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Carbohydrate-based cancer vaccines: target cancer with sugar bullets

Chang-Cheng Liu · Xin-Shan Ye

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Abstract With the booming development of glycobiology and glycochemistry, more and more structures of tumor-associated carbohydrate antigens (TACAs) are identified. Their broad expression and high specificity in cancer make them important targets to develop cancer vaccines or immunotherapies. However, most of the TACAs are T cell-independent antigens, they cannot elicit a powerful enough immune response to prevent or treat cancer. Immunotolerance and immunosuppression are more easily induced due to their endogenous properties and the declining immunity of the patients. This review summarizes the recent efforts to overcome these obstacles: coupling the carbohydrate antigens to proper carriers such as proteins or some small molecule carriers, and chemically modifying the structures of the TACAs to enhance the immunogenicity of TACAs and break the immunotolerance.

Keywords Carbohydrate-based anti-cancer vaccine · Immunotherapy · Immune tolerance · Antigen · Carrier

Introduction

The first reported cancer immunotherapy can date back to 1983 when William Coley injected live or inactivated *Streptococcus pyogenes* and *Serratia marcescens* into tumor tissues and a tumor-eliminated immune response was evoked [1]. Advances in passive immune treatments with anti-tumor monoclonal antibodies [2] strengthen our confidence that cancer vaccines, which can induce a durable and long-lasting response in patients may be accomplished. Unlike traditional prophylactic vaccines, most cancer vaccines (except vaccines targeting tumor-associated viral agents such as human papilloma virus or hepatitis B virus, *etc.*) are mainly used to remove some very incipient tumors and the residues after surgery or chemotherapy and keep them from relapsing.

Cancer vaccines can be divided into two groups: undefined-antigen vaccines and defined-antigen vaccines [3]. Tumor cells, which were inactivated through hypochlorous acid oxidation, UVB-irradiation, or repeat cycles of freeze and thaw and so on were tested as undefined-antigen vaccines [4]. Tumor cells, which were modified to express co-stimulatory molecules and/or cytokines could be more immunogenic [5–7]. Their efficacy has been tested in several clinical trials targeting different tumor types, including colon cancer [8] and melanoma [9]. Dendritic cell (DC)-based vaccines are another kind of undefined-antigen vaccines. Tumor antigens were loaded on DCs [10], and then, as professional antigen presenting cells (APCs), DCs were able to present or cross-present the antigens to helper T (Th) cells or cytotoxic T cells (CTL) by major histocompatibility complex (MHC) class II or I molecules, respectively. Clinical trials targeting melanoma were reported [11], and Provenge, a DC vaccine for advanced prostate cancer attained license in the US, being the first veritable cancer vaccine [12]. Allogeneic tumor cells or DCs have been used in place of autologous ones, which are individualized-tailored and cannot be generally applied [10]. Heat-shock proteins (HSPs) are a kind of multifunctional proteins, capable of eliciting innate and adaptive antitumor immunity by signaling transduction, participating in antigen processing and presenting [13]. Some kinds of HSPs can bind to their receptors and mediate internalization by APCs [14].

C.-C. Liu · X.-S. Ye (✉)
State Key Laboratory of Natural and Biomimetic Drugs,
School of Pharmaceutical Sciences, Peking University,
Xue Yuan Road No. 38,
Beijing 100191, China
e-mail: xinshan@bjmu.edu.cn

Vaccines based on autologous tumor-derived proteins or peptides complexed with HSPs have been evaluated in clinical trials targeting different cancers [15–17].

Large numbers of tumor associated antigens (TAAs) have been described [18], making it possible to develop defined-antigen cancer vaccines, which can elicit a more specific response and avoid the risk of inducing autoimmunity compared with undefined ones. However, antigens expressed on tumor cells may vary between different patients and if the antigen is not necessary for the growth or spread of the tumor cells, the tumor cells can evolve to lose it to escape from the immune surveillance. If the targeted antigens are also expressed by healthy tissues, undesired severe side-effects will be produced [19]. Therefore, the selection of antigens is very important in vaccine design. Patients involved in clinical trials or applications should also be carefully chosen. Inappropriate design of that may be the reason of failures in clinical trials [20].

Tumor-associated carbohydrate antigens (TACAs), which are expressed uniquely or excessively on the surface of the tumor cells [21], featuring truncation and sequence alteration of the sugar chains and/or over sialylation of cell-surface glycolipids and *O*- and *N*-linked glycoproteins, are important compositions of TAAs. The abnormal glycosylation has been shown to correlate with various cancer development stages including invasion and metastasis [22–24], and abnormal glycosylation in primary tumors may be a signal of poor prognosis [25]. Many TACAs are expressed at a fetal development stage, but only very low level of TACAs can be detected in normal tissues of adults. Therefore, TACAs may be good targets for cancer vaccination. A number of TACAs have been identified, including: the mucin related (*O*-linked) GalNAc (Tn), sialyl Tn (STn), Thomsen-Friedenreich (TF) and so on; the blood group Lewis related lewis^X, Lewis^Y, sialyl lewis^X, sialyl Lewis^A and so on; the glycosphingolipids Globo H, stage-specific embryonic antigen-3 (SSEA-3) and so on; the sialic acid-containing glycosphingolipids, the gangliosides GM2, GD2, GD3, fucosyl GM1 and so on; and the polysialic acid (PSA) [26–29]. There are some large and extensively branched *N*-linked β-1,6-*N*-acetylglucosamine oligosaccharides on the surface of tumor cells [30–32]. The tri- or tetra-antenna oligosaccharides increase the cell surface terminal sialylation, and are typically found in the initial stages of viruses or oncogenes-induced carcinogenesis [33, 34]. Different malignant tissues express different kinds and amounts of TACAs [35].

