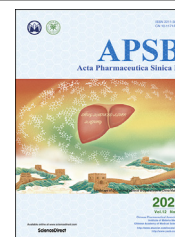




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ORIGINAL ARTICLE

# Fully synthetic Tn-based three-component cancer vaccine using covalently linked TLR4 ligand MPLA and iNKT cell agonist KRN-7000 as built-in adjuvant effectively protects mice from tumor development



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## KEY WORDS

Cancer vaccine;  
Tn antigen;  
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**Abstract** We present a new strategy for self-adjuvanting vaccine development that has different types of covalently-linked immunostimulants as the carrier molecule. Using Tn antigen as the model, a three-component vaccine (MPLA-Tn-KRN7000) containing the TLR4 ligand MPLA and the iNKT cell agonist KRN7000 was designed and synthesized. This expands fully synthetic self-adjuvanting vaccine studies that use a single carrier to one with two different types of carriers. The corresponding two-component conjugate vaccines Tn-MPLA, Tn-KRN7000 and Tn-CRM197 were also synthesized, as controls. The immunological evaluation found that MPLA-Tn-KRN7000 elicits robust Tn-specific and T cell-dependent immunity. The antibodies specifically recognized, bound to and exhibited complement-dependent cytotoxicity against Tn-positive cancer cells. In addition, MPLA-Tn-KRN7000 increased the survival rate and survival time of tumor-challenged mice, and surviving mice reject further tumor

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attacks without any additional treatment. Compared to the glycoprotein vaccine Tn-CRM197, the two-component conjugate vaccines, Tn-MPLA and Tn-KRN7000, and the physical mixture of Tn-MPLA and Tn-KRN7000, MPLA-Tn-KRN7000 showed the most effect at combating tumor cells both *in vitro* and *in vivo*. The comparison of immunological studies in wild-type and TLR4 knockout mice, along with the test of binding affinity to CD1d protein suggests that the covalently linked MPLA-KRN7000 immunostimulant induces a synergistic activation of TLR4 and iNKT cell that improves the immunogenicity of Tn. This work demonstrates that MPLA-Tn-KRN7000 has the potential to be a vaccine candidate and provides a new direction for fully synthetic vaccine design.

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## 1. Introduction

Tumor vaccination, which is to activate and strengthen the immune system of patients, is a powerful and practical tool in cancer treatment<sup>1,2</sup>. Tumor-associated carbohydrate antigens (TACAs) overexpressed on cancer cell surfaces are highly correlated with tumor metastasis and progression, and thus have been plausible targets for cancer vaccine development<sup>3</sup>. However, due to weak T cell-independent immunogenicity, TACAs alone are usually not able to activate T helper (Th) cells to elicit sufficient immune responses for cancer therapy<sup>4</sup>. An effective method to address such limitations is the covalent conjugation of TACAs with an immunogenic carrier. This strategy makes the conjugates as a whole that can be recognized and presented by antigen-presenting cells (APCs), improving the TACA-specific immune responses and switching them from T cell-independent to T cell-dependent<sup>5,6</sup>.

The immunogenic carrier, which can activate the immune system and augment immunogenicity, is crucial for TACA-based synthetic vaccines. Early research focused on protein carriers, typically platforms such as KLH<sup>7,8</sup>, BSA<sup>9,10</sup>, TTox<sup>11,12</sup>, and CRM197<sup>13,14</sup>. In the past several decades, various glycoprotein vaccines have been studied in preclinical and clinical trials<sup>15,16</sup>. However, some limitations, including “epitope suppression”, random conjugation manner, and uncontrollable equivalents of TACAs hindered their further development<sup>17–19</sup>. These deficiencies negatively affect the quality and immunization efficacy of glycoprotein vaccines. Moreover, glycoprotein vaccines usually need to be co-administered with an additional adjuvant, which may produce unwanted side effects<sup>20</sup>.

To solve issues associated with the glycoprotein vaccine, fully synthetic self-adjuncting TACA-based vaccines have recently been pursued. This type of vaccine possesses homogeneous and well-defined chemical structures, and this feature is helpful for quality control, structure–activity relationship analysis, and further optimization. In addition, the carrier proteins and external adjuvants used in glycoprotein vaccine are not needed, avoiding the adverse effects induced by protein and adjuvant. Typically, the ligands of Toll-like receptors, invariant natural killer T (iNKT) cell agonists, or zwitterionic polysaccharides are used as built-in adjuvants and delivery carriers to construct fully synthetic self-adjuncting TACA vaccines. To date, numerous this kind of vaccines have been explored. For example, TACA-MPLA<sup>21,22</sup>, TACA-KRN7000<sup>23,24</sup> and TACA-PS A1<sup>25,26</sup> all elicit TACA-specific IgG/IgM antibodies production and exhibit cytotoxicity against tumor cells. Despite the progress achieved toward the

