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Syntheses and Immunological Evaluation of Self-Adjuvanting Clustered *N*-Acetyl and *N*-Propionyl Sialyl-Tn Combined with A T-helper Cell Epitope as Antitumor Vaccine Candidates

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Abstract: Sialyl-Tn (STn) is a tumor-associated carbohydrate antigen (TACA) rarely observed on healthy tissues. We synthesized two fully synthetic *N*-acetyl and *N*-propionyl STn trimer (triSTn) vaccines possessing a T-helper epitope and a TLR2 agonist, since the clustered STn antigens are highly expressed on many cancer cells. Immunization of both vaccines in mice induced the anti-triSTn IgG antibodies, which recognized triSTn-expressing cell lines PANC-1 and HepG2. The *N*-propionyl triSTn vaccine induced the triSTn-specific IgGs, while IgGs induced by the *N*-acetyl triSTn vaccine were less specific. These results illustrated that *N*-propionyl triSTn is a valuable unnatural TACA for anticancer vaccines.

Tumor cells are distinguished from normal cells by the display of aberrant cell surface levels and types of carbohydrate domains, called tumor-associated carbohydrates antigens (TACAs).[1] Because TACAs are prevalent on cancer cell surfaces, they represent a promising class of active immunotherapy epitopes.[2] However, these TACAs are T-cell independent antigens to exhibit poor immunogenicity and difficult to induce immunoglobulin (Ig) class switching. A typical strategy used to address the disadvantages of TACAs involves conjugating them to a strong immunogenic carrier protein, such as keyhole limpet hemocyanin (KLH).[3] Conversely, carrier proteins might suppress an antibody response against the TACA epitopes.[4]

The sialyl-Tn (STn, NeuAcα(2,6)GalNAcα-O-Ser/Thr) antigen is a TACA richly expressed on mucins in a number of epithelium-derived tumors (e.g., breast, pancreas, prostate, gastric and ovarian) and is rarely observed on adult healthy tissues.[5,6] STn correlates with invasion and aggression potential.[5] STn has been used to develop anticancer vaccines such as STn-KLH conjugate, Theratope, for treating metastatic breast cancer.[7] However, Theratope was failed in a phase 3 clinical trial; extensions in the overall survival and time to disease progression did not reach statistical significance.[8] Theratope induced the high levels of anti-KLH and anti-STn IgGs but low level of anti-mucin IgGs in patients (anti-KLH/anti-STn/anti-ovine submaxillary mucin (OSM)=64:64:1), suggesting many anti-STn IgGs could not recognize mucins and therefore cancer cells.

The use of the clustered STn should be necessary to provoke the efficient anti-tumor response, since STn is expressed as clusters on mucins. Here, we focused the trimeric STn clusters observed in the mucins of most adenocarcinomas.[9] The monoclonal antibodies B72.3 and MLS102, which primarily recognize STn clusters, displayed a high specificity to tumor cells against normal cells.[9a,b] The STn trimer (triSTn) is, therefore, a promising target for recognizing epithelial cancers.

Self-adjuvanting strategy[10] offers an attractive approach to circumvent the anti-carrier immune response by using a built-in adjuvant without using carrier proteins. Toll-like receptor (TLR) 2 agonists, lipopeptides, have been employed in many self-adjuvanting vaccines.[11] Boons *et al.* revealed that a tumor-associated MUC1 glycopeptide vaccine composed of TLR1/2 ligand (Pam_3CSK_4) and a T-helper cell epitope induced high titers of IgGs and Pam_3CSK_4 was more potent than TLR2/6 ligand (Pam_2CSK_4) as an adjuvant.[11a] In addition, TLR2 agonists proved to induce anti-tumor immune responses.[12] We therefore used Pam_3CSK_4 as an adjuvant for the construction of STn-based vaccines to overcome the poor immunogenicity of STn.