To apply TACAs to cancer vaccines, the first problem is how to obtain them sufficiently and expediently. In early stage, the carbohydrate epitopes were mainly obtained by the separation from tumor tissues [36], which was time-consuming and difficult to get homogeneous materials. Recent development of oligosaccharide assembly strategy such as automated solid-phase synthesis [37], programmable chemoselective one-pot synthesis [38], and pre-activation based one-pot synthesis [39] has basically solved this problem, making the carbohydrate antigens available with high purity.

The second problem is the poor immunogenicity of TACAs. To some extent, most TACAs are endogenic and induce immunotolerance before vaccination. According to the nature of carbohydrate epitope, TACAs are mostly T cell-independent type II antigens which cannot be presented to T cells and cannot induce a T cell response. Without the help of T cells, class switch from IgM to IgG and enhanced recall memory response cannot be generated. The IgM antibody appears firstly to respond to initial exposure to antigen. However, due to its low affinity and short lifespan, it is inadequate to defeat the cancer.

Once TACAs are conjugated to a proper carrier (*e. g.* protein), the antigens may be treated in a T cell dependent way by the immune system (Fig. 1). When injected into the body, the conjugate vaccine may be captured and phagocytized by DCs and other APCs. The DCs process the captured antigen and assemble it to MHC class I and II molecules. The maturation of DCs still requires some "danger signals" sensed by pattern recognition receptors (PRRs) on the surface of them. Signaling through PRRs leads to the up-regulation of MHC and co-stimulatory molecules. The DCs will migrate from the tissues to the lymph nodes [40].

In the lymph nodes, the DCs interact with the naïve T cells whose T cell receptor (TCR) can recognize the antigen loaded in the MHC molecules of the DCs. With the co-stimulatory signals provided by interaction of CD80/86 and OX40L of the DCs with CD28 and OX40 of the T cells, the Th cells are activated [41], and some of them differentiate to memory T cells. The CTLs can also be activated by cross-presentation of the MHC class I molecules [42] with the help of Th cells.

B cells can be activated by cross-link of B cell receptors (BCRs), which combine with repeating cognate epitopes of the antigens, and turn to IgM-secreting B cells. Meanwhile, they can also process antigens and assemble them to MHC class II molecules. Once the B cells have bound sufficient antigen molecules, they will migrate to the boundary of the B and T cell zones, which place activated Th cells will also move to [43]. When antigen-specific Th cells encounter antigen-presenting B cells, the interaction between TCR and antigen-MHC II complex can lead to up-regulating of co-stimulatory molecules *e. g.* CD40L on the surface of Th cells which will bind CD40 on the B cells, inducing cytokines (*e.g.* IL-4) secretion by Th cells. The double combination and cytokines result in B cell proliferation, differentiation, and class switching. Some high-affinity IgG antibody-secreting B cells and long-living memory B cells are generated.

The secreted antibodies will induce an anti-cancer effect through several possible mechanisms. Antibodies can

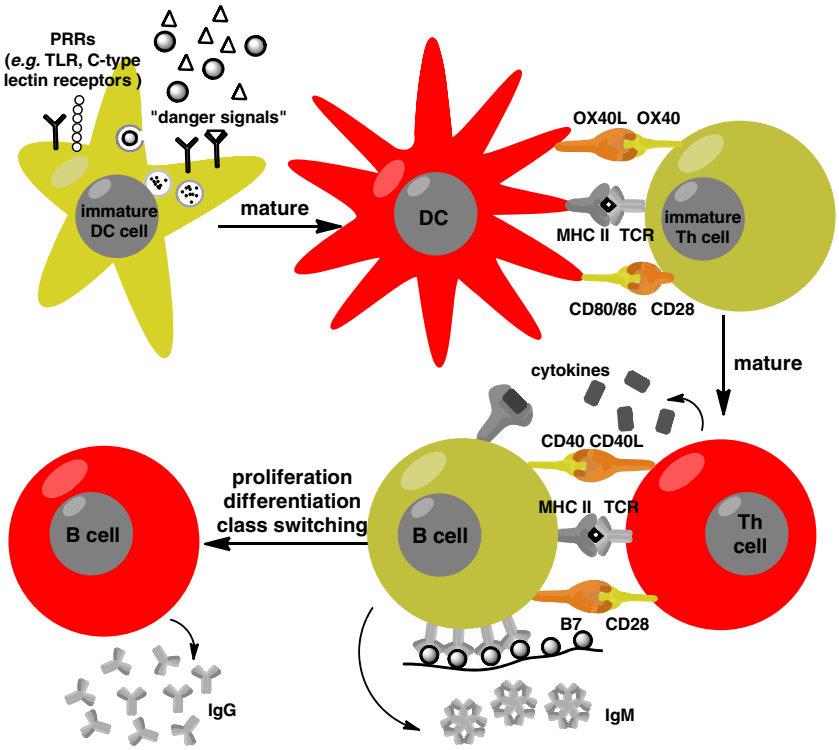

Fig. 1 A possible pathway of T-dependent antigen presentation and antibody generation. DCs capture and process antigens and then present antigens to Th cells and activate them. The maturation of DCs is stimulated by signals from PRRs. Th cells interact with B cells and induce the proliferation and differentiation of B cells. The class switching from IgM to IgG also happens meanwhile

