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Tumor-associated *O*-glycans of MUC1: Carriers of the glyco-code and targets for cancer vaccine design

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Abstract

The transformation from normal to malignant phenotype in human cancers is associated with aberrant cell-surface glycosylation. It has frequently been reported that MUC1, the heavily glycosylated cell-surface mucin, is altered in both, expression and glycosylation pattern, in human carcinomas of the epithelium. The presence of incomplete or truncated glycan structures, often capped by sialic acid, commonly known as tumor-associated carbohydrate antigens (TACAs), play a key role in tumor initiation, progression, and metastasis. Accumulating evidence suggests that expression of TACAs is associated with tumor escape from immune defenses. In this report, we will give an overview of the oncogenic functions of MUC1 that are exerted through. TACA interactions with endogenous carbohydrate-binding proteins (lectins). These interactions often lead to creation of a protumor microenvironment, favoring tumor progression and metastasis, and tumor evasion. In addition, we will describe current efforts in the design of cancer vaccines with special emphasis on the synthetic MUC1 glycopeptide vaccines. Analysis of key factors that govern structure-based design of immunogenic MUC1 glycopeptide epitopes are described. The role of TACA type, position, and density on observed humoral and cellular immune responses is evaluated.

Keywords

MUC1; Tumor-associated carbohydrate antigens; Lectins; Vaccines; Immune response

1. MUC1 structure and function in normal cells

Mucin 1 (MUC1, CD227) is a type I transmembrane glycoprotein consisting of two subunits (Fig. 1). A large, heavily glycosylated extracellular N-terminal domain (MUC1-N) that

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Conflict of interests

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protrudes from the cell surface up to 200 – 500 nm and an intracellular C-terminal domain (MUC1-C) that is non-covalently interlinked through a degenerative sequence [1–3]. It is expressed in the apical and basolateral surfaces of most secretory glandular epithelium [4] but is occluded from mesenchymal cells and skin epithelium [5]. The extracellular mucin-like domain of each allele contains a variable number of identical 20-amino acid tandem repeats (VNTR, HGV**TS**APD**T**RPAPG**ST**APPA) that can range from 20 to 120 repeats with five potential sites for post translational *O*-linked glycosylation at serine (Ser) and threonine (Thr) residues [5, 6]. Various monosaccharides comprise the extensively branched *O*-glycan chains, such as *N*-acetylgalactosamine (GalNAc), galactose (Gal), *N*-acetylglucosamine (GlcNAc), *N*-acetylneuraminic acid (sialic acid, Neu5Ac), and fucose (Fuc) (Fig. 1). Alpha *O*-linked GalNAc is always the first glycan attached to Ser or Thr residues of MUC1 tandem repeats, and in normal cells GalNAc is supplemented by additional sugar moieties to form eight main core structures (Fig. 2)[7]. These are further modified, giving rise to extended dense clusters of MUC1 with variable termini. T-synthase enzyme (core 1 β3-galactosyltransferase or β3GalT), chaperoned with oligomeric endoplasmic reticulum-localized Cosmc protein, facilitates synthesis of the core I structure (Galβ1,3-GalNAcα-*O*-Ser/Thr) [8]. Core 2 GlcNAc transferases (C2GnTs) extends the core I structure by adding GlcNAc in a β1–6 linkage to the existing GalNAc of core I structure [9]. Core 1 and 2 structures are acceptors for a wide range of glycosyltransferase, and elongation of the C6-branch of core 2 is the most frequently found form of MUC1 [5]. Lengthening of the GalNAc moiety by addition of GlcNAc in β1,3-linkage, followed by addition of a second GlcNAc in β1,6-linkage results in formation of core 3 and 4 structures, respectively [10]. Core 1–4 structures comprise the primary glycan structures observed in humans. The extensive and diverse array of glycan permutations from the MUC1 polypeptide backbone (apomucin) serve as a protective barrier for epithelium, and in addition plays a functional role in monitoring the extracellular environment and signal transduction into the cell [11].

Alternatively, co-translational *N*-linked glycosylation links the amidic nitrogen of asparagine to GlcNAc at five possible sites [12]. Four of these sites are in the degenerative region of MUC1-N, whereas one site is located in the extracellular region of MUC1-C [12]. The biantennary chain composition builds and branches with sugar variations inclusive of mannose, xylose, and 12 others common glycans. *N*-linked glycosylation plays a role in secretion, protein folding, and trafficking of MUC1 [12].

2. Tumor-associated MUC1

In cancer, tumor-associated MUC1 (TA MUC1) becomes redistributed over the entire surface of the cell due to a loss in apical-basal polarity [3, 11]. Additionally, the glycosylation pattern of the extracellular N-terminal domain of TA MUC1 differs from that of MUC1 expressed on normal cells. The long-branched glycan chains are truncated and mostly exhibit core 1 *O*-glycans, that reveal the underlying tandem repeat region(s) (Fig. 1) [3, 13, 14]. Cessation of core 2 structures stems from a loss of core 2 β6-GlcNAc transferase activity [15] and/or mutations in Cosmc chaperone [7, 16, 17]. Additional implications regarding cell homeostasis and development of an alkaline pH of the Golgi lumen are proposed in creation of the core 1 structures [17, 18]. Some truncated glycans of TA MUC1 are capped by sialic acid, mostly due to the overexpression of α2,6- and α2,3-sialyl

transferases [19, 20]. Consequently, the most common TACAs formed from incomplete synthesis are GalNAcα-*O*-Ser/Thr (Tn, Thomsen Nouveau, CD175), Neu5Acα2,6-GalNAcα-*O*-Ser/Thr (sTn, sialyl Tn, CD175s), Galβ1,3-GalNAcα-*O*-Ser/Thr (TF, Thomsen-Friedenreich, CD176, T antigen) and Neu5Acα2,6- and Neu5Acα2,3-Galβ1,3-GalNAcα-*O*-Ser/Thr (2,6-sTF, 2,3-sTF) (Fig. 1 and 2). Their expression levels have been used as biomarkers of poor prognosis [21]. While oncofetal TF antigen is virtually absent from healthy tissue, most TACAs are found expressed in low amounts in normal tissue but increased amounts in premalignant and malignant tissue [3, 22]. Tn and sTn antigens are found in breast, prostate, colon, respiratory, pancreas, ovarian [23, 24], and gastric cancers [7, 25]. The Tn antigen is expressed in >90% of breast cancers [7, 26], 10–90% of other forms of cancers and is prevalent in 25–70% of premalignant tissues in the colon [7]. Many epithelial cancers (>80%) display sTn antigen and its expression is associated with decreased overall survival of patients [27]. TF and sTF are both expressed on breast cancer, while TF is more prevalent in gastric, colon, pancreas, ovary, prostate, and stomach cancers and is also found in 90% of cancer types [28, 29]. Tn, sTn, and TF antigens are found co-expressed on tumor cells and their expression correlates to disease progression from their involvement in tumor invasion, metastasis, and evasion of the immune system [19]. Sialylated forms of Tn and TF glycans have been found to play key roles in immune suppression [24, 30].

In addition to truncated *O*-glycans, the expression of specific sialyl and fucosyl transferases generates sialyl-Lewis antigens, NeuAcα2,3-Galβ1,3-(Fucα1,4)-GlcNAc-R (sLe^a, sialyl-Lewis^a) and NeuAcα2,3-Galβ1,3-(Fucα1,3)-GlcNAc-R (sLe^x, sialyl-Lewis^x) (Fig. 1 and 2) [7, 28]. Sialyl-Lewis^a structures are found in >50% of colon, stomach, and pancreas cancers, along with lung, liver, breast, and mesothelioma cancers [7]. Whereas, sialyl-Lewis^x structures are found in >90% of pancreas and stomach cancers, along with colon, esophagus, ovary, and breast cancers [7].

Broad distribution of TA MUC1 on both primary tumors and metastasis, including cancer stem cells, has rendered MUC1 as a widely explored target in many diagnostic and (immuno)therapeutic approaches [31–33]. Based on certain criteria, such as therapeutic function, immunogenicity, and cancer cell specificity, MUC1 was listed by the National Cancer Institute Translational Research Working Group as the second most promising target in cancer research from a list of 75 tumor-associated antigens [34].

3. Cell-surface TACA-lectin interactions: Role in tumor progression, metastasis and immune evasion

Carbohydrate-binding proteins, lectins, are the main binding partners of TACAs on the surface of the cell [35, 36]. These interactions often lead to creation of a pro-tumor microenvironment, favoring tumor progression and metastasis [37]. In addition, glycan-protein recognition may contribute to tumor progression through evasion of antitumor immune-responses [38–40]. It has recently been proposed that the tumor glyco-code may be considered as novel immune checkpoints [41]. Common human cell surface lectins that bind to TACAs of MUC1 belong to three main groups: C-, S- and I-type lectins [42] (Fig. 3).