Modifying the C5 *N*-substituent of the sialic acid in the sialic acid-containing antigens has been shown to impact their immunogenicity.[13] In those approaches, unfortunately, some of the antibodies produced only recognized the modified antigens, not the parent antigens. Whereas, *N*-propionyl STn glycoconjugate vaccine induced the production of antibody which can recognize natural STn epitope and was more immunogenic.[13b-f] Here, we tested the use of unnatural *N*-propionyl triSTn as a TACA antigen to construct a vaccine, expected to generate a more effective triSTn-specific immunogenic response.

We designed the anti-triSTn vaccines **1** and **2**, which are composed of the *N*-acetyl or *N*-propionyl triSTn antigens in the middle, the T-helper cell peptide (TT830-843 from the tetanus toxoid, QYIKANSKFIGITE),[14] and Pam_3CSK_4[15] (Figure 1). The universal human and mouse T-helper cell epitope was indispensable for the production of IgG antibodies via the adaptive immune response. In comparison to the construction of other self-adjuvanting glycopeptide vaccines through the linear solid phase synthesis,[11] the advantages of our design were facilitation of assemblies of the triSTn antigens with the T-cell epitope and the adjuvant via convergent synthesis. Copper click azide-alkyne cycloaddition (CuAAC) and thioether ligation were employed to build the vaccine **1** and **2**, since the resulting

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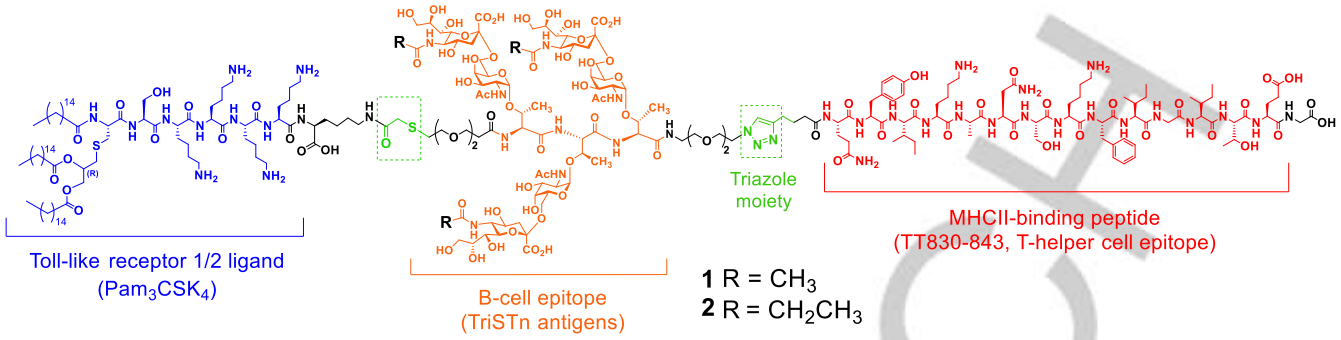
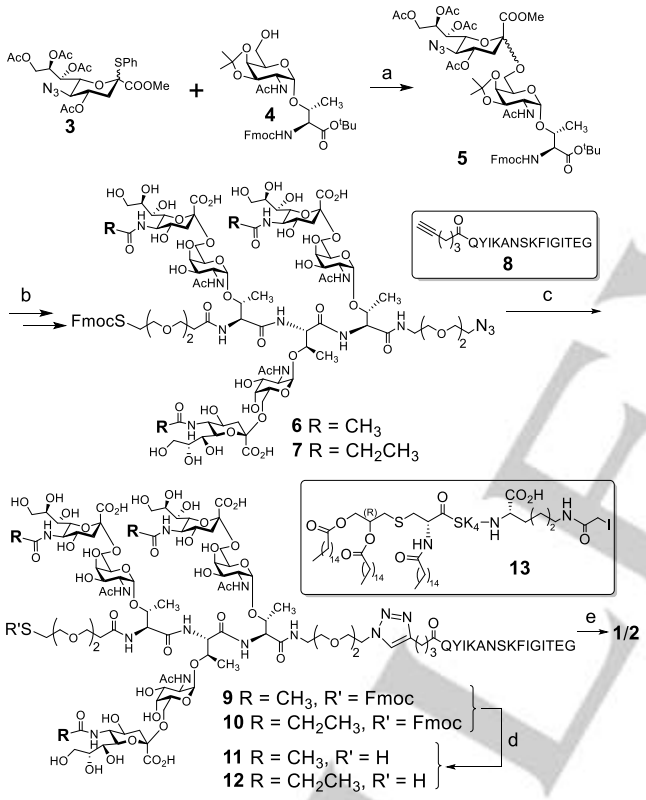