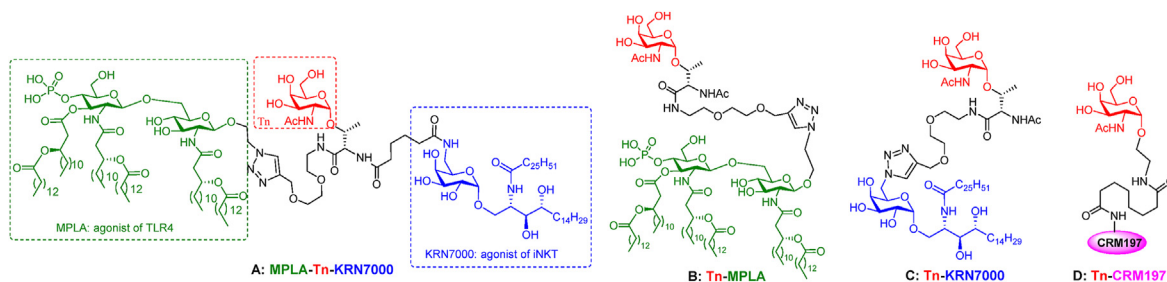
TACA-based vaccine, low immunogenicity and efficacy deter them from further clinical applications. Therefore, more effective strategies to increase the immunogenicity of TACA vaccines remains a high priority.

Based on our previous research on fully synthetic self-adjuncting vaccines<sup>27–29</sup>, we hypothesize that covalently coupling TACA with two different types of immunostimulants that synergistically activate a distinct pattern of cell-signaling molecules has the potential to address the deficiency of low immunogenicity in TACA vaccine development. To this end, the natural human TLR4 ligand isolated from *Salmonella minnesota*<sup>30</sup>, a monophosphoryl-lipid A (MPLA) with three lipid chains, was selected as a type of immunostimulant. Although many self-adjuncting vaccines based on the *Neisseria meningitidis* MPLA containing four lipid chains have been reported<sup>21</sup>, *S. minnesota* MPLA has not yet been used as a built-in adjuvant. KRN7000 was selected as another immunostimulant; it has been widely used for fully synthetic vaccines in recent years<sup>31</sup>. These two immunostimulants were simultaneously coupled with the Thomsen-nouveau (Tn) antigen ( $\alpha$ GalNAc-Ser/Thr), one representative epitope for therapeutic cancer vaccine development, to produce a three-component conjugate (MPLA-Tn-KRN7000, Fig. 1A). The coupling sites and the linkers between TACA and immunostimulant are significant to the efficacy of self-adjuncting vaccines. To avoid the adverse effects to the activity of MPLA and KRN7000, the 1-*O*-position of MPLA and 6-*N*-position of KRN7000 were selected as coupling sites in our design based on previous work<sup>23,32–34</sup>. 1,2,3-Triazole and bifunctional suberic acyl, both of which have been safely used in previous work for the construction of synthetic TACA-based vaccines<sup>22,28,29</sup>, were used as the linkers. We also designed the corresponding two-component vaccines Tn-MPLA, Tn-KRN7000 and the semisynthetic glycoprotein Tn-CRM197 (Fig. 1B–D). The immune effects and anti-tumor efficacies of these conjugate vaccines and the physical mixture of Tn-MPLA and Tn-KRN7000 were evaluated and compared.

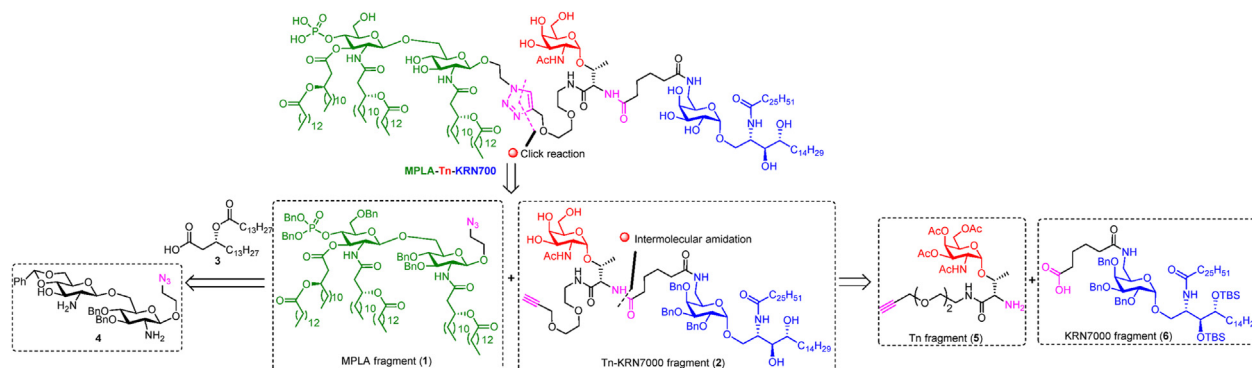
## 2. Results and discussion

### 2.1. Retrosynthetic analysis

Retrosynthetic analysis of the designed three-component conjugate MPLA-Tn-KRN7000 is shown in Fig. 2. The MPLA fragment (1) and Tn-KRN7000 fragment (2) were obtained by dismantling the 1,2,3-triazole group. The MPLA fragment was