3.1. C-type lectins

C-type lectins, the largest and most diverse of the lectin families, share a carbohydrate recognition domain (CRD) signature motif that binds glycans in a calcium dependent manner [43]. Selectins, macrophage galactose lectin (MGL), and dendritic cell (DC)-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) are examples of common human C-type cell surface lectins that bind to TACAs of MUC1. Most members of this family function as adhesion molecules, and all are considered to engage as signaling receptors and contribute to different steps of the metastatic spread of cancer.

Selectins are perhaps the best characterized molecules among the C-type lectins [44]. They are single-chain transmembrane glycoproteins that are found on the surface of endothelial cells (E-and P-selectins), platelets (P-selectins), and leukocytes (L-selectins), and are known to participate in lymphocyte and leukocyte rolling, an early step in the extravasation process [45]. Carbohydrate ligands on tumor cells exploit the selectin-dependent mechanism mediating cell tethering and rolling interactions that progress distant organ metastasis. Sialylated and fucosylated ligands, such as sialyl-Lewis antigens (sLe^a and sLe^x), are recognized by selectin CRDs. The increased levels of these antigens on TA MUC1 lead to enhanced interactions with selectins on endothelial cells, thus contributing to metastatic progression [19, 28, 46–48].

Macrophage galactose lectin (MGL) is the best studied of the multiple C-type lectins expressed exclusively on macrophages and dendritic cells (DCs) [49]. It is a homotrimer cluster protein, and is thought to have a similar structure to the asialoglycoprotein receptor (ASGPR) [50]. However, the carbohydrate-recognition profile specificity of MGL differs significantly from that of ASGPR. ASGPR recognizes tri- or tetra-antennary glycans containing both Gal and GalNAc, whereas MGL recognizes only GalNAc structures [51]. The specificity of MGL for GalNAc residues with exposed C3- and C4-hydroxyl groups [50] explains the ability of the receptor to bind to tumor-associated forms of MUC1 bearing Tn [52] and sialyl-Tn antigens [53], but not to more complex and branched *O*-glycan structures of MUC1 found on normal cells. It has been shown that MGL is expressed by M2-like tumor-associated macrophages (TAMs) which are known to promote tumor growth and metastasis [54]. Thus, interaction of MGL with MUC1-Tn and MUC1-sTn is likely to modulate the TAM phenotype and/or activity, leading to metastatic extravasation. MGLs preferential expression on tolerogenic APCs found at carcinoma tumor sites [55, 56], further indicates their role as an immunomodulator within the tumor microenvironment contributing to tumor-induced immune tolerance [57, 58]. Yet, MGL is able to internalize epitopes that positively instruct dendritic cell differentiation and subsequent T cell responses. MGL targeting a MUC1-Tn glycopeptide consisting of three tandem repeats, lead to modulation of DC maturation and initiation of strong CD8+ T cell immune response [59]. These findings demonstrate that MGL activation can be used for anticancer vaccination strategies. Furthermore, it has been shown that the nature of the MGL receptor dictates intracellular processing, and depending on the structure of the ligand and its affinity for MGL, the resulting immune responses can differ significantly [60–63]. Thus, glycan-based CLR targeting offers he ability to manipulate the glycan density and their presentation, allowing the design of effective multivalent binding systems. Considering the crucial role of tumor-

associated forms of MUC1 with MGL in tumor immunology, an in-depth understanding of this interaction is essential for it to be exploited for cancer vaccine strategies.

DC-SIGN is predominantly expressed on DCs where it functions as an adhesion receptor and mediates binding and internalization of pathogens through interaction with *N*-linked high mannose oligosaccharides [64]. However, DC-SIGN participates in cell-cell interactions through binding with endogenous ligands, such as Lewis antigens [65]. Indeed, glycan-modified (Le^x or Le^b)-coated liposomes were shown to boost T cell responses by targeting DC-SIGN receptors on DCs [66]. Consequently, DC-SIGN interaction with TA MUC1 plays a role in the adhesion of tumor cells to endothelial cell. Correlation of antitumor response failure has been linked to suppression of DCs functional maturation caused by DC-SIGN bridging interactions between DCs and the Lewis glycans present on some colorectal carcinoma cells [67]. In addition, it has been found that interaction of TA MUC1 with DC-SIGN leads to induction of regulatory T cells (Tregs) [68]. This functional suppression induces immune evasion, that favors metastasis of colon cancer [65].

3.2. S-type lectins (Galectins)

Galectins are a growing class of ß-galactoside-specific animal lectins [69, 70]. Of the 15 members of this family found in mammals, galectin-1 and galectin-3 are the most ubiquitously expressed types. In some tissues, only galectin-1 or galectin-3 is expressed, while both galectins are often co-expressed in immune cells [71]. Galectins are defined by a common CRD, and their capacity for bivalent or multivalent binding, which permits formation of galectin-glycoprotein networks called “lattices” that regulate cell-surface glycoprotein organization and signaling. Galectins are differentially expressed in cancer [72]. A number of important roles in cancer initiation and progression [73–75], as well as in tumor-immune escape [76, 77] have been associated with these two galectins. TF antigen of TA MUC1 has been shown to be actively involved in tumor metastasis, promoting several key cell-cell interactions *via* association with galectin-3 (Fig. 4) [29, 78, 79]. The interaction between TF antigen and galectin-3 represents an important early step in heterotypic cancerendothelial adhesion and the formation of intravascular metastatic deposits [80, 81]. Furthermore, binding of galectin-3 to TA MUC1, predominantly its extracellular domain, induces MUC1 cell surface polarization, and increases MUC1– epidermal growth factor receptor (EGFR) interaction [82]. This interaction leads to EGFR activation and likely makes an important contribution to EGFR associated tumorigenesis and cancer progression. Furthermore, galectin-3-MUC1-induced cancer cell homotypic aggregation increases cancer cell survival by preventing the initiation of cellular anoikis [83]. Analysis of the molecular recognition features of galectin-3 binding to TF antigen revealed enhancement in affinity for the TF antigen linked to MUC1, as it exists in its natural cellular context [84]. The dissociation constants for interaction of galectin-3 and the glycosylated MUC1 fragments measured by isothermal titration calorimetry decreased up to 10 times in comparison to that of the free TF disaccharide [84]. The most notable feature of the binding of MUC1 glycopeptides to galectin-3 was a shift from a favorable enthalpy to an entropy-driven binding process. Similarly, structural analysis of binding of avian galectin-3 to TF-threonine conjugate showed transient interaction between galectin-3 and amino acid threonine [85].

These additional lectin-peptide scaffold contacts may contribute to the high selectivity of endogenous lectins for their natural counter-receptors.

Galectins are also heavily involved in regulation of immune functions [86]. Galectin-1 and galectin-3 bind to the discrete sets of glycoproteins on the surface of T cells [87] and trigger T cell death [88, 89]. It was suggested that galectin-3 may serve as a ligand for MUC1 expressed by activated T cells, and this interaction could potentially modulate immune effector functions [90]. Galectins expressed by tumor cells exhibit tolerogenic effects, that facilitate cytokine imbalance, and induction of anergy, deletion of antigen-reactive T cells, and stimulation of suppressive Tregs [19, 90]. Targeted inhibition of galectin-1 and galectin-3 has demonstrated a strong immunosuppressive effect on T cells [91, 92].

3.3. I-type lectins

Siglecs (sialic acid binding Ig-like lectins) are a family of lectins that are mostly expressed by cells of the immune system [93, 94]. They play a key role in mediating cell-cell interactions *via* recognition of different sialylated glycoconjugates which can lead to the activation or inhibition of the immune response [95]. Signaling through cytoplasmic immunoreceptor tyrosine-based activation motif (ITAM)-bearing siglecs usually results in cell activation, while engagement of intracellular immunoreceptor tyrosine-based inhibition motif (ITIM)-bearing receptors is usually inhibitory. Interaction of sTn of TA MUC1 with siglecs *via* ITIM motifs reduces anti-tumor immunity and thus is thought that these interactions play a central role in tumor evasion [96]. For example, recognition of sialylated glycans on cancer cells as non- or altered-self, by natural killer (NK) cells and their mediated cytotoxicity as part of an initial immune response to the tumor, is blocked when Siglecs-7 and Siglec-9 are recruited to the heavily sialylated tumor cells [97]. MUC1-sTF binding to Siglec-9 on monocytes and macrophages induced the release of factors that can promote tumor growth and modulate the microenvironment [96]. These macrophages are also characterized by increased expression of programmed cell death-1 (PD-L1) ligand, an immune check point inhibitor [41]. While the specific mechanistic role of MUC1 sLe^x epitopes on the immune system remains unclear, they are known to bind with Siglec-9 [98]. However, research thus far seems to have established that siglecs interaction with sialylated epitopes on MUC1 likely inhibit the immune response.

