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A Synthetic Glycopeptide Vaccine for the Induction of a Monoclonal Antibody that Differentiates between Normal and Tumor Mammary Cells and Enables the Diagnosis of Human Pancreatic Cancer

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Abstract: *In studies within the realm of cancer immunotherapy, the synthesis of exactly specified tumor-associated glycopeptide antigens is shown to be a key strategy for obtaining a highly selective biological reagent, that is, a monoclonal antibody that completely differentiates between tumor and normal epithelial cells and specifically marks the tumor cells in pancreas tumors. Mucin MUC1, which is overexpressed in many prevalent cancers, was identified as a promising target for this strategy. Tumor-associated MUC1 differs significantly from that expressed by normal cells, in particular by altered glycosylation. Structurally defined tumor-associated MUC1 cannot be isolated from tumor cells. We synthesized MUC1–glycopeptide vaccines and analyzed their structure–activity relationships in immunizations; a monoclonal antibody that specifically distinguishes between human normal and tumor epithelial cells was thus generated.*

The introduction of vaccination against bacterial infections marked a breakthrough in human medicine.[1] Immunization with attenuated microbes elicited immune reactions in form of selective antibodies, which efficiently neutralize the attacking microbe. Aside from the curing effect, an immunological memory is installed, which provides long-term protection against the pathogen. The induced antibodies are also valuable tools for diagnosis of the corresponding infection. Concerning the treatment of cancer, therapeutic antibodies directed against factors overexpressed in tumor cells[2] or loaded with cytotoxic drugs[3] are powerful tools, and markers in the form of therapeutically inefficient antibodies are important for diagnosis,[4] but not effective for certain types of tumors. However, active immunization against tumor diseases has not been achieved thus far. Tumors, in contrast to microbes, are endogenous tissues and thus protected by the self-tolerance of the immune system. To overcome this fundamental problem hindering immunization against cancer, the immune system must be activated to differentiate between self and altered-self structures exposed on the surface of malignant cells.[5] For the development of an active vaccination against cancer, an altered-self structure typical for tumor cells must be identified. The tumor-associated mucin MUC1 is such a typical cancer antigen.[6]

MUC1 is a heavily glycosylated protein expressed on many epithelial tissues, for example, in breast, colon, prostate, and pancreas tissues and in the ovaries.[6] Its extracellular part contains a domain that consists of 20 to 100 repetitive copies of the sequence PAHGVTSAPDTRPAPGSTAP, including five O-glycosylation sites at serine or threonine. Normally, MUC1 carries long glycans, which force the protein to adopt an extended conformation. Owing to the dense glycosylation, the peptide backbone is inaccessible to the immune system. The glycans of tumor-associated MUC1 are much shorter and often prematurely sialylated[7] owing to a down-regulated β-1,6-*N*-acetyl-glucosaminetransferase and up-regulated sialyltransferases in tumor cells. As a consequence, the peptide backbone of MUC1 on tumor cells is accessible to the immune system.[7b,8] To utilize this structural difference for immunotherapy, the immune system must be activated specifically against these tumor-associated MUC1 structures. This can only be achieved with very specific tumor-associated MUC1 antigens. As the transferase activities in tumor cells are never completely down- or up-regulated, normal as well as tumor-associated glycans can occur even on a single MUC1 molecule. The application of such glycoproteins within the scope of an immunotherapy could cause severe autoimmune reactions against normal MUC1. As pure tumor-associated MUC1 antigens cannot be isolated from tumor cells, they must be obtained by chemical total synthesis, which provides access to pure tumor-associated MUC1 glycopeptide antigens.[9] In particular, solid-phase peptide synthesis (SPPS) affords glycopeptide epitopes with different glycosylation patterns for the investigation of structure–activity relationships. However, these synthetic glycopeptides are endogenous and hence not sufficiently immunogenic structures. Therefore, in an antitumor vaccine, the tumor-associated MUC1 glycopeptides must be combined with immune-stimulating components to break the self-tolerance of the immune system. In particular, MHC class II T-helper epitopes are

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mandatory for the switch to IgG-producing B-cells within an adaptive immune response and for a long-term immunological memory.

