-PD1 antibody, demonstrated a markedly improved in vivo expansion, but clinical responses were modest (Heczey et al. 2017). Surprisingly, there was an increasing expansion of $CD33^{+}/CD163^{+}$ myeloid cells that may have played a role in hampering the anti-tumor response.

Glycopeptide-specific CARS: Tn-MUC1

The Tn-MUC1 glycoform is widely expressed in ovarian serous carcinoma, lung, castrate-resistant prostate cancer, breast cancer and multiple myeloma, among others. Tn-MUC1 is naturally internalized by the macrophage galactose-type C-type lectin (MGL) and Tn-glycopeptides are presented in HLA Class I and II complexes by dendritic cells (Napoletano et al. 2007). High-affinity glycopeptide-specific antibodies have been developed to target Tn-MUC1 (Sorensen et al. 2006; Tarp et al. 2007). 5E5 mAb binds with an affinity of 1.7 nM and can lyse breast cancer cells via complement mediated and antibody-dependent cellular cytotoxicity (Lavrsen et al. 2013).

Tn-MUC1-specific CAR-T cells, which comprise the variable domains of the 5E5 mAb, can eliminate pancreatic and leukemia in xenograft models, and, similar to the original antibody, display cancer-specificity and negligible reactivity against normal tissues (Posey et al. 2016). Of note, other CARs have been developed from the SM3 mAb discussed earlier, which target MUC1 and rely upon aberrant glycosylation; however, these CARs are not glycopeptide-targeting CARs but instead react with the MUC1 peptide backbone exposed as a result of aberrant glycosylation (Wilkie et al. 2008). In comparison to hapten-targeting CAR-T cells, anti-glycopeptide CARs can target tumor cells with increased affinity and can target proteins that are essential for driving oncogenic features of the cell, such as MUC1. ScFvs, such as anti-TAG72 CC49, are prone to increased aggregation due to repetitive antigenic nature of TAG72 on multiple O-linked surface proteins and likely to diminish effector functions of the CAR-T cells (Whitlow et al. 1993). In addition, the 5E5-based CAR is a second-generation receptor, providing co-stimulation that enhances T cell survival and persistence and prolongs durable anti-tumor responses.

Improving cancer immunotherapy by targeting the tumor glycoproteome

Systematic glycan discovery of additional O-glycopeptide targets

As mentioned previously, the mutations that exist in all cancer cells provide ideal targets for the endogenous T cell responses through the TCRs, but some cancers exhibit low mutational burden and other escape antigen presentation; thus, tumors become less or non-immunogenic. In the context of CAR-T cells, the identification of cell surface cancer-specific epitopes, and not epitopes with shared expression on healthy tissues, is key to obtaining an on-target anti-tumor immune attack in solid tumors. Abnormal glycosylation provides differentially expressed, immunogenic epitopes that are promising targets for antibody-based therapies, such as CAR-T cells and high affinity mAbs to O-glycopeptide epitopes on tumor-associated proteins.

Recent progress in O-glycoproteomics has greatly increased our insight into the O-glycoproteome. Currently, approximately 5000 human O-glycoproteins and over 15,000 O-glycosites are known (Steentoft et al. 2013; Levery et al. 2015). In addition, specific truncated O-glycosylation sites can be elucidated through immunoprecipitations with lectins or defined anti-Tn or anti-STn antibodies of primary tumors or serum from cancer patients (Campos et al. 2015). Based on our current understanding, only a very small subset of these O-glycosites appear to present suitable combination epitopes and induce immune responses; however, there could be a number of reasons for this prediction. Among the most obvious reasons are difficulties in discovery and characterization, as exemplified with the FDC-6 antibody (Matsuura and Hakomori 1985).

One method to systematically generate O-glycopeptide specific mAbs employs genetically engineered cells (SimpleCells) that express homogeneous cancer-associated O-glycoforms (Steentoft et al. 2013). The engineered cells are used as an unlimited source of immunogen and as a screening platform, where mAbs reactive with the engineered cells but not the isogenic wildtype cells can be selected for further characterization. In a proof of concept study, a mAb targeting a truncated O-glycoepitope in FXYD5, known to be upregulated in many cancers, was developed. Similar to the 5E5 mAb targeting Tn-MUC1, this mAb exhibited restricted cancer-specific reactivity (Steentoft et al. manuscript in prep). We are therefore cautiously optimistic that additional combined Tn/STn-glycopeptide epitopes can be identified and mAbs can be generated to develop safe and effective CAR-T therapies.

