Review

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The Development of Vaccines from Synthetic Tumor-Associated Mucin Glycopeptides and their Glycosylation-Dependent Immune Response

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Abstract: Tumor-associated carbohydrate antigens are overexpressed as altered-self in most common epithelial cancers. Their glycosylation patterns differ from those of healthy cells, functioning as an ID for cancer cells. Scientists have been developing anti-cancer vaccines based on mucin glycopeptides, yet the interplay of delivery system, adjuvant and tumor associated MUC epitopes in the induced immune response is not well understood. The current state of the art suggests that the identity, abundancy and location of the glycans on the MUC backbone are all key parameters in the cellular and humoral response. This review shares lessons learned by us in over two decades of research in glycopeptide vaccines. By bridging synthetic chemistry and immunology, we discuss efforts in designing synthetic MUC1/4/16 vaccines and focus on the role of glycosylation patterns. We provide a brief introduction into the mechanisms of the immune system and aim to promote the development of cancer subunit vaccines.

Keywords: Immunotherapy, anti-tumor vaccines, glycoconjugate, subunit vaccines, glycopeptide antigens

1. Introduction

Despite the enormous progress in cancer treatments, the number of cancer deaths increased in 2020 to over 10 million worldwide.[1] (WHO, February 2021) These data call the scientific community to develop new therapeutics. At the beginning of the 20th century, the Nobel laureate Paul Ehrlich postulated that the immune system is involved in carcinogenesis and controlling tumor progression.[2] Despite the complex self-regulatory barriers that the immune system experiences in fighting cancer ('the cancer immunoediting hypothesis'),[3] immune-based cancer therapies, such as active immunization or passive antibody therapies like immune checkpoint inhibitors, were proven to be effective clinically.[4,5] Ideally, the targeted therapy should effectively overcome cancer-induced immune suppression and induce a selective attack on tumor cells without damaging healthy tissue.[6] In this regard, the membrane mucin glycoproteins are promising target structures. While mRNA vaccines[7] recently proved to induce efficient anti-viral (Covid-19) immune responses, their efficiency against tumor tissues in personalized medicine remains limited.[8] While vaccination with viral mRNA induces biosynthesis of viral proteins with appropriate post-translational modification, administration of tumor cell mRNA does not initiate the biosynthesis of protein components with tumor-associated posttranslational habitus. In this respect, the

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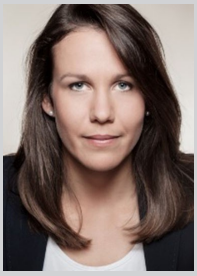

Natascha Stergiou studied Biomedical Chemistry at the Johannes Gutenberg University in Mainz. Her thesis was about the design and immunological evaluation of MUC1 anti-cancer vaccines and a MUC1-specific monoclonal antibody, focusing on the application in a preclinical breast cancer model. In June 2018, she received her Ph.D.; the GDCh awarded her thesis in the pharmaceutical/medical chemistry field. She received the junior breast cancer research award by the Claudia von Schilling Stiftung for her work on preventive immunization and antibody monitoring in breast cancer. Based on her interest in learning new techniques and strategies to evaluate disease-specific targeting with monoclonal antibodies, she went to the Tracer Center Amsterdam as a Post-Doc, where she currently investigates newly developed monoclonal antibodies with ^{89}Zr-Immuno-PET-imaging.

Adele Gabba received her BSc (2012) and MSc (2014) in Chemistry from Insubria University in Italy and her PhD (2019) from National University of Ireland Galway under the supervision of Prof. P. V. Murphy. Her Ph.D. project focused on the synthesis of multivalent glycoclusters to target the Human Macrophages galactose C-type lectin (MGL) for vaccine development and on MGL structural elucidation. In the second half of 2020 she joined the group of Prof. P. Besenius in the Johannes Gutenberg University of Mainz and worked on the ERC funded project CoG SUPRAVACC. She was awarded a Marie Skłodowska Curie individual fellowship to explore the combinatorial dendritic cells' receptors engagement with polymeric antigens, and she is currently working at Massachusetts Institute of Technology in the group of Prof. L. Kiessling.

Moritz Urschbach studied Biomedical Chemistry at the Johannes Gutenberg University Mainz, obtained his B.Sc. degree in 2017 and completed his M.Sc. in 2018. He subsequently joined the group of Prof. P. Besenius as a graduate student where he is currently focusing on the development of self-assembling glycopeptide anti-tumor vaccines. As such he is also part of the DFG funded Collaborative Research Center "Nanodimensional polymer therapeutics for tumor therapy" (CRC 1066).

Horst Kunz studied chemistry at the Humboldt University Berlin and at the Johannes Gutenberg University Mainz. He completed his Ph.D. with Prof. L. Horner on organophosphorus chemistry in 1969, and his 'Habilitation' on protecting group chemistry in 1977. He was appointed Associate Professor of Organic Chemistry in 1979 and Full Professor of Bioorganic Chemistry in 1988 at the Johannes Gutenberg University Mainz. His research interests concern stereoselective reactions, syntheses of alkaloids, peptides and carbohydrates, in particular, syntheses of glycopeptides. He was awarded the Max Bergmann Medal in 1992, the Emil-Fischer Medal in 2000, and the Adolf Windaus Medal in 2001. In 1998, he was elected corresponding member of 'Sächsische Akademie der Wissenschaften zu Leipzig' (Saxony Academy of Science). He became Senior Research Professor in 2010 and retired in 2013.

Edgar Schmitt studied Biology at the Johannes Gutenberg University Mainz, completed his Ph.D. in 1986 and his 'Habilitation' (venia legendi for Immunology) in 1997 at the University Medical Center Mainz. He has held the professorship for cellular immunology since 2002 at the Institute for Immunology and since 2003 is the deputy head of the institute. During his career, he was involved as a speaker in various large transregional research projects and has been a member of the Research Center for Immunotherapy executive board since 2007. His research focus is on T- and B-cell immunology and the development of cancer vaccines. His collaboration with Prof. Horst Kunz for more than 20 years led to numerous high-impact publications in the field of glycopeptide cancer vaccines. Together with Prof. Pol Besenius, he has continued working on new delivery systems for the tumor-associated glycopeptide vaccines.

Pol Besenius was born and raised in Luxemburg, and studied Chemistry in Vienna and Glasgow. He received his Ph.D. from the University of Strathclyde in Glasgow (2008) under the supervision of Prof. D. Sherrington and Prof. P. Cormack. As Marie-Curie Fellow Pol undertook postdoctoral studies at the Eindhoven University of Technology with Prof. A. Palmans and Prof. E.W. "Bert" Meijer (2008-11). He started his independent research group at the Organic Chemistry Institute in Münster supported by a Liebig Fellowship in 2011, and moved to the Johannes-Gutenberg University of Mainz in 2015 to take up a Professorship in Macromolecular Chemistry. Pol was elected as young fellow to the North Rhine-Westphalian Academy of Sciences and Arts (2013–14). In 2018 he was awarded an ERC Consolidator Grant, and since 2018 he acts as editor for the newly launched Thieme journal 'Organic Materials'. His research interests include synthetic macromolecular chemistry, self-assembly in water and at interfaces, responsive supramolecular polymers, viromimetic particles and synthetic vaccines.

cell surface membrane mucins with a tumor-typical structure remain most promising antigens for anti-tumor vaccines.

Mucins 1, 4, and 16 (MUC1, MUC4, and MUC16) are strongly overexpressed in most epithelial cancers including cancers with high incidence and high mortality[9] as breast, ovarian, lung, colorectal, prostate, pancreas, and stomach.[10–12] In most cases, MUC overexpression has been shown to correlate with resistance to apoptosis and promote tumorigenesis and metastasis.[13,14] In many scenarios, it led to poor prognosis. The expression level of mucins is altered in malignancies, but also abnormal glycans can be found on the MUC surface.[15,16] Due to a reduced glycosyltransferase activity with a simultaneously increased sialyltransferase activity in the tumor tissue,[17] the mucin glycans differ remarkably from mucins on healthy cells. These aberrant structures, referred to as tumor-associated carbohydrate antigens (TACAs), carry much shorter glycan chains. As a consequence, the peptide backbone on tumor cells is accessible to the immune system, for instance, to humoral antibodies that can be induced by active immunization or to monoclonal antibodies developed explicitly against tumor-associated (TA) mucins.[18,19] Specific TA mucin (MUC1/MUC4/MUC16) antigens can neither be obtained from tumor cell lysates as an antigen for an immunization, nor can the tumor cell lysate be used directly as a vaccine. A possible autoimmune reaction against healthy epithelial tissue poses too great a danger. To avoid this problem, chemically defined TA MUC glycopeptide structures can be obtained synthetically as tumor-associated antigens (TAAs) in high purity.[20–23] In more than 34 years of research in the design of glycopeptide vaccines,[24] we have investigated the preparation and immunological evaluation of MUC vaccine constructs with diverse glycosylation patterns, which elicit antibodies with different features in terms of isotype, strength and specificity.[8,25–27] This review discusses recent MUC1, MUC4, and MUC16 subunit cancer vaccine candidates, focusing on the importance of glycosylation.[28–33] Furthermore, a brief introduction into the mechanisms of the immune system and the design of subunit vaccines is provided for the interdisciplinary readership of Chemical Record.

2. Tumor-Associated Carbohydrate Antigens (TACAs)

Glycosylation is one of the most abundant posttranslational protein modifications and is indispensable for countless biological processes like cell differentiation or signal transduction. When chemically linked to cell surface proteins, glycan patterns build unique structural features, which are presented towards the extracellular space and thereby act as specific recognition motifs. These are decisive for cellular identity. However, the composition of those patterns is not directly determined by a genetic blueprint but a perfectly orchestrated process involving numerous glycosyl-transferases and glycosidases. Therefore, it is not surprising that oncological transformations, and altered cellular enzyme activity, are accompanied by aberrant protein glycosylation.[34,35] The resulting carbohydrate motifs are referred to as "tumor-associated carbohydrate antigens" (TACAs). Whereas long carbohydrate chain mucin-type *O*-glycans are present under physiological conditions, TACAs are found to be truncated, less branched, and more likely to be sialylated (Figure 1).[36,37]

Generally, *N*-GalNAc is the first saccharide to be transferred onto the protein backbone, which is subsequently elongated by β1,3-galactosyltransferase to give the core 1 motif (Galβ1,3-GalNAcα-*O*-Ser/Thr). Reduced expression of core 2-β1,6-*N*-acetylglucosamine transferase, responsible for elongating core 1, results in an accumulation of the latter. Springer and coworkers therefore identified the Thomsen nouveau (T_N, GalNAcα-*O*-Ser/Thr) and the Thomsen-Friedenreich (T, core 1) antigen (Figure 1) as general carcinoma autoantigens.[38] Overexpression of sialyltransferases further results in sialyl T_N (ST_N) and sialyl T (ST) derivatives. Besides core glycans, the alteration of terminal glycan structures is a common feature of malignant transformations. Upregulated fucosylation activity[39] causes accumulation of Lewis a/b or Lewis x/y and, most importantly, their sialylated derivatives $SLe^{a/b/x/y}$. The immunological evaluation relies on the synthesis of glycopeptides presenting defined TACA motifs. In this regard, synthetic organic chemistry still outperforms chemoenzymatic or biotechnological approaches that lack regioselectivity in the presence of multiple serine and threonine residues in MUC sequences. Chemists have optimized sophisticated strategies towards fully synthetic mucin glycopeptides over the last decades. The critical step usually involves the glycosylation of a protected amino acid building block, which is subsequently used in solid-phase peptide synthesis. We refer the interested reader to comprehensive reviews covering the organic synthetic approach using protecting group strategies[40–42] or methodologies that rely on chemoenzymatic routes.[43–47]

Pathogen recognition receptors located on antigen-presenting cells (APCs), the sentinels of the immune system, and antibody-producing B cells can recognize TACAs and trigger an efficient immune response.[48,49] In this sense, cell surface glycans can function as cellular IDs.[50] The structural identification of these unique fingerprints characterizing tumors as well as pathogens have allowed chemists to synthesize structural analogs that are presented to the immune system and evoke an adaptive immune response against those cells displaying the 'cancer or pathogen ID'.

In the presented tutorial-type review, we discuss how the identity, abundancy, and location of the glycans on the MUC backbone influence the cellular and humoral immune re-

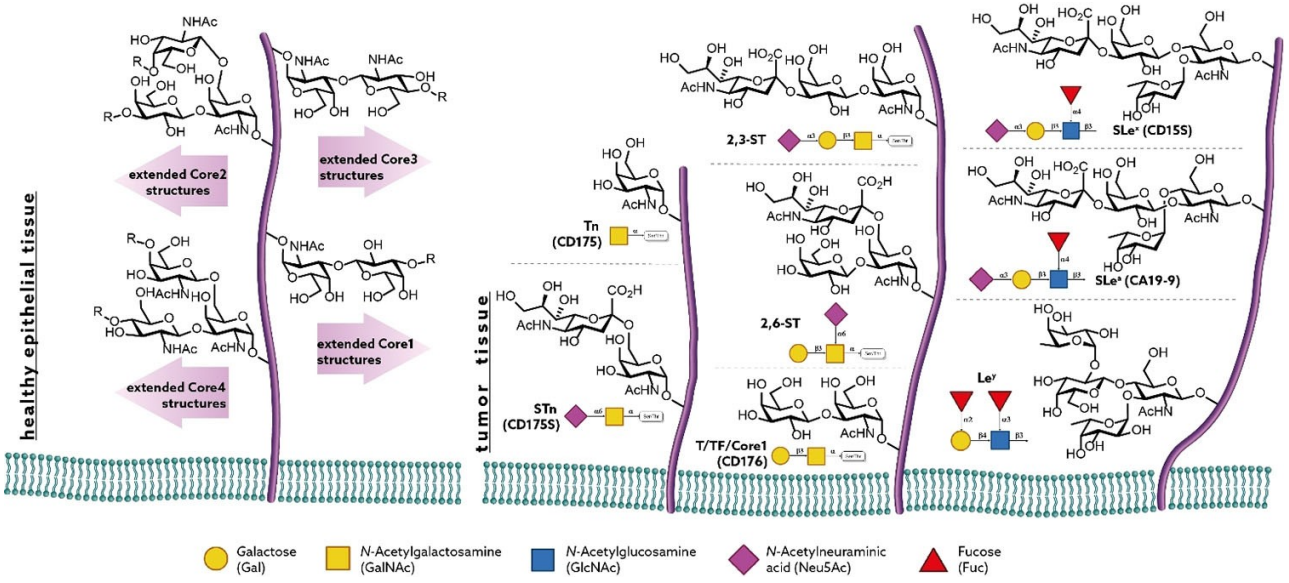

Figure 1. Schematic and molecular representation of mucin type *O*-glycosylation patterns in physiological (left, R = long and partially branched glycan chains) and tumor tissue (right).

sponse. Based on some of our own activities in more than two decades of research in the development of MUC glycopeptide anti-tumor vaccines, we bridge synthetic chemistry and immunology, to focus particularly on the role of glycosylation patterns in the development of cancer subunit vaccines.

3. Immunization as an Active Therapy to Stimulate the Immune System in the Fight against Cancer

Immunization as an immunotherapeutic strategy can re-initiate the body's immune system in recognizing TAAs. It can be given prophylactically or as immediate treatment. The most important cells to be targeted are (a) B cells, which will produce TAA-specific antibodies and could develop long-term protection; (b) various T helper (Th) cells that modulate the strength, duration, and efficiency of the immune response; and (c) cytotoxic T lymphocytes (CTLs), that are capable of directly attacking the tumor tissue.[51]

Carbohydrates play a double function in TAA vaccines. On the one hand the TACAs function as antigens themselves and on the other hand they influence the conformation of the peptide backbone,[52] which are recognized by the immune cells. These mucin based glycopeptide structures present a multivalent glycopeptide scaffold, and thus are ideal ligands for B cell receptors. They cross-link the receptors expressed on the surface of B cells and induce the vaccine endocytosis. B cells are at this point partially activated. They differentiate in plasma B cells and can already produce TAA antibodies. This immunological response is defined as a T cell-independent pathway, and the antibodies arising from it, IgM and IgD, are short-lived and have a comparatively low affinity for 'their' antigen. They are therefore not particularly specific. To achieve long-lasting immunity and specific antibody responses, other players of the immune system need to be involved in such a humoral response. This activation process is called T cell-dependent immune response (Figure 2): After injection, the vaccine construct encounters the APCs at the injection site, for instance, dendritic cells (DCs). The glycopeptide moiety will be recognized by receptors located on the surface of APCs (e.g. DC-SIGN, MGL, Selectins, Siglec)[53,54] triggering antigen endocytosis. Once inside the cell, the vaccine construct is lysed and brought again to the APC surface, where the peptide fragments are bound to the major histocompatibility complex II (MHC II) and presented to naïve $CD4^+$ helper T cells. The response of helper T cells does not only depend on the receptor-ligand interaction. Co-stimulatory molecules presented by the APCs and the cytokine environment during activation drive a Th1, Th2 or Th17 cell response. Th1 cell activation is for example promoted by TNF-α and Th2 cell activation by IL-4. In section 4, the role of synthetic immunostimulants and -modulators for the design of vaccines is discussed further.[55,56] After binding to the MHC II-associated antigen, Th cells differentiate into activated T cells (e.g. Th1 and Th2) and release cytokines such as IFN-γ (Th1) and IL-4 (Th2), which, together with co-stimulatory signals (CD40L/CD40, CD28/B7) control the differentiation of B cells into antibody-producing plasma cells and memory B cells. Maturation involves the induction of isotype class-switch recombination. The Th cell cytokines determine the immuno-

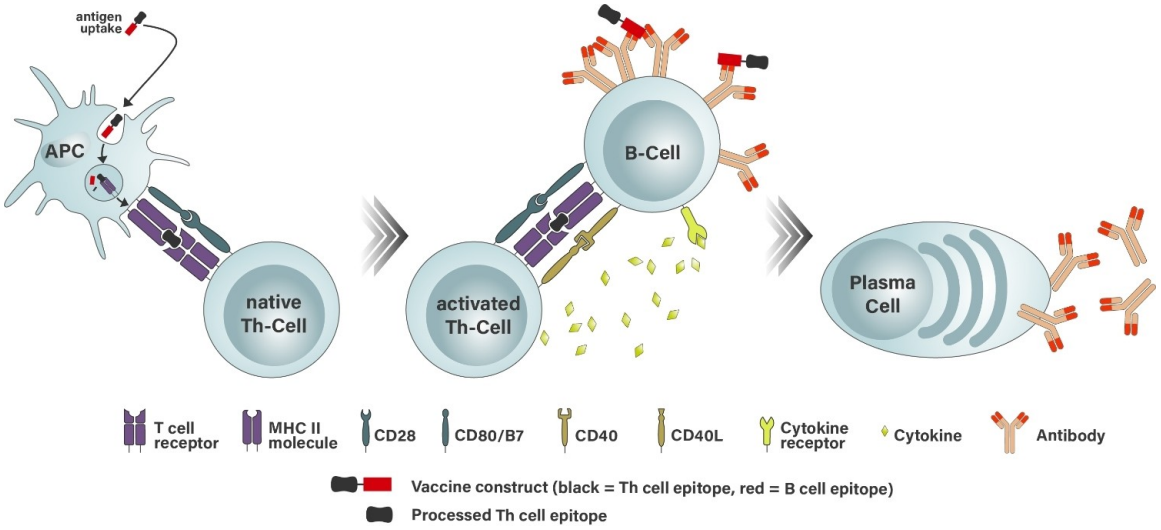

Figure 2. Schematic illustration of anti-MUC antibody induction by a synthetic vaccine construct. APCs internalize the vaccine molecules by endocytosis, followed by antigen processing. The resulting fragments are loaded onto MHC-II molecules and presented to Th cells. (left) The activated Th cells support the differentiation of B cells (which also present the Th epitope vaccine fragment on MHC-II) into antibody-secreting plasma cells. (middle to the right).

globulin isotype, preferentially in the direction of the specific long-lasting IgG isotype family (e.g. IgG1, IgG2a, IgG2b, IgG2c, IgG3). The IgG antibodies will undergo a somatic hypermutation in the region of the antigen-binding site by DNA polymerases which are induced by the binding of the Th cell to the B cell. This point mutation will contribute to the affinity maturation of the antibodies resulting in a roughly hundred-fold increase of the antibody affinity at the end of this process. Furthermore, Th1 can create a cellular immune response by releasing IFN-γ and TNF-α.[57,58] Th cells are therefore responsible for initiating and maintaining the humoral and cellular immune responses against TAAs.

The development of the B-cell memory is critical for the long-term success of an immunization. Memory B cells are characterized by the rapid production of high levels of high-affinity antibodies.[59] In tumor immunology, the anti-tumor role of B cells and their neutralizing tumor-specific antibodies has long been underestimated. Antibodies initiated with glycopeptide vaccines represent the most specific weapon. The bound tumor-specific antibodies can (i) disrupt the function of the TACA, (ii) activate the complement cascade (CDC),[60] and (iii) cause the attack of natural killer cells (NKs) through antibody-dependent cell cytotoxicity (ADCC). The tumor antigen uptake by local APCs and the presentation of the processed antigen is suppressed in the tumor microenvironment. The antibodies bound to the tumor cells can simplify the presentation and cross-presentation of the TAAs by DCs, which reactivate CTLs. In addition to their role in the rapid production of high-affinity antibodies, memory B cells can also act as efficient antigen presenting cells, promote T cell expansion and subsequent memory formation.[61] They represent a versatile weapon in the fight against tumors.

4. Vaccine Design

The structure of the vaccine should include decisive components needed to engage all the cell receptors involved in the immune response. To create a specific antigen-dependent antibody response following vaccination, it is crucial to understand that the TA glycopeptides function as B cell epitopes. To achieve the needed T-B cell interaction, the B cell epitope/glycopeptide (hapten) is conjugated to Th cell epitopes in the form of proteins or peptide epitopes, which serve as carriers (hapten-carrier-principal)[62–64] to enhance the immunogenicity via activation of the adaptive immune system. B and Th cell epitopes must be linked, which in most applications is achieved using a covalent, flexible, non-immunogenic spacer, to make sure that the TAA conformation is not affected. B cells need to process and present the Th cell epitope coupled to them after receptor-mediated endocytosis of their epitope and thus recruit the necessary T cell help ('linked recognition').[65] A prominent example is the tetanus toxoid (TTox) carrier protein, which is strongly immunogenic when conjugated to MUC glycopeptides.[66] However, the synthesis and analysis of these glycoconjugate vaccines is associated with challenges, and a complete characterization of the TTox-conjugated glycopeptide vaccines is essential for clinical application. Therefore, choosing immunodominant oligopeptide domains from the TTox protein enables the production of fully synthetic, molecularly defined constructs.

When choosing the Th cell peptide, it must be ensured that the peptide has already been identified as a T cell epitope and that there are no MHC restrictions. A very promising peptide is p30, a precisely defined 20 amino acid partial sequence of the full TTox protein (tt947-966).[67] p30 is considered a 'universal' immunogenic Th cell epitope since it is recognized by all MHC haplotypes,[68] and is also found to bind to many human leukocyte antigen (HLA) molecules such as DR7, DR9, DRw11, DPw2, and DPw4. The p30 20mer peptide sequence contains three different Th cell epitopes, each of which is recognized in connection with different MHC-II molecules.[69] When comparing the immunological response between a TTox protein conjugate and a p30 molecular conjugate of the same glycopeptide,[70] the TTox conjugate reveals more than a 133fold stronger IgG titer.[71,72] Both vaccines preferentially induce IgG1, but the binding affinity toward T47D cells was twice as high for the TTox derived antisera. In addition to p30, TTox as a whole protein has other 'universal', immunodominant Th cell epitopes (p2 and p4) in its amino acid sequence.[69] The high density of Th cell epitopes in the protein carrier enables increased activation of Th cells. Next to tetanus toxoid carrier proteins, diphtheria toxoid CRM_{197}, keyhole limpet hemocyanin (KLH), immunogenic oligopeptide sequences thereof or synthetic universal epitopes have been used (see also section 6). The interested reader is further referred to recent literature examples[73–76] and review articles[77–82] for glycoconjugate vaccines which cover the use of various Th cell epitopes more extensively.

As mentioned before, tumor cells can actively suppress an immune response, e.g. through the reduction or even absence of APC proliferation in the tumor environment, down-regulation of the MHC molecules on the APCs and associated reduced antigen presentation.[83–85] Various clinical and pre-clinical studies also report the need to use adjuvants to stimulate APCs e.g. via Toll-like receptors (TLRs) like TLR2, TLR3, TLR4 and TLR9.[86] New clinical studies using immunostimulatory adjuvants such as the TLR-agonists CpG-ODNs (TLR9) and Poly(I:C) (TLR3) have proven their therapeutic potential as vaccine adjuvants and direct anti-tumor substances.[87] In the past, vaccines have often been formulated as mixtures of antigen plus adjuvant.[88,89] Challenges arise if the release kinetics of independently diffusing entities are not controlled. Covalent linkage of the different antigen and adjuvant components has emerged as a promising approach resulting in 'self-adjuvanting' vaccines (Figure 3), which have the advantage that APCs activated by the adjuvant are the same APCs exposed to the antigen.[90]

To date, researchers have pursued various strategies to overcome immunosuppressive mechanisms. Not only the use of built-in or external adjuvants such as the TLR ligands,[88,89,91,92] but also the multivalent presentation of antigens on polymers as carrier materials[93,94] and their use as

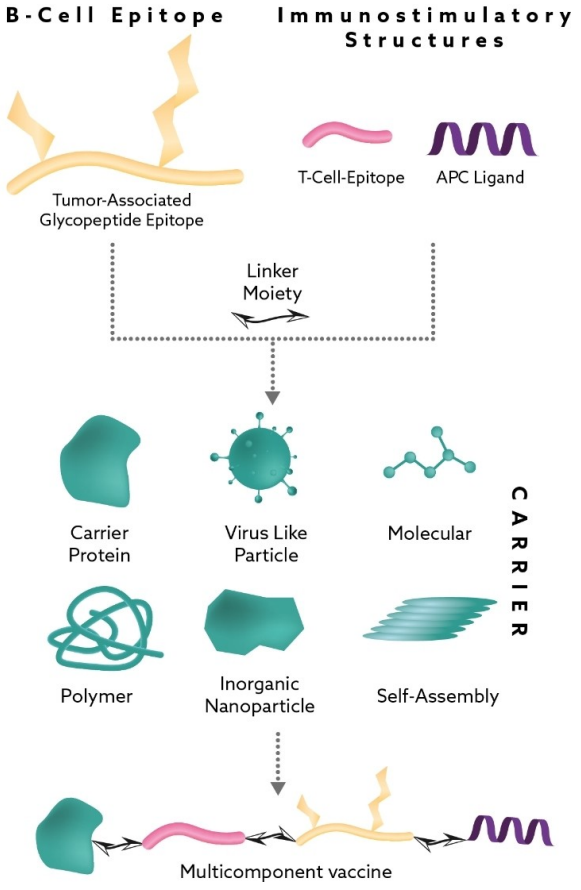

Figure 3. Design of a glycopeptide anti-tumor vaccine. The B-cell glycopeptide epitope requires additional immunostimulatory structures to elicit an adequate antibody response. As co-presentation of the components is crucial, the B and T cell epitopes must be linked to each other directly or be presented on the same carrier. Note that the carrier can also fulfil the task of an immunostimulatory structure (e.g. TTox carrier protein acts as Th epitope).

delivery systems[89,94] are successful strategies to overcome endogenous immune-suppressive mechanisms that can occur when using altered endogenous TAA. Another promising strategy is to use ligands to target APCs such as macrophages and DCs.[95] Adjuvant APC stimulation thereby results in the induction of cytokines, increased expression of MHC II, membrane-bound co-stimulatory signals (e.g. CD80, CD86), all promoting Th differentiation.[96] The type of APC stimulation is further crucial for the quality of Th differentiation in terms of polarization.[97]

The mechanisms mentioned above require spatial proximity of B and T cell epitope and immunostimulant, to ensure the simultaneous uptake of the components. The use of flexible, inert 'spacer molecules' serves to spatially separate the adjuvants and the Th cell epitope from the B cell epitope, the glycopeptide, in order to exclude a potential influence on the

glycopeptide conformation. In addition, optimization concerning the type of vaccine injection (subcutaneous vs. intraperitoneal), dose, and frequency of the immunization is important. In our lab recent immunizations using MUC1-glycopeptide vaccines in mice are done 3–4 times in total, every two weeks, with 10 μg of the vaccine. Based on our experience, a stronger immune response is achieved through immunization when working with adjuvants such as CFA (Complete Freund's Adjuvant) or IFA (Incomplete Freund's Adjuvant), which form a water-in-oil emulsion with the vaccine. This usually results in efficient uptake of the antigen by the relevant immune cells, such as DCs and macrophages. In addition, there is a non-specific activation of macrophages by the emulsifier contained in CFA and IFA.[98] Many preclinical studies are performed with CFA for primary immunization. CFA is toxic for mice and not permitted in humans. Our experience shows that primary immunization in CFA does not lead to stronger humoral immune response than in IFA if suitable vaccine formulations are used. To evaluate the performance of the designed vaccine, careful analyses after the last immunization are necessary: detection of the amount of initiated TAA-specific antibodies (enzyme-linked immunosorbent assay, ELISA), determination of the antibody isotypes (ELISA), and the specific binding of the induced antibodies to human cancer cells expressing the TAA as target (fluorescence-activated cell sorting, FACS). In the case of vaccines with adjuvants that target specific immune cell types, an *ex vivo* analysis of the proliferation and activation of those target cells is necessary.[88,95]

5. Structures of MUC1/MUC4/MUC16 on Epithelial Cells and Tumor Cells

5.1. The Mucin MUC1

MUC1 also described as Episialin, EMA, CA15-3, is a transmembrane member of the mucoprotein family. It was the first mucin to be characterized structurally.[99] It is expressed on almost all epithelial tissues. In healthy cells, the extensive, negatively charged glycans form a mucinous gel and create a physical barrier that protects the underlying epithelia from drying out, changing the pH value, pollutants, and microbes.[100] The glycoprotein consists of two peptide fragments, the long extracellular N-terminal subunit (MUC1-N) and the short C-terminal subunit (MUC1-C) protruding into the cell interior. Both subunits are connected extracellularly via stable hydrogen bonds in the SEA domain ('Sea urchin sperm protein,' Enterokinase, Agrin) forming a heterodimeric complex. MUC1-N is located on the cell surface and consists of proline (P), serine (S), and threonine (T)-rich 20 amino acid-long repeating domains (PAHGVTSAPDTRPAPGSTAP) with five possible *O*-glycosylation sites, which are also known as 'variable number of tandem repeat' (VNTR) region (Figure 4B).[101] Proline acts as a helix breaker and favors the elongated form of MUC1. In MUC1, the tandem repeat is repeated 20 to 120 times.[102] The MUC1-C consists of a transmembrane domain and a cytoplasmic domain. The cytoplasmic tail contains highly conserved serine and tyrosine residues, phosphorylated by growth factor receptors and intracellular kinases, and thus offer binding sites for other signaling molecules such as PI3K, NF-κB, and β-catenin.[103] In healthy tissues, MUC1 is heavily glycosylated in the extracellular subunit and the glycosylation pattern varies greatly depending on the glycosyltransferases expressed in the tissue. Three serines and two threonines provide five potential *O*-glycosylation sites in each tandem repeat at which polysaccharide side chains can enzymatically be built up in the Golgi apparatus.[104] The structure of the saccharide chains always begins with *N*-acetyl-galactosamine, to which the specific glycosyltransferases can attach additional components such as galactose, fucose, or sialic acid (*N*-acetylneuraminic acid). A distinction is made between various typical carbohydrate structural units described as core structures.[103] *O*-Glycosylation correlates with the biological properties of MUC1 to protect the cell surface from chemical and mechanical effects.[105] The peptide backbone is shielded by the dense glycosylation, thus preventing the protein from undergoing proteolysis. Compared to human MUC1 (huMUC1), murine MUC1 (muMUC1) contains a shortened VNTR region (16 instead of 20 amino acids) and is only 34% similar to the human sequence.[106]

MUC1 is overexpressed in epithelial tumors such as breast, ovarian, pancreatic, prostate, and colon cancer and glycosylated aberrantly in the tumor tissue due to reduced glycosyltransferase activity and increased activity of sialyltransferases. This creates the already mentioned TACAs. Due to the aberrant glycosylation in the MUC1-N domain, TA MUC1 differs significantly in biochemical properties and its cellular distribution from MUC1, expressed in healthy epithelial cells. Based on this, the TA MUC1 can be referred to as a neoantigen. The shortened carbohydrate side chains of TA MUC1 lead to a loss of cell polarity, i.e., TA MUC1 is no longer expressed apically but ubiquitously on the surface of a tumor cell.[107] The loss of cell polarity can induce the detachment of a tumor cell from the tissue and thus favor the formation of metastases.[108,109] TA MUC1 in breast cancer cells mainly carries (sialylated) T_N- or T-antigens as basic building blocks, since the glycosyltransferase core-2 β1,6-*N*-acetylglucosamine transferase is not expressed at all or is expressed to a reduced extent, thus preventing the formation of core 2-*O*-glycans.[38,110] MUC1 overexpress the sLe^x and sLe^a (Figure 1) epitopes in colon cancer cells and shows a decreased *O*-acetylation.[111] In addition, TA MUC1 is strongly sialylated, which prevents the elongation of glycan side chains. The

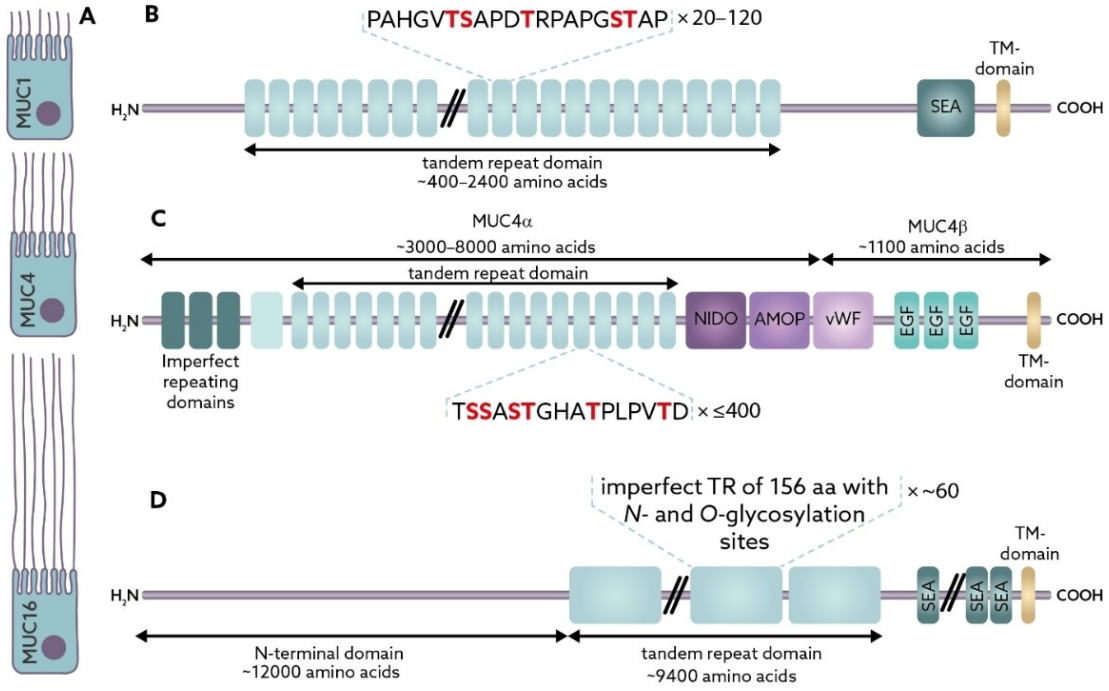

Figure 4. Schematic comparison of the structural features of MUC1, MUC4 and MUC16. A: relative sizes of the mucins on epithelial cells. Schematic structure of MUC1 (B), MUC4 (C) and MUC16. Short intracellular C-termini are followed by a transmembrane (TM) domain followed by differing structural domains like Sea urchin sperm protein, enterokinase, agrin (SEA), epidermal growth factor-like (EGF) domain, unique von Willebrand factor (vWF)-, adhesion-associated- (AMOP), and nidogen-like (NIDO) domains. The highly glycosylated tandem repeat (TR) domains are dominating the structure.

α2,3- and α2,6-sialyltransferases-I are overexpressed here in breast cancer cells. This results in more sialylated core 1 glycans such as the ST_N and ST antigen.[112] The shortened saccharide side chains also have the consequence that the conformation of the peptide backbone changes since the carbohydrates have a decisive influence on the shape of the glycopeptide.[52] Partial sequences of the peptide backbone adopt a 'turn' conformation[113] and protrude from the otherwise linear polypeptide. This makes immunodominant epitopes accessible to immune cells or antibodies.[114,115] In fact, antibodies against TA MUC1 were detected in the serum of breast and ovarian cancer patients.[116–118] Clinical studies have been initiated to evaluate the use of antibodies to MUC1 and of immunogens based on MUC1 for immunotherapy of patients.[10] In this way, peptide epitopes were identified within the VNTR region immunodominant in B and T cell responses. The epitope with the most potent immunological effect contains the PDTRP partial sequence.[119–121] In addition, hypoglycosylation enables the cleavage and release of MUC1-N (soluble MUC1 (sMUC1)) by extracellular proteases.[122] An evident overexpression of TA MUC1 is a clear indication of tumor progression and an increased risk of metastasis. Accordingly, an increase in the expression of TA MUC1 correlates with a poor prognosis.[123,124]

5.2. The Mucin MUC4

Like MUC1, MUC4 on healthy cells is heavily glycosylated. It comprises two subunits connected by a putative cleavage site (GDPH): the extracellular MUC4α subunit and the transmembrane subunit MUC4β (Figure 4C).[125] MUC4α includes a variable number of tandem repeats (TRs) of 16 amino acids. This sequence, TSSASTGHATPLPVTD, is repeated up to 400 times arising in an 850 kD subunit that is predicted to extend >2 μm above the cell surface in the apical region. The numerous serine and threonine residues within the tandem repeats offer multiple sites for *O*-glycosylation. MUC4β includes a domain rich in *N*-glycosylation sites.[126]

Although MUC4 belongs to the membrane-bound mucins; it can be detected in milk and saliva, possibly an effect of an included cleavage site.[127] MUC4 is the first mucin to be expressed in normal lung epithelial tissue during development. It is also present in squamous epithelial cells of the esophagus, small bronchi, bronchioles and also occurs on the digestive tract, colon, salivary glands and cornea.[125] In cancer malignancies, both MUC4 abundancy and glycosylation patterns are altered. MUC4 expression is dysregulated in colon adenocarcinoma,[128] reports of upregulation were also found for breast,[129,130] lungs,[131] ovarian,[132] pancreas[133,134] cancer, and acute myeloid leukemia.[135] There are few examples, such

as mucoepidermoid carcinomas of the salivary gland, where overexpression correlates with a better prognosis.[136,137] However, in most scenarios, overexpression correlates with poor clinical outcomes, and MUC 4 expression level can be used as a prognostic marker.[11,138] So far, MUC4 was better explored as a therapeutic target for pancreatic cancer (PC).[139] PC has among the lowest survival rates and, in 2020, has caused over 460,000 deaths worldwide. PC is primarily asymptomatic. Therefore, when the diagnosis occurs, most patients have already developed distant metastases so that the removal of the primary tumor load can hardly rescue such a patient.

MUC4 is practically absent in healthy pancreas cells while is overexpressed in pancreatic cancer (PC) even in its early stages, pancreatic intraepithelial neoplasm.[140] Its unique presence in PC, and correlation with poor prognosis, led to the development of downregulation strategies that are beyond the scope of this review but were comprehensively discussed by Jain, Batra and coworkers.[141] Similar to other types of cancer and other members of the mucin family, PC mucins are aberrantly glycosylated.[142] SLe^a (Figure 1), also known as CA19-9, is found in the serum of 75% of pancreatic cancer patients. Patients with increased sLe^a levels suffer from a poorer prognosis as compared to those with average grades.[143,144] Other TACAs detected on pancreatic cancer tissue are the T antigen, T_N and ST_N. However, there are no studies that report the MUC4 glycosylation sites and patterns in different types of cancer.

Aberrant glycosylation of MUC4 and evidence of MUC4-specific autoantibodies in PC patients reinforce the hypothesis that MUC4 may be an excellent early-stage biomarker for PC and can be used for vaccine development.[141,145] Antibody production using recombinant proteins do not offer the possibility to investigate the molecular role of the carbohydrates. On the contrary, chemical synthesis allows to obtain pure tumor associated glyocpeptide antigens with a defined chemical structure[20] and explore the correlation between antigen structure and induced immune response systematically.

5.3. The Mucin MUC16

With a MW of 3–5 million Da, MUC 16 is the largest representative of the mucin family.[146] Discovered initially by Bast, Colvin, Knapp and coworkers in 1981[147] when developing monoclonal antibodies against ovarian cancer and named tumor antigen CA125, the structure was identified as part of the new mucin 16 as late as 2001.[148,149] The MUC16 protein is composed of three domains, namely a large N-terminal region comprising ~12000 amino acids with Pro/Ser/Thr making more than 47%[150] and is extensively *O*- and *N*-glycosylated, followed by a tandem repeat domain-containing about 60 repeats of 156 amino acids, rich in serine and threonine (Figure 4D). The repeats incorporate 54 sea urchin sperm protein, enterokinase, agrin (SEA) domains and are interrupted by two Ankyrin (ANK) and 14 leucine-rich repetitions. Contrary to MUC1 and MUC4, the repeat amino acid sequence is not identical but homologous and harbors multiple *N*-glycosylation sites. Two conserved cysteines, which are twenty amino acids apart from each other, are likely to form disulfide bridges.[149] The C-terminus encompasses the juxtamembrane domain, a transmembrane domain, and a short cytoplasmatic region. Notably, the juxtamembrane domain contains two additional SEA domains. One harboring putative cleavage sites that lead to proteolysis produces an extracellular fragment, also known as the CA125 antigen, released into the extracellular space. The actual site of cleavage is still a matter of ongoing investigations.[151]

Unfortunately, knowledge about structural properties of MUC16 as well as about structure-related functions is still incomplete and the current literature on this subject is divided e.g. concerning the cleavage site. This is all the more unfortunate because MUC16 seems to have a prominent role in multiple cancer types, being the third most frequently mutated gene in most cancer types[152] and overexpressed in various malignancies. Remarkably, over 80% of ovarian cancers[153] overexpress MUC16. In addition, the mucin was found overexpressed in pancreatic, esophageal, colorectal, and breast cancer.[154]

Especially in ovarian cancer, the released CA125 fragment is harnessed by laboratory medicine as a serum tumor marker shortly after its initial discovery and is widely used today.[155] It correlates with tumor progression or regression, making it a well-suited for monitoring purposes. Still, due to the limited sensitivity and specificity of the applied Abs, the biomarker doesn't perform well in screenings.[156] A modulating role in tumor cell growth, motility, invasiveness, and tumorigenesis has been attributed to MUC16 in ovarian[157] and pancreatic[158] cancer. Further, MUC16 expression mediates immunoprotective effects on cancer cells via multiple mechanisms, facilitating metastasis as well. The cell surface-bound MUC16 can prevent NK cell lysis of cancer cells via blockage of the immune synapse formation.[159] Additionally, the soluble MUC16 fragment inhibits NK cell cytotoxicity in the surrounding environment by downregulating the expression of activating CD16, a receptor involved in antibody-dependent cellular cytotoxicity (ADCC).[160] Interaction with the inhibitory Siglec-9 receptor further diminishes NK cell response.[161]

Besides the CA125 portion, the C-terminal part of MUC16 has moved into focus in recent years. It is the only part, which remains connected to the tumor cell, potentially responsible for oncogenic signaling and enabling precise targeting or immunization. The ectodomain fragment, especially its most proximal region, plays a critical role in the oncogenic behavior of MUC16.[157,162] Site-specific *N*-glycosy-

lation of this domain was shown to be a key aspect for oncogenic interactions responsible for invasion and xenograft growth.[163] The effects are induced involving Galectin-3 mediated formation of complexes with the epidermal growth factor receptor (EGFR). Another study could also attribute the adverse effects to the retained C-terminal fragment in an *in vivo* mouse model.[162]

As for other mucins, the nature of the glycan structures presented on the protein backbone changes upon malignant transformation. T_N and ST_N antigens are reported in ovarian cancer and core 1 and core 2 structures. Besides *O*-glycans, differences in the *N*-glycosylation pattern are reported as well. Bisecting bi-antennary and non-fucosylated glycans are decreased, whereas an increase of core-fucosylated bi-antennary monosialylated motifs has been observed.[164,165]

6. Importance of Glycosylation Pattern in MUC Vaccines

In the last decades, it was disclosed that TACAs and MUC protein backbone are interdependent and indispensable for vaccine development. On the one hand, naked MUC peptide epitopes have low immunogenicity and induce immune tolerance: MUC tandem repeats do not catalyze vaccine internalization via APCs surface receptors. Examples of immunization with 'naked' MUC epitope, both as carrier protein conjugates or as multiple tandem repeat peptides, failed to produce long-lasting antibodies and cytotoxic T lymphocyte. For example Tecemotide (L-BLP25, Stimuvax®), a vaccine candidate developed by Merck KGaA and based on a twenty-five amino acid unglycosylated MUC1 sequence conjugated to 3-*O*-deacyl-4′-monophosphoryl lipid A, did not meet the primary endpoint in phase 3 clinical trial, with the administration of tecemotide after chemoradiotherapy for patients with unresectable stage III non-small-cell lung cancer.[166] On the other hand, glycans on their own do not bind to MHCs and, apart from a few remarkable examples, are only able to evoke a weak T cell-independent immune response. Still, it is reported that they stabilize peptide binding to MHC I,[167,168] and facilitate the construct uptake by triggering endocytosis upon binding with a wide variety of receptors on APCs. The role of carbohydrates is not limited to the binding purposes. Reported studies suggest that the chemical structure of the glycans and their attachment to the peptide backbone play a vital role in the immunological outcome. Furthermore, the dogma that carbohydrates alone cannot act as immunogens was challenged by Andreana and coworkers that used a vaccine construct entirely composed by carbohydrates to generate high level anti-T_N antibodies.[169] The antigen was conjugated onto a zwitterionic polysaccharide construct, and was reported to induce an MHC class II-mediated T cell response in the absence of a protein carrier.[170,171]

Given the lack of comprehensive studies where the vaccine carrier platform and adjuvant type are kept constant, while glycan type and glycosylation site are varied systematically, it is still impossible to draw a definitive conclusion and decipher the glyco-code that governs immunity in anti-tumor glycoconjugate vaccines. In the following paragraphs, we provide a collection of literature examples and discuss how the differences of TACAs type and glycosylation sites on MUC1, MUC4 and MUC16 subunit vaccines influence the immunological outcome.

6.1. TA MUC1 Vaccines

MUC1 is the TAA with the greatest interest in vaccine development. Numerous reviews have already been published, which provide an overview of different adjuvants and delivery systems,[8,172] and MUC1 based vaccine approaches in clinical trials.[173,174] The interested reader is referred to these comprehensive review articles. Here, we deliberately only give an overview of our own synthesized MUC1 vaccines and, as mentioned in the introduction, focus on the importance of glycopeptide design and glycosylation pattern, which reflect the structure of TA-MUC1 variants. Vaccines with different glycosylation pattern are compared, which were tested under the same conditions. In the following examples, the humoral immune response after induction by various TTox vaccines was analyzed to evaluate, which glycosylation pattern caused the strongest and most selective antibody production. The glycopeptides are always a C-terminal variant of the tandem repeat sequence of TA MUC1 (PAHGVTSAPDTRPAPG-STAP-PA) extended by two amino acids with five potential *O*-glycosylation sites,[177,178] which are derived from the VNTR ('variable number of tandem repeats') region. Different glycosylations on the two immunodominant domains PDTRP[52,179] and GSTAP,[66] which are considered to be binding motifs for anti-TA MUC1 glycopeptide antibodies,[72] are compared. The simplest and most common TACA is the T_N building block[180] and its sialylated form ST_N.[181] Both building blocks were first chemically linked to a serine, inserted as *O*-glycosylated amino acids in position 17 of the 22 amino acid long peptide sequence and formed the MUC1 (22) $S^{17}T_N$-TTox vaccine,[95] and the MUC1 (22) $S^{17}ST_N$-TTox vaccine (Figure 5).[72] It was also studied whether double glycosylation in a peptide sequence with a T_N component at the beginning of the sequence and one at the end of the sequence leads to a conformational change of the same and a changed immune response. The glycosylation is at position 6 of the VNTR sequence and position 18 in the GSTAP motif of the VNTR sequence and forms the MUC1 (22) $T^{6}T_N$-$T^{18}T_N$-TTox vaccine (Figure 5).[175] In addition, a 2,3-ST was

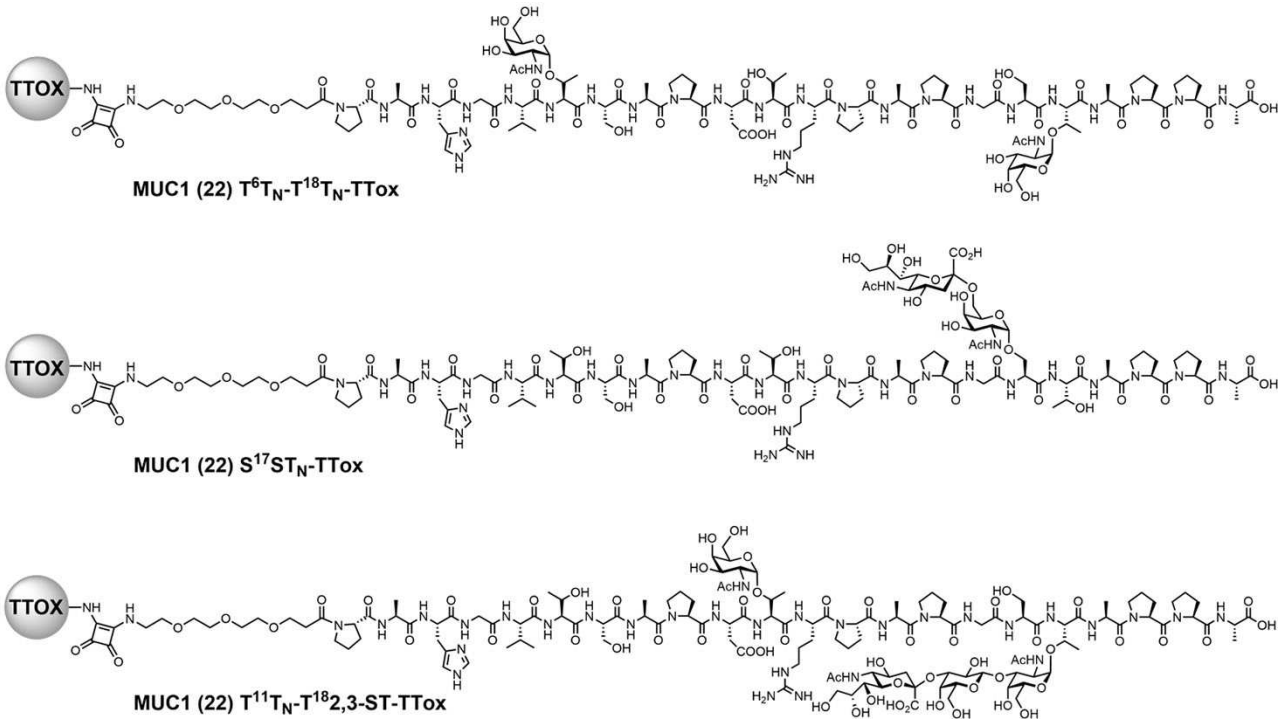

Figure 5. Partial molecular structure of selected TACA-MUC1-TTox conjugates: MUC1 (22) $T^{6}T_{N}$-$T^{18}T_{N}$-TTox vaccine,[175] MUC1 (22) $S^{17}ST_{N}$-TTox vaccine[72] and MUC1 (22) $T^{11}T_{N}$-T^{18}2,3-ST-TTox vaccine.[176]

synthesized and conjugated to the threonine at position 18 in the amino acid sequence and formed the MUC1 (22) T^{18}2,3-ST-TTox vaccine (Figure 5).[176]

