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Authors: Horst Kunz, David Strassburger, Markus Glaffig, Natascha Stergiou, Sabrina Bialas, Pol Besenius, and Edgar Schmitt

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Synthetic MUC1 antitumor vaccine with incorporated 2,3-sialyl-T carbohydrate antigen inducing strong immune responses with isotype specificity

D. Straßburger,[a] M. Glaffig,[a] N. Stergiou,[b] S. Bialas,[a] P. Besenius,[a] E. Schmitt,[b] H. Kunz*[a]

Dedication ((optional))

Abstract: The endothelial glycoprotein MUC1 is well known to underlie alterations in cancer by means of aberrant glycosylation accompanied by changes in morphology. The heavily shortened glycans induce a collapse of the peptide backbone and enable accessibility of the latter to immune cells, rendering it a tumor-associated antigen. We report synthetic vaccines based on MUC1 tandem repeat motifs comprising tumor-associated 2,3-sialyl-T antigen, conjugated to the immunostimulating tetanus toxoid. Immunization with these vaccines in a simple water/oil emulsion produced a strong immune response in mice to which stimulation with complete Freund's adjuvant (CFA) was not superior. In both cases high levels of IgG1, and IgG2a/b were induced in C57BL/6 mice. An additional glycosylation in the immunodominant PDTRP domain lead to improved binding of the induced antisera to MCF-7 breast tumor cells compared to the mono-glycosylated peptide vaccine.

MUC1 has been identified as promising tumor-associated antigen. It is found on the surface of epithelial cells throughout the human body.[1] It is a membrane-bound glycoprotein and features a highly glycosylated extracellular domain containing a variable number of tandem repeats (VNTR) of proline-, serine- and threonine-rich repetitive sequences of 20 amino acids (PAHGVTSAPDTRPAPGSTAP). The O-linked saccharides make up 50-90% of the molecular weight[2] and cause the brush-like morphology of the glycoprotein protruding 200-500 nm[3,4] from the apical cell membrane. Tumorigenesis involves profound alterations in the metabolism-affecting enzyme expression levels. The decrease of β-1,6-*N*-acetylglucosaminyltransferase[5] activity and upregulated α-2,3- (ST3Gal-I) and α-2,6-sialyltransferases (ST6GalNAc-II) result in a dramatic shortening of glycan chains on tumor-associated (TA)-MUC1 preferentially terminated with sialic acid.[6–8] The hypoglycosylation provokes the collapse of the hydrophobic peptide backbone rendering it accessible to the immune system.

Tumor-associated MUC1 also interacts with receptors and growth factors pushing the proliferation of the tumor further.[9] TA-MUC1 can also bind to selectins on blood vessel endothelial cells facilitating metastasis.[10,11]

An anti-tumor vaccine is thought to prime the organism's immune system to enable the recognition of tumor-associated structures and activate self-defense mechanisms to eliminate the malignancy. Such an immune response should address solid tumors as well as circulating malignant cells, thus lowering the tumor burden and the risk of metastases. Nevertheless, a cancer immunotherapy through treatment with a vaccine holds the inherent risk of an autoimmune response due to the endogenous origin of the epitope and the associated self-tolerance. In this case the generated antibodies do not distinguish between healthy and tumor cells due to a lack of antibody selectivity.

In comparison to peptide epitopes carbohydrate antigens induce low immune responses mainly due to the involved T-cell-independent mechanisms.[12,13] Among the tumor-associated carbohydrate antigens (TACAs) the trisaccharide (2,3)-sialyl T-antigen (2,3-ST) was found the most abundant in different tumor cell lines e.g. T47D[14] and HAT-29[15]. Furthermore, highly sialylated MUC1 is found in invasive and metastasizing cancers.[16] An immune response directed against these structures should have a high therapeutic impact on neoplastic tissues. A supreme objective of such a vaccine is to avoid the worst-case scenario of auto-immune reaction with fatal outcome for the patient by thorough design of the vaccine. Recent experiments with transgenic mice expressing human MUC1 showed no auto-aggressive side effects although the generated antibodies exhibit almost equivalent binding properties towards human breast tumor cells.[17]