Figure 1. The fully synthetic vaccines **1** and **2**.



Scheme 1. Synthesis of the vaccines **1** and **2**. (a) ICl, $In(OTf)_3$, EtCN, MS4A, -78 °C, 3 h, 94% (based on recovery of **4**), α/β = 11/1; (b) see the Supporting Information; (c) **8**, $CuSO_4$, sodium ascorbate, 80% $DMSO_{(aq)}$, rt, 5 h, and then DOTA, rt, 40 min; 67% for **9**, 68% for **10**; (d) i) 50 mM LiOH, H_2O, rt, 30 min; ii) 90 mM HOAc/H_2O; iii) 20 mM TCEP, H_2O, rt, 30 min, 84% for **11**, 72% for **12**; (e) **13**, Et_3N, DMSO, 40 °C, 15 h, 30% for **1**, 31% for **2**.

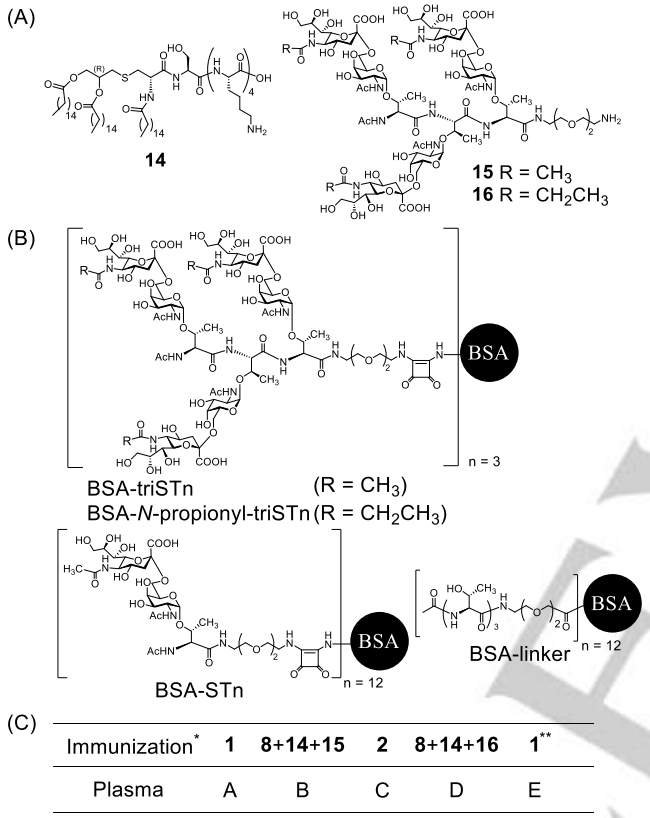
triazole[11e] and thioether[16] moieties have the low immunogenicity.

A simple and reliable method to construct the *N*-acetyl and *N*-propionyl STn monomer is of primary importance in the preparation of the triSTn antigens. Previously, Guo *et al*.[13c] and Ye *et al*.[13f] employed the *N*-trifluoroacetyl (*N*-TFA) sialyl donor to obtain the *N*-TFA STn monomers in good yields with reasonable stereoselectivity (α/β = 4/1). Here, glycosylation of **4** with C5-azido sialyl donor **3** by using ICl and $In(OTf)_3$ as a promoter afforded the C5-azido STn monomer **5** in good yield with high α-selectivity (α/β = 11/1) (Scheme 1).[17] The triSTn antigens **6** and **7** were readily prepared from compound **5** in good yields (**6**: 33% and **7**: 22% in 13 steps) via modifications of functional groups, introduction of linkers, and an iterative peptide coupling process (Scheme S1). Conjugation between **6**/**7** and T cell peptide **8** by click reaction was then investigated (Table S1). Compounds **6**/**7** smoothly reacted with **8** by using appropriate amounts of $Cu(SO_4)_2$ and sodium ascorbate in 80% $DMSO_{(aq)}$ (oxygen-free) to give **9** and **10** in moderate yields, respectively. It should be noted that use of the non-degassed solvents in the CuAAC reaction led to the significant production of an unidentified by-product (Figure S9). Since the removal of Fmoc group of **9** with 20% piperidine/DMF gave a considerable amount of by-products, **9**/**10** were treated with 50 mM $LiOH_{(aq)}$ to afford the mixture of the free thiol and the disulfide-linked dimer, which was treated with tris(2-carboxyethyl)phosphine (TCEP) to afford the free thiol **11**/**12** in good yields, respectively. Coupling of thiol **11**/**12** with **13** was then carried out according to Kunz and Li (Table S2).[11d] The use of excess amount of nucleophile **11**/**12** compared to electrophile **13** in DMSO (oxygen-free) in the presence of Et_3N was critical to obtain the coupling products. The vaccines **1** and **2** were thus obtained in approximately 30% yields. Although significant amount of the disulfide-linked dimers was formed, they were readily converted to **11**/**12** by TCEP treatment.

To examine the necessity of covalent attachment among the three components (trisTn or *N*-propionyl-triSTn, T-helper cell epitope, and adjuvant) in vaccines **1** and **2**, compounds **14**-**16** were synthesized for control experiments (Figure 2A). TriSTn, *N*-propionyl-triSTn, STn, and linker were conjugated to bovine serum albumin (BSA) through a squarate linker[18] to afford BSA-triSTn, -*N*-propionyl-triSTn, -STn, and -linker for ELISA assays (Figure 2B). (The synthesis of **14**-**16** and the preparation of the BSA glycoconjugates are described in the Supporting Information).

Vaccines **1**, **2**, mixture (**8**+**14**+**15**), and mixture (**8**+**14**+**16**) in phosphate-buffered saline (PBS) were intraperitoneally administrated to wild-type BALB/c mice on day 1, 14, 28, and

42. The first immunizations were performed using complete Freund's adjuvant (CFA), the others with incomplete Freund's adjuvant (IFA). Blood was collected from each mouse before immunization on day 0 (blank controls) and on days 8, 21, 35, and 49. The blood samples were used to prepare plasma by the standard method. The immunogenicities of vaccine **1**, mixture (**8**+**14**+**15**), **2**, and mixture (**8**+**14**+**16**) were evaluated using the corresponding plasma samples A-D, respectively (Figure 2C). Mouse immunized with vaccine **1** in PBS without an external adjuvant provided plasma E.



(C)

Immunization*	1	8+14+15	2	8+14+16	1**
Plasma	A	B	C	D	E

Figure 2. Various preparations for immunization and BSA-glycoconjugates for ELISA assays. (A) compounds **14**-**16**. (B) BSA-triSTn, -*N*-propionyl-triSTn, -STn, and -linker conjugates. (C) The vaccines were evaluated in wild-type BALB/c mice to generate corresponding immune responses (plasma samples A-E). *The first immunization was administered with complete Freund's adjuvant (CFA), the others with incomplete Freund's adjuvant (IFA). ** All progress of the immunization was administered only with PBS buffer without CFA or IFA.

The IgG antibody titers on day 0 and day 49 are shown in Figure 3. No detectable anti-triSTn IgG titers were presented before immunization. As depicted in plasma A (Figure 3A), the high anti-triSTn IgG titer was induced by vaccine **1**. On the contrary, the mixture (**8**+**14**+**15**) afforded a negligible immune response to the triSTn epitope (plasma B). These results clearly demonstrated that the covalent conjugation in vaccine **1** was critical for the production of IgGs. Moreover, plasma E obtained by administration with vaccine **1** in the absence of an external adjuvant (CFA) showed the slightly higher anti-triSTn IgG titer than plasma A. The results were consistent with the studies by Kunz and Li *et al*, *i.e.*, the inclusion of CFA led to inhibition of the antibody production with self-adjuvanting vaccines.[19, 11d-e] These results indicated that self-adjuvanted vaccines have high potential for the practical application.

Noteworthy, the binding epitope of anti-triSTn IgGs induced by vaccine **1** was carbohydrate part of **14** because anti-linker IgG titers were very low in A and E. Although the anti-triSTn IgGs elicited by vaccine **1** recognize extensively triSTn epitope but concomitantly monoSTn. As mentioned above, STn antigen was observed on a few normal cells, though it was more highly expressed on cancer cells.[6] To avoid the problem of autoimmunity in a new STn-based vaccine, the desired immune response should be specific against the triSTn epitope.

Plasma C produced by vaccine **2** possessing unnatural-type *N*-propionyl triSTn also exhibited a significant immune response against *N*-propionyl and *N*-acetyl triSTn epitopes with the higher specificity to triSTn and a negligible immune response to the linker epitope (Figure 3B). The IgGs raised by vaccine **2**, therefore, have the cross-reactivity to natural-type *N*-acetyl triSTn epitope. Though the titer of plasma C was lower than plasma A, the IgG titer ratio of triSTn to monoSTn (7.3:1, Figure 3B) in plasma C was 2.2-fold higher than the same ratio in plasma A (3.3:1, Figure 3A). The self-adjuvanting strategy was also effective in *N*-propionyl triSTn antigen (plasma C versus D, Figure 3B). The IgM antibody titers of plasma A-D on day 49 also gave similar results (see the Supporting Information).

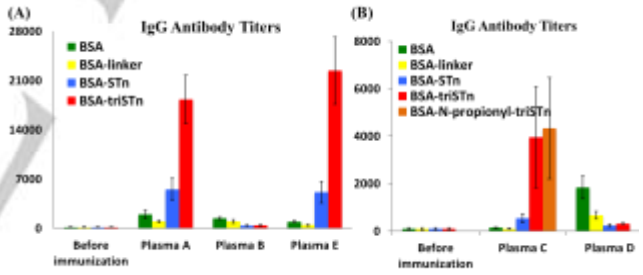


Figure 3. IgG antibody titers of (A) plasma A, B, and E, and (B) plasma C and D after four immunizations (day 49) with various preparations and before immunization (day 0). ELISA plates were coated with BSA-triSTn for the anti-triSTn IgG titers, BSA-*N*-propionyl-triSTn for the anti-*N*-propionyl-triSTn IgG titers, BSA-STn for the anti-STn IgG titers, BSA-linker for the anti-linker IgG titers, or BSA for the nonspecific-binding IgG titers. Error bar represented standard error of the mean.

The differences in the immune responses induced by vaccines **1** and **2** were further explored by measuring the IgG and IgM titers in plasma A and C on days 0, 8, 21, 35, and 49 (Figure 4A-D). The IgG and IgM titers against the triSTn epitope in plasma A and C gradually increased with the boost immunizations. In plasma A, IgG titers were boosted after third and fourth immunizations on days 28 and 42, whereas IgM titers were boosted after second immunization on day 14. Interestingly, on days 35 and 49 in plasma A, IgGs revealed higher selectivity to triSTn against STn than IgMs (Figure 4A and 4C), suggesting affinity maturation in IgGs was promoted after third and fourth immunizations of vaccine **1**.

In plasma C, both IgG and IgM titers induced by vaccine **2** were boosted after first and second immunizations (Figure 4B and 4D). On the day 21, plasma C showed 2-fold higher triSTn-specific IgG titers (3151 for triSTn) compared to plasma A (titers: 1467 for triSTn). These results suggested that *N*-propionyl triSTn is more immunogenic than *N*-acetyl triSTn. However, the same phenomenon was not observed after third and fourth immunization. Although the mechanistic details were not clear, the slight structural difference between *N*-propionyl and *N*-acetyl triSTns resulted in the significant difference in immune responses.

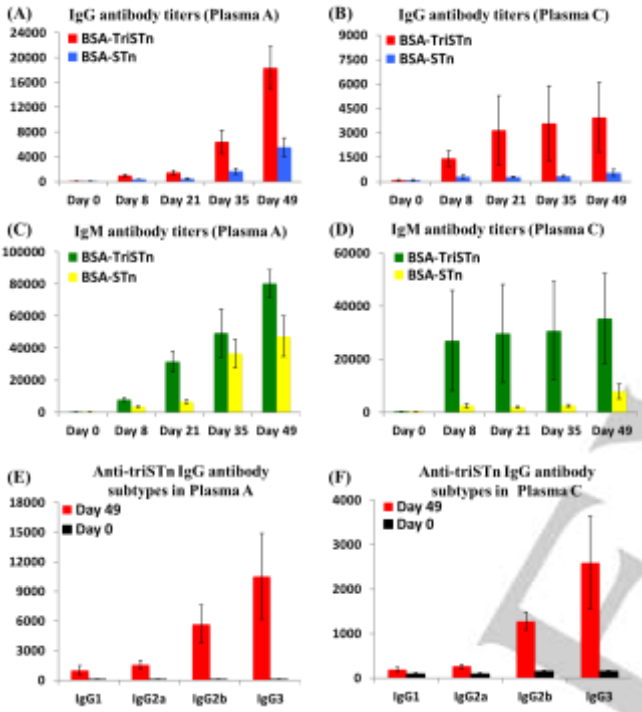


Figure 4. The IgG and IgM antibody titers measured in plasma samples A and C on days 0, 8, 21, 35, and 49 (A-D) and the anti-triSTn IgG antibody subtypes in plasma A and C on days 0 and 49 (E-F). ELISA plates were coated with BSA-triSTn to measure the anti-triSTn antibody titers and BSA-STn to measure the anti-STn antibody titers. Error bar represented standard error of the mean.

IgG subclasses exhibit substantial differences in immune functions and cytotoxicity against the target tumors. The IgG2b and IgG3 isotypes of the anti-triSTn antibodies were observed predominantly in both plasma A and C, whereas a small amount of the IgG2a was also found (Figure 4E-F). The mouse IgG3 is primarily anti-carbohydrate antibodies.[20] The production of IgG2b and IgG2a suggested vaccines **1** and **2** induced the adaptive T cell-mediated immunity to implement an IgG subclass switching.

Flow cytometric analysis revealed that the anti-triSTn IgGs in plasma A and C effectively recognized human pancreatic cancer cell line PANC-1 and human hepatic cancer cell line HepG2 (Figure 5). Strong fluorescence intensities were observed in PANC-1 and HepG2 treated with plasma A or C (mean fluorescence intensity (MFI); PANC-1-A: 830,339, PANC-1-C: 1,008,248, HepG2-A: 925,487, HepG2-C: 889,697). PANC-1 expresses clustered STn and STn,[21] whereas HepG2 shows specific expression of clustered STn.[22] Our results indicated that the IgGs induced by vaccines **1** and **2** recognized clustered STn antigens on cancer cells, though the IgGs might also recognize monoSTn on cancer cells. In addition, the IgGs induced by **1** and **2** showed similar affinity to PANC-1 and HepG2, despite of the lower titers of anti-triSTn IgGs elicited by 2 (Figure 3 versus Figure 5). As described, the IgG ratio of anti-KLH/anti-monoSTn/anti-OSM obtained from the Theratope phase 3 clinical trial was 64:64:1. These results suggested that anti-monoSTn IgGs barely recognize the native STn and the clustered STn, since OSM contains both clustered and unclustered forms of the STn and is considered to be representative of native STn present on tumor-associated mucin.[8a] The present study indicates the triSTn antigens can induce antibodies that can recognized the clustered STn on cancer cells and therefore provides the opportunity to solve the issue of Theratope.

