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Towards a Self-Adjuvanting Multivalent B and T cell Epitope Containing Synthetic Glycolipopeptide Cancer Vaccine

Olivier Renaudet,[a] Lbachir BenMohamed,[b] Gargi Dasgupta,[b] Ilham Bettahi,[b] and Pascal Dumy*[a]

Malignant tumor cells are characterized by the overexpression of altered glycoproteins or glycolipids resulting from the deregulation of glycosylation processes.[1] The identification of these tumor-associated carbohydrate antigens (TACA) has largely contributed to the development of cancer diagnostic and immunotherapy. Particularly, TACA present strong antibody determinants (B cell epitopes) which are primarily targeted by tumor-specific antibodies (Abs). Although TACA are rightly considered as tremendous potential targets for cancer vaccines, their poor immunogenicity still hampers their use as therapeutic vaccines. To address this challenge, both careful rational design and robust chemical procedures should be considered to construct TACA-based vaccine prototypes capable of promoting a strong and selective Ab response against tumor cells.

In the last decade, intensive research has focused on the development of molecularly defined TACA-based vaccine prototypes.[2] These studies have clearly defined that not only the display of TACA, but also their nature and molecular formulation are crucial to improve immunity against tumors. First, a multivalent presentation of TACA, either on carrier protein[3] (for example keyhole limpet hemocyanin) or on synthetic delivery systems containing CD4+ T helper (Th) cell epitope[4] (for example multiple antigen glycopeptide) is required to elicit strong B cell responses and raise high affinity tumor-specific Abs. In addition, it was established that priming and sustaining of both Ab and CD8+ cytotoxic T cell (CTL) responses, the latter also crucial in cancer immunity, requires CD4+ Th cell help.[5] This suggests that an ideal cancer vaccine formulation must incorporate B cell, CD4+, and CD8+ T cell epitopes to ensure both humoral and cellular eradication of tumors.[6] Finally, these synthetic multivalent vaccines should be delivered together with potent and safe external immunoadjuvants to ensure an early and strong immunity.[2d] To avoid the potential toxicity related to most of external adjuvants, especially often in immunocompromised cancer patients, recent reports have highlighted that palmitoyl-tailed B and T cell epitope peptides delivered in adjuvant-free saline are clinically safe, eliciting strong, long-lasting, and multivalent protective immunity.[7]

However, except for a few studies reporting the synthesis of up to three-component multivalent or "polytope" vaccines,[5,8] no molecular constructions have been designed so far on the basis of these overall structural features. This is presumably because of inherent difficulties,[9] despite recent progresses in the synthesis, assembly, and formulation of oligosaccharide and glycoconjugate biomolecules.[10] In this communication, we report for the first time on the design, synthesis, safety, immunogenicity, and protective efficacy of a prototype, molecularly defined, fully synthetic, self-adjuvanting multivalent glycolipopeptide (GLP) cancer vaccine.

As illustrated on Figure 1, our GLP vaccine prototype associated four essential components displayed on a molecular delivery system: 1) a cluster of TACA B-cell epitope; 2) a CD4+ Th peptide epitope; 3) a CD8+ CTL peptide epitope; and 4) a palmitic acid (PAM) that serves as a built-in immunoadjuvant. We first focused on developing a controlled assembly of such a multiepitopic molecule based both on oxime and disulfide bond formation.

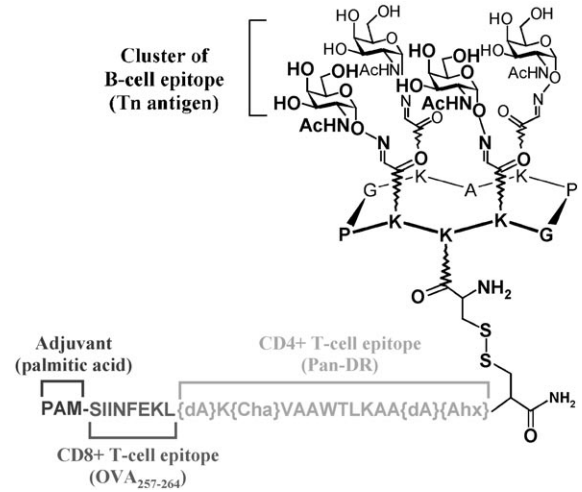

Figure 1. Illustration of the palmitoyl-tailed chimeric B-Th-CTL vaccine prototype (with dA: L-alanine; Cha: cyclohexyl alanine; Ahx: L-2-aminohexanoic acid).