Limiting tumor escape

One benefit of targeting differential glycans by CAR-T cells proposed here is the limit on tumor escape. For TCR-based immune responses, including adoptive cellular immunotherapies, tumors often escape through loss of proteins involved in MHC assembly and antigen processing, such as B2-microglobulin, HLA variants, TAP1 or TAP2 (Patel et al. 2017). For CD19 CAR-T therapies, tumor escape occurs mostly in the form of CD19 loss or splice variants of CD19 (Sotillo et al. 2015), and these isoforms pre-exist treatment (Fischer et al. 2017), suggesting that CAR-T treatment

eradicates the $CD19^+$ cells and allow those cells with $CD19^-$ isoforms to outgrow. By contrast, glycans and combination glycopeptide epitopes are the products of biochemical pathways that lead to full glycosylation and require many enzymes, including some with redundancy. It may be more difficult for tumors to escape broadly targeting anti-glycan CAR-T cells than CARs targeting peptide or glycopeptide epitopes. For instance, there are approximately 20 human GalNAc polypeptide transferases that generate the initial Tn antigens of the O-glycome and tumors would, in many cases, need to lose several of these transferases in order to not generate any cell surface Tn antigens. In addition, while sialylation or sulfation might silence the epitope, Tn antigen expression promotes oncogenic features in the absence of other tumorigenic drivers (Radhakrishnan et al. 2014) and silencing of Tn antigen expression could be a major disadvantage for the tumor cell. The ability to limit tumor escape may translate to all glycan hapten-targeting CAR-T cells, where target glycans are generated by several enzymes that exhibit a level of redundancy and are modified on more than one glycoprotein (e.g., polysialylation of NCAM and SynCAM1 by ST8Sia2 and ST8Sia4), but it may not apply to glycopeptide-targeting CAR-T cells, in which loss or mutation of the protein backbone would promote tumor escape (e.g., loss of MUC1 for Tn-MUC1 targeting CAR-T cells).

Reducing the immunosuppressive tumor microenvironment

A different angle on glycosylation and CAR-T therapy that should be mentioned is the expression of glycans in the tumor microenvironment. The tumor microenvironment can stunt anti-tumor immune responses either through the enrichment of immunosuppressive cells (e.g., Tregs, MDSCs, M1 macrophages), which present inhibitory markers and secrete anti-inflammatory cytokines, or through the presentation of physical barriers to effector T cell infiltration, including dense glycocalyx and tumor-induced stroma. In glioblastoma, the cellular surface glycocalyx influences the sensitivity of glioma stem cells and the resistance of differentiated glioma cells to cytotoxic T cells (Bassoy et al. 2017). Therefore, glioma-specific (e.g., EGFRvIII (O'Rourke et al. 2017) or IL13Rα2 (Brown et al. 2016)) CAR-T cells could be engineered to edit the surface glycocalyx of differentiated glioma cells, a strategy that could increase the anti-tumor efficacy. This approach, applied through anti-HER2-sialidase conjugates, desialylated tumor cells in an antigen-specific manner, reduced inhibitory interaction with siglec receptors, and enhanced binding to activating NK receptors (Xiao et al. 2016). Engineering combinatorial anti-tumor approaches to enhance migration through dense, tumor-induced stroma, where T cell migration is normally impaired, could include induction of additional integrins or inducible expression of collagenase or heparanase in response to oncofetal fibronectin expression. Degradation of the heparan sulfate proteoglycans (HPGs) is a fundamental process for the trafficking and accumulation of T cells to the tumor site and CAR-T cells lose the expression of heparanase during the ex vivo manufacturing process, leading to an impaired ability to degrade the extracellular matrix. Induction of heparanase expression enhanced tumor infiltration and improved survival in neuroblastoma xenograft models (Caruana et al. 2015).

Conclusions

Increasing CAR-T safety and success in solid tumors will require tumor-specific antigens. In this review, we provided an overview of the budding field of glycan-directed CAR-T therapies, including new efforts to generate high affinity O-glycoepitope antibodies as antigen-recognizing domains of CARs. We also proposed that glycan-targeting is a mechanism to overcome the tumor escape that is observed with peptide-targeting CARs, such as anti-CD19 CAR-T cells. Lastly, we proposed the use of glycocalyx-modifying strategies to alter the tumor microenvironment and improve the impact of CAR-T cells in solid tumors.

Acknowledgements

Research in the authors laboratories were supported by a research grant from Tmunity Therapeutics Inc. (ADP), The Danish Research Council (DFF-4004-00,397B) (CS), The Lundbeck Foundation (R221-2016-438) (CS), Læge Sofus Carl Emil Friis og hustru Olga Doris Friis' Legat (UM) and the Danish National Research Foundation (DNRF107) (CS and UM).

Conflict of interest statement

C.S., D.M. and T.R.K. have no conflict of interest. C.H.J. reports grants from Novartis, outside the submitted work. In addition, C.H.J. has a patent 9464140, 9161971, 8975071, 8916381 and 8906682, with royalties paid by Novartis, is a scientific founder of Tmunity Therapeutics, a biotech dedicated to developing engineered T cells for therapy of cancer, infections and autoimmunity, and has a founders stock from Tmunity. A.D.P. is an inventor on intellectual property owned by the University of Pennsylvania and receives research funding from Tmunity Therapeutics, Inc. for the development of glycan-directed CAR-T cells. U.M. has conflict of interest. U.M. is an inventor on patents on mAb 5E5 owned by the University of Copenhagen.

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