4. Cell-surface TACAs: Targets for cancer immunotherapy

TACAs of MUC1 are antigenic due to the lack of their expression on healthy cells, making them ideal targets for anti-tumor immune prevention and therapy. Indeed, a broad spectrum of natural anti-TACA antibodies are present in human serum [99–102]. These anti-glycan antibodies are IgM type, however, recently the IgG type cancer-associated auto-antibodies have been detected toward distinct *O*-glycopeptide epitopes [103]. The presence of circulating naturally occurring anti-MUC1 antibodies have been correlated with better disease prognosis in cancer patients [104–106].

Envisioned vaccine design strategies based on TA MUC1 need to consider features such as prophylactic or therapeutic approaches. The overall goal in preventative cancer vaccine design is to intervene before natural immunosurveillance is sequestered and prevent

precancerous tumors from shifting into a microenvironment that favors regulatory and immunosuppressive mechanisms [107–109] (Fig. 5). This is based on the hypothesis of cancer immune surveillance or “tumor editing,” that incorporates three different outcomes for cancer progression: tumor elimination, equilibrium with the immune system, and escape from immune control [110]. However, the general objective in therapeutic cancer vaccine design differs, as established tumors have already escaped immune surveillance. These vaccine concepts aim to induce strong antigen-specific T cell responses. Therapeutic vaccines and checkpoint inhibitors are designed to restore immunosurveillance [31, 105, 111, 112]. The future of immunotherapy lies in various combinations of drugs that modulate the tumor microenvironment and strengthen natural cancer immunosurveillance (and induce regression of tumors). Regardless of the approach, the vaccine candidate should trigger both humoral and cellular immunity in order to generate an effective MUC1 anti-cancer immune response [113]. In addition to an antigen (target for the immune response), and an adjuvant (antigen immune enhancers), current vaccines incorporate novel carrier formulations for more efficient delivery and additional stimulus, initializing a more potent immune response[114].

Earlier attempts at immunization with nonglycosylated MUC1 were not successful as mice failed to produce high levels of anti-tumor cytotoxic T lymphocytes (CTL) and IgG antibodies due to the relatively low immunogenicity and immune tolerance of MUC1 [115–117]. The immune tolerance of non-glycosylated MUC1 can be overcome by use of MUC1 glycopeptide epitopes and/or specialized adjuvants and novel formulations in vaccine constructs. Glycopeptides bearing TACAs appear to be recognized as foreign or “abnormal-self,” while non-glycosylated MUC1 peptides, are perceived as “self” [118]. These data also point out that MUC1 glycopeptides bearing TACAs could be considered as safe and effective cancer vaccines. However, we must bear in mind that tumor cells display a collective array of TA MUC1 epitopes, while that is not mirrored in the current synthetic TACA-MUC1 peptide vaccine designs. Many current vaccine constructs include a single type of TACA-MUC1 epitope that may elicit specific anti-MUC1 antibodies, however, the lack of natural glycan variety, may result in the inability of the vaccine to generate an extended adaptive immune response. Nevertheless, the increased immune stimulation has been attributed to the glycan(s) type, and site(s) of attachment, and/or glycosylation induced conformational changes within the three immunodominant peptide epitopes (PDTRP, GSTA, and GVTS) [119–123]. However, rules that govern the immunogenicity of the three *O*-glycosylated peptide motifs within the MUC1 tandem repeat sequence are still poorly defined [124–127].

There are several examples of synthetic TACA-MUC1 vaccine designs developed over the last several years that assist in specificity aid eliciting an anti-antigen response [31, 120, 122, 128, 129]. The first prophylactic cancer vaccine clinical trial based on a non-viral antigen, MUC1, in healthy individuals at-risk for colon cancer has been recently been completed [130]. The study identified 13 fully-human IgG type antibodies with specificity for several tumor-associated MUC1 epitopes with a wide range of binding affinities. These antibodies are promising candidates for development of immunotherapeutic drugs. This review focuses on the MUC1 *O*-glycopeptide vaccines bearing Tn, sTn, TF, and sTF antigens, in combination with carrier platforms (bacterial toxins and virus-like particle), adjuvants such

as those targeting pattern recognition receptors [Toll-like receptors (TLRs) and C-type lectin receptors (CLRs)], and/or novel nanoparticle-based delivery systems (Fig. 6).

4.1. Protein carriers: TTox/BSA/KLH

Strong antigen-specific immune responses were induced when synthetic mono- and multiglycosylated MUC1 epitopes were paired with BSA or TTox as protein carriers in the presence or absence of a spacer between the two components [122]. These conjugates bearing Tn, sTn, TF, and/or sTF proved to be efficient vaccines, inducing very strong immune responses in mice with predominant IgG isotype antibodies.

BSA-conjugated glycopeptide vaccines, based on MUC1 20-mer tandem repeat sequence bearing either Tn or TF antigen at Thr9 (PDT*RP region), displayed a specific enhanced immune response [131]. On the contrary, the same MUC1 sequence, when bearing Tn antigen at Ser15 (GS*TA) elicited a lower immune response [131]. These differences were attributed to the conformational differences amongst Ser and Thr *O*-glycosylation points [132–136]. The glycosidic linkage of GalNAc-α-Thr is rather rigid in solution compared to GalNAc-α-Ser. Overall, higher antibody specific titers were generated when both the PDT*RP and GS*TA region were glycosylated with either two Tn antigens, or with one TF and one Tn antigen. Even though divalent glycan presentation elicited high IgG antibody levels, it is important to recognize that the monoglycosylated PDT*RP epitope bearing TF antigen produced almost the same antibody titer levels. Favorability for the TF antigen was seen in both glycan positions. By keeping Tn or TF antigen at Thr within the PDT*RP epitope and introducing sTn or 2,6-sTF glycans at Ser within the GS*TA epitope similar results were obtained [133]. It was concluded that TF favors the PDT*RP region while sialoglycans show immune preference in the GS*TA region. These BSA-conjugated glycopeptide vaccines induced IgG-isotype antibodies, which bound to TA MUC1 expressed on MCF-7 tumor cells. The role of glycosylation within GS*T*A epitope was further evaluated with either BSA or TTox-conjugated to extended 22-mer MUC1 epitopes bearing sTn glycan at either Ser or its neighboring Thr residue [137]. The TTox vaccine conjugate bearing the sTn glycan at the Thr residue was selective for not only tumor cell lines expressing TA MUC1 but also for native tumor tissues, showing for the first-time the diagnostic value of these antibodies. Further extension of MUC1 to include the PDTRP epitope surrounded by two STAPPA motifs (27-mer epitope), and glycosylation with the Tn antigen within the GS*TA epitope, resulted in a strong immune response that demonstrated the IgG class switch [121]. The 27-mer conjugated vaccine induced antibodies that bind with high specificity to TA MUC1 on tumor cells and was successfully used in diagnosis of pancreatic cancer. Recently, a 22-mer MUC1 peptide sequence was functionalized with the Tn antigen at Thr within the PDT*RP region and sTn at Thr within the GST*A region and coupled to TTox [138]. The vaccines induced strong immune responses in wild-type and human MUC1-transgenic mice without autoimmune side effects again the self-antigen. Vaccination with TTox carrier conjugates containing 2,3-sTF within the GST*A region either with or without Tn glycan attached to Thr within PDT*RP region induced robust immune responses [119]. The observed class switch to IgG subtype indicates an acquisitioned immunological memory. Although the incorporation of Tn antigen within PDT*RP region had less impact on the total antibody titers, breast cancer tumor cells

(MCF-7) incubated with the antisera from mice immunized with the divalent MUC1 vaccine showed increased antibody binding. This was attributed to induction of a more tumor-selective epitope conformation influenced by glycosylation. These results support the hypothesis that the glycosylation pattern plays a key role in tumor specificity.

TF antigen mimetics (fluorine-substituted a nalogues in the 6 and 6' positions of the pyranose rings) were incorporated within GVT*S epitope of MUC1 20-mer sequence and conjugated to TTox [139]. These two component synthetic vaccines overrode natural tolerance of the immune system and induced a sufficiently strong immune response in mice. Highly-specific antibodies selectively targeted the tumor-associated MUC1 structures and strongly bound breast cancer cells of the MCF-7 cell line.

Recently, a synthetic MUC1 glycopeptide library consisting of 18 predesigned epitopes of different lengths (20-, 40-, 60-, and 100-mer) carrying Tn, sTn, TF, and/or sTF glycans were used to generate novel anti-MUC1 monoclonal antibodies [140] that utilized BSA and keyhole limpet hemocyanin (KLH) conjugated protein carriers. Two novel antibodies with pre-designed carbohydrate specificities for GalNAc with either an unsubstituted *O*-6 position or its sialylated version were established. Both antibodies recognized specific *O*-glycan structures at the PDT*RP motif, however, one antibody recognized Tn, T, and 2,3-sTF glycans, while the other antibody recognized sialic acid at the same position (sTn, 2,6-sTF, and di-sialylated TF).

A synthetic 7-mer triglycosylated-Tn MUC1 vaccine utilizing only the immunodominant PDT*RPAP region in conjugation with PEG, diethyl squarate linker, and BSA afforded similar IgG antibody levels as the non-glycosylated version [141]. However, when MCF-7 breast cancer cells were challenged with the antisera from the triglycosylated MUC1 vaccine there was stronger binding to the cells in comparison to the non-glycosylated version. The overall findings suggest that use of the immunodominant PDT*RP region in a vaccine design, glycosylated or not, assists in overcoming weak immunogenicity [141].

Enhancement of vaccine specificity can be further achieved by including multi-component architecture in vaccine design. Mannose receptors expressed on the surface of DC and APC cells provide an integral bridge between innate and adaptive immunity. Thus, it was hypothesized that attaching mannose ligands to MUC1 glycopeptide vaccines would lead to more efficient internalization and presentation to T_H-cells and ultimately to endocytosis. The vaccine design included a 22-mer MUC1 peptide sequence with Tn anchored at Ser in the GS*TA region, conjugated to TTox at the *C*-terminus, and either a di-mannose or a tetra-mannose receptor ligand at the *N*-terminal side of a MUC1 epitope [142]. The mannosylated vaccine induced much stronger specific IgG immune responses in mice than the non-mannosylated vaccine control. The tetravalent mannosyl candidate was recognized better by APCs resulting in antibody-dependent cell cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) [142].

4.2. Virus-like particle carriers: RNA Bacteriophage Qβ viral carrier

Due to the inherently weak nature of TACA-MUC1 epitopes alone in generating strong T cell responses and long-lasting memory cells, there is need for T helper cell protein carriers.

Bacteriophage Qβ viral carriers offer safe immunostimulatory effects that can display a repetitive array of antigens on its immunogenic surface that produce an effective immune response. Additionally, these virus-like particles (VLPs) self-assemble into viral envelope proteins in which TA MUC1 peptides can be inserted using covalent conjugation. Several MUC1 glycopeptides were synthesized, made up of one full length tandem repeat region of MUC1 (20- and 22-mer peptides), that were monoglycosylated with the Tn antigen in the PDT*RP or GST*A region [143]. These Qβ vaccine constructs elicited strong immune responses in wild type mice, however, IgG antibodies from tolerant human MUC1 transgenic (Tg) mice did not bind tumor cells strongly. Ultimately, the 9-mer sequence SAPDT*RPAP was identified as immunogenic and a critical protective epitope capable of inducing an immune response in the immunotolerant environment. A Qβ-MUC1 vaccine conjugate with SAPDT*RPAP sequence induced a strong IgG antibody response and exhibited high tumor binding and killing activities. The antibodies were selective toward human breast cancer tissues, suggesting its high translational potential. This same research group recently developed vaccines of a similar design utilizing the TF and sTn antigens which also elicited strong immune responses in immune-tolerant human MUC1.Tg mice [144]. The Tn and sTn glycosylated MUC1 vaccines displayed similar IgG binding levels against the cancer cells. The TF glycosylated MUC1 vaccine produced about four times higher titer levels than the other constructs. Additionally, the Qβ-MUC1-TF vaccine showed *in vivo* efficiency in a lung tumor mice model [144] and has great potential as anticancer vaccine.

4.3. Self-adjuvating multicomponent vaccines

The inherent immunogenicity of carrier proteins is known to suppress the immune response toward the desired MUC1 glycopeptide epitopes. Thus, a new approach incorporates TLRs ligands as part of novel fully synthetic self-adjuvating vaccines. TLRs are expressed on immune cells, macrophages and DC, and act as a bridge between innate and adaptive immunity [145, 146].

Boons and co-workers have designed a tricomponent vaccine that incorporates a TLR2 ligand, $Pam3CysSerK_4$, MUC1 peptide sequence bearing Tn antigen within the TSAPDT*RPAP epitope, and an additional T helper cell epitope derived from poliovirus [147, 148]. This vaccine candidate elicited a robust IgG antibody response able to kill cancer cells by antibody-dependent cell-mediated cytotoxicity (ADCC). The superior properties of the vaccine candidate were also attributed to its ability to produce cytokines at the site where the vaccine interacts with immune cells, thus facilitating maturation of relevant immune cells. Furthermore, it was found that the presence of $Pam3CysSerK_4$ type of TLR ligand and an additional T cell epitope is essential for optimal activity and high titers of IgG [149]. The immune response of the same tricomponent vaccine with a novel Tn antigen mimic (a-methyl serine), was evaluated [150]. The incorporation of the Tn glycan mimetic resulted in more proteolytically resistant construct and an unchanged conformation, however, the immunogenicity didn’t exceed that of the threonine analogue.

It was found that replacement of the TLR2 ligand with a TLR9 ligand, CpG oligodeoxynucleotide (CPG-ODN 1826) was not beneficial [151]. The IgG antibody

response of the TLR2 candidate indicated a mixture of Th1/Th2 immune response with high antibody titers, whereas the TLR9 design produced much lower levels. The incorporation of an sTn antigen at the Thr9 position in the TLR2 based vaccine Construct led to potent humoral and cellular immune responses [152]. Interestingly, the levels of IgG stimulation compared to the Tn glycosylated epitope [151], were lower and the IgM levels were higher, highlighting the superiority of Tn glycosylated vaccine version. Recently, the same group reported use of longer MUC1-derived peptide sequence that incorporates a Tn antigen within the PDT*RP region [153]. This longer MUC1 sequence contains multiple epitopes that can activate B-cells, cytotoxic T-lymphocytes, and helper T cells. It was hypothesized that the exogenous T helper cell epitope is not necessary for efficient immune activation. Ultimately, it was shown that by omitting the artificial linkers and exogenous T helper cell epitopes this vaccine candidate was able to activate T cells and induce high IgG titer levels. This study highlights the importance of the MUC1 peptide sequence (proper length) and glycosylation (glycan position and/or combination of glycans) in eliciting humoral and cellular immune responses.

TLR2 receptor ligands were utilized by the Payne group [154–156] in development of self-adjuvating vaccines with or without exogenous helper T cell epitopes. The ability of lipopeptide, Pam_2Cys (MALP-2 analog) to induce specific antibodies when conjugated to the 20-mer nonglycosylated (GVTSAPDTRPAPGSTAPPAH) or pentaglycosylated MUC1 epitope (GVT*S*APDT*RPAPGS*T*APPAH) *via* a PEG spacer was evaluated [154]. The pentaglcosylated MUC1 bears Tn or TF antigens. The glycosylated vaccine constructs were shown to induce high titers of class-switched IgG antibodies despite the lack of helper T cell epitopes. More importantly, only the Tn glycosylated vaccine candidate displayed cross-reactivity, while the TF glycosylated antibodies were specific and only recognized the epitope to which they were raised. The inclusion of the helper T cell epitope, PADRE, in this two-component vaccine construct with use of the extended 27-mer MUC1 epitope glycosylated at Thr in the PDT*RP region and at Ser in the GS*TA region with a Tn antigen boosted the antibody response and resulted in elevated cytokine production [157].

Li and co-workers [158], utilized a 20-mer MUC1 tandem repeat sequence with a Tn antigen in the PDT*RP region conjugated to the fibroblast stimulating lipopeptide 1 (FSL-1, Pam_2-CGDPKHPKSF) that supports recognition through TLR2 and TLR6 with or without helper T cell epitopes. Immunological results indicate that the tricomponent glycosylated vaccine design stimulated humoral and cellular immune responses that were almost 1.5 times higher than the candidate without the T helper epitope and the antibodies generated from tricomponent vaccine mice bound MCF-7 cancer cells much more effectively.

4.4. Novel delivery systems (self-assembled, nanoparticles, liposomes, polymeric)

Since pathogens such as viruses and bacteria often display multiple copies of ligands on their surfaces, the immune system prefers their multivalent displays. Consequently, when designing a vaccine, it is advantageous to apply a multivalent approach to stimulate B cell receptor crosslinking that ultimately, results in enhanced antibody production. Recently, novel nanostructured scaffolds decorated with multiple copies of an antigen for eliciting robust immune responses have been designed [159]. This review highlights the architecture

and immune response of a diverse set of recently developed nanostructured MUC1 vaccine scaffolds.

Peptide-based self-assembly principle has been used in the field of drug delivery[160, 161], and most recently in vaccine design. Self-assembling peptide, Q11 (Ac-QQKFQFQFEQQ-Am) [162], was conjugated to a tandem repeat sequence of MUC1 glycosylated with a Tn antigen at either the PDT*RP region or the GST*A region or at both positions [163]. These vaccines elicited a high level of antibody response without any adjuvant. They appeared to act through a T cell independent pathway and were associated with the activation of cytotoxic T cells. Most recently, a novel design was proposed by the same group [164]. The synthetic monoglycosylated MUC1 epitopes with Tn linked at Thr in the PDT*RP region were conjugated to a Nap-GFFYK, an unnatural D-amino acid nanovector carrier [164]. These vaccine candidates aggregated into particles of varied diameter (40 to 200 nm) and produced high IgG titer levels that were MUC1-Tn specific. The Nap-nanovector alone was not immunogenic and the IgG class switch was verified by immunizing mice with only MUC1-Tn, confirming they had high levels of IgM antibodies while the nanovector vaccine had minimal IgM antibodies. It was also concluded that complement dependent cytotoxity (CDC) pathway was developed. Additionally, when antisera antibodies from the nanoparticle vaccine immunized mice where presented against MUC1 expressing MCF-7 tumor cells, high binding was observed [164]. Humoral and cellular immune responses were induced as verified by cytokine release analysis.

Hyperbranched polymers form well-defined multivalent platforms that offer unique opportunities for enhancing immunogenicity by optimal multiple antigen presentation. The Kunz research group [165], developed such a fully synthetic multivalent MUC1 polymer vaccine based on hyperbranched polyglycerol (HPG). This globular polymeric carrier, with dendrimer-like structure, was chosen because of its favorable properties, biocompatibility, non-immunogenicity, and solubility in water. In addition to HPG, the vaccine conjugates contained a 22-mer MUC1 glycopeptide with Tn antigen conjugated in the GST*A region or the diglycosylated version with additional Tn conjugated in the PDT*RP region and tetanus toxoid T cell epitope P2 (QYIKANSKFIGITEL). Two types of the HPG-based vaccines were prepared, one carrying 3 monoglycosylated MUC1 epitopes, and other with 6 diglycosylated MUC1 epitopes, offering a more dense antigen presentation. To understand the immunological potential of these vaccines, mice were immunized with the two different vaccines paired with adjuvant. As a control, a diglycosylated MUC1 peptide conjugated to BSA was used. The more dense vaccine candidate produced higher IgG titer levels overall, but it was noted that the end-point titer levels didn’t reach levels elicited by other vaccines that use TTox. However, the MHC-II mediated immune response was observed, and immunological memory was developed. The vaccine carrying the diglycosylated MUC1 sequence consistently had higher IgG subtype titer levels in comparison to the monoglycosylated one, and in addition, exhibited the ability of inducing ADCC and CDC cytotoxicity. Lastly, the antibodies induced by the multivalent vaccine (diglycosylated version) displayed higher binding percentages to epithelial tumor cells.

Liu and co-workers [166], developed a polymer based vaccine that encased the TTox T cell epitope helper with an immunostimulant γ-polyglutamic acid (γ-PGA), and glycosylated

MUC1 sequences with Tn antigen in the PDT*RP and GS*TAP regions. In their studies they made five vaccine combinations that included, 1) one diglycosylated MUC1 tandem repeat sequence that was extended with seven *N*-terminal lysines, γ-PGA, and TTox, 2) same as 1, with an adjuvant, 3) same as 1, without lysine *N*-terminal extensions and adjuvant, 4) same as 1 without γ-PGA and an adjuvant added, and 5) a positive control of the MUC1 sequence covalently attached to the TTox epitope. The multilayer spherical vaccine candidates were all determined to have a hydrodynamic radius of about 355 nm. The first and fifth vaccine models produced equivalent amounts of IgG antibody titers and the third and fourth vaccines elicited the worst immune responses. Surprisingly, the second vaccine model that includes Freund's adjuvant produced about half the amount of IgG antibodies as vaccines one and five. It was proposed that Freund's adjuvant may destruct the multilayer architecture, resulting in a lower immune response. Importantly, the first vaccine model also induced high levels of IgM antibodies, pointing out that the IgM – IgG switch may not have completely happened. When the mouse antisera were incubated with MCF-7 cells, only the first vaccine design showed binding to the tumor cells and successfully killed MCF-7 cells.

The cationic hydrogel type of nano-based scaffold was used in conjunction with MUC1 peptides bearing Tn antigen within the GST*A region and T cell helper P2 (QYIKANSKFIGITEL) epitope derived from TTox [167] or unmethylated cytidine-phosphate-guanosine (CpG) as internal adjuvant for TLR stimulation. The self-assembled hydrodynamic radius of the nanogel vaccines were between 65.4 and 66.7 nm. While both vaccines were able to elicit co-stimulatory cell markers of the immune system, the CpG version was able to induce T cell proliferation. The CpG version vaccine held a higher overall quality of derived antibodies as confirmed by their selective binding for T47D cancer cells.

Functionalized gold nanoparticles carrying CD4+ T cell peptide epitope P30 (FNNFTVSFWLRVPKVSASHLE) from Tetanus toxoid and MUC1 glycosylated with Tn antigen in the GS*T*A and PDT*RP regions were described by Cai and coworkers [168]. It was determined that the glycopeptide-functionalized gold nanoparticles led to an antibody isotope switch from Th2 to Th1 mediated cellular response. Notably, when the gold nanoparticles were removed from the vaccine, the major immune response was Th2 mediated. Antigen mimics based on O → S/Se replacement at the glycosidic linkage were explored in the design of MUC1-functionalized gold nanoparticles by the Corzana group [169]. The MUC1 epitope consisted of a hexapeptide sequence APDT*(O/S or OSe)-RP. The vaccine conjugate tested without any adjuvant, resulted in a significant humoral immune response. Importantly, the mice antisera recognize cancer cells in biopsies of breast cancer patients with high selectivity.

Recently two groups decorated liposome surfaces with an immune complex-targeting ligand, L-rhamnose (Rha) epitope [170, 171]. Rha epitope was used to improve antigen uptake of a glycopeptide sequence containing a CD8+ T cell epitope. Both groups used 20-mer glycosylated MUC1 epitope with Tn antigen attached in the PDT*RP region, but different TLR targeting ligands, Pam_3Cys [170] or Pam_3CysSK_4 [171]. When the Rha epitope was present in the vaccine the IgG titer levels were twice as high as the vaccine without it, demonstrating that CD4+ T cell help was stimulated more effectively in the presence of the

Rha epitope. However, the choice of TLR2 ligand was equally important for *in vivo* enhancement of the response [171]. Liposomes decorated with natural killer T cell (NKT) agonist, α-galactosylceramide (α-GalCer) were prepared in a similar manner [172]. The presence of α-GalCer was expected to activate NKT cells to release large quantities of various cytokines, which may help B cells to proliferate and undergo antibody class switching. The glycosylated 21-mer MUC1 liposomes with Tn antigen in the PDT*RP region was used in preparation of the *N*-terminally palmitoyl lipid analogs, Pam-MUC1 or Pam_2-MUC1. The vaccine designs that didn't have α-GalCer used Pam_3CSK_4 immunostimulating adjuvant instead. Mouse models vaccinated with α-GalCer constructs expressed higher levels of inflammatory cytokines towards Th1 and Th2 subtypes and antibodies from the immunized mice elicited antisera that bound better to MCF-7 cells than the other vaccine models. They were also able to determine that Pam_2-MUC1 significantly boosted the immunodominant response and it was determined to be the result of the conjugated palmitoyl lipid chains effectively mediating complement lysis. Promising lipid-dependent immunological responses were also observed when the TLR1/2 agonist Pam_3CSK_4 was used as an adjuvant.

5. Summary and future perspectives

Several therapeutic MUC1 vaccine designs have shown promise in early clinical trials, nevertheless the observed therapeutic effects were insufficient to reach clinical use. The immune tolerance and heterogeneity of TACA-MUC1 on the surface of tumor cells played a pivotal role in the unsuccessful clinical outcomes. Several glycopeptide epitopes carrying one or multiple TACAs were evaluated in recent studies in wild type and MUC1.Tg mouse models. In addition to varied peptide epitope sequences, different type of TACAs, and their position and density, adjuvants and novel delivery systems were employed in the vaccine designs. Although results were not always easy to compare due to the multiple variables in the experimental design, it has been shown that a diverse array of MUC1 antigen preparations were able to trigger humoral and cellular immunity. However, a better understanding of the immunological determinants of an extended adaptive immune response is still needed. The glycan(s) type, and site(s) of attachment, and/or glycosylation induced conformational changes within the immunodominant MUC1 peptide epitopes are foreseen to play a key role in achieving the full potential of MUC1 glycopeptide anti-cancer vaccines.

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ABBREVIATIONS

TACA(s)	tumor associated carbohydrate antigen(s)
MUC1	Mucin 1
Ser	Serine
Thr	Threonine

MUC1-N	*N*-terminal MUC1
MUC1-C	*C*-Terminal MUC1
GalNAc	*N*-acetylgalactosamine
Gal	Galactose
GlcNAc	*N*-acetylglucosamine
Neu5Ac	*N*-acetylneuraminic acid
Fuc	Fucose
Tn	GalNAcα-*O*-Ser/Thr, Thomsen Nouveau, CD175
TF	Galβ1,3-GalNAcα-*O*-Ser/Thr, Thomsen-Friedenreich, CD176, T antigen
sTn	Neu5Acα2,6-GalNAcα-O-Ser/Thr, sialyl Tn, CD175s
sTF	Neu5Acα2,6- and Neu5Acα2,3-Galβ1,3-GalNAcα-*O*-Ser/Thr, sialyl TF
Cosmc	Core 1 β3GalT specific molecular chaperone
α	Alpha
β	Beta
T-synthase	Core 1 β3galactosyltransferase, β3GalT
Glu	Glucose
ST6GalNAc-I	α2,6-siayltransferase-1
C2GnTs	core 2 GlcNAc transferases
EGFR	epidermal growth factor receptor
ST3Gal-I	α2,3-sialyl transferase
MHC	Major histocompatibility complex
sLe^a	NeuAcα2,3-Galβ1,3-(Fucα1,4)-GlcNAc-R
sLe^x	NeuAcα2,3-Galβ1,3-(Fucα1,3)-GlcNAc-R
NK	Natural killer
DC	dendritic cell
DC-SIGN	Dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin
MR	Mannose receptor

CRD	Carbohydrate recognition domain
S-type lectin	Galectins
Siglecs	Sialic acid-binding immunoglobulin-type lectin, I-type lectins
MGL	Macrophage galactose lectin
T cell	T lymphocyte
T_H	T helper cells
mAbs	Monoclonal antibodies
CD4+	Cluster of differentiation 4 T cells
CD8+	Cluster of differentiation 8 T cells
VCAM-1	vascular cell adhesion proton 1
Tregs	regulatory T cells
IgM	immunoglobulin M
IgG	immunoglobulin G
TTox	tetanus toxoid
ITIM	tyrosine-based inhibition motif
PD-L1	programmed cell death-1
TAM	tumor-associated macrophages

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HIGHLIGHTS

- MUC1 is aberrantly glycosylated and overexpressed in epithelial cancers.
- TACAs of MUC1 include truncated *O*-glycans and their sialylated counterparts.
- Endogenous lectins are receptors for TACAs.
- TACAs are targets for prophylactic and immunotherapeutic cancer vaccine design.
- Factors that govern the design of synthetic cancer vaccines are described.

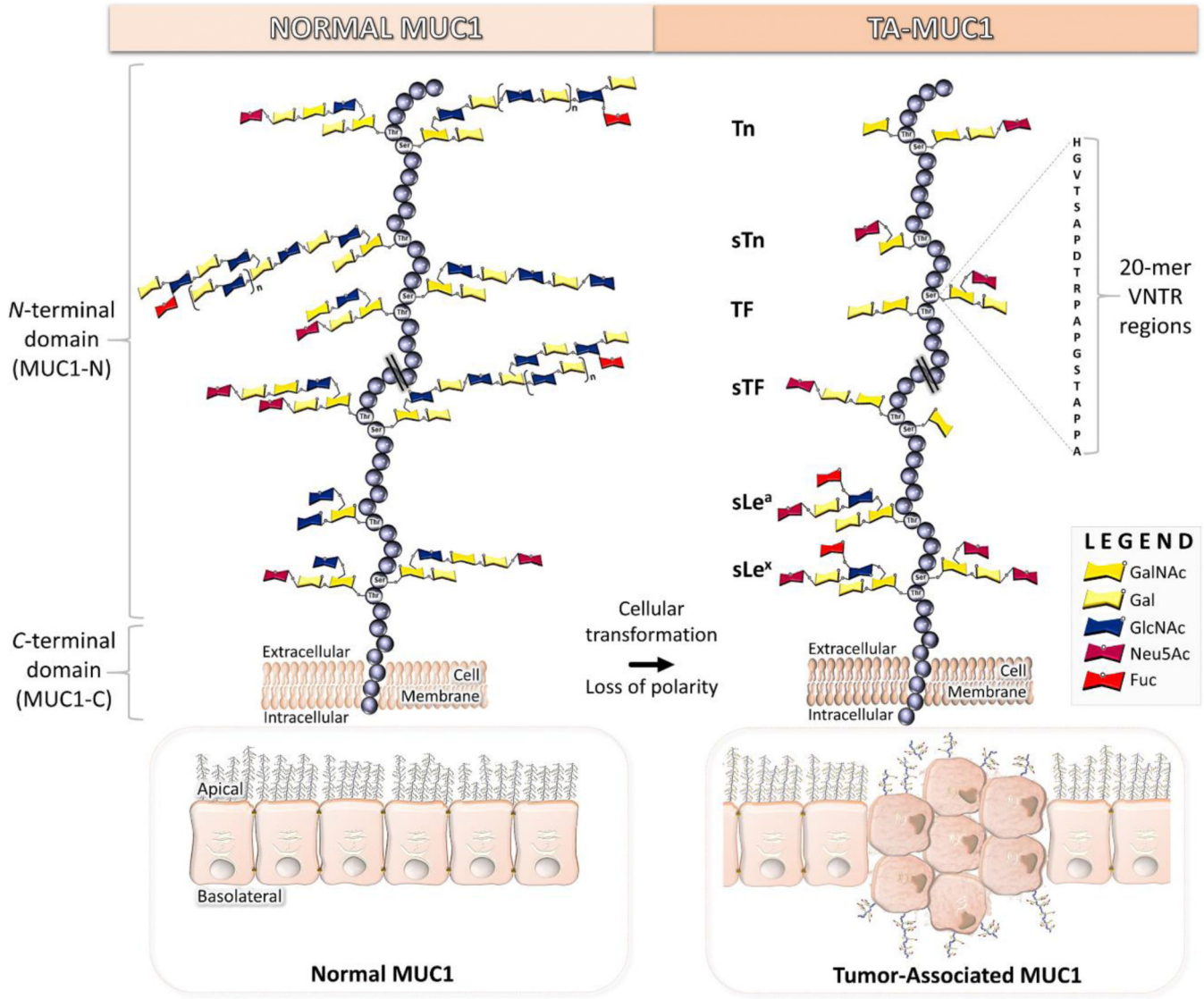

Fig. 1.
Structural differences between normal and tumor-associated MUC1.