In comparison with classical synthetic methods, such chemoselective procedures offer several advantages: 1) each counterpart (carbohydrate, peptide backbone, and lipid) are synthesized separately, thus preventing critical manipulations of multiple protecting groups and activating reagents; 2) oxime liga-

[a] Dr. O. Renaudet,[+] Prof. P. Dumy
Département de Chimie Moléculaire
UMR-CNRS 5250 & ICMG FR 2607
Université Joseph Fourier
38041 Grenoble Cedex 9 (France)
Fax: (+33) 4-7651-4946
E-mail: pascal.dumy@ujf-grenoble.fr

[b] Dr. L. BenMohamed,[+] Dr. G. Dasgupta, Dr. I. Bettahi
Laboratory of Cellular and Molecular Immunology
Department of Ophthalmology
University of California Irvine
School of Medicine
Irvine, CA 92697-4375 (USA)

[+] These authors contributed equally to this research.

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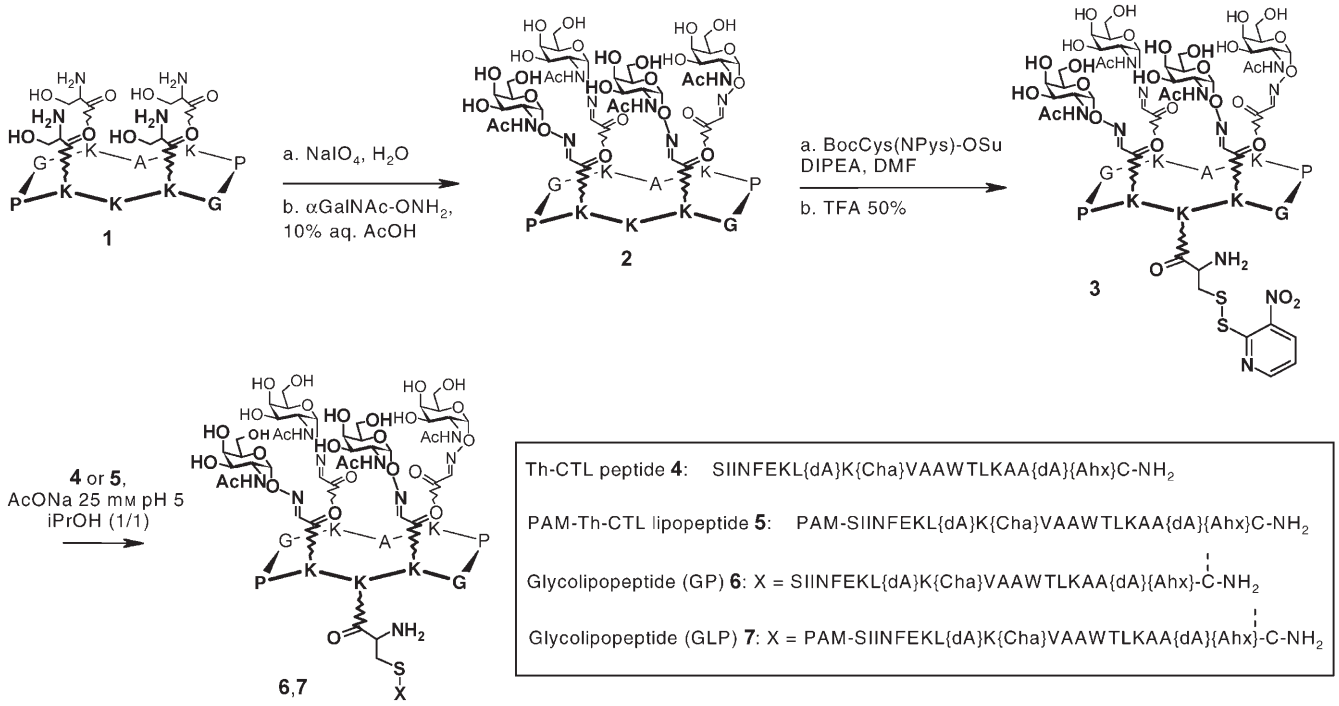
tion and disulfide bond formation are compatible, reproducible, and highly chemoselective coupling chemistries which permit construction of the molecular assembly from deprotected building blocks under mild aqueous conditions in a controlled manner; 3) the resulting multivalent GLP vaccine prototypes are molecularly defined and water soluble molecules allowing their in vivo delivery in adjuvant-free saline for immunogenicity and protective efficacy studies.