There is strong evidence that the glycosylation sites within the MUC1 tandem repeat and the incorporated TACA determine specificity and selectivity of the induced immune response.[18–20] It was shown that the saccharides cause conformational changes in the peptide epitopes[21] impacting on the generated antibodies. In order to enhance the mandatory selectivity, the 2,3-sialyl T-antigen most frequently occurring on carcinomas was incorporated into the B-cell epitope of the vaccine on threonine (T^{18}) of vaccine **V1** whose sequence comprises the VNTR domain. The 20mer tandem repeat was expanded by proline and alanine in order to complete the STAPPA motif known to be a CD8+ T-cell epitope.[22,23] In the second vaccine **V2**, a Tn antigen was attached additionally to threonine 11 within the immunodominant PDTRP region. There is evidence that glycosylation at this site has a strong impact on morphology[24] and on receptor binding.[25] Although in patients suffering from e.g. mammary carcinoma, low antibody levels against TA-MUC1 were detected,[26,27] they lack a strong autonomous humoral response due to suppression by the tumor. To overcome this hurdle in the synthetic vaccine, the B-cell epitope was

[a] D. Straßburger, M. Glaffig, S. Bialas, Prof. Dr. P. Besenius, Prof. Dr. H. Kunz
Institut für Organische Chemie
Johannes Gutenberg-Universität Mainz
Duesbergweg 10-14, 55128 Mainz
E-mail: hokunz@uni-mainz.de
[b] N. Stergiou, Prof. Dr. E. Schmitt
Institut für Immunologie
Johannes Gutenberg-Universität Mainz
Obere Zahlbacher Straße 67, 55101 Mainz

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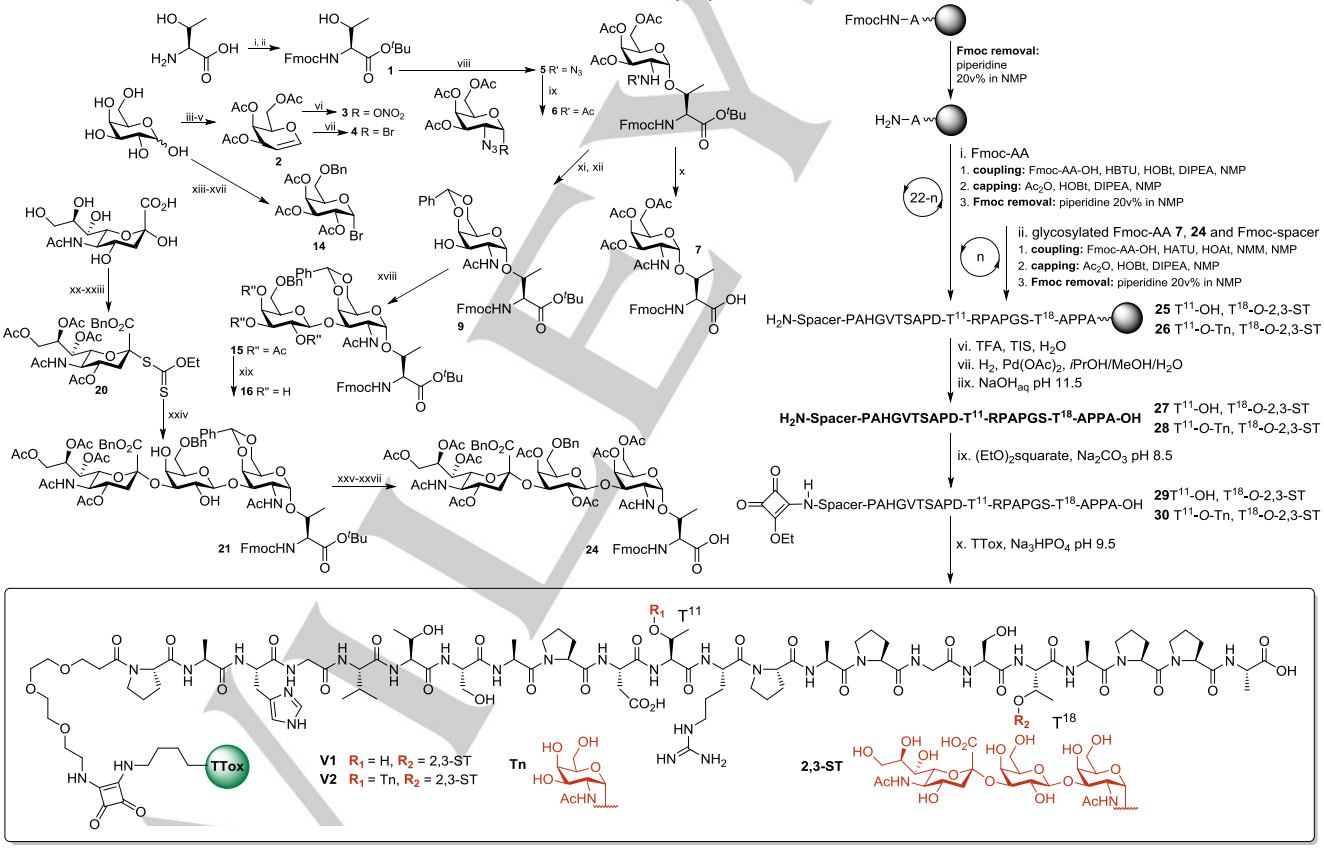
conjugated to the strongly T-cell-stimulating tetanus toxoid (TTox) which is an established immunostimulant and applicable in human immunotherapy.

The biomimetic synthesis of the TACAs Thr-Tn **7** and Thr-2,3-ST **24** starts from L-threonine, D-galactose and *N*-acetyl neuraminic acid.[28] Peracetylated D-galactose was treated with HBr in acetic acid. Subsequent elimination with zinc yielded galactal **2**. Through azidonitration[29] the neighboring effect-lacking azide was introduced on C-2. The generated nitrate ester **3** on C-1 was converted to α-bromide **4** as the glycosyl donor. Glycosylation of acceptor **1** was achieved with silver perchlorate/carbonate.[30] Simultaneous reduction of the azide **5** and conversion to the acetamide **6** were achieved by treatment with zinc in acetic acid/acetic anhydride mixture in THF. In the case of Thr-2,3-ST the acetyl protecting groups were removed with catalytic sodium methoxide in methanol.[31] The galactosyl donor **14** was synthesized from galactose. Positions 1',2' and 3',4' were blocked as acetonides and a benzyl ether was installed on C-6. Acidic cleavage of the acetal protecting groups and subsequent peracetylation, followed by treatment with HBr in acetic acid yielded the second glycosyl donor **14**. In order to address the 3-position of Tn **9**, acetyl protecting groups were removed with catalytic sodium methoxide in methanol and positions 4 and 6 were masked as benzylidene acetal. The glycosylation was carried out under FISCHER HELFERICH conditions to afford the intermediate T-antigen **15** in excellent yields. The sialyl donor **20** was prepared from *N*-acetylneuraminic acid (NANA) by exhaustive acetylation, benzylation of the carboxylic function by reaction of the cesium salt with benzyl bromide. Treatment with acetyl chloride and subsequent substitution with potassium xanthate yielded donor **20**. Sialylation of the selectively deacetylated acceptor **16** was performed using methyl sulfenyl bromide, prepared *in situ* from the disulfide, and silver triflate.[32] The acetal group of product **21** was acidolytically removed and replaced by acetyl groups. For the introduction in solid phase syntheses (SPPS), *tert*-butyl esters of both, Thr-Tn **7** as well as Thr-2,3-ST **24**, were removed using TFA in DCM. A Tentagel *R* TRT resin preloaded (0.17 mmol/g) with Fmoc-L-alanine was used as the solid support. It should be mentioned that some modifications and optimizations of the deprotection procedures (vii) and (viii) were made (Scheme 1): For removal of the benzyl group, Pd(0) was prepared *in situ* from the Pd(II) acetate in a mixture of 2-propanol, water and acetic acid in order to avoid the



Scheme 1 On the left: synthesis of the tumor-associated carbohydrate antigens Thr-Tn **7** and Thr-2,3-ST **24** (**i**) Fmoc-OSu, $NaHCO_3$, acetone, 97% (**ii**) DCC, CuCl, ^tBuOH, 65% (**iii**) Ac_2O, $HClO_4$ (**iv**) HBr, AcOH, 98% (**v**) Zn, AcOH, 87% (**vi**) CAN, NaN_3, MeCN, 40% (**vii**) LiBr, MeCN, 70% (**viii**) $AgClO_4$, Ag_2CO_3, toluene/DCM, 44% (**iix**) Zn, AcOH, Ac_2O, THF, 55% (**ix**) NaOMe/MeOH 68%. (**x**) DCM, TFA, H_2O, 91% (**xi**) NaOMe, MeOH, 68% (**xii**). α,α-dimethoxytoluene, *p*-TosOH, MeCN 82%. (**xiii**) $Hg(CN)_2$, MS3 Å, CH_3NO_2, DCM, 92%. (**xix**) NaOMe/MeOH, 75% (**xx**) Ac_2O, pyridine, quant. (**xxi**) 1.Cs_2CO_3, EtOH/H_2O, 2.BnBr, DMF, 67% (**xxii**) AcCl, H_2O, quant. (**xxiii**) KEX, EtOH, 82% (**xxiv**) MeSBr, AgOTf, MS 3 Å, MeCN/DCM, 53% (**xxv**) AcOH, 70%. (**xxvi**) Ac_2O, pyridine, DMAP, 97%. (**xxvii**) TFA, anisole, quant. On the right: SPPS Synthesis of MUC1 tandem repeat glycopeptides and subsequent protecting group manipulations. On the bottom: final TA MUC1 tetanus toxoid conjugate vaccines.

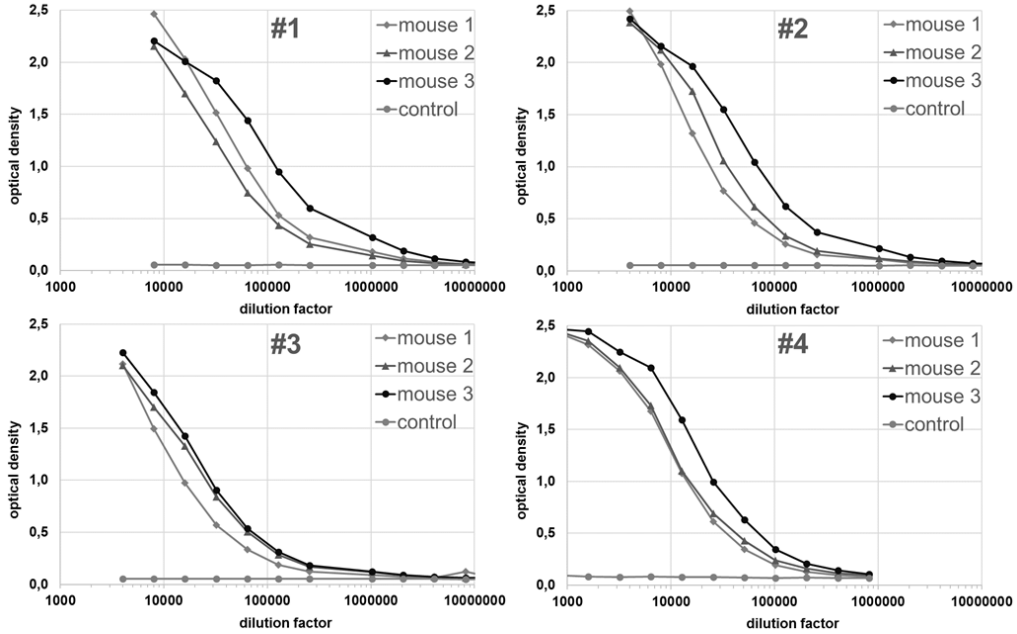


Figure 1. ELISA dilution curve of the sera collected from mice treated with vaccines five days after the third immunization. **#1**: **V1** first CFA/s.c., boost IFA/i.p. BALB/c (3 mice), **#2**: **V1** first CFA/s.c., boost IFA/i.p. C57BL/6 (3 mice), **#3**: **V1** only IFA, C57BL/6 (3 mice), **#4: V2** first CFA/s.c., boost IFA/i.p., BALB/c (3 mice), OD at 410 nm.

formation of catalytically inactive palladium aggregates. The deacetylation was carried out in dilute aqueous solution of 4 mM sodium hydroxide. The pH of 11.5 of the reaction was steadily monitored until a constant pH indicated complete conversion. Subsequent semipreparative HPLC yielded the amino-terminated compounds **27** and **28**. The conjugation of the amino-terminated B-cell epitopes was achieved via coupling of their squarate monoamides[33] **29** and **30** coupling which allows for a chemoselective linkage of the glycopeptides to lysines of TTox.

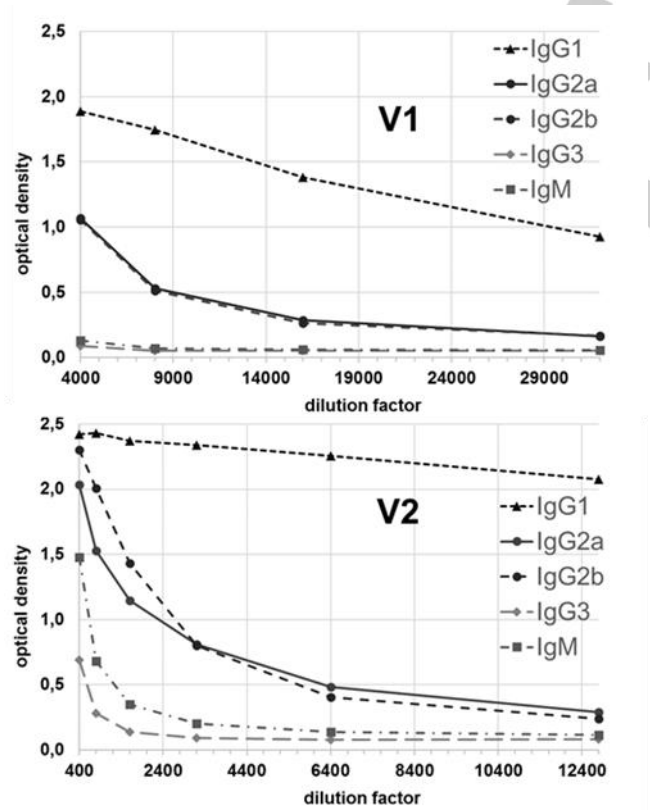


Figure 2. Isotype analysis of selected sera from BALB/c mice treated with **V1** (#1) and **V2** (#4) five days after third immunization, OD at 410 nm.

This concept enables a multivalent presentation of the glycopeptide epitope considered crucial for an effective immunostimulation. Ultrafiltration over a polyether sulfone membrane (exclusion limit 30 kDa) followed by lyophilization of the supernatant yielded vaccines **V1** and **V2** (Supporting Information). Conjugation of the glycopeptides to BSA was achieved by an analogous procedure and gave the coatings for ELISA experiments **C1** and **C2** (Supporting Information).

The first immunizations were performed by subcutaneous injection of a formulation of the vaccines **V1** and **V2** in complete Freund's adjuvant (CFA), a water-in-oil emulsion containing inactivated *mycobacteria tuberculosis*. CFA is an effective adjuvant used for boosting the immune response, but its usage limited to animal models due to its severe side effects. Therefore **V1** was additionally tested in mice when CFA was replaced by incomplete Freund's adjuvant (IFA, a simple water-in-oil emulsion) in the first immunization. Immunizations were performed three times in groups of three mice each. The antisera were collected from the tail veins five days after the last administration. The yielded anti-sera were tested for antibody levels by ELISA and cell affinity by flow cytometry.

BALB/c wild-type mice generally generate IL-4-dominated immune responses via MHC II restricted T helper 2 cell support and, therefore, the activation of a humoral immune response with high titers of the IgG1 antibody type.[34] For the purpose of investigating the differences in immune responses, **V1** was also applied in mice of the C57BL/6 strain. The sera collected from BALB/c and C57BL/6 mice showed high titers for both vaccines indicating a strong overall immune response (see Supporting Information). Titer levels of BALB/c mice treated with vaccine **V2** (T^{18} 2,3-ST and T^{11} Tn, #4) did not significantly differ from those of mice immunized with the mono-glycosylated vaccine **V1** (T^{18} 2,3-ST), both administered with CFA. Additionally, in groups of C57BL/6, **V1** was applied with (#2) and without CFA (#3). Unexpectedly, only insignificant differences were observed in direct comparison. We hypothesize, that CFA is not necessary to enhance the efficiency when working with TTox conjugated vaccines. Furthermore, glycosylation of threonine 11 within the immunodominant domain (**V2**) enhanced the immunogenicity most likely due to a more tumor-selective conformation of the

glycopeptide epitope.[24,25] The presence of IgG1 antibodies indicates the specificity of the induced immune response. Increased levels of both IgG2a and IgG2b after immunization of BALB/c mice with **V2** compared to **V1** (Figure 2) should activate antibody-dependent cell-mediated cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) as a result of the response to TA-MUC1. The development of T-cell dependent immune responses is necessary for the switch from IgM to IgG mandatory for the generation of a long term immunological memory against tumor-associated MUC1 glycopeptides found on epithelial cancers. The induced antibodies are not directed against the squarate linker as was shown by complete neutralization with the glycopeptide epitopes.[20].