**Figure 1** Structures of designed three and two-component vaccines.



**Figure 2** Retrosynthetic analysis of MPLA-Tn-KRN7000 conjugate.

constructed through a fatty chain (**3**) and the key disaccharide with a 2-azidoethyl group (**4**). The Tn-KRN7000 fragment was disassembled as a Tn derivative with a propargyl and an amino group (**5**) and a KRN7000 derivative with a free carboxyl group (**6**). All these fragments can be afforded from commercially available starting materials, such as D-glucosamine, D-galactose, and D-galactosamine. The protecting groups, which greatly affect the coupling reaction, are crucial during the preparation of the fragments. To accurately complete the assembly of MPLA-Tn-KRN7000, the hydroxyl groups on Tn and the lipid of KRN7000 were protected by acetyl and TBS groups, respectively, which could be selectively removed in subsequent reactions. The hydroxyl groups on the carbohydrate ring of MPLA and KRN7000 were protected by benzyl groups, which could be simultaneously removed after the final assembly.

## 2.2. Preparation of MPLA-Tn-KRN7000 conjugate

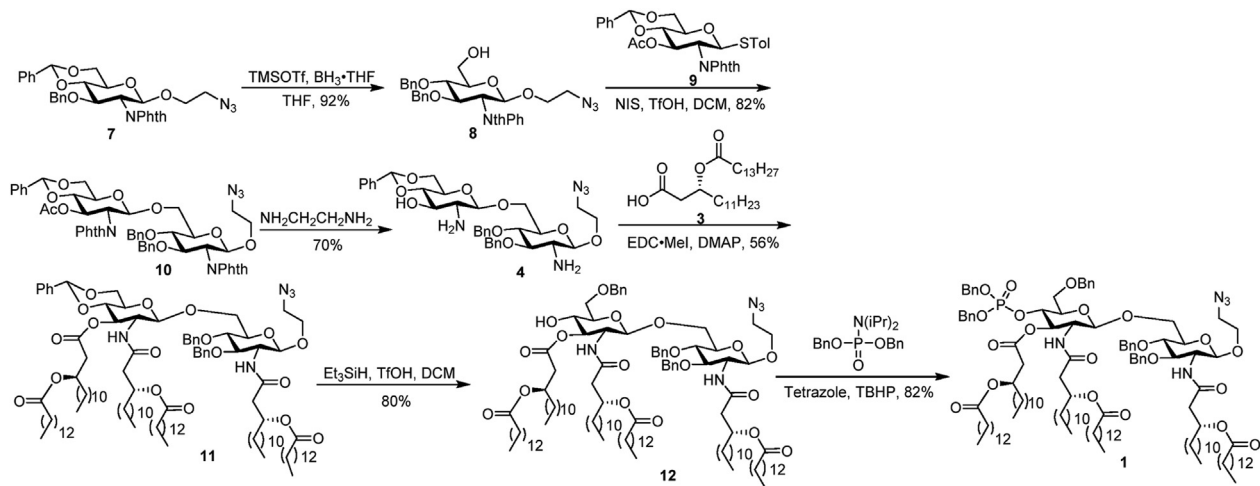
### 2.2.1. Synthesis of MPLA fragment (1)

The synthesis of MPLA fragment (**1**) started from D-glucosamine derivative **7**, which was prepared according to reported procedures with seven steps<sup>35,36</sup> (Supporting Information). The benzylidene ring in **7** was opened regioselectively under trimethylsilyl trifluoromethanesulfonate (TMSOTf) and  $\text{BH}_3 \cdot \text{THF}$  conditions to afford **8** with an exposed hydroxyl group at the C-6 position (Scheme 1). Glycosylation of **8** with **9**, a glycosyl donor obtained using a similar method to previous work<sup>37</sup>, was then performed using trifluoromethanesulfonic acid (TfOH) as the catalyst in the presence of *N*-iodosuccinimide (NIS), giving the fully protected disaccharide **10** (82% yield). The doublet signal with a coupling constant of 8.8 Hz at  $\delta$  5.11 ppm in the  $^1\text{H}$  NMR spectrum, which was corresponded to the resonance of hydrogen atom at the C'-1 position of **10**, confirmed the newly generated glycosidic linkage was  $\beta$ -conformation. Thereafter, simultaneous removal of *N*-