In the last few years, we have developed new cyclodecapeptide scaffolds based on the TASP (template assembled synthetic protein) model.[11] These regioselectively addressable functionalized template molecules (or RAFT) exhibit two independent and chemically addressable domains. This structural feature allows sequential and regioselective assembly of biomolecule-based ligands and biologically functional units to confer both recognition and effector properties by preventing steric hindrance.[12] In particular, we have recently demonstrated that a molecularly-defined RAFT core displaying clustered Tn antigen analogue represents an effective nonimmunological carrier molecule.[13] This preliminary study has thus provided a serious molecular basis for the design of next generation synthetic TACA-based cancer vaccines.

In this study, we took advantage of the synthetic versatility of the RAFT platform, and synthesized a GLP prototype cancer vaccine **7** and its corresponding glycopeptide (GP) structural analogue **6**, used as positive control (Scheme 1). Both GLP and GP molecules are composed of a RAFT core displaying clustered Tn analogues as B-cell epitopes to ensure an efficient Ag delivery to antigen presenting cells (APCs). For this purpose, we report herein an efficient ligation strategy that allows the incorporation of carbohydrates and lipopeptide on the RAFT core. This strategy first requires the preparation of aminooxy-lated carbohydrate to be assembled onto the cyclic decapeptide template displaying glyoxoaldehyde functions.[12b] The GalNAc moiety containing the aminooxy function at the alpha anomer position was prepared as a Tn analogue following our recently described procedure.[14] The RAFT core **1** was assembled from an orthogonally protected linear decapeptide which was prepared on the acido labile SASRIN resin using Fmoc/*t*Bu strategy under standard activation with PyBOP/DIPEA (benzotriazole-1-yloxytripyrrolidinophosphonium hexafluorophosphate/diisopropyletylamine). The glycine was linked to the C-terminal end to secure the subsequent cyclization step from epimerization. Cyclization of the linear peptide in solution followed by successive steps of regioselective deprotection and coupling of serine residues afforded **1**. After oxidative cleavage of serines with sodium periodate, the aminooxy αGalNAc was coupled as a cluster on the upper domain of **1** by oxime ligation. This chemoselective coupling reaction occurred in an aqueous solution of acetic acid to provide the pure tetravalent glycocluster **2**. A cysteine residue bearing the activating group *S*-3-nitro-2-pyridinesulfenyl (Npys) was finally incorporated at the free lysine side chain pointing below the RAFT core **2**. After acidolysis of the Boc protecting group with trifluoroacetic acid, the pure and stable compound **3** was used without further purification. The presence of a Npys group enables the regioselective conjugation of any biomolecule containing free thiol group through disulfide bridge formation.[15]

The $OVA_{257-264}$ peptide (SIINFEKL)[16] was used as a target CD8(+) T-cell epitope and synthesized in line with the Pan-DR universal CD4(+) Th peptide (PADRE, (dA)K-(Cha)VAAWTLKAA(dA)(Ahx) with dA: l-alanines, Cha: l-cyclohexyl alanine and Ahx: aminocaproic acid,[17] see Scheme 1). The PADRE $CD4^+$ Th epitope helps to prime and sustain both B



Scheme 1. Chemoselective assembly of glycopeptide (GP) **6** and glycolipopeptide (GLP) **7** vaccine prototypes.