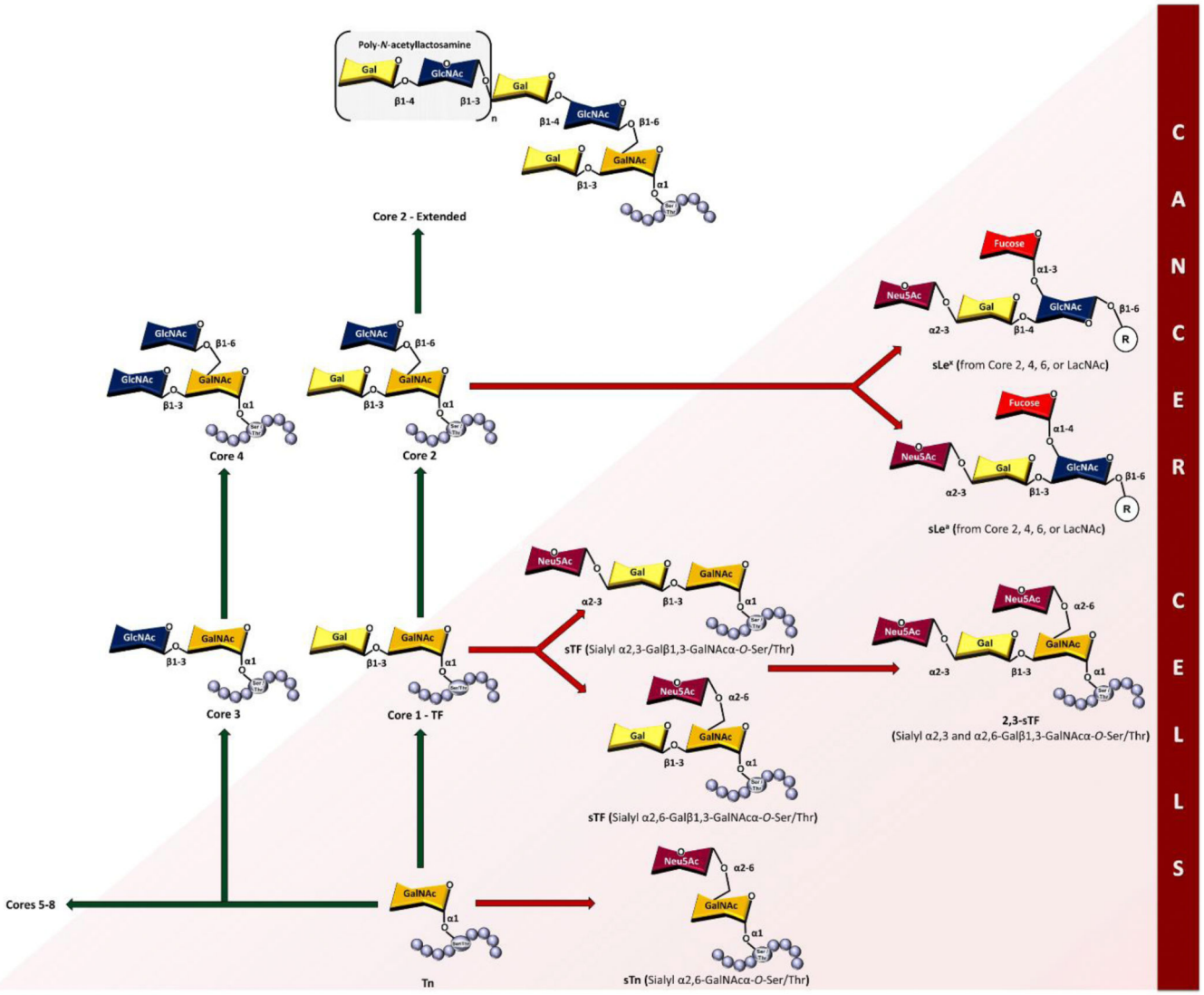

Fig 2.
Core *O*-glycan and TACA structures.

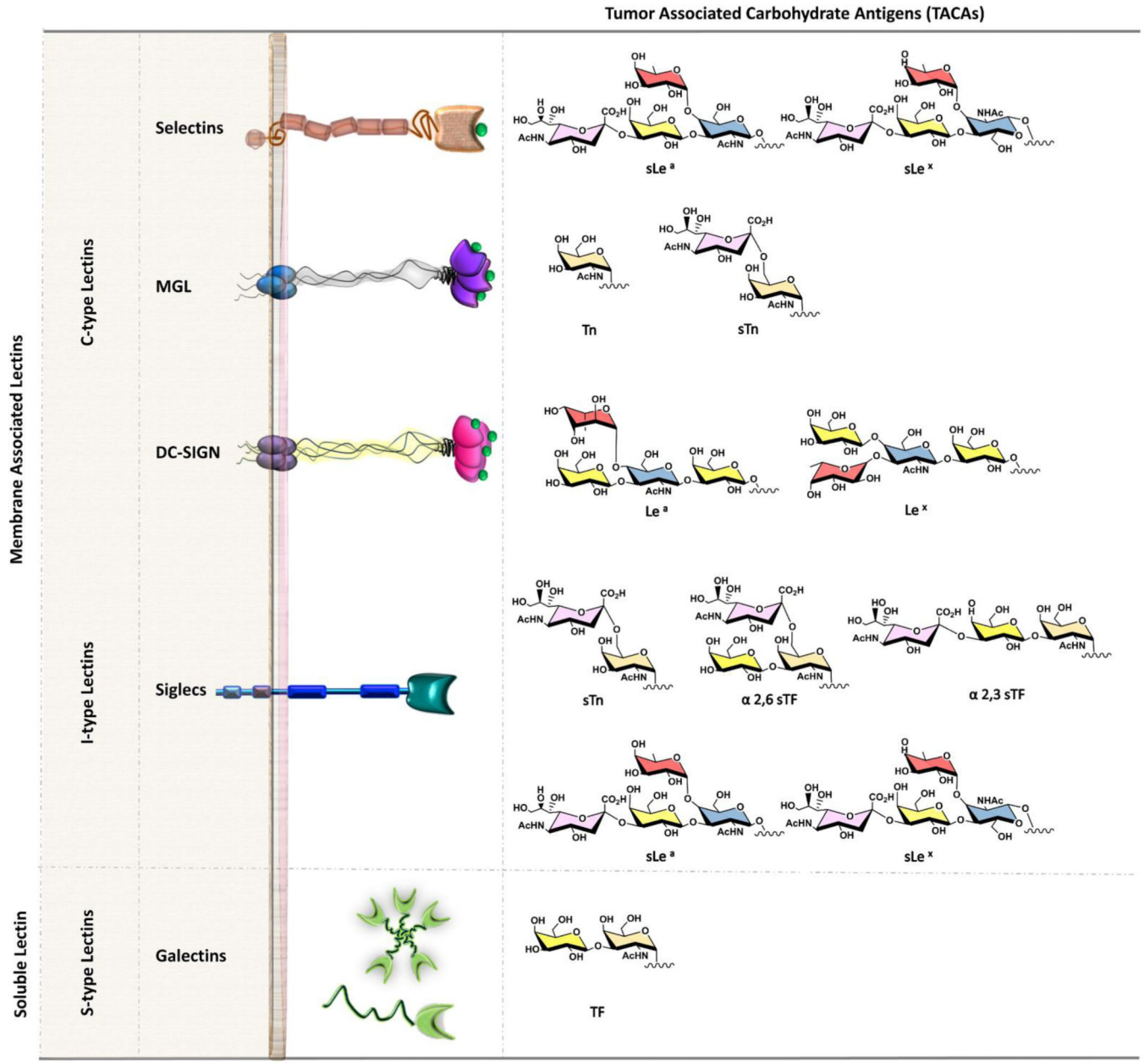

Fig. 3.
Ligands for TA MUC1 and the TACAs that ligate them.

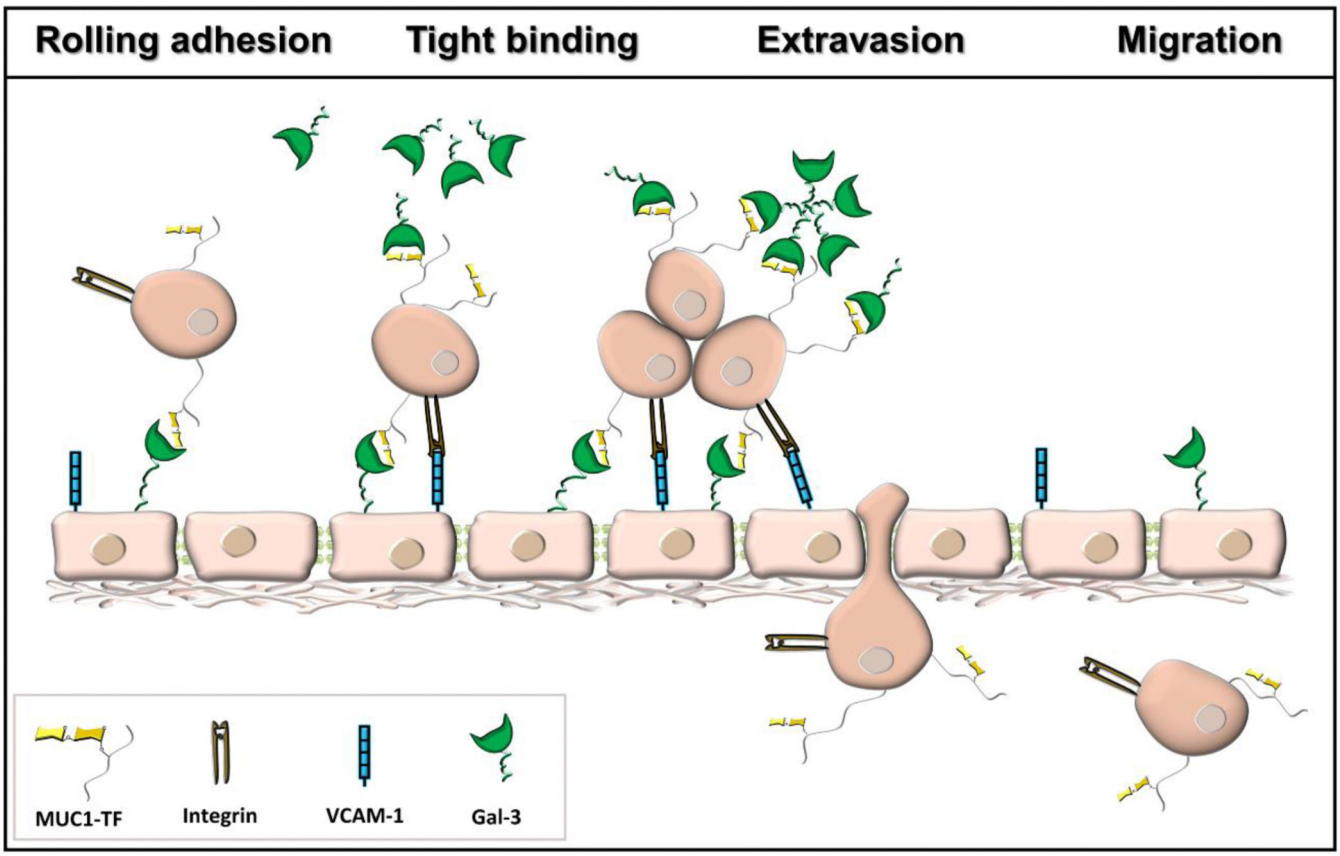

Fig. 4.