inhibit the development of the cancer since the antigen binding fragments of the antibodies can target some TACAs which are important for signaling, invasion or metastasis of the tumor cells. When the tumor cells are opsonized by two or more antibodies, complement dependent cytotoxicity (CDC) and/or antibody dependent cellular cytotoxicity (ADCC) will be activated and kill the tumor cells [44]. The complement system plays a crucial role in the innate immunity and is able to induce tumor cell lysis and phagocytosis through a complex network [45, 46]. The interactions of constant fragment (Fc) domain of antibodies and Fcγ receptors on effector immune cells including natural killer (NK) cells, macrophages and granulocytes can activate ADCC [47] and the effector cells can cause direct lysis of the target cell.

Most carbohydrate-based anti-cancer vaccines consist of two main parts: the carrier and the carbohydrate epitope. Here, we will try to summarize the recent efforts to develop effective carbohydrate-based cancer vaccines from these two aspects.

Design of carriers

Protein carriers

Covalently conjugating carbohydrate epitopes with immunogenic proteins has been successfully applied to many infectious pathogens including *Haemophilus influenzae* type b, *Neisseria meningitides*, and *Streptococcus pneumoniae* [35]. Immunogenic proteins are also widely examined as carbohydrate-based cancer vaccine carriers.

Early stage researches were involved in vaccines containing a single type of TACAs either isolated or synthesized, such as mucin-related Tn, TF and STn [48–50], gangliosides GD2, GD3 and GM2 [36, 51, 52], and Globo H [53–55]. Though animal trial data showed promise, most of them ended up with disappointing results in human studies. A phase III study of applying ganglioside GM2-keyhole limpet hemocyanin (KLH) with QS-21 as the adjuvant to patients with stage II melanoma showed that the vaccine was ineffective and could even be detrimental to stage II melanoma patients [56]. STn-KLH plus QS-21 named Theratope was tested in more than 1000 women with distant metastatic breast, and the results confirmed its safety but no survival benefit was presented [57].

Clustering of TACAs on the carrier protein may be helpful because of the BCRs cross-link-induced activation nature of B cells. More to the point, cancer mucins are likely to abundantly express several Tn, STn or TF molecules in a row on the tandem repeat scaffold [58, 59], so clustering can mimic this configuration and induce antibodies with higher affinity to the natural antigens. Several studies have proved the efficiency of this strategy. STn(c)-KLH (clustered STn-KLH) vaccine and TF(c)-KLH vaccine both adjuvanted with QS21 were tested in prostate cancer patients and evoked higher titers of IgM and IgG antibodies [60, 61].

Another way to mimic the natural epitope is to conjugate not only the TACAs but also the simplified related mucin

peptides to the carrier protein. An additional advantage of this method is that antibodies against the mucin can be also induced. Livingston and co-workers synthesized a series of varying degree Tn glycosylated MUC1 and MUC2 peptides and conjugated them to KLH. Tn-MUC1-KLH produced significantly higher antibody titers against Tn and MUC1 than Tn(c)-KLH conjugate and unglycosylated MUC1-KLH conjugate, respectively [62]. Kunz and co-workers reported a STn-MUC1-tetanus toxoid (TT) vaccine, which induced extraordinarily high titers of antibodies in wild-type mice [63, 64]. A fluorine-substituted TF-based vaccine was also investigated in this way [65].

For a certain kind of cancer such as melanoma, different patients or different melanoma cells within the same patient may express heterogeneous TACAs, so vaccines based on combinations of different TACAs, especially several TACAs closely associated with a particular cancer type, may evoke a broader spectrum and more efficient response [66]. A physical mixture of GD3-KLH, Lewis^Y-KLH, MUC1-KLH, MUC2-KLH, and QS-21 induced strong antibody responses against the carbohydrate epitope individuals [67]. And a heptavalent KLH conjugate vaccine containing TF(c), Tn, STn, MUC1, GM2, Globo H and Lewis^Y got similar results [68]. However, this approach needs a large amount of carrier proteins. If the carrier specific T-cells overload, response against the individual antigen will decrease. Difficulty in quality control and low conjugation efficiency also limit its application [67, 69–71]. Therefore, unimolecular polyvalent vaccines are better solutions.

Conjugating Tn, Globo H and Lewis^Y to KLH through a single polypeptide backbone induced antibodies against each of the three haptens [72, 73]. Vaccine containing prostate tumor-associated antigens Tn, TF, STn, Lewis^Y, and Globo H was proved to be promising in preliminary immunologic evaluations [74, 75]. Simultaneously, a complicated unimolecular hexavalent vaccine containing breast tumor-associated antigens Tn, TF, STn, GM2, Globo H and Lewis^Y was synthesized and evaluated (Fig. 2) [75].