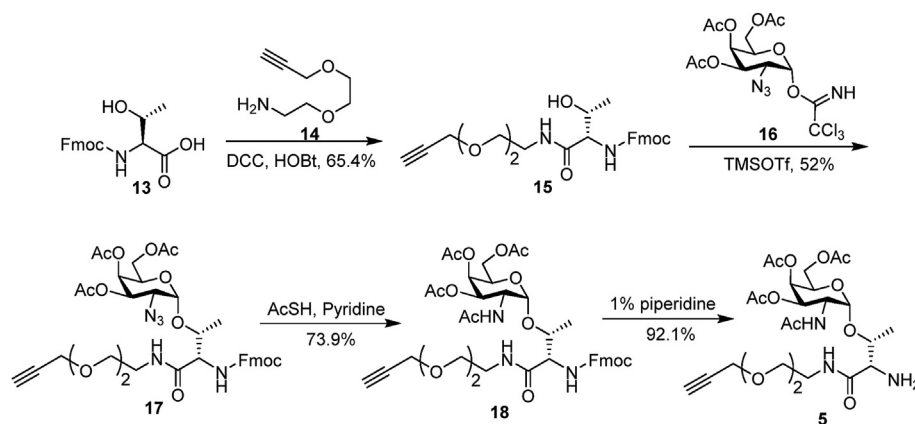
phthalyl (Phth) and *O*-acetyl groups in **10** with ethylenediamine in refluxing methyl alcohol conditions afforded the key intermediate **4**, which was ready for lipid installation. The chiral fatty acid **3**, synthesized according to published procedures from commercially available (*R*)-epichlorohydrin<sup>38</sup>, was simultaneously installed to the free amino and hydroxyl groups of **4** using 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide methiodide (EDC·MeI) and 4-dimethylaminopyridine (DMAP) as promoters, delivering **11** in a 56% yield. Next, the benzylidene ring was selectively opened under  $\text{Et}_3\text{SiH}$  and TfOH conditions, affording intermediate **12** with a free hydroxyl group at C'-4 position. Finally, phosphorylation was performed using the phosphoramidite method to form the desired MPLA fragment **1**. The signal of the hydrogen atom at C'-4 position in the  $^1\text{H}$  NMR spectrum shifted from  $\delta$  3.60 ppm to  $\delta$  4.40 ppm, confirming the success of this reaction.

### 2.2.2. Synthesis of Tn fragment (5)

The Tn fragment (**5**) was synthesized from commercially available *N*-fluorenylmethoxycarbonyl (Fmoc)-L-threonine (**13**), as shown in Scheme 2. First, compound **13** and 2-(2-(prop-2-yn-1-yloxy)ethoxy)ethan-1-amine (**14**) reacted under the influence of 1,3-dicyclohexylcarbodiimide (DCC) and 1-hydroxybenzotriazole (HOBT) to produce the chain linker (**15**). The glycosyl donor **16** with an azido group was afforded with four steps using D-galactosamine hydrochloride as raw material<sup>39,40</sup> (Supporting Information). Note that the azido group in **16** is crucial to obtain the  $\alpha$ -linkage product<sup>41</sup>, the same conformation to natural Tn antigen. Afterward, glycosylation of **15** with **16** was performed under the influence of trimethylsilyl trifluoromethanesulfonate (TMSOTf), delivering **17** in a 52% yield. The doublet signal with a coupling constant of 3.6 Hz at  $\delta$  5.28 ppm in the  $^1\text{H}$  NMR spectrum, which was corresponded to the resonance of hydrogen atom at the C-1 position in **17**, demonstrated the glycosidic linkage was  $\alpha$ -conformation.



Scheme 1 Synthesis of MPLA fragment 1.



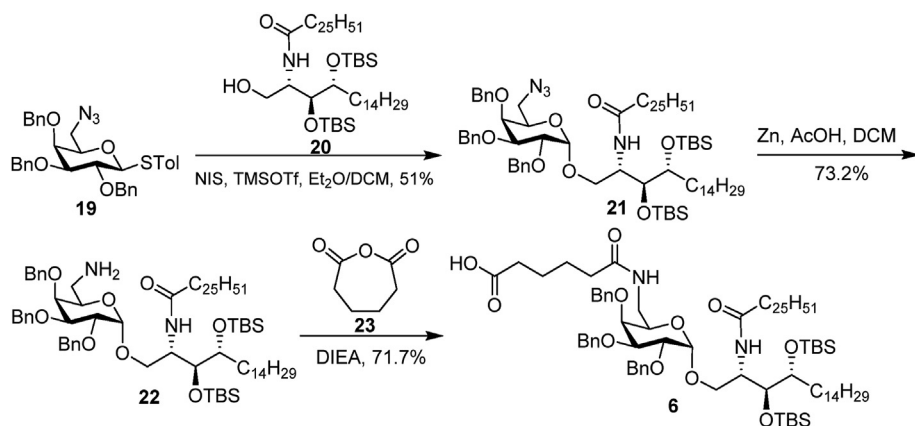
Scheme 2 Synthesis of Tn fragment 5.