and CTL responses.[7] A cysteine residue was linked to the C-terminal end of the resulting Th-CTL chimeric peptide **4** to provide the anchoring site for further conjugation on the lower face of the RAFT core **3**. The resulting Th-CTL-RAFT GP backbone **4** was extended at the N-terminal end with a palmitic acid moiety (PAM) to confer the self-adjuvanting property of the resulting GLP molecule. Molecules tailed with fatty acid moieties target and induce maturation of dendritic cells, when delivered in adjuvant-free saline.[7] Lipopeptide construction was carried out in two steps using a previously reported protocol that provides high yield, molecularly pure, and water soluble compounds.[6] Unlike the first generation of lipopeptide synthesized on solid support, the palmitic acid moiety was introduced in the chimeric Th-CTL **4** in solution after synthesis and purification of the peptide backbone to afford PAM-Th-CTL lipopeptide **5**. This method is compatible with water or saline formulation and solubility of lipopeptides with hydrophobic sequences, such as PADRE epitope. Indeed, full solubility was obtained when PAM-Th-CTL **5** was formulated in water or in PBS solutions at concentrations as high as 5 mg mL^{-1}. Chimeric Th-CTL peptide **4** and lipopeptide **5** were eluted as a single peak on analytical RP-HPLC and exhibited the expected molecular weight and sequence when analyzed by mass spectrometry.

Chimeric Th-CTL peptide **4** or chimeric PAM-Th-CTL lipopeptide **5** were next assembled to **3**. The disulfide bond formation was performed under argon gas in a degassed solution of isopropanol and sodium acetate buffer to ensure good solubility and under dilution to minimize the intermolecular dimer formation of chimeric epitopes. In both cases, the reaction course showed a clean crude reaction mixture and completeness within one hour, as assessed by analytical reverse phase HPLC (Figure 2). Glycopeptide (GP) **6** and glycolipopeptide (GLP) **7** were obtained after HPLC purification over a C_{18} or C_5 column, respectively. Although the crude reaction mixtures emphasize the efficiency of the ligation protocol, we assume that the moderate yields (≈48–65%) recovered after purification might be due to the precipitation of the GP **6** or GLP **7** on the semi-preparative column. These well-defined, water soluble synthetic constructs were finally analyzed by electrospray mass spectrometry (ES-MS) and their purity determined by analytical HPLC (Figure 2B and C). As expected, ES-MS analysis revealed signals corresponding to the multicharged ions of **6** and **7**.

We next investigated the safety and immunogenicity of this multivalent B-Th-CTL, self-adjuvanting GLP **7**. Three groups of twenty BALB/c mice each were immunized subcutaneously at the base of the tail with: 1) the GLP **7**, delivered in adjuvant-free saline (Group 1); the GP analogue **6** delivered with cytosine-phosphate-guanine (CpG_{1826}) adjuvant (positive controls, Group 2); and saline alone (nonimmunized controls; Group 3). Unlike the GP/CpG_{1826}, the GLP vaccine was well tolerated with no local or general adverse reactions, such as local inflammation at the sites of injection or weight loss, recorded in immunized mice. Two weeks following the second immunization, RAFT-specific Abs, PADRE-specific $CD4^+$ T cell, and $OVA_{257-264}$-specific $CD8^+$ T cells were studied. To determine whether the Abs developed against the Tn cluster displayed in our vaccine construct can recognize the native form of Tn antigen displayed on human tumor cells, we first performed a flow cytometry based binding assay between the immune serum IgG