Protein-based cancer vaccines are most widely investigated, and some of them have entered clinical trials. However, protein carriers have many drawbacks. Firstly, poor reproducibility of the conjugation reactions and non-site-specific coupling of the TACAs may cause heterogeneities and ambiguities of the vaccines in structure and composition, resulting in varieties of immune responses in patients. Secondly, the linker and/or the carrier protein may be immunogenic, leading to “epitope suppression” of the TACAs [76–79].

Small molecule carriers

Depending on the growing insight into the immune system, vaccines are designed to target appropriate immune cells and activate them to achieve a more powerful and certain response with some small molecule carriers.

DCs are the most potent professional APCs *in vivo*. They can monitor the environment and sense “danger signals” through PRRs. There are many kinds of PRRs on DCs, such as Toll-like receptors (TLR), nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) and C-type lectin receptors (CLR). Their natural ligands are some pathogen-associated molecules, including double-stranded RNA, lipopolysaccharide, lipopeptide, polymannose and so on [80]. Without such a stimulus, DCs may induce peripheral tolerance by T cell deletion, anergy or production of regulatory T cells or immunomodulatory cytokine-secreting T cells [81]. PRRs are important targets of new vaccine adjuvants and many existing adjuvants are ligands of PRRs, such as aluminum salts to NLRP3, CpG to TLR9 and so on [82]. PRRs have many signaling pathways resulting in different immune responses, so the immune responses can be modulated to target specific pathogens.

PRRs are good targets for vaccine design. For example, mannose receptor (MR), a kind of CLRs, is a potent antigen-uptake receptor with specificity for glycan structures. MR can bind to polysaccharides terminated in mannose, fucose, or *N*-acetylglucosamine, which are abundant on microbes and some endogenous glycoproteins, and subsequently contribute to their internalization and clearance [83]. Targeting MR with mannosylated carriers, mannan-conjugates or anti-MR monoclonal antibody conjugates may induce stronger Th and CTL responses [84]. Immunization of mice with polysaccharide mannan-MUC1 (protein) vaccines, which were conjugated under oxidative or reductive conditions could elicit Th1-type (generating high CTLs but low antibodies) or Th2-type (high antibodies but low CTLs) responses, respectively [85, 86]. Especially, the oxidized mannan-MUC1 can protect mice challenged by tumor cells [87, 88]. Clinical trials showed the mannan-MUC1 vaccine was safe and could induce both cellular and humoral immune responses in advanced cancer patients [89].

Th cells play a critical role in the immune system. Some peptide epitopes can generate effective Th cells responses in the general human population. For example, a synthetic, non-natural Pan HLA DR binding epitope (PADRE) can bind to the majority of common MHC II types of human and then be presented to epitope-specific Th cells and be capable of eliciting a more powerful T cell help. Therefore, PADRE is a good carrier in vaccine construct [90, 91]. Some peptide epitopes such as chicken ovalbumin (OVA) peptide (257-264) can bind to MHC class I molecules and then activate CTLs in a similar way [92].

Incorporating the above small molecular immune epitopes and/or immunostimulants into the carriers to replace the protein carriers has been extensively explored.

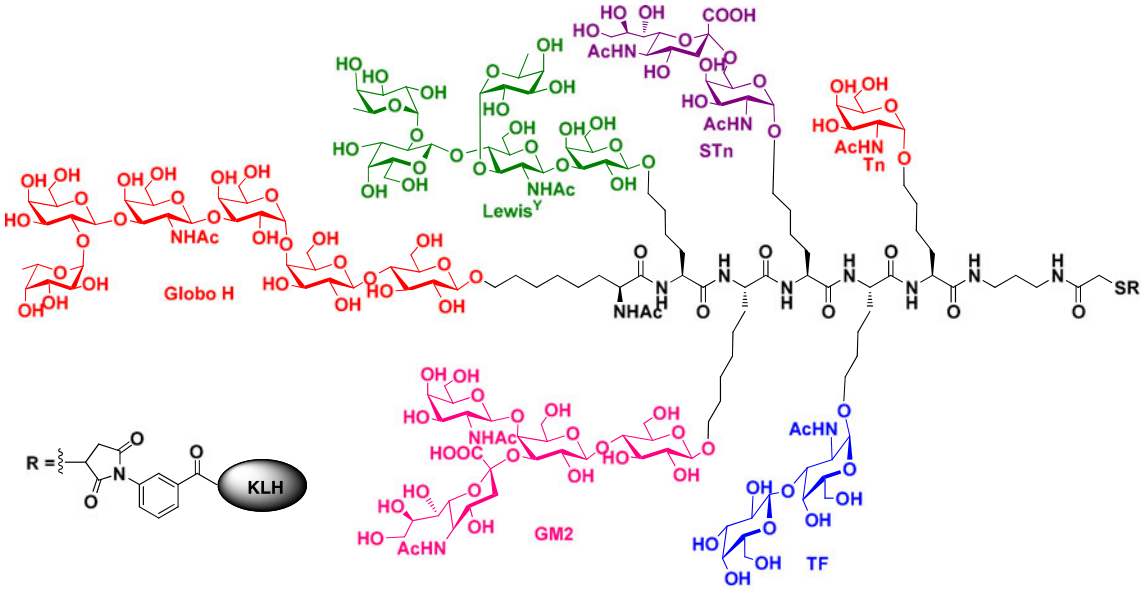

Fig. 2 An unimolecular hexavalent vaccine containing breast tumor-associated antigens Tn, TF, STn, GM2, Globo H and Lewis^Y