Treatment of **17** with thioglycolic acid and pyridine produced **18**. Then, removal of the Fmoc-protecting group by piperidine gave the target fragment **5** in a 92.1% yield.

### 2.2.3. Synthesis of KRN7000 fragment (6)

The synthesis of KRN7000 fragment (**6**) proceeded from properly protected D-glucosamine derivative **19** (Scheme 3), prepared using D-galactose as raw material and an approach similar to that

in the literature<sup>42–44</sup> (Supporting Information). The chiral lipid chain **20**, which was used as the glycosyl acceptor, was synthesized as previously described<sup>45</sup>. For glycosylation, several reaction conditions were screened to selectively form  $\alpha$ -conformation glycosidic linkage (Supporting Information Table S1), which is the same as natural KRN7000. The reaction performed with NIS and TMSOTf under 0 °C gave the best result, affording **21** in a 51% yield. The doublet signal with a coupling



Scheme 3 Synthesis of KRN7000 fragment 6.

constant of 2.6 Hz at  $\delta$  4.83 ppm in  $^1\text{H}$  NMR spectrum corresponded to the resonance of hydrogen atom at the C-1 position in **21**. This result confirmed that the newly formed glycosidic linkage was  $\alpha$ -conformation. After the azido was converted to amine group promoted by zinc powder and acetic acid, acylation of **22** was proceeded with hexanedioic anhydride, generating fragment **6** that had a free carboxylic acid group for attachment with **5**.

#### 2.2.4. Assembly of MPLA-Tn-KRN7000 conjugate

With the desired three fragments in hand, assembly of the MPLA-Tn-KRN7000 conjugate was performed as shown in Scheme 4. As fragment **1** is more complicated to obtain, fragments **5** and **6** were first coupled to minimize the loss of **1**. The two-component intermediate **2** containing fragments **5** and **6** was obtained in the presence of EDCI and HOBt in a 78.5% yield. After removal of TBS groups with  $\text{BF}_3 \cdot \text{Et}_2\text{O}$ , all acetyl groups of the methyl carboxylate in the resultant intermediate were deprotected by sodium methoxide to produce **24**. Then, compound **24** was coupled with fragment **1** through a click reaction using cuprous iodide (CuI) as the catalyst and *N,N*-diisopropylethylamine (DIEA) as the base to give the key intermediate **25**, which was characterized with  $^1\text{H}$ ,  $^{13}\text{C}$ , 2D,  $^{31}\text{P}$  NMR and HRMS. Finally, removal of all benzyl groups through hydrogenolysis catalyzed Pd under  $\text{H}_2$  atmosphere furnished the target MPLA-Tn-KRN7000 conjugate in an 85.8% yield.

### 2.3. Preparation of Tn-MPLA and Tn-KRN7000 conjugates

#### 2.3.1. Synthesis of Tn-MPLA conjugate

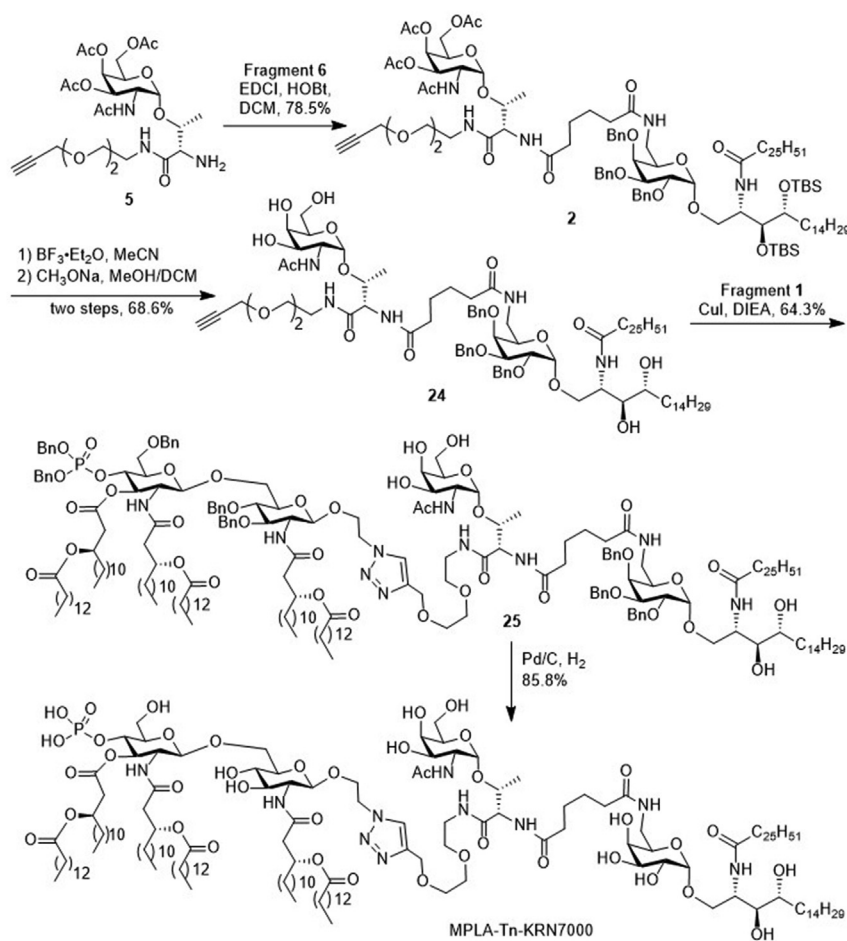
The synthesis of Tn-MPLA conjugate was depicted in Scheme 5. The free amino group in fragment **5** was first acylated by acetic anhydride using pyridine as the promoter, producing intermediate **26** in a 76.2% yield. Subsequently, all acetyl groups in the methyl carboxylate were removed with sodium methylate, giving **27** in an 86% yield. After the coupling of **27** and **1** through a click reaction, the hydrogenolysis of **28** was performed to give the desired conjugate of Tn-MPLA.