Herein, we show that vaccines consisting of synthetic MUC1 glycopeptides as B-cell epitopes and tetanus toxoid (TTox) as the immune-stimulating carrier meet these requirements. After immunization of BALB/c mice, they elicited extraordinarily high titers of IgG antibodies, which bind to various human tumor cells with high affinity. To investigate the properties of the induced antibodies in cellular assays and to evaluate their potential as diagnostic tools in clinical practice, a monoclonal tumor-associated MUC1 antibody GGSK-1/30 was produced by hybridoma technology.

Comparative studies with MUC1-expressing normal epithelial cells demonstrated that the monoclonal antibody binds to tumor-associated MUC1 with high specificity, but ignores MUC1 on normal cells and has an exceptional diagnostic value.

The selectivity of the immune response induced by an antitumor vaccine depends on the MUC1 B-cell epitope. To achieve high tumor selectivity, the synthetic glycopeptide epitope must accurately mimic the tumor-associated MUC1 and must not reflect the structures of MUC1 exposed on normal cells. The common element of the MUC1 B-cell epitope investigated in the present study is the tandem repeat sequence of MUC1.

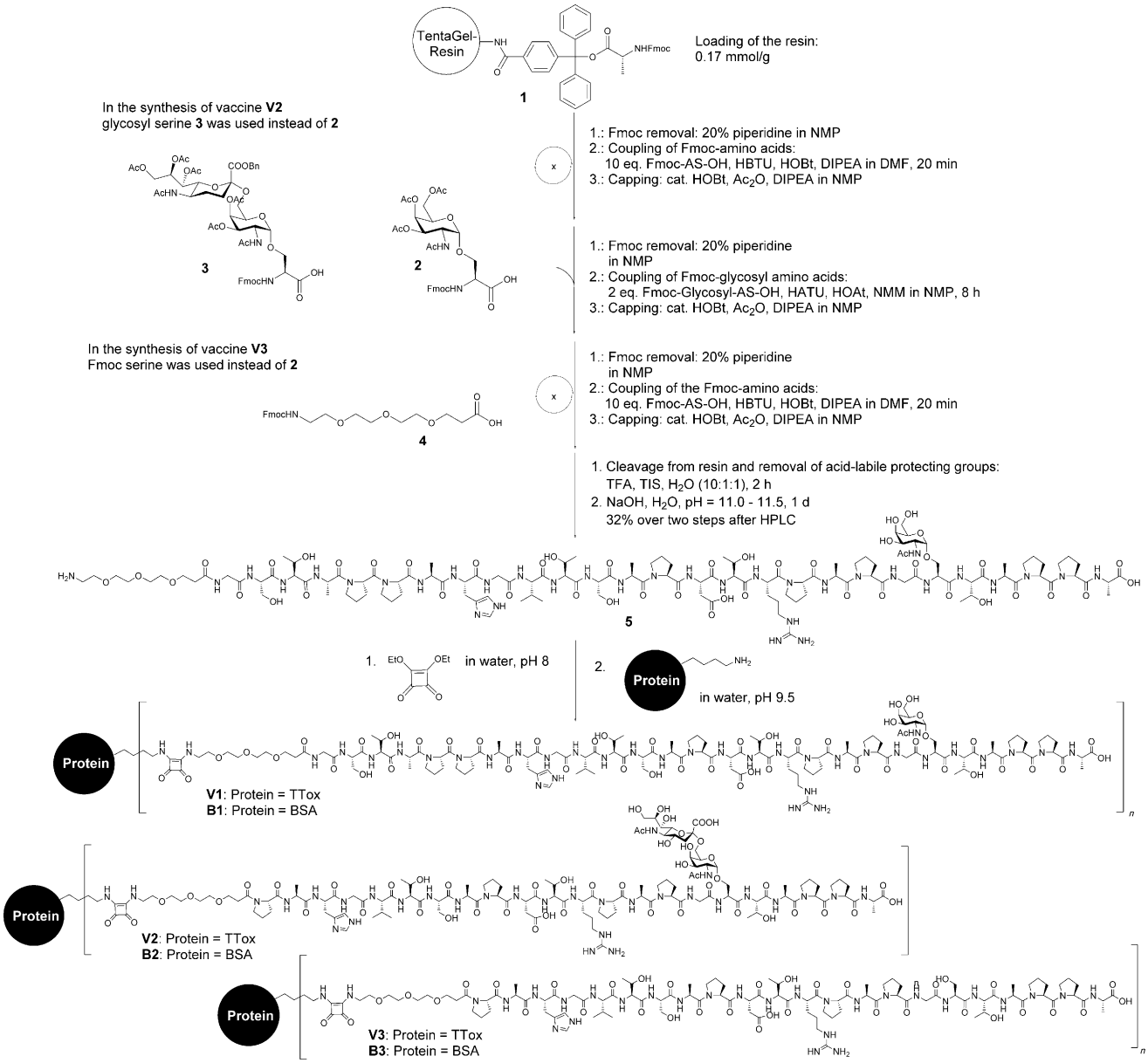
Three MUC1 (glyco)peptide antigens were obtained by solid-phase synthesis according to an Fmoc procedure[9b,10] (Scheme 1; see also the Supporting Information) and conjugated to tetanus toxoid or bovine serum albumin (BSA). In a new design compared to our previously described MUC1–TTox vaccines,[11] the tandem repeat was extended at the C-terminus by Pro and Ala to complete the STAPPA motif (**PA**HGVTSAPDTRPAPGSTAP-**PA**) and further varied by N-terminal extension, as it is known from NMR studies that the glycosylated STAPPA motif adopts a helical conformation and induces a turn in the PDTRP epitope, a preferred binding site of tumor-induced anti-MUC1 antibodies.