Pam_3Cys is a synthetic lipopeptide derived from the *N*-terminus of lipoprotein from *Escherichia coli*. As a ligand of TLR2, it has been widely used as a built-in adjuvant since it was reported in 1990 [93]. Its structure-activity relationships were explored and Pam_3CysSK_4, a stronger TLR agonist, was discovered [94]. A Pam_3Cys-Tn vaccine was fully-synthesized then tested on mice, and high titers of IgM were detected [95]. Danishefsky and co-workers attached the trimeric cluster of Tn [62], Lewis^Y, trimeric cluster of Lewis^Y [96], or the unimolecular polyvalent hapten [74, 75] to Pam_3Cys, and detected a IgM antibody response in mice. When KLH was incorporated, antibody class switching happened. Recently, Li and co-workers conjugated one, two, or four Tn-MUC1 to Pam_3Cys based on click reactions [97].

The monophosphoryl lipid A (MPLA) is a detoxified form of endotoxin lipopolysaccharide [98]. It can interact with TLR4, which results in cytokine secretion [99], and then induce a T cell-mediated immunity. Therefore, MPLA was investigated as a built-in adjuvant. The MPLA-GM3/GM3 derivative conjugates elicited robust IgG antibody responses without any external adjuvants in mice [100], whereas the application of external adjuvant, for example Titermax Gold, even reduced the immune responses to the MPLA conjugates.

Vaccines composed of Th cell epitope and B cell epitope were also designed and synthesized. Tn or trimeric Tn(c) was linked to Th cell epitope PV (derived from polio virus) or PADRE through a non-immunogenic polylysine scaffold (Fig. 3a). These multiple antigen glycopeptide (MAG) vaccines were tested on mice and nonhuman primates with alum or QS-21 as the adjuvants [101–104]. These vaccines were able to elicit good titers of IgG antibodies and improve the survival rate of tumor-bearing mice [102, 103]. IgG titers of MAG:Tn3-PV were higher than those of Tn(c)-KLH under the same adjuvant system, proving that presenting the TACAs in a MAG scaffold is an effective way to enhance the efficiency of the vaccines [103, 104]. Dumy and co-workers investigated a new kind of scaffolds called regioselectively addressable functionalized templates (RAFTs) to attach clustered Tn antigens to one or two Th cell epitope PV sequences (Fig. 3b). *In vitro* and *in vivo* evaluation showed that RAFT is a promising scaffold as a nonimmunogenic vaccine carrier. Immunizing mice with alum induced IgG antibodies, which recognized the native Tn antigen on tumor cells [105]. Kunz and co-workers synthesized a vaccine consisting of STn-MUC1 and a Th epitope $OVA_{323\text{-}339}$ and immunized mice in combination with complete Freund's adjuvant, resulting in a strong and highly specific humoral immune response against the tumor-associated structure [106]. In a similar way, they designed a divalent Tn/TF-MUC1-PADRE vaccine. The vaccine elicited high titers of IgG antibodies to both of the two TACAs. The antiserum of mice recognized human breast cancer cell line MCF7 [107].

In 2005, Boons and co-workers reported a three-component cancer vaccine that contained a B cell carbohydrate epitope Tn, a Th cell epitope YAF (derived from an outer-membrane protein of *Neisseria meningitides*) and the TLR ligand Pam_3Cys. Immunizing mice in phospholipid-based liposomes, the vaccine elicited low-to-moderate titers