#### 2.3.2. Synthesis of Tn-KRN7000 conjugate

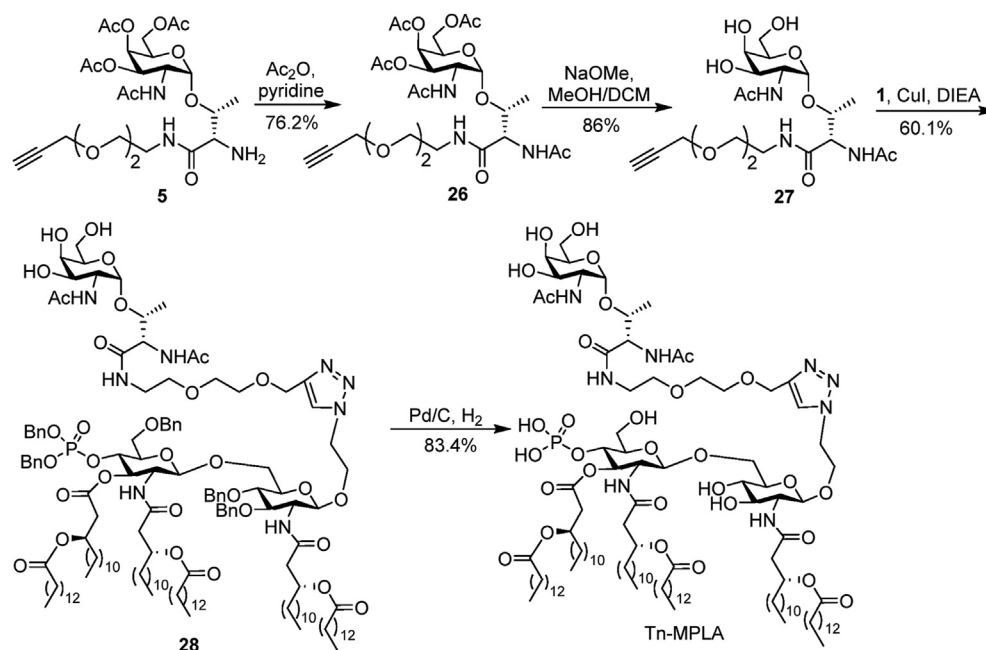
1,2,3-Triazole was also used as the linker between Tn and KRN7000. The synthesis of Tn-KRN7000 conjugate was directly started from intermediate **21**, as shown in Scheme 6. The TBS groups in **21** were selectively deprotected by  $\text{BF}_3 \cdot \text{Et}_2\text{O}$  to produce KRN7000 derivative **29**. Then, the desired conjugate of Tn-KRN7000 was afforded through a click reaction and hydrogenolysis as described for Tn-MPLA.

#### 2.3.3. Preparation of Tn-CRM197 and Tn-HSA

The positive control Tn-CRM197 and the coating antigen Tn-HSA, which was used in enzyme-linked immunosorbent assays (ELISA) to determine Tn-specific antibodies, were also synthesized (Scheme 7). Triazolyl moiety, used in MPLA-Tn-