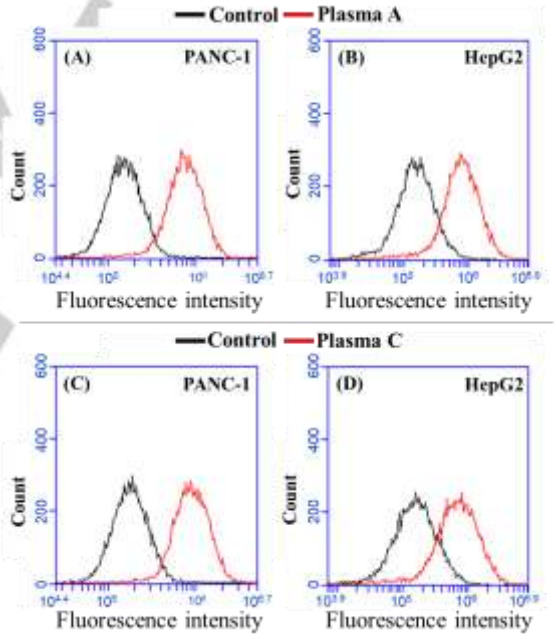


Figure 5. Flow cytometry analysis of the binding between plasma A and C (1:50 diluted) collected on day 49 and the human pancreatic cancer cell line PANC-1 (A and C) or human hepatic cancer cell line HepG2 (B and D). Alexa Fluro®488-goat anti-mouse IgG (1:50 diluted) was used as a secondary antibody for staining. Control: mice plasma before immunization (black color); Plasma A or C: mixed plasma from a group (n = 5) of mice (red color).

The use of glycopeptide vaccines provides another possibility to solve the issue of Theratope. Previously, Kunz et al. reported that MUC1 glycopeptide vaccines having monoSTn induced IgG1-type antibodies that recognized human breast cancer cell lines T47D and MCF-7 as well as the breast tumor tissues, which expressed tumor associated MUC1.[23] They also generated IgG1-type monoclonal antibody GGSK-1/30 based

on MUC1 glycopeptide vaccination. GGSK-1/30 bound to MUC1-expressing tumor cells T47D, MCF-7, and PANC-1. GGSK-1/30 also bound to the pancreatic cancer tissues, whereas the normal cells showed almost no binding.[24] High specificity of GGSK-1/30 to tumor associated MUC1-expressing cancer cells is provably owing to the specific recognition to the glycopeptide epitope.

In summary, *N*-propionyl triSTn vaccine **2** induced anti-triSTn IgG antibodies that efficiently recognize cancer cells expressing clustered STn. Additionally, vaccine **2** elicited a triSTn-specific immune response that can minimize autoimmunity. Self-adjuvanting *N*-propionyl triSTn fulfills the requirements for a next-generation STn-based vaccine without the use of a carrier protein as a means for avoiding the weaknesses of Theratope.

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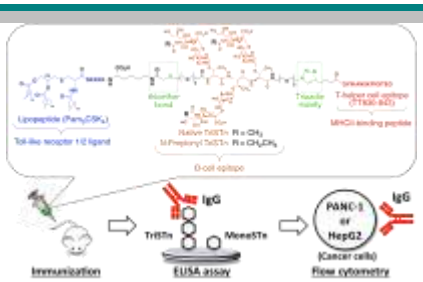
COMMUNICATION

The effective anti-STn antibodies were generated by two fully synthetic *N*-acetyl and *N*-propionyl triSTn vaccines including the built-in T-helper epitope and adjuvant. The *N*-propionyl triSTn vaccine elicited a triSTn-specific immune response.



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Synthesis and Immunological Evaluation of Self-Adjuvanting Clustered *N*-Acetyl and *N*-Propionyl Sialyl-Tn Combined with A T-helper Cell Epitope as Antitumor Vaccine Candidates