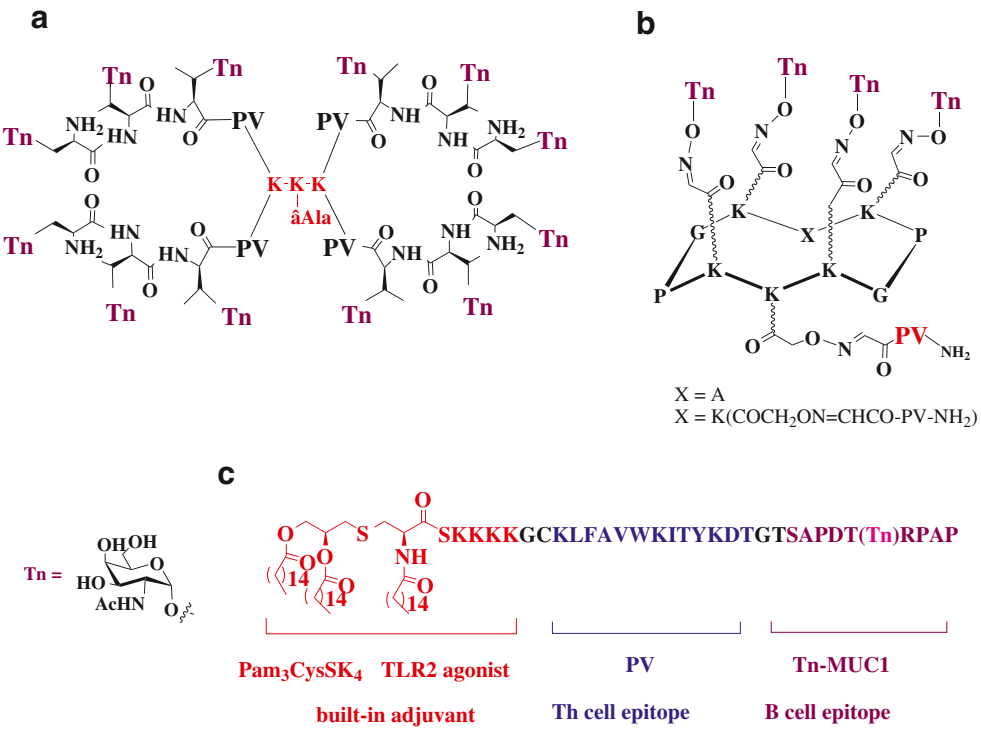

Fig. 3 Structures of representative small molecule carriers for cancer vaccines. **a** A two-component vaccine incorporating Th cell epitopes and B cell epitopes based on MAG scaffold. **b** A two-component vaccine incorporating Th cell epitopes and B cell epitopes based on RAFT scaffold. **c** A three-component vaccine incorporating a built-in adjuvant, a Th cell epitope and a B cell epitope

of IgG antibodies with or without the external adjuvant QS-21 [108]. Subsequently, an optimized three-component vaccine consisting of Tn-MUC1, PV (instead of YAF) and Pam_3CysSK_4 (instead of Pam_3Cys) was synthesized (Fig. 3c) [109]. The vaccine elicited antigen-specific CTLs and high titers of IgG antibodies, which can mediate ADCC [110, 111]. When an immunosilent lipopeptide was used instead of Pam_3CysSK_4, the IgG titers reduced seriously, proving that TLR engagement was very important. Co-administered with Pam_3CysSK_4, it elicited high titers of IgG again, but the antiserum did not recognize the cancer cells. Therefore, covalent attachment of the built-in adjuvant is necessary [112].

A four-component vaccine containing a cluster of Tn based on RAFTs, a Th cell epitope (PADRE), a CTL epitope ($OVA_{257-264}$) and Pam_3Cys was constructed. This vaccine also induced strong Th cell and CTL responses and was effective in preventing mice challenged with MO5 cell line from death [113].

Galili and co-workers described an antibody-mediated antigen uptake mechanism to enhance the uptake of the vaccines by APCs [114, 115]. There are large numbers of anti-Gal-α(1-3)-Gal-β(1-4)-GlcNAc/Glc (α-Gal) antibodies in humans, constituting about 1 % of serum IgG. The antibodies can attach to APCs by the interaction between the Fc portion of the antibodies and Fcγ receptors on APCs. Vaccines containing α-Gal can be recognized by the antibodies on APCs and then internalized, processed and presented. Considering the high titers of anti-L-rhamnose (Rha) antibodies in human serum, Sucheck and co-workers incorporated Rha in the vaccine and then tested the vaccine in mice that pre-immunized with Rha-OVA or not [116]. It was found that anti-Tn titers of Rha-YAF-Tn were higher in mice pre-immunized with Rha-OVA than mice without pre-immunization, showing the potential of Rha-conjugation in vaccine design.

Other carriers

Zwitterionic polysaccharides can be taken up and degraded by APCs, and then presented to Th cells by MHC II molecules, leading to Th cell activation in the absence of proteins [117, 118]. A zwitterionic capsular polysaccharide consisting of a tetrasaccharide repeating unit (~120 units), PS A1, derived from commensal anaerobe *Bacteroides fragilis*, can elicit an immune response similar to that of exogenous proteins [119, 120]. Andreana and co-workers designed and synthesized an entirely carbohydrate vaccine candidate by site-specifically conjugating Tn antigen to PS A1 (Fig. 4a) [121]. The conjugate elicited high titers of antibodies without an immune stimulant. The anti-Tn-PS A1 sera contained notable amounts of IL-17A, which illustrated

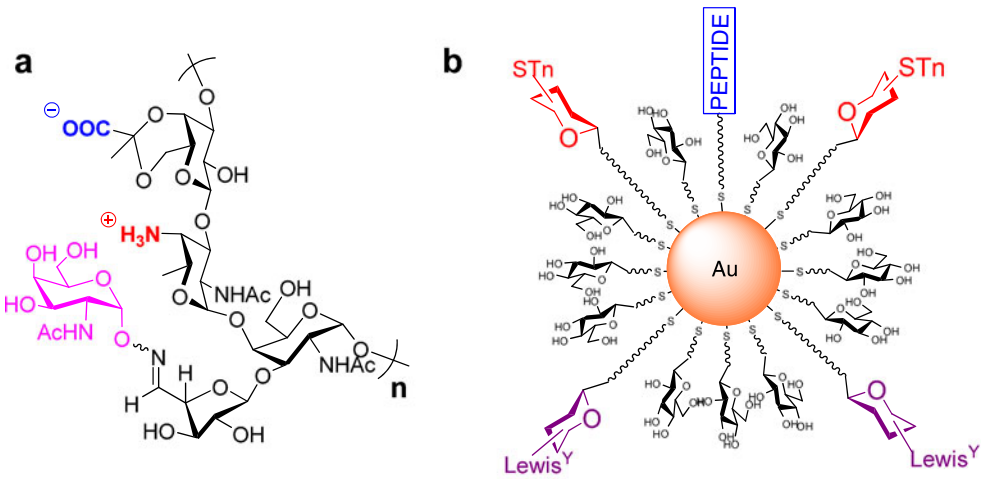

Fig. 4 Structures of two vaccines with other carriers. **a** Structure of Tn-zwitterionic polysaccharide (PS A1) conjugate. **b** A vaccine incorporating Tn, Lewis^Y and Th cell epitopes based on a nanoparticulate carrier

that the carrier elicited a unique antitumor Th17 response [122].