Conclusion

The results of the immunological evaluation provide strong evidence that robust immune responses are induced through vaccination with both synthetic (T18 2,3-ST)-vaccines **V1** and **V2**. Surprisingly co-administration with CFA did not show a benefit for **V1**. Human MCF-7 breast tumor cells incubated with the antisera induced by **V2** (containing the second glycan in the PDTRP domain) showed increased binding of the antibodies in the flow cytometry (Figure 3). Although the incorporation of Tn antigen at threonine 11 has less impact on the total antibody titers, their affinity significantly increases probably due to an improved turn-type conformation in the PDTRP domain [24,25] This supports the hypothesis that the glycosylation pattern plays a key role in tumor specificity. The observed class switch to IgG subtypes, indicates an acquisitioned immunological memory and the generated antibodies show high affinity to tumor cells.

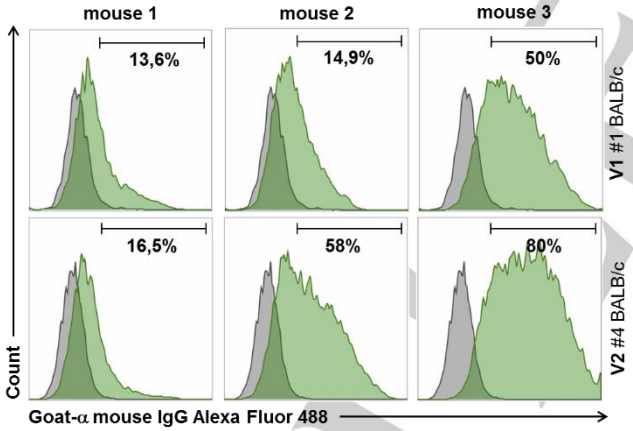


Figure 3. Flow cytometry analysis of the binding of the antisera (after the 3rd immunization) to MCF-7 breast tumor cells.

Thus, the synthetic glycopeptide vaccines are promising, potent for the induction of antitumor-directed immune responses in vivo, in particular regarding the comparability of the murine and the human immune system. These results are highly encouraging for the translation into human cancer therapy, not least because the glycopeptide vaccines no longer need the co-stimulant CFA which is not applicable in humans.

The mice used were 8 weeks old. All mice used for this study were bred and housed in a specific pathogen-free colony at the animal facility of Johannes Gutenberg University following institutionally approved protocols (permission was obtained from the Landesuntersuchungsamt Koblenz, reference number: 23 177-07/G 08-1-019).

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Keywords: MUC1 • synthetic antitumor vaccine • 2,3-sialyl-T antigen • glycopeptide • regioselective sialylation

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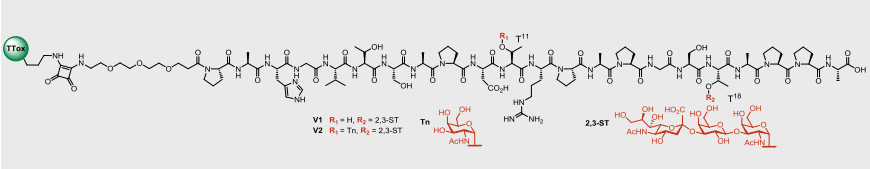

Synthetic antitumor vaccines based on human tumor-associated MUC1 glycopeptides incorporating 2,3-sialyl T antigen and Tn antigen induce a strong immune response in BALB/c and C57BL/6 mice with antibody subtype selectivity. Control experiments indicate that CFA is not superior to IFA and the generated antisera effectively bind to MCF-7 tumor cells. These results suggest that glycosylation in the PDTRP domain positively effects the immunological response.

D. Straßburger, M. Glaffig, N. Stergiou, S. Bialas, P. Besenius, E. Schmitt, H. Kunz**

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Synthetic MUC1 antitumor vaccine with incorporated 2,3-sialyl-T carbohydrate antigen inducing a significant immune response with isotype specificity