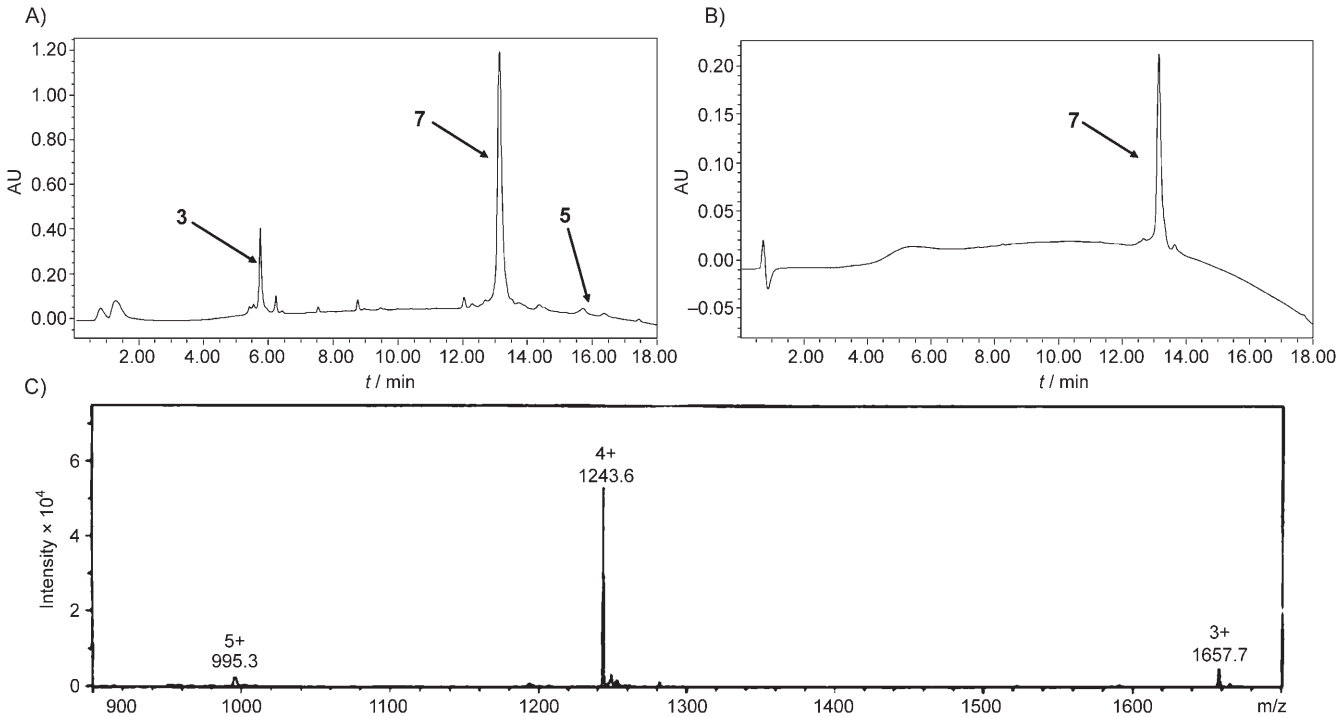


Figure 2. Analysis of glycolipopeptide **7**: A) Example of HPLC profile of the crude reaction mixture of disulfide bond formation between RAFT carrier **3** and lipopeptide **5** after 1 h. B) HPLC profile of pure glycopeptide **7**; C) ES-MS (positive mode) of **7** after purification.

and breast cancer cell line MCF7. Figure 3 shows the binding profile of the immune serum IgG collected from mice immunized with either GP **6** + CpG_{1826} adjuvant (Figure 3A) or GLP **7** in adjuvant-free saline (Figure 3B), and nonimmune serum IgG collected nonimmunized control mice with MCF7 cells. Both GP and GLP immune sera, tested at a 1:250 dilution, showed a strong positive shift of mean binding (22.74 and 24.06 respectively, Figure 3C) when compared with the nonimmunized sera at the same dilution (4.54). This result clearly indicates that mice immunized by the GLP **7** or GP **6** + CpG_{1826} elicit IgG antibodies capable of recognizing human tumor cell lines expressing Tn carbohydrate antigen. Unlike their recognition of tumor cells, immune sera did not recognize nontumor cells (that is, T 2 cell line, not shown). As tested in ELISA, serum IgG induced by GLP **7** and GP **6** specifically recognized the RAFT glycocluster. In addition, the GLP vaccine **7** induced strong PADRE-specific $CD4^+$ T cell and $OVA_{257-264}$-specific $CD8^+$ T cells responses, highlighting correct APCs processing and T-cell presentation of both Th and CTL epitopes displayed by the prototype GLP structure.[18]

We next explored the protective efficacy of this multivalent B-Th-CTL GLP prototype vaccine **7**, using the MO5/BALB/c tumor mouse model.[19] Ten days after the last immunization, mice in all three groups were challenged subcutaneously with MO5 cells in their right flank and monitored for tumor growth (Figure 4A) and survival for up to 90 days (Figure 4B). The tumor growth was measured and recorded until the tumor volume reached a maximum limit of 2800 mm^3, when mice were euthanized and scored as "dead". As indicated in Figure 4A, after MO5 cell challenge in nonvaccinated control mice, there was a lag of ~35 days for tumor growth to initiate. In the GLP **7** immunized group, none of the twenty mice developed a tumor in the monitoring period of 90 days after inoculation of MO5 cells. In contrast, in the GP **6** + CpG_{1826} immunized positive control group, 4 out of 20 mice developed tumor whereas 16 mice remain tumor free up to 90 days. Therefore the survival rates are 100% in GLP **7** vaccinated mice, whereas 80% survived in GP **6** + CpG_{1826} immunized mice (Figure 4B). In contrast, the survival rate in nonimmunized control group was less then 30%. Together, these results demonstrate that vaccination with a totally synthetic self-adjuvanting GLP prototype vaccine **7**, induces a strong protection against tumors.

In conclusion, this report consists, to our knowledge, a first demonstration of the synthesis, safety, immunogenicity, and protective efficacy of a new generation GLP cancer vaccine containing four components: 1) a clustered carbohydrate B-cell epitope, 2) a CD4+ T cell epitope peptide, 3) a CD8+ T-cell epitope peptide, and 4) a build-in adjuvant lipid moiety. The strength of induced antitumor B and T cell protective immuni-