Nanoparticulate carriers are widely investigated to enhance the efficacy of therapeutic agents or vaccines [123]. Ojeda and co-workers constructed a series of gold multifunctional glyconanoparticles (GNPs) incorporating STn, Lewis^Y antigens and Th epitopes (TT) (Fig. 4b) by reducing gold salts in the presence of thiol functionalized neoglycoconjugates through a self-assembly process [124]. The GNPs were water soluble, stable to enzymatic degradation and easy to be modified. Preliminary data of *in vivo* test on mice indicated that reactive antisera, which were able to detect STn and Lewis^Y epitopes on the surface of GNPs were generated.

Modifications on TACAs

Metabolic oligosaccharide engineering-based immunotherapies

Although TACAs are upregulated on the surface of tumor cells, some of them also exist in fetal tissues and some normal tissues of adults. As a result, tolerance is often induced. Chemical modifications of the TACAs may break the immunotolerance and enhance the immunogenicity of the TACAs.

The oligosaccharides on cell surface can be engineered to display unnatural structures by metabolic engineering [125, 126]. Cancer immunotherapies utilizing this technology can be divided into two types: passive type or active type. Passive immunotherapies usually need some exogenous antibodies. Jennings and co-workers incubated leukemic cells with a biosynthetic precursor *N*-propionylmannosamine, resulting in the unnatural *N*-propionyl sialic acid residue on the cell surface. Monoclonal antibody (mAb) 13d9 which recognized α-(2-8)-linked *N*-propionylated PSA could kill the tumor cells through ADCC. The metastasis of leukemic cells was effectively controlled *in vivo* [127]. *N*-Butyrylmannosamine could be metabolized by human melanoma SK-MEL-28 cells. Along with complement, the cells could be lysed by anti-GD3Bu mAb 2A and GD3Bu-KLH-induced polyclonal antiserum. This treatment approach, which utilized precursor and specific antibodies could effectively protect mice from tumor grafting [128].

However, due to the complex preparation processes and high cost, the application of mAb is limited. Guo and co-workers developed an active treatment strategy based on metabolic engineering. The patients are firstly immunized with a structure-modified TACA vaccine, and then take the correspondingly modified biosynthetic precursor to engineer the rapidly proliferating tumor cells to express the artificial antigen instead of the natural one. Thus the trained immune system can recognize and kill the tumor cells [129]. This strategy contains two important points: firstly, the TACA analogue vaccines should be able to evoke a powerful and specific immune response; secondly, the modified biosynthetic precursor should be able to be metabolized and expressed on the tumor cells [130].

A series of GM3 analogues with *N*-modified sialic acid residues were synthesized and conjugated to KLH. Studies in mice indicated that *N*-phenylacetyl GM3-KLH induced an outstanding immune response, much stronger than that of natural GM3-KLH [130]. The corresponding biosynthetic precursors, *N*-modified mannosamines, were synthesized and incubated with several tumor cell lines, and the results showed that only *N*-phenylacetyl-D-mannosamine was efficiently incorporated to GM3 on the surface of tumor cells. The

engineered tumor cells could be killed by the *N*-phenylacetyl GM3-KLH-induced antiserum in the presence of complement [131]. STn has also been investigated in a similar way [132, 133], and some substituted *N*-phenylacetyl STn analogues, which are more immunogenic, have been presented [134].

It should be noted that this strategy may have some shortcomings. Firstly, as sialic acid participates in many crucial biological processes, the expression of artificial sialic acids on normal cells may cause some side effects [125]. Secondly, the patients will have to take the modified mannosamine after immunization to bioengineer the tumor cells, making it more complicated than traditional vaccines.

Cross-reactivity based immunotherapies

Most of the antibodies used or evoked in metabolic oligosaccharide engineering immunotherapies have low affinities to the natural TACAs, while some structure-modified antigens can induce antibodies, which recognize not only the modified antigens but also the natural antigens on tumor cells. Applying these cross-reactive antigens to cancer vaccines can avoid the risks caused by metabolic engineering. The extent of modification must be precisely controlled to make the antigen immunogenic enough to break tolerance and capable of inducing cross-reactive antibodies at the same time.

Long chain PSA exists on the surface of group B *Neisseria meningitides*, *Escherichia coli* K1 and small cell lung cancer cells, making it a vaccine target. However, it is often tolerated by the immune system due to prenatal exposure of polysialylated glycoproteins to immune system. Jennings and co-workers replaced the *N*-acetyl groups of PSA by *N*-propionyl ones and then conjugated it to TT. This conjugate elicited a bactericidal immune response in mice [135, 136]. The conjugate of *N*-propionylated PSA and KLH was tested on patients with small cell lung cancer. The antibody titers were much higher than that of unmodified PSA-KLH conjugate and most of the IgM antibodies cross-reacted with unmodified PSA and small cell lung cancer cell lines and were bactericidal against group B meningococci, but no cross-reactive IgG antibodies and complement-dependent lysis of tumor cells were demonstrated [137, 138]. A modification of GD3-based vaccine, GD3-lactone-KLH, was prepared by treating GD3-KLH conjugate under acid conditions. Clinical trials showed that GD3-lactone-KLH vaccine was more immunogenic than GD3-KLH, capable of inducing higher titers of IgM and IgG antibodies, which could strongly cross-react with GD3 and lyse the GD3 positive cell line SK-Mel-28 [139]. GD2-lactone-KLH vaccine also got promising results in patients with melanoma [140].