[12] Furthermore, the position of glycosylation was shifted from Thr6 to Ser17. The different MUC1 B-cell epitopes were compared in mouse immunizations to determine the effect of glycosylation and peptide sequence on the tumor selectivity of the induced anti-MUC1 antibodies. Vaccine **V1** was built up as a 27 mer peptide sequence by N-terminal elongation. The PDTRP domain is thus surrounded by two STAPPA motifs. For comparison with a related 22 mer glycopeptide vaccine,[13] a T_N antigen serine was introduced at Ser22 (corresponding to Ser17 in **V2**). The B-cell epitope of vaccine **V2** comprises the 22 mer sequence substituted with a sialyl T_N antigen at Ser17. Its synthesis has already been described.[10a] To clarify the role of glycosylation as an antigenic component and a factor influencing the conformation of the peptide,[10b,12b] a TTox vaccine **V3** was formed as the non-glycosylated 22 mer for the first time. After completion of the solid-phase synthesis, the (glyco)peptides were detached from the resin with trifluoroacetic acid/triisopropylsilane/water (10:1:1). Under these conditions, concomitant removal of all acid-labile protecting groups from the glycopeptides was achieved. After purification by preparative HPLC, the (glyco)peptides were isolated in yields of 29 to 88%. The removal of the carbohydrate protecting groups was accomplished in aqueous NaOH at pH 11.5.[11a] Purification by preparative HPLC yielded the 27 mer glycopeptide of **V1** in 89% and the 22 mer glycopeptide of **V2** in 53% yield.

For the construction of the glycopeptide vaccines, tetanus toxoid (TTox) was applied as a strongly immunogenic carrier. It contains potent T-helper cell epitopes, which initiate T-helper cell stimulation. Furthermore, TTox is widely used in human medicine. It is efficient in humans and in mice. For the coupling to TTox,[11a] the N-terminal spacer amino group was reacted with diethyl squarate (see the Supporting Information) in water at pH 8.0.[14] The squarate monoamide was then reacted with the protein at pH 9.5. As had been established for analogous MUC1–TTox vaccines,[11a] the coupling to TTox as well as to BSA resulted in the multivalent presentation of the MUC1 epitopes.

For immunological evaluation, three mice[25] per group were immunized three times with vaccines **V** at intervals of three weeks. The first immunization was carried out with complete Freund's adjuvant (CFA). The two boost immunizations were performed with incomplete Freund's adjuvant (IFA), a simple water-in-oil emulsion. Five days after each boost, blood was drawn from each mouse and analyzed for glycopeptide-specific antibodies in ELISA on microtiter plates coated with the corresponding glycopeptide–BSA conjugates **B**.[15] All vaccines elicited high titers of IgG antibodies against the glycopeptides after the second boost. The titers at half-maximal absorbance (see the Supporting Information) ranged from 10000 to 200000. Isotype-specific ELISA showed that mainly IgG1 and small amounts of IgM antibodies were induced (see the Supporting Information), thus suggesting that all vaccines trigger an adaptive T-cell-mediated immune response by affinity maturation of the antibodies and establishment of an immunological memory.[16]

Using tumor-associated MUC1-expressing human breast cancer cells T47D,[17] the binding of the antisera to epithelial tumor cells was determined by flow cytometry. The antibodies induced by the non-glycosylated vaccine **V3** showed almost no binding to the tumor cells (Figure 1). In contrast, vaccine **V2**, which has the same peptide sequence but bears a sialyl T_N antigen at Ser17, induced antibodies exhibiting a high binding rate to the T47D tumor cells. These results provide evidence that a tumor-associated glycan within the B-cell epitope is mandatory for the induction of a large fraction of the B-cell repertoire recognizing aberrantly expressed MUC1 on human tumor cells. In agreement with these results, vaccine **V1**, with an N-terminally elongated peptide sequence and the small T_N antigen at Ser22, elicited antibodies that exhibited moderate binding to T47D tumor cells (Figure 1), reminiscent of the low binding of antibodies induced with a MUC1 22 mer glycopeptide vaccine carrying two T_N antigens in the VTSA and GSTA regions.[13] These findings suggest that both the peptide sequence and the tumor-associated glycosylation are crucial for the induction of tumor-binding antibodies.

To investigate the binding of antibodies induced by vaccine **V2** in greater detail and to have unlimited access to such an antibody, hybridomas were generated by fusing spleen cells of an immunized mouse with murine myeloma



Scheme 1. Solid-phase synthesis, removal of the *O*-acetyl groups of the glycopeptide antigens, and subsequent coupling to carrier proteins by reaction with diethyl squarate.

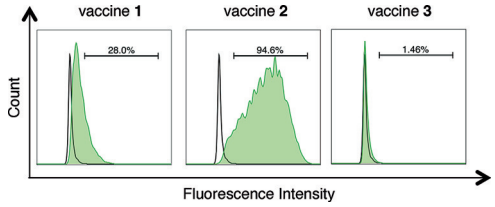


Figure 1. Binding of the induced antisera to human T47D breast cancer cells. For each vaccine, a representative result of the flow-cytometry analysis of one immunized mouse is shown. The graphs in green show the results for the vaccine-induced antisera (dilution of the antisera: 1:500), whereas the graphs in black show negative controls (serum of a non-immunized mouse, dilution: 1:500).

cells in polyethylene glycol (see the Supporting Information). Cloning and screening of these hybridomas resulted in a monoclonal antibody GGSK-1/30 that binds with a high rate to the synthetic MUC1 B-cell epitope that was present in the TTox vaccine used for vaccination. The IC_{50} value is about 0.06 ng mL^{-1} (see the Supporting Information). Isotyping revealed that mAb GGSK-1/30 is of the IgG1 isotype. Flow cytometry of the tumor-associated MUC1-expressing human breast cancer cell lines T47D and MCF-7[18] showed that mAb GGSK-1/30 binds to these tumor cells with high rates (Figure 2 A). Detection with goat anti-mouse IgG1, labeled with Alexa Fluor 488, stained 99 % of the MCF-7 and 94 % of the T47D cancer cells. The binding of mAb GGSK-1/30 to human pancreatic cancer cells PANC1[19] was also determined. Although these cells express tumor-associated MUC1 at a significantly lower level than MCF-7 and T47D cells,[20] the antibody still binds to 74 % of the PANC1 cells. As pancreatic cancer is a most devastating disease and its specific

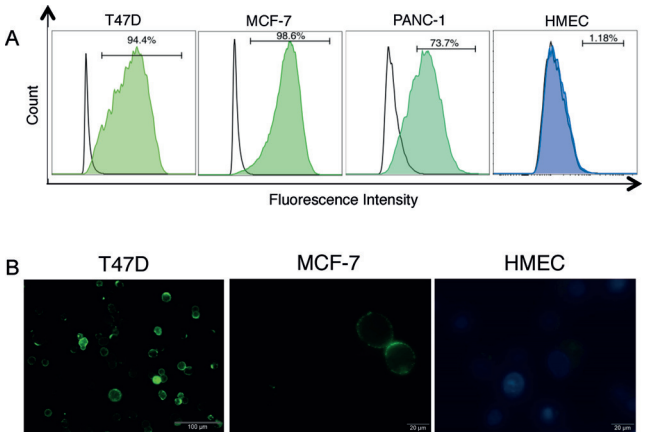


Figure 2. A) Binding of the monoclonal antibody GGSK-1/30 to human cancer cells detected through staining with secondary goat anti-mouse IgG1 antibody labeled with Alexa Fluor 488. Concentration of the monoclonal antibody: 1 μg mL^{-1}. The colored graphs show its binding to different cell lines (from left to right: T47D, MCF-7, and PANC1) and to normal human epithelial cells (very right). Negative controls (incubation only with the secondary antibody) are shown in black. B) Fluorescence-microscopy staining of breast cancer cells (left: T47D, magnification: ×20; middle: MCF-7, ×100) and normal epithelial cells (right: HMEC, visualized with DAPI, ×100).

diagnosis in clinical routine is still unsolved, these properties are of particular interest.

However, the key requirement for the successful diagnostic use of the monoclonal antibody and the therapeutic application of the anti-tumor vaccines is that the induced antibodies bind exclusively to tumor-associated MUC1 on cancer cells and not to MUC1 on benign epithelial cells. Binding to healthy tissues could cause misdiagnosis or severe autoimmune reactions. To investigate the binding specificity of mAb GGSK-1/30, human mammary epithelial cells (HMEC) were used. These cells express normally glycosylated MUC1 on their cell surface[21] and thus serve as a model for normal epithelial tissues. Prior to flow cytometry, the MUC1 (CD227) expression of the HMEC cells was tested by the polymerase chain reaction and gel electrophoresis (see the Supporting Information). Flow-cytometry analysis showed that mAb GGSK-1/30 does not bind to normal MUC1 on HMEC (Figure 2 A). These binding results of GGSK-1/30 were confirmed by fluorescence microscopy of MCF-7 and T47D tumor cells as well as by the staining of HMEC cells with a goat anti-mouse IgG1 linked to Alexa Fluor 488 (Figure 2 B). To render the HMEC cells visible, their nuclei had to be stained with the blue fluorescent dye 4′,6-diamidino-2-phenylindole (DAPI). In a cross experiment, both T47D tumor cells and normal epithelial cells (HMEC) marked with a proliferation dye were combined and stained with GGSK-1/30. Again only the tumor cells were stained with high intensity, whereas the normal cells (HMEC) showed almost no binding. These promising findings suggest that the binding of mAb GGSK-1/30 is highly specific. Human tumor cells are clearly recognized, but normal human epithelial cells are not affected.

It then appeared particularly interesting whether the antibody is also capable of specifically staining epithelial tumor tissues from human patients. Pancreatic cancer is a severe problem. Five years after its diagnosis less than 10 % of the patients have survived.[22] The reasons for the poor prognosis are that often no symptoms occur at the onset of the disease, and that no appropriate routine tests are available for the specific diagnosis. Keeping this in mind, mAb GGSK-1/30 was used as diagnostic tool for staining different pancreatic cancer tissue sections from patients.

Figure 3 Aa shows the staining of a ductal pancreatic adenocarcinoma.[22] A characteristic of this cancer is that the tumor cells grow around secretory glands in several layers. These are exactly the areas that are intensely recognized by mAb GGSK-1/30. The staining of a mucinous cystadenocarcinoma in Figure 3 Ab also shows the specificity of mAb GGSK-1/30: Whereas the tumor cells around secretory glands are recognized with high specificity, the normal tissue is not affected. It should be emphasized that this cell section was obtained by a fine-needle biopsy, that is, with minimal invasive effort.

In conclusion, synthetic MUC1 glycopeptide antitumor vaccines with TTox as the immune-stimulating carrier are currently the most potent MUC1 vaccine candidates. They induce outstandingly high titers of tumor-associated MUC1-specific IgG antibodies compared to other antitumor vaccines.[23] The multiple presentation of the MUC1 glycopeptides on TTox enhances the immunogenicity of the vaccine. The reason for the superior accuracy of the immune response, in particular of that induced by vaccine **V2**, can be attributed to the structure of the MUC1 glycopeptide epitope. Many factors, including the length and the segment of the peptide sequence, the tumor-associated carbohydrate antigen, the

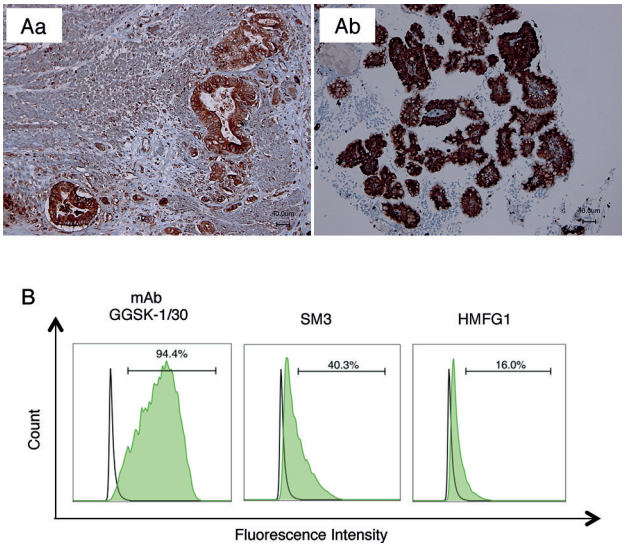


Figure 3. A) Staining of pancreatic cancer sections with the monoclonal antibody GGSK-1/30 and the goat anti-mouse IgG antibody, conjugated to horseradish peroxidase for the oxidation of 3,3′-diaminobenzidine to form a red-brown polymer. Concentration of mAb: 2 μg mL^{-1}. Aa) Staining of a pancreas adenocarcinoma (magnification: ×100). Ab) Staining of a mucinous cystadenocarcinoma of the pancreas (×100). B) Binding of different anti-ta-MUC1 antibodies to T47D cancer cells. Graphs in green show the binding to T47D cancer cells (left: mAb GGSK-1/30 induced by **V2**, 1 μg mL^{-1}; middle: antibody SM3, 1 μg mL^{-1}; right: antibody HMFG1, 1 μg mL^{-1}). Negative controls shown in black.

position of the linkage to the peptide, the spacer used to separate the epitopes from the carrier, and the coupling ratio of the antigen and the immunogenic carrier, are crucial for the efficiency of the vaccine. It is therefore advantageous to synthesize the MUC1 glycopeptides on solid phase, because it gives access to MUC1 epitopes with different, but exactly specified glycosylation patterns and simplifies the study of structure–activity relationships. The superior selectivity of the immune response that is induced against a well-defined, purely synthetic epitope is demonstrated by the binding ratios of commercially available tumor-associated MUC1 antibodies compared to those of the monoclonal antibody described in this work (Figure 3B). The antibodies SM3[24] or HMFG1[7a] were induced against partially deglycosylated and delipidated MUC1, respectively, from human milk. Owing to the microheterogeneity of these antigens, the induced antibodies are not sufficiently precise in distinguishing between normal and tumor-associated MUC1. In contrast, the synthetic MUC1 B-cell epitope of vaccine **V2**, against which the antibody GGSK-1/30 was induced, obviously appropriately reflects the structure of the aberrantly glycosylated MUC1 exposed on epithelial tumor cells. Therefore, the induced antibody binds exclusively to tumor-associated MUC1 on tumor cells and not to normal epithelial cells.

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Keywords: antitumor vaccines · glycopeptides · MUC1 · tumor cell recognition · tumor-selective monoclonal antibodies

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