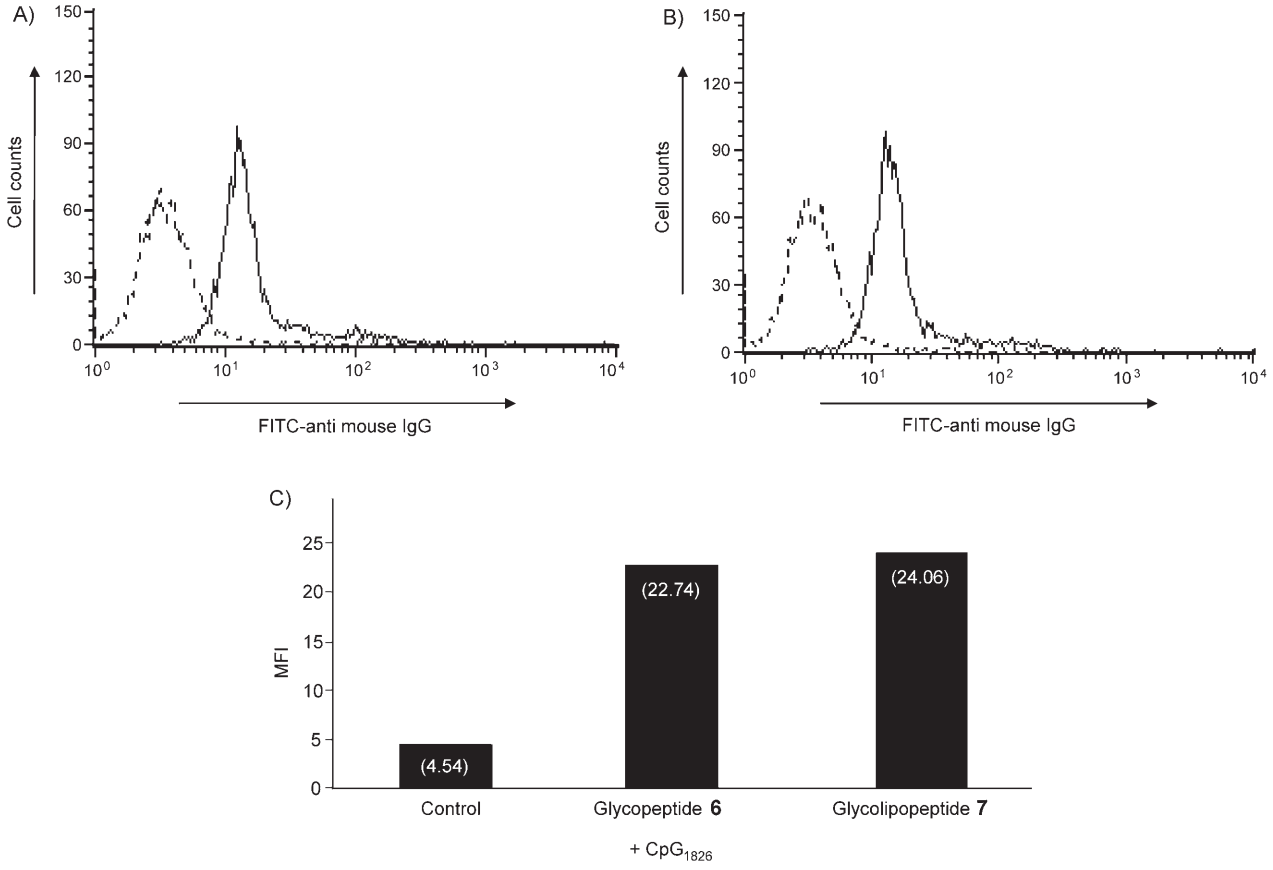


Figure 3. Recognition of breast cancer cell line (MCF7) bearing Tn antigen by mice sera immunized either with A) Glycopeptide **6** + CpG_{1826} or B) Glycolipopeptide **7**. MCF7 cells (4×10^5 cells) were incubated with 10 μL of immunized mice sera (solid line) or non-immunized mice sera (broken line) at 1:250 dilution and the binding was detected by flow cytometry using FITC labeled anti-mouse IgM. The mean fluorescence intensity (MFI) were calculated and shown in C).

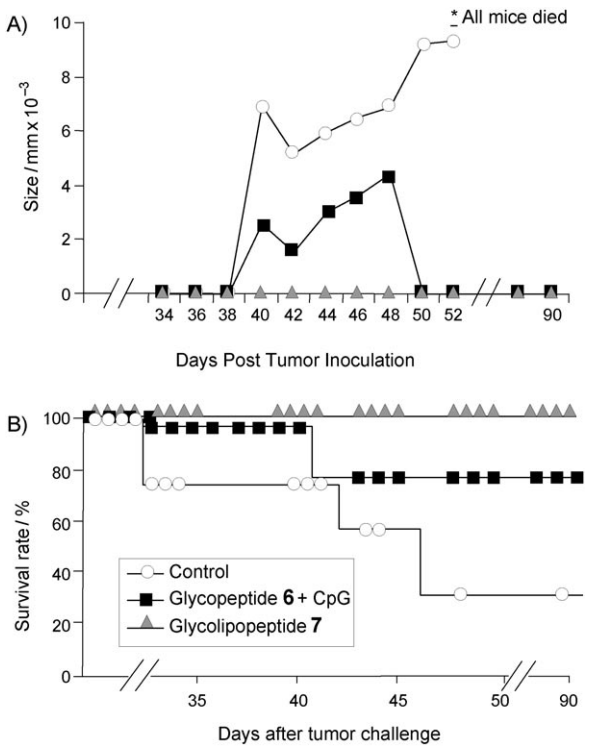


Figure 4. Glycolipopeptide vaccine **7** induces protective immunity against tumors: The survival rate of the mice immunized in each case three times with 200 mg of glycopeptide **6** and CpG1826 (25 mg) glycolipopeptide **7** or PBS (control). The C57BL/6 mice were subcutaneously charged with 5×10^3 MO5 melanoma cells given into the flank.

ty, together with the modularity of construction, saline solubility, and safety of this totally synthetic self-adjuvanting GLP, provide a molecularly defined vaccine formulation that could open new perspectives in human cancer immunotherapy.

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