Bundle and co-workers synthesized a series of modified antigens by changing the *O*-linked oligosaccharides to *S*-linked ones, including *S*-linked trimannose [141] and tetramannose [142] against *Candida albicans*, trisaccharide [142] against *Shigella flexneri*, GM3 [143] and GM2 [144] against cancer, and conjugated them to TT. These vaccines evoked antibodies cross-reactive to the native antigens, but the antibody titers did not show significant increase relative to those of unmodified vaccines. Hoffmann-Röder and co-workers modified the TF antigen by fluorine-substituting the hydroxyl groups [145–147]. When the two 6-hydroxyl groups were both substituted by fluorine, the disaccharide was conjugated to TT, the acquired vaccine induced a strong cross-reactive immune response in mice [65, 148].

Several *N*-modified STn derivative-KLH conjugates were investigated. The sera from immunized mice were screened by a high-throughput glycan microarray immunoassay. The results indicated that the STn analogues were more immunogenic than STn, especially *N*-propionyl STn, which evoked two times more antibodies than STn when it was conjugated to KLH [149]. Nearly the same time, our group systematically examined the modifications of STn antigen. We synthesized more than 40 STn analogues by substituting the two *N*-acetyl groups with azido, free amino groups or other *N*-acyl groups, replacing the carboxylic group by hydroxamic acid, hydroxymethyl or methyl ester, and altering the configuration of the glycosidic bond or even the glycoform. After a competitive ELISA screening *in vitro*, the analogues with positive response were conjugated to KLH and then mice were immunized with these conjugates. Three fluorine-containing STn analogue conjugates (Fig. 5) displayed outstanding results with 3~5 times higher antibody titers than those of natural STn-KLH. And the antisera selectively recognized STn-positive tumor cells [150]. We also synthesized a *S*-linked STn analogue [151], and the related evaluation is underway in our group.

Conclusion

Cancer can be considered as a kind of immune system disorder to some extent. Although malignant cells may be initiated by a complicated network of multiple causes, a healthy immune system may control and eliminate them. A cancer vaccine may help to construct a tumor-specific immunity.

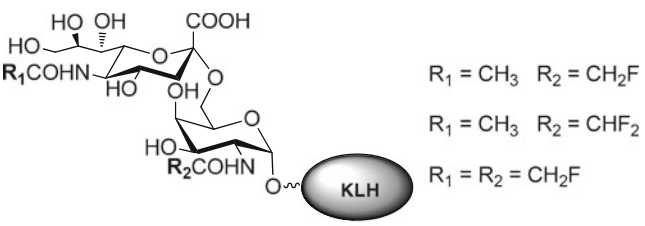

Fig. 5 Structures of three fluorine-containing STn-KLH conjugates which induced 3~5 times higher antibody titers than those of natural STn-KLH

Conjugating carbohydrate epitopes to a proper carrier is a breakthrough in carbohydrate-based vaccine design. This strategy is based on our growing knowledge of the immune network. Protein carrier-based vaccines are most widely used and several of them have entered clinical trials. Many carriers can help the vaccine to target needed immune cells for a desired immune response. Small molecule carrier-based vaccines are simpler and can be fully-synthesized, making them homogeneous and chemically well-defined. These vaccines get exciting outcomes in animal level. But most involved carbohydrate epitopes are unmodified, which may be difficult to overcome the immunotolerance. Combining modified cross-reactive epitopes may get a better result.

Most TAAs are self-antigens and tolerated by the immune system, and regarding TACAs, the carbohydrate epitopes are of poor immunogenicity due to their T cell-independent properties. Therefore, it is very important to break the immunotolerance and enhance the immunogenicity of the TACAs in cancer vaccine design and immunotherapy. Modified TACAs can be considered exogenous by the immune system and initiate a stronger response than the natural ones. When combining cancer cell glycoengineering, this strategy showed great potential. However, the security of glycoengineering needs to be further investigated cautiously and deeply. Applying cross-reactive antigens can avoid this question and be simpler in clinic. Derivatives of TACAs were systematically and subtly designed to invoke a cross-reactive response. The rational design of cross-reactive antigens can be guided by the structure of neutralizing antibodies or mimicking the natural presentation of TACAs on the cancer cells, for examples, restricting the conformation of TACAs to enhance the affinity to neutralizing antibodies or clustering the antigens and presenting the antigens along with related peptides.

In spite of many efforts, there are still no carbohydrate-based cancer vaccines approved into clinical use, and many cancer vaccine candidates failed in clinical trials. We hope that more rational designs of carriers, carbohydrate haptens, and clinical trial protocols may provide our body guard with more efficient sugar bullets to defeat cancer.

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