Another MUC1 (22) $T^{11}T_{N}$-T^{18}2,3-ST-TTox vaccine[176] was intended to evaluate whether the additional glycosylation with T_N on threonine at position 11 in the immunodominant PDTRP motif changes the immune response. Furthermore, the humoral immune response after immunization with a MUC1 (22) – TTox vaccine without glycosylation[182] was tested. All TTox vaccines were immunized in BALB/c WT mice as described in section 4. Glycosylation with ST_N in the STAPPA motif on S^{17} induced the strongest glycopeptide-specific titer. Only a weak IgG response was elicited with its non-sialylated analog, T_N, glycosylated on the same serine. If both motifs (PDTRP and STAPPA) in the VNTR sequence are glycosylated with one T_N each, the antibody response to the vaccine is increased again. The glycosylation of T^{18} with 2,3-ST in the STAPPA motif leads to a stronger humoral immune response than glycosylation with T_N, but less than the corresponding ST_N vaccine. If, in addition to the STAPPA motif, another site in the 2,3-ST vaccine with T_N in the PDTRP motif is glycosylated, this does not lead to an increase in the IgG titer.

The Ig isotype determination clearly shows that the use of different glycopeptides as TACAs leads to different humoral immune responses with respect to the formation of different Ig isotypes. Glycosylation of S^{17} with ST_N or T_N and glycosylation of T^{18} with 2,3-ST induced only an IgG1 response. As soon as a second saccharide was incorporated into the sequence, an IgG2a and IgG2b response was induced in addition to an IgG1 response. This observation parallels results of vaccination in mice with fully synthetic p30-MUC1 vaccine conjugates even when administered in PBS without additional adjuvant.[70] The conclusion is also supported by studies using vaccinations with multivalent MUC1 glycopeptide vaccines conjugated with TLR 2 ligands, to obtain antisera, which were reported to initiate the killing of MCF-7 tumor cells.[60]

The Th cells that control the IgG class change of the B cells must consequently experience a different activation by vaccines with double glycosylation than by vaccines with single glycosylation. An IgM response could not be detected in any serum after the fourth immunization. This suggests that all vaccines triggered an adaptive Th-mediated immune response and led to the establishment of immunological memory.[183] The specific binding of the induced antibodies to TA MUC1 was tested on human breast cancer cells; the antisera showed the strongest binding with the MUC1 (22) $S^{17}ST_{N}$-TTox vaccine or with the doubly glycosylated MUC1 (22) $T^{11}T_{N}$-T^{18}2,3-ST-TTox vaccine. The lowest binding to the TA MUC1 was shown by the antisera, which were induced by the

T^6T_N-$T^{18}T_N$ glycopeptide and by the peptide only glycosylated with 2,3-ST. The binding analyses show that in the case of additional glycosylation outside the STAPPA sequence, great attention must be paid to the positioning of the second glycan. Glycosylation at the beginning of the sequence revealed antisera that were not specific for the TA glycosylation pattern of human breast cancer cells. However, additional glycosylation ($T^{11}T_N$-T^{18}2,3-ST) in the second immunodominant region, the PDTRP sequence, appears to map a glycosylation pattern that is more common on breast cancer cells. In a control experiment, immunization with a non-glycosylated 22mer MUC1 sequence was carried out. The 'naked' peptide sequence was recognized as an antigen. High IgG titers, including very high IgG2a, moderate IgG2b, and IgG1, but no IgM titers were induced. This result suggests that the choice of the 22 amino acid long peptide sequence used from the tandem repeat of the TA MUC1 is immunogenic. The peptide sequence of a glycopeptide used as a tumor-selective antigen is of great importance[184] because it contains the immunodominant regions. Importantly, the antisera induced by the 'naked' peptide sequence itself did not show any binding to human breast cancer cells. This result highlights that the 22 amino acid long peptide sequence of the tandem repeat of TA MUC1 only represents promising TAAs when decorated with TACAs. This also provides evidence that the induced antibodies are specific to both the immunodominant regions of the TA MUC1 sequence and the TA glycan. It was further shown that after incubation of the antisera obtained from vaccinations with the TA MUC1 glycopeptides, the antigen-binding sites of the induced antibodies were blocked. Importantly, the same antisera were not neutralized with the non-glycosylated MUC1 peptide sequence.[185] These combined results suggest that a MUC1-glycopeptide carrying ST_N in the STAPPA motif conjugated to TTox,[72] represents the most effective vaccine to date. However, it should be noted that this vaccine only induced IgG1 titers in the BALB/c model. By immunizing C57BL/6 J mice, Ig2b titers were induced, which trigger the molecular anti-tumor mechanisms, antibody-dependent cell-mediated cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC)[186–189] in the murine model. Preventive vaccination significantly inhibited breast tumor progression in a C57BL/6 based autochthonous preclinical mouse model.[190] Further analysis showed that the induced antisera recognize the expression of TA MUC1 in human biopsies of invasive breast cancer tumors of various stages, depending on the number of metastatic lymph nodes.[72]

Consequently, the MUC1 (22) $S^{17}ST_N$-TTox conjugate was used to generate a particularly selective monoclonal antibody, GGSK-1/30,[191] using the hybridoma technique.[184] This monoclonal antibody was radiochemically labeled with ^{89}Zr and preclinically evaluated *in vivo* as a diagnostic agent for breast cancer.[192] Furthermore, the specific binding of the mAb GGSK-1/30 to human breast and pancreatic cancer biopsies was proven. The GGSK-1/30 showed stronger binding to human breast and pancreatic cancer cells and cancer tissue of fine needle biopsies than the commercially available monoclonal antibodies SM3 and HMFG1.[184] SM3 and HMFG1 were induced against partially deglycosylated MUC1 from human milk. Due to the microheterogeneity of these antigens, the induced antibodies are not sufficiently specific to distinguish between physiological MUC1 and tumor-associated MUC1. Therefore, the presented data using the GGSK-1/30 mAb support the need for and huge potential of molecular glycopeptides with a defined glycosylation pattern as B cell epitopes in subunit vaccines.

6.2. TA MUC4 Vaccines

MUC4 glycopeptides synthesis has been extensively explored.[4,193,194] The synthetic knowledge laid a solid basis for the exploration of MUC4 subunit vaccines. Barchi and his group prepared a vaccine construct based on 3–5 nm gold nanoparticles covalently coated with a 28-residue peptide from the complement derived protein C3d, and MUC4 glycopeptide B cell epitopes.[195] The 16 amino acid glycopeptides (TSSASTGHATPLPVTD) were obtained through solid-phase synthesis in which serine and threonine bearing a single T antigen were incorporated in a the MUC4 sequence to achieve mono incorporation at different positions of serine and threonine residues (S^5T, T^6T, $T^{10}T$) and homogeneous bisincorporation (S^5T-T^6T). After immunization experiments in mice, both IgG and IgM antibodies were detected. The monofunctional peptides induced almost identical production for IgG and IgM, while the construct with two consecutive glycosylation sites suppressed the response. $T^{10}T$ induced a higher production of IgG over IgM, suggesting isotype switching. The antisera showed strong binding with their respective immunogen, although also bound to the unglycosylated peptide. Unpublished data of the authors claim that mice with implanted 4T1 breast tumor cells had a longer survival rate when vaccinated with the S^5T construct, compared to vaccinations using constructs in which the T antigen was linked to different positions.[196]

The MUC4 (16) S^5T-KLH vaccine was evaluated as a potential lead, and high antibody titers were determined by ELISA assays after vaccination in mice and rabbits.[196] Next to the glycosylated MUC4 (16) S^5T and unglycosylated MUC4 (16) epitopes, various domains of the MUC4 TR were synthesized: the N-terminal half TSSASTGH, and C-terminal half ATPLPVTD, as well as the glycosylated TSSASTGH-S^5T. After vaccination with a MUC4 (16) S^5T-KLH, ELISA assays were used to show that the polyclonal antiserum interacts strongly with the MUC4 glycopeptide and unglycosylated

peptide, with a slight preference of 1.2:1 for the glycopeptide epitope. However, the antiserum was shown to bind primarily to C-terminal half, which led the authors to suggest that the carbohydrate may redirect the response to the unglycosylated C-terminal domain of the MUC4 TR. Further, the authors were able to show that the affinity purified serum bound to $MUC4^+$ human pancreatic tumor cell lines CAPAN-2 and HPAC.

Westerlind and co-workers observed a similar recognition pattern in vaccination studies. In their investigation, two MUC4 glycopeptides carrying the tumor-associated T and ST_N antigens on S^2 and S^5, were used as B cell epitopes conjugated with a tetanus toxoid sequence to serve as Th epitope in murine immunization studies.[197] To further explore the antibody specificity, the sera were screened on a microarray exposing a broad set of MUC4 glycopeptides with different glycans and glycosylation sites. Antibody isotype analysis revealed a predominant production of IgG1, and the microarray results of both constructs suggest that a naked C terminal domain (ATPLPVTD) is needed for antibody binding: the sera bind to the naked peptide or to peptides glycosylated in the N-terminal region, while peptides glycosylated on T^{10} were only weakly recognized. It is worth noting that the presence of T antigen on both serines at position 2 and 5 leads to a strong immune response, whereas in the work of Barchi and his group glycosylation on consecutive amino acids serine and threonine at position 5 and 6 does not. Furthermore, Westerlind and collaborators did not observe binding of the antisera to CAPAN-1 and CAPAN-2 (MUC4 +) pancreatic tumor cells. This also suggests that the unglycosylated C-terminal domain is the dominant binding epitope of the induced antibodies, and further explains the failure to stain tumor cells. One has to keep in mind that a comparison of different immunization studies, in particular when the vaccine conjugation chemistry and carrier constructs are different, is not always possible. However, the presented studies and different outcomes clearly highlight the impact of precisely tailored glycosylation patterns on the immune response and antibody production.

6.3. TA MUC16 Vaccines

Recently, an epithelial ovarian cancer (EOC) immunopeptidome study identified MUC16-derived peptides as being among the most frequently presented HLA class I antigens, which do not occur in benign tissues.[198] They are presented on many different HLA allotypes, and $>85\%$ of the identified peptides behaved immunogenically. The isolation of high-affinity CTL recognizing MUC16 was problematic.[199,200]

Although the immunogenicity of CA125, as part of MUC16, has been known for a long time, there are very few examples of antibodies that recognize epitopes with known structure. The strategy of using defined synthetic MUC16 antigens is also rarely applied for vaccination purposes and in the discovery of specific antibodies. This can be attributed to the slow progress of gaining structural information about the parent protein. MUC16-related immunotherapy was mainly focused on antibodies against the CA125 region. Oregovomab,[201,202] a high-affinity mouse monoclonal antibody against CA125, and Abagovomab,[203] an anti-idiotypic antibody, were both assessed in phase II and III clinical trials in the treatment of ovarian cancer. Both did not exhibit significant therapeutic benefits. Examples for vaccination and the generation of antibodies against defined epitopes are rare. David, Clausen and coworkers developed a vaccine based on a linear tandem repeat antigen expressed by *E. coli*, which was subsequently enzymatically decorated with multiple T_N glycans.[204] Combined with Freund's adjuvant, the antigen did elicit a potent serum IgG response in the mouse model. However, no glycoform-specific mAbs were isolated, and the identified mAb against the immunogenic epitope only showed significant affinity for the non-glycosylated peptide.

Recently, the vaccination with synthetic *N*-glycosylated ectodomain peptide antigens of MUC16 were shown to result in the generation of antibodies against specific MUC16 glycosylation sites.[163] The authors incorporated *N*-glycosidic chitobiose (GlcNAc-β-(1→4)-GlcNAc-β-*N*-Asn) as carbohydrate antigen representing the minimal unit attached to asparagine according to a glycome analysis. The glycopeptides were bound to a keyhole limpet hemocyanin (KLH) carrier and subsequently used to generate novel *N*-glycosylation site dependent antibodies by immunizing mice. They were shown to be reactive with glycosylated MUC16 epitopes, but not with glycosylated MUC16-irrelevant negative control peptides. Application of the generated antibodies inhibited ovarian cancer growth *in vivo* and tumor cell invasion by blocking the glycosylation-dependent oncogenic behavior of the MUC16 ectodomain resulting from MUC16-Galectin-3 interactions. The retained extracellular domain of MUC16 has also been targeted using chimeric antigen receptor CAR T cells since 2010. Second-generation CARs[205] and IL-12 secreting armored CARs[206,207] were shown to elicit anti-tumor activity with improved survival in orthotopic xenotransplant ovarian cancer tumor models and in syngeneic models of ovarian peritoneal carcinomatosis, respectively.

The MUC16 CA125 is still a gold standard biomarker in terms of ovarian cancer diagnosis despite sensitivity and specificity issues. A study analyzing the binding epitopes of classical OC125 and M11 mAb, which are both currently used in clinical CA125 assays for ovarian cancer found that a glycosylated recombinant tandem repeat does not exhibit distinct binding in comparison to non-glycosylated fragments.[208] This raises the question of how antibodies directed against glycopeptide epitopes may improve the issues

mentioned above. However, compared to MUC1 and MUC4, there has not been much effort to apply synthetic molecular defined MUC16 glycopeptide epitopes in order to generate specific immune responses by vaccination, which does not allow us to hypothesize or comment on the efficacy of such an approach.

7. Conclusions

Mucin1/4/16 are promising tumor antigens due to their overexpression in over 90% of epithelial tumors and their involvement in tumor progression. Their tumor-associated structures differ significantly from the physiologically expressed glycoproteins on healthy tissues. Due to the aberrant glycosylation in tumor cells, specific MUC1/4/16-peptide backbone epitopes are accessible as neoantigens for antibodies and immune effector cells while these structures are absent on healthy tissues. Precise mapping of the tumor associated glycosylation pattern is crucial for the generation of high IgG antibody titers, which specifically bind to human cancer cells. Notably, the glycosylation pattern has an enormous influence on the induced humoral immune response. This indicates a high structural antigen selectivity of the generated immune response. The experimental data on the vaccine design show that one needs to choose an antigen structure for the B-cell epitope that is very different from the physiological structure of MUC1 on healthy cells. The synthetic glycopeptide epitope must mimic a tumor associated structure. In addition to the antigen structure, its combination with universal Th cell epitopes is necessary to induce a long-lasting and effective anti-tumor response for the general population, independent of MHC restrictions. With the immunological understanding and knowledge about cancer immune surveillance, vaccines should contain suitable adjuvants to obtain a broader immune response that can be adapted to the individual phenotype of different epithelial cancers. The examples of synthetic MUC1 vaccines clearly show why the precise and at the same time modular vaccine design is of great importance.

Many active immunotherapies against TA-MUC1 have already been tested, but no clinical study has shown the expected anti-tumor effect in patients. Nevertheless, the findings repeatedly confirmed that high anti-TA MUC1 IgG levels in breast cancer patients correlated positively with improved overall survival. Although TA MUC4 and TA MUC16 seem to be promising targets, the number of studies investigating glycopeptide epitopes for vaccination is limited compared to MUC1. Significant immune responses have already been shown against MUC4 derived glycopeptides and the resulting antibodies exhibit glycosylation pattern specificity. The size and the homology between the tandem repeats of MUC16 render the development of defined vaccine constructs more tedious. The focus on targeted therapy with known anti-CA125 antibodies has shown limited success in clinical trials. This may be attributed to the fact that MUC16 is released into the extracellular space after cleavage and, thus, the amount presented by the cell is reduced. An insufficient antibody selectivity and specificity must also be considered, indicating a significant drawback of the MUC16 tumor marker detection.

In summary, the published research data in the last two decades suggest that taking advantage of a TA MUC1/4/16-based immunization has the potential to inhibit the progression and metastasis of epithelial tumors. The development of therapeutic vaccines inducing specific humoral immune responses against tumor-associated mucines with a defined glycosylation pattern therefore shows increasing potential for clinical studies. Secondly, we further point out that the pharmaceutical industry continues to pursue targeted antibody therapy to treat cancer, which in the case of tumor-associated mucines underlines the potential of using synthetic glycopeptide vaccines for the generation of monoclonal antibodies. The further improvement of anti-TA MUC1/MUC4/MUC16 glycopeptide antigens, the development of innovative vaccine carrier materials and modular delivery platforms, as well as the generation of various antibody derivatives will pave the way towards successful clinical applications in the future.

8. Abbreviations

TA	Tumor-associated
TAA	Tumor-associated antigen
TACA	Tumor-associated carbohydrate antigen
Th cell	T helper cell
CTLs	Cytotoxic T Lymphocytes
MHC	major histocompatibility complex
APC	antigen presenting cell
DC	dendritic cell

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