**Scheme 4** Assembling of MPLA-Tn-KRN7000 conjugate.



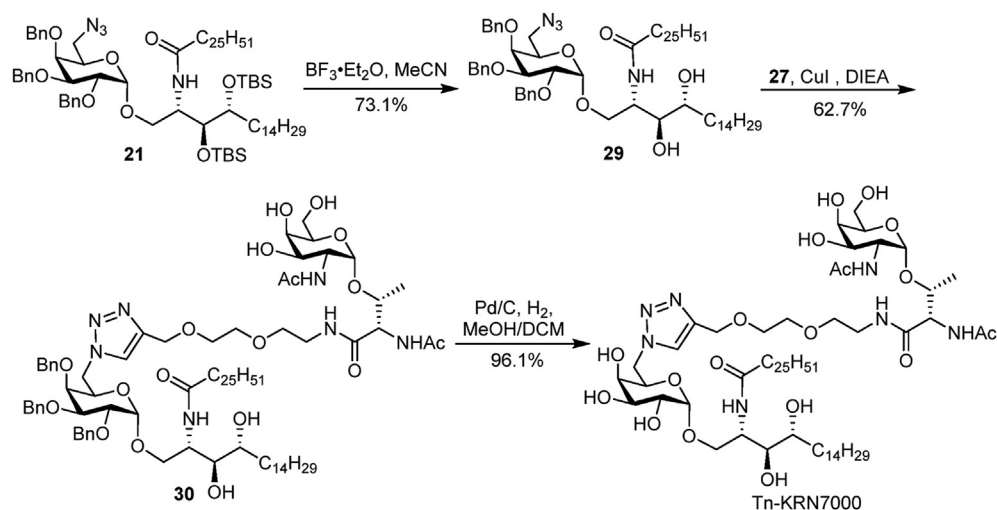
**Scheme 5** Synthesis of Tn-MPLA conjugate.

KRN7000, Tn-MPLA and Tn-KRN7000, may affect the immunological activity of synthetic glycoprotein vaccines<sup>46</sup>. Thus, a flexible linker that not only had a high reactivity but also a negligible effect on the immunogenicity was used in Tn-CRM197 and Tn-HSA. The synthesis of these two glycoproteins started from a free amine group-equipped galactosamine derivative **31**, which was prepared using our previously reported procedure with five steps<sup>29</sup>. Treatment of **31** with a disuccinimidyl suberic acid (**32**) yielded the active ester **33**, and then **33** reacted with CRM197 or HSA proteins in 0.1 mol/L PBS buffer (pH = 7.8). The crude products were purified with dialysis and followed lyophilization, affording the desired Tn-CRM197 or Tn-HSA conjugate. The epitope ratios of these two conjugates were analyzed by MALDI-TOF MS (Supporting Information). Results showed that the levels of antigen loading

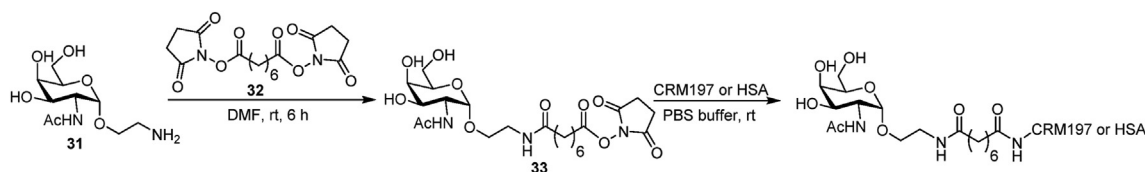
meet the standards for glycoprotein vaccines or capture reagents applied in ELISA<sup>33</sup>.

#### 2.4. Immunological studies of Tn-CRM197, Tn-MPLA, Tn-KRN7000, MPLA-Tn-KRN7000, and the mixture of Tn-MPLA and Tn-KRN7000 in wild-type C57BL/6 mice

The immunological evaluation of Tn-CRM197, Tn-MPLA, Tn-KRN7000, MPLA-Tn-KRN7000 conjugates, and the mixture of Tn-MPLA and Tn-KRN7000 (Tn-MPLA/Tn-KRN7000; Tn-MPLA and Tn-KRN7000 in molar ratios of 1:1) commenced with female C57BL/6 mice (6–8 week-old). All animal studies were conducted in accordance with the Guiding Principles in the Care and Use of Animals (China) and were approved by the Laboratory Animal Ethics Committee of Guangzhou University of