Galectin-3 interaction with TF antigen of TA MUC1 promote heterotypic cancer-endothelial adhesion and cancer cell homotypic aggregation. Integrins and VCAM-1 are additional mediators in the adhesion process.

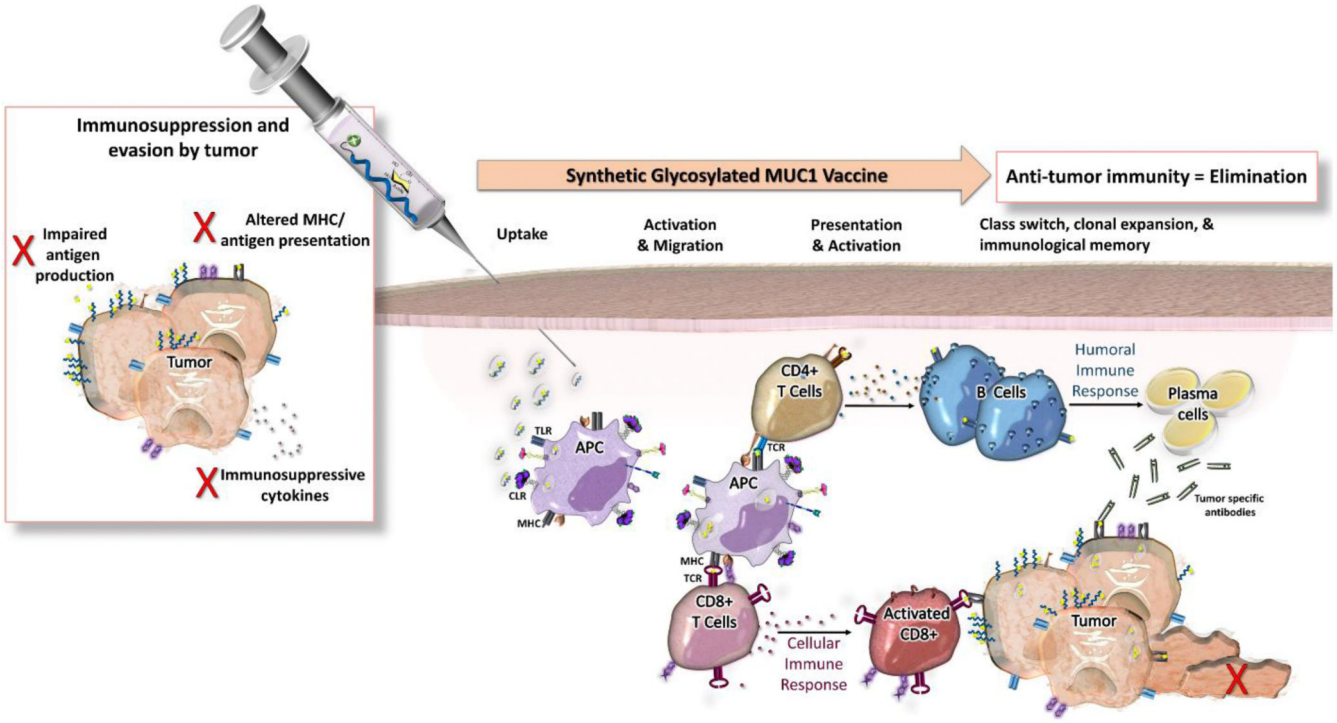

Fig. 5.

Cancer vaccines either aim to prevent precancerous tumors from shifting into a microenvironment that favors regulatory and immunosuppressive mechanisms (preventive) or to restore immunosurveillance (therapeutic).

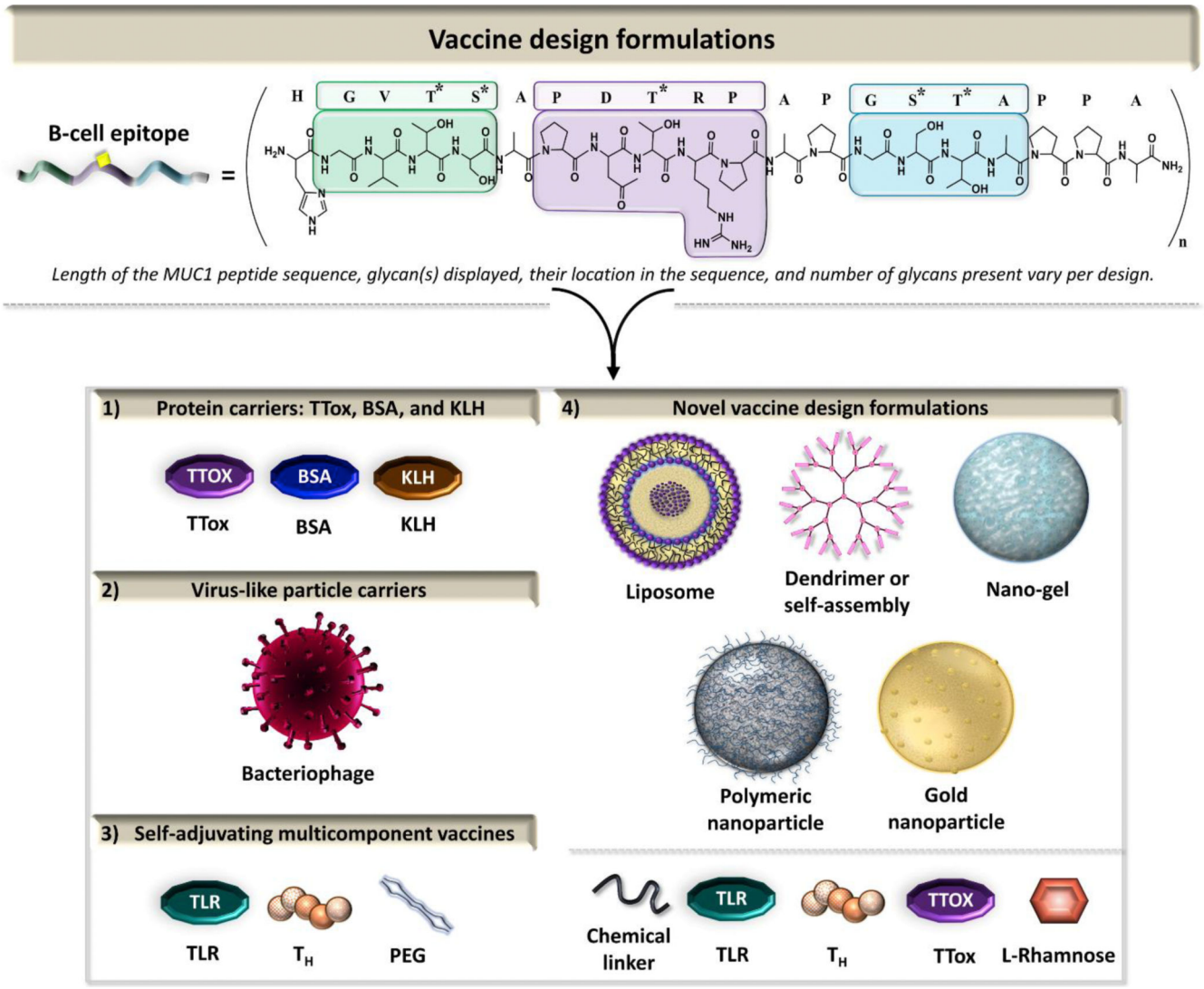

Fig. 6.
Vaccine formulations represented in this review.