Antitumor Vaccines

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A Fully Synthetic Four-Component Antitumor Vaccine Consisting of a Mucin Glycopeptide Antigen Combined with Three Different T-Helper-Cell Epitopes**

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Abstract: *In a new concept of fully synthetic vaccines, the role of T-helper cells is emphasized. Here, a synthetic antitumor vaccine consisting of a diglycosylated tumor-associated MUC1 glycopeptide as the B-cell epitope was covalently cross-linked with three different T-helper-cell epitopes via squaric acid ligation of two linear (glyco)peptides. In mice this four-component vaccine administered without external immune-stimulating promoters elicit titers of MUC1-specific antibodies that were about eight times higher than those induced by a vaccine containing only one T-helper-cell epitope. The promising results indicate that multiple activation of different T-helper cells is useful for applications in which increased immunogenicity is required. In personalized medicine, in particular, this flexible construction of a vaccine can serve as a role model, for example, when T-helper-cell epitopes are needed that match human leukocyte antigens (HLA) in different patients.*

The discovery in the late 19th century that treatment with inactivated viral or bacterial pathogens leads to protection against infectious diseases marked a great discovery in modern medicine.[1,2] Although vaccination is a powerful tool, its use is currently limited to protecting against infectious diseases caused by bacterial, protozoic, and viral pathogens. Cancer is a major cause of death in the developed world. However, protection from cancer by means of vaccination is challenging, since the human immune system has evolved to differentiate between exogenous and endogenous structures. In the case of cancer it has to distinguish between self and altered-self, that is, neoplastic cancer cells. A promising target for this differentiation is the mucin MUC1,[3] a highly glycosylated protein that occurs on the surface of epithelial cells of many tissues, for example, in breast, pancreas, ovary, and colon epithelium. The main part of MUC1 consists of an extracellular domain, which contains numerous (20 to 120) tandem repeats, each of which is a 20-mer sequence PAHGVTSAPDTRPAPGSTAP with five potential O-glycosylation sites at serine and threonine. MUC1 on normal cells is heavily glycosylated. Due to the down-regulated glycosyl transferase activities[5] in tumor cells, the carbohydrate side chains of MUC1 in tumor cells are much shorter than those on normal cells. These truncateded tumor-associated carbohydrate antigens (TACAs) make the peptide backbone of MUC1 accessible for the immune system.[3,6]

Since normal MUC1 may occur next to tumor-associated MUC1 even within a single tumor cell, the isolation of pure tumor-associated MUC1 from tumors is not possible. Nowadays, the use of solid-phase glycopeptide synthesis provides access to chemically pure structures of tumor-associated MUC1 glycopeptides in sufficient quantities. In contrast to classic vaccines (e.g. against rabies)[7] where the activity of the pathogenic material must be reduced, the opposite is the case for antitumor vaccines based upon the endogenous tumor-associated MUC1. Its immunogenicity is not strong enough for sufficient activation of the immune system against the tumor. In order to achieve a strong immune response, the tumor-associated MUC1 glycopeptide has to be linked as a B-cell epitope to immune-stimulating components. Especially MHC class II T-helper-cell epitopes are needed for activation of T-helper cells, because only these cells promote via their co-stimulatory signals (cytokines and costimulatory receptors) the appropriate activation of B-cells and induce their maturation into antibody-producing plasma cells.

Extraordinarily high titers of tumor-specific MUC1 antibodies are generated when MUC1 B-cell epitopes are linked to tetanus toxoid, a detoxified protein derived from *Clostridium tetani* that contains many potent T-helper-cell epitopes.[8,9] One disadvantage of these conjugates is that they cannot be characterized well once the glycopeptide is coupled to the protein. Furthermore, these immunizations were carried out with complete Freund's adjuvant (CFA), a strong immune-stimulating material that activates several pathways of the innate immune system. Nowadays, its use is prohibited in humans due to severe adverse reactions. In addition, commercial use of tetanus toxoid and proteinogenic vaccines is expensive. Most of the expense was associated with the cold chain for storage.[10] For these reasons efforts have been expended to develop fully synthetic multicomponent vaccines.[11,12] For example, the immune-stimulating Toll-like

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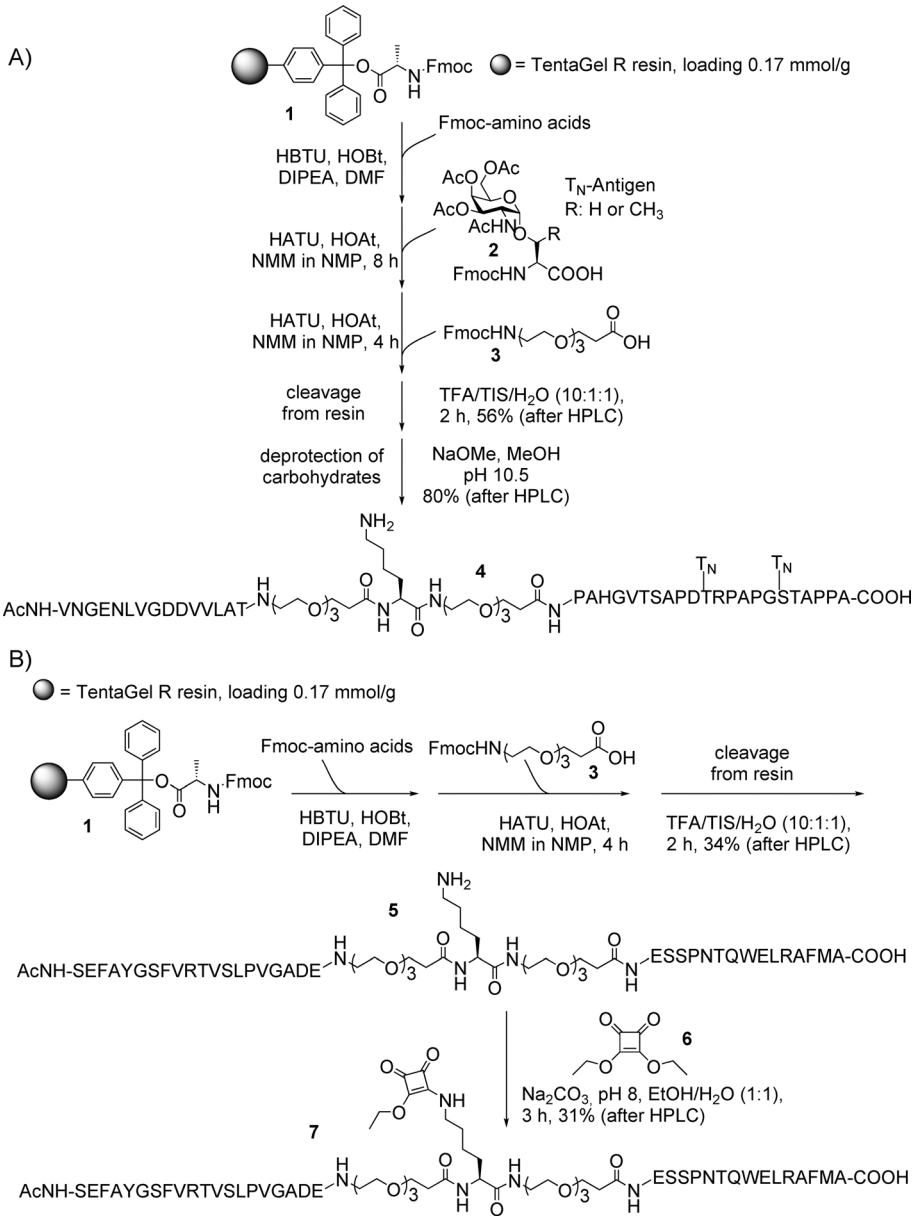
receptor 2 (TLR2) agonist Pam_3CysSK_4 has been coupled to T-helper-cell epitopes and tumor-associated B-cell epitopes and resulted in considerable immune responses in animal studies.[13–16] In other studies the tumor-associated MUC1 glycopeptides as well as T-helper-cell epitopes were conjugated to polymeric carriers in order to enhance the immunogenicity of antitumor vaccines by multivalent antigen presentation.[17,18]

Since the quality of the immune response crucially depends on the efficacy of the T-helper-cell epitope, we here describe the synthesis of a first example of a fully synthetic four-component glycopeptide vaccine, which contains up to three different T-helper-cell epitopes. Such a structure enhances the activation of B-cells by different T-helper cells and leads to significantly higher titers of tumor-binding IgG antibodies in mice than those obtained from an analogous vaccine with only one T-cell epitope.

The specificity of antibodies induced by these vaccines is crucially determined by the B-cell epitope. It has to mimic the tumor-associated structure of MUC1 accurately and must not induce antibodies that bind normal MUC1 in order to avoid autoimmune reactions. As a basis for the B-cell epitope, the tandem repeat sequence extended by two amino acids on the C-terminus (a 22-mer peptide) was selected (PAHGVTSAPDTRPAPGSTAPPA). This sequence contains the immune-dominant domains PDTRP and GSTA, which both are considered preferred binding sites for anti-MUC1 antibodies.[19] The conformation of MUC1 tandem repeat peptides is distinctly influenced by O-glycosylation.[20] In the present work, two glycan side chains were installed at the peptide sequence, one T_N antigen at threonine-11 and a second T_N antigen at serine-17, so that both immune-dominant domains were glycosylated (see Scheme 1 A).

Although there are different ways to activate the immune system and to generate antibody-producing B-cells, the most efficient proceeds via activation by T-helper cells, because they mediate affinity maturation and antibody isotype switching. Both are crucial for the generation of highly specific antibodies that selectively recognize the tumor cells and induce antibody-dependent cytotoxicity (ADCC). Furthermore, T-helper cells are involved in the induction of memory B-cells, which permit long-term immunity. For the construction of the vaccine, three different MHC class II peptides were selected, which are active in mice and in humans activating T-helper cells and induce a T-cell-mediated immune response due to their cytokine profile. These epitopes are reported to activate T-helper cells which produce a cytokine milieu consisting of interleukin 4 (IL-4), interleukin 2 (IL-2), and interferon γ (IFN-γ). Two of the epitopes are derived from *Yersinia pestis* (epitope 1: 14-mer peptide (VNGENLVGDDVVLAT, cytokine induction: IFN-γ > IL-2 > Il-4, T-cell proliferation: +++);[21] epitope 2: 15-mer peptide (ESSPN-TQWELRAFMA, cytokine induction:



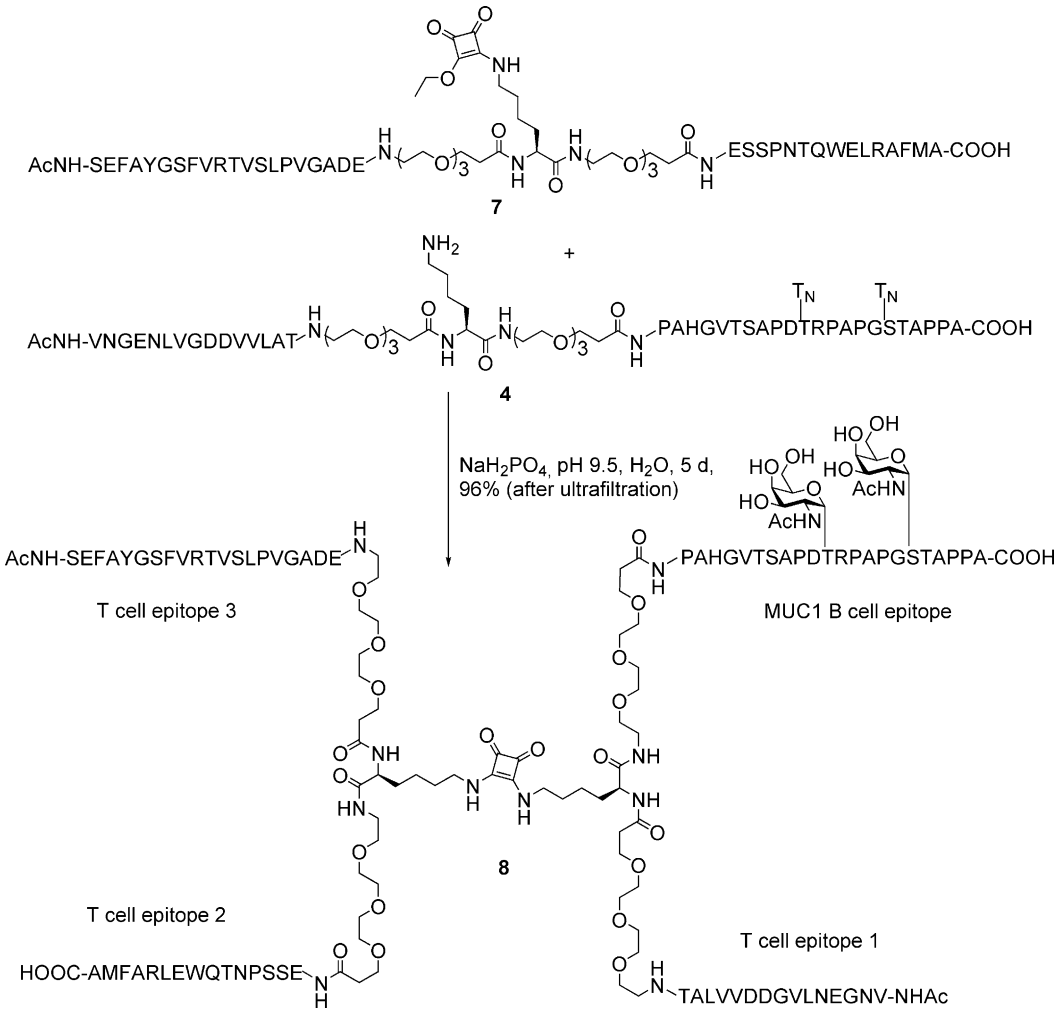
Scheme 1. Solid-phase synthesis and subsequent removal of the O-acetyl protecting groups of glycopeptide **4** (A). Solid-phase synthesis of peptide **5** followed by subsequent reaction with diethyl squarate (**6**) led to the squaric acid monoamide ethyl ester **7** (B).

IFN-γ ≫ IL-2 > IL-10 > IL-4, T-cell proliferation: ++).[22] The third is a 20-mer peptide (SEFAY-GSFVRTVSL-PVGADE, cytokine induction: IL-4 ≫ IFN-γ, T-cell proliferation: +)[23] derived from *Mycobacterium tuberculosis*.

For optimal efficiency of the vaccine, all four components are linked covalently. Nevertheless, the B-cell epitope glycopeptide should be presented in its natural conformation. Therefore, the B- and T-cell epitopes of the vaccine were separated via flexible, water-soluble, non-immunogenic triethylene glycol spacers[24] **3**, which ensure that the individual (glyco)peptides do not influence each other in their conformation. The syntheses of the linear (glyco)peptide conjugates **4** and **5** were achieved by 9-fluorenylmethoxycarbonyl (Fmoc)-based solid-phase peptide synthesis starting from an Fmoc-alanine-loaded TentaGel trityl resin **1** (Scheme 1).[8] The Fmoc amino acids were coupled using HBTU/HOBt, whereas the coupling of the Fmoc-*O*-glycosyl amino acid building blocks (T_N antigen, **2**) and spacers was accomplished with the more reactive HATU/HOAt coupling reagents. A central lysine was inserted between the two spacers. It serves as the anchor for later cross-linking reactions. To prevent further reactions at the N-terminus, the (glyco)peptide conjugates were acetylated with acetic anhydride. After completion, the (glyco)peptide conjugates were detached from resin with concomitant removal of all acid-labile protecting groups on the amino acid side chains using trifluoroacetic acid/triisopropylsilane/water (10:1:1). The peptide conjugate **5** was obtained in a yield of 34% after preparative HPLC. The glycopeptide conjugate (Scheme 1A) also was purified by preparative HPLC and the O-acetyl groups were removed from the glycans using catalytic NaOMe in methanol (pH 10.5). The product was purified by preparative HPLC to give the free glycopeptide conjugate **4** in an overall yield of 45% (see the Supporting Information). For coupling the two conjugates the ε-amino group of the lysine of **5** was reacted with diethyl squarate (**6**) in sodium bicarbonate solution (pH 8)[25] in water/ethanol to give squaric acid monoamide ethyl ester **7**, which was purified by preparative HPLC (Scheme 1B, Supporting Information). The linear glycopeptide **4** was reacted with **7** to build up the target vaccine **8** in Na_2HPO_4 buffer (pH 9.5).[26] The crude product was purified by ultrafiltration to afford vaccine **8** in 96% yield (Scheme 2).

It should be noted that in vaccine **8** the epitopes are connected in a certain pattern: While the T-cell epitope 1 (*Y. pestis*) and the *M. tuberculosis* epitope (T-cell epitope 3) are arranged with the glycopeptide antigen in a linear C→N terminus mode, the glycopeptide is coupled to the T-cell peptide 2 in a branched N→N terminus fashion, which is supposed to be more resistant to degradation.[27] This pattern can be varied for reasons of optimization. The described principle of construction also affords the possibility of the multiple presentation of any of the epitopes.

In order to evaluate the potential of the triple T-helper-cell epitope vaccine **8**, a comparison with vaccine **4**, which contains just the *M. tuberculosis* T-helper-cell epitope was carried out. Three female Balb/c mice per group (age of 12 weeks) were immunized four times at intervals of three weeks with one of the vaccines.[32] Administration was performed



Scheme 2. Ligation of glycopeptide **4** and peptide **7** to give vaccine **8**.

intraperitoneally (50 μg glycopeptide in 40 μL of a simple water-in-oil emulsion) without external immune-stimulating components. Five days after each booster immunization blood was collected from the tail vein of each animal and screened through enzyme-linked immunosorbent assay (ELISA) for vaccine-induced serum antibodies of the IgG isotype. The microtiter plates were coated with the conjugate of the 22-mer MUC1 glycopeptide antigen to bovine serum albumin (BSA, see the Supporting Information).[28,29] Five days after the first booster immunization antigen-specific IgG antibodies were detected in the sera of all mice. The titers were significantly higher in the group of mice immunized with the triple T-helper-cell epitope four-component vaccine **8** (Figure 1 A). This enhanced potency was even more pronounced after the third booster immunization. The end-point titer values for vaccine **8** (mouse 2 and 3) were in the range of about 30 000, approximately eight times higher than the end-point titers induced by the two-component vaccine **4** (Figure 1 B). Compared to conjugates containing the whole tetanus toxoid protein, the elicited IgG titers of vaccine **8** are about one order of magnitude lower.[8,9] However in comparison to other fully synthetic vaccines[16–18] evaluated by similar ELISA techniques the IgG titers elicited by vaccine **8** are higher. In a further ELISA experiment the isotypes of the antibodies were analyzed using isotype-specific secondary antibodies. This analysis revealed that vaccine **8** induced in two of three mice mainly antibodies of the IgG1 isotype and antibodies of IgM isotype in significantly lower amounts (Figure 2 A). The predominant occurrence of IgG1 antibodies gives evidence that vaccine **8** actually elicits adaptive T-cell-mediated immune responses triggered predominantly through IL-4,[30,31] which also resulted in the formation of an immunological memory with long-term immunity. To check whether the antibodies are able to bind tumor-associated MUC1 on cells, the antisera induced by vaccines **8** and **4** were incubated with cells of the MUC1-expressing human breast cancer cell line T47D. After washing, T47D cells recognized by the induced antibodies were stained with a fluorescence-labeled goat anti-mouse IgG secondary antibody, and the fluorescence intensity of the cells was determined by flow cytometry. The antisera induced by vaccine **4** in mouse 3 showed only moderate binding to the T47D cells, whereas the antisera induced by **8** in mouse 2 showed a high binding affinity to tumor-associated MUC1 on the cell surface and stained 71 % of all the T47D cells (Figure 2 B).

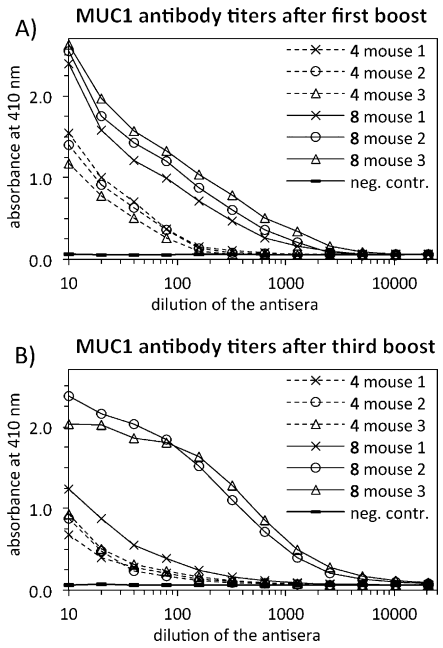


Figure 1. Antibody titers of vaccine **4** and **8** after the first (A) and third (B) booster immunizations.

The fully synthetic four-component antitumor vaccine containing a tumor-associated MUC1 glycopeptide as the B-

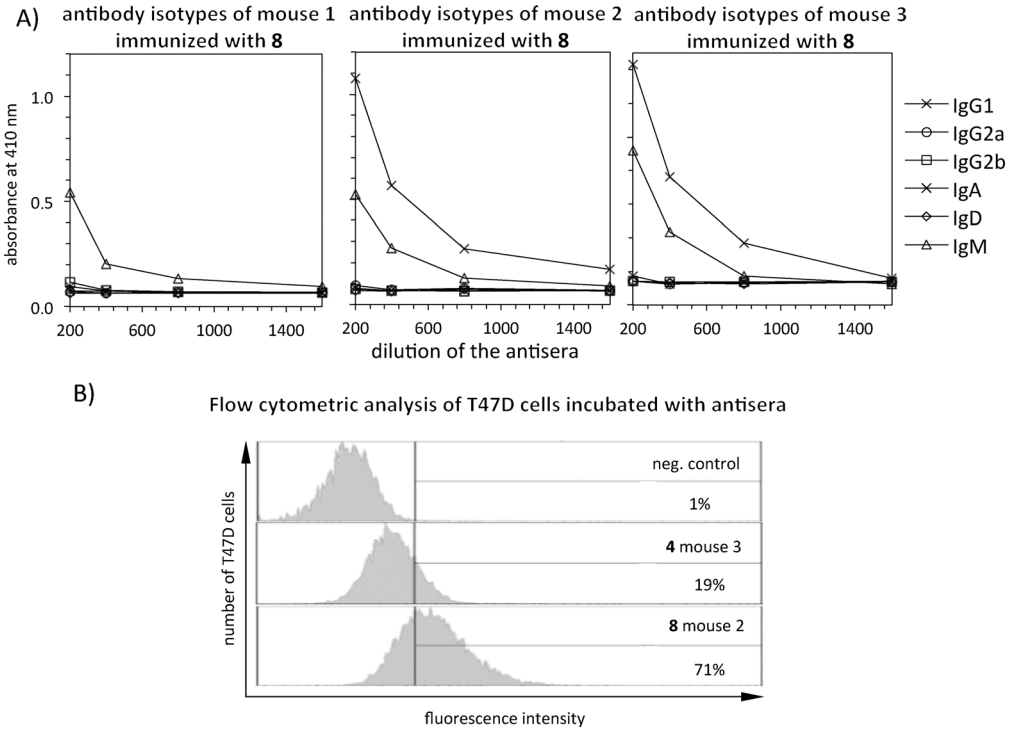


Figure 2. Analysis of the antibody isotype induced by vaccine **8** (A) and flowcytometric analysis of the binding of induced antibodies to human breast cancer cells of the cell line T47D (B, negative control: serum of a non-immunized mouse).

cell epitope and three different bacterial T-helper-cell epitopes elicits adaptive T-cell-mediated immune responses and predominantly protective IgG antibodies that recognize tumor-associated MUC1 on human breast cancer cells. The branched structure of this vaccine is also expected to be more stable towards metabolic degradation. The synthesis of this novel vaccine type succeeded by the criss-cross formation of a squaric diamide at the ε-amino groups of lysine linkers within the linear two-component conjugates which contain a B-cell epitope glycopeptide/T-cell epitope peptide or a T-cell epitope/T-cell epitope peptide combination, respectively. To maintain the conformation of the epitopes within the vaccine, all four components were separated by flexible, water-soluble triethylene glycol spacers. The antibody titers induced by this four-component vaccine were about one order of magnitude higher than those induced by a vaccine exposing a single T-helper-cell epitope. These results reveal that the concept of activating a number of different T-helper cells is promising and may also be attractive for other applications in which enhanced immune responses gain the therapeutic benefit of drugs. An optimized selection of the built-in T-cell epitopes should make it possible to modulate the cytokine milieu in a way which is most efficient in inducing the desired immune reaction. In addition, the described four-component vaccine concept offers a variety of modifications. For example, the B-cell epitope can be combined with two T-cell epitopes and an immune-stimulating lipopeptide of the Pam_3Cys type acting as a built-in adjuvant. Furthermore, the flexible assembly of the vaccine could serve as an approach for personalized medicine, when HLA-matched T-helper-cell epitopes are needed for different patients.

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