**Scheme 6** Synthesis of Tn-KRN7000 conjugate.



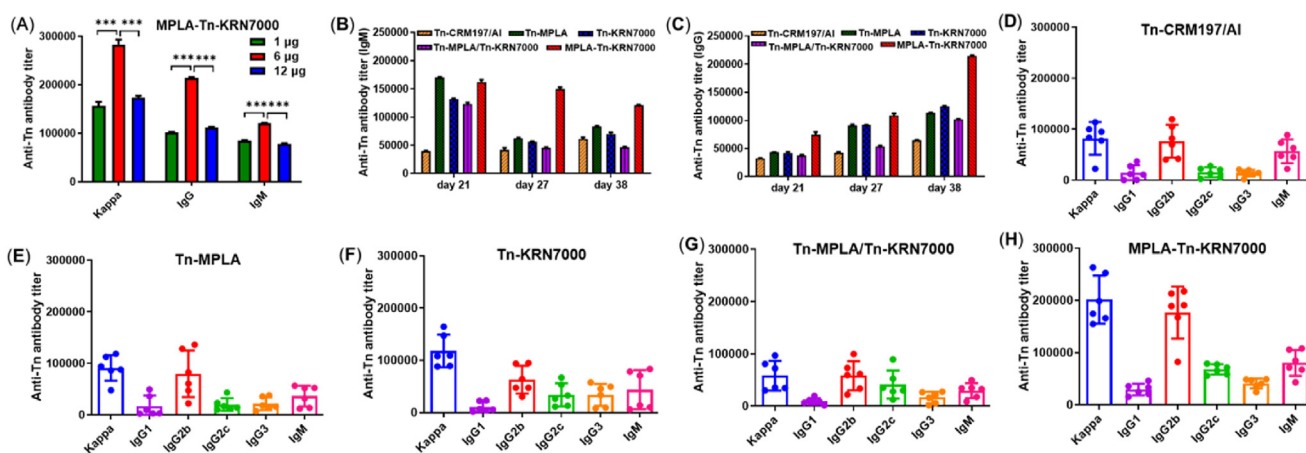
**Scheme 7** Synthesis of Tn-CRM197 and Tn-HSA conjugates.

Chinese Medicine (No. IITCM-20190135) on June 1st, 2019. The fully synthetic conjugate samples were administrated with liposomes, which were prepared with conjugate vaccines, cholesterol and 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC) in molar ratios of 10:50:65 using the sonication dispersion method. Liposomes are contributed to the enhancement of solubility and immunogenicity of conjugates. As glycoprotein vaccine is more effective co-administrated with an external adjuvant, the semi-synthetic Tn-CRM197 conjugate was immunized with an emulsion. The emulsion was freshly prepared by thoroughly mixing Tn-CRM197 and a clinically used alum adjuvant (Al) in PBS buffer (pH = 9.6).

For immunization, Tn-MPLA, Tn-KRN7000, Tn-MPLA/Tn-KRN7000 and MPLA-Tn-KRN7000 were administrated with a dose of 6  $\mu$ g of Tn per 0.1 mL liposome, the concentration that we found the most effective for MPLA-Tn-KRN7000 in a preliminary experiment (Fig. 3A and Supporting Information Fig. S7). The average liposome diameter of Tn-MPLA, Tn-KRN7000, Tn-MPLA/Tn-KRN7000, and MPLA-Tn-KRN7000 is  $1272.7 \pm 273.2$ ,  $1413.3 \pm 130.8$ ,  $1248.3 \pm 193.1$ , and  $1459.3 \pm 364.3$  nm (Support Information Tables S2–S5 and Figs. S3–S6). The Tn-CRM197 conjugate was administrated with 1.7  $\mu$ g Tn per 0.1 mL emulsion, a dose that elicited robust Tn-specific immune responses with an alum adjuvant<sup>24</sup>. Groups of six mice were injected subcutaneously with freshly prepared liposomes or emulsion on Days 1, 14, 21, and 28. Each mouse was bled before initial immunization (Day 0, blank controls) and on Days 21, 27 and 38 post-first immunization. Antisera were

prepared with standard protocols. The titers of kappa, IgM, IgG and IgG isotypes (IgG1, IgG2b, IgG2c, and IgG3) antibodies were detected using ELISA assay.

The total titers of IgM and IgG antibodies in pooled sera collected from Days 21, 27, and 38 post-first immunization of each group were measured. Fig. 3B reveals that Day 21 antisera of all groups elicited highly Tn-specific IgM titers, and that Tn-MPLA, Tn-KRN7000, Tn-MPLA/Tn-KRN7000 and MPLA-Tn-KRN7000 groups were higher than Tn-CRM197/Al. As we know, IgM is the primitive antibody induced in response to a foreign antigen. This result reveals that all fully synthetic conjugates could rapidly elicit a stronger immune response than the semisynthetic glycoprotein Tn-CRM197 with Al. IgG antibodies of the Day 21 antisera were also detected and titers further increased after the third and fourth immunizations (Fig. 3C), revealing that long-term secondary immune responses were triggered. After the fourth immunization, the titers of IgG antibodies induced by Tn-MPLA, Tn-KRN7000 and MPLA-Tn-KRN7000 were higher than those of the Tn-CRM197/Al group, suggesting that MPLA, KRN7000 and the conjugate of MPLA and KRN7000 were more effective immunostimulants than CRM197. Day 38 IgG antibody titers in mice receiving MPLA-Tn-KRN7000 reached up to 214,631, nearly twice as high as Tn-MPLA and Tn-KRN7000 groups (113,550 and 125,076, respectively), revealing that our three-component conjugate containing two different types of immunostimulants was more effective than corresponding two-component vaccines. One possible reason is that the covalently linked MPLA and KRN7000 used as a



**Figure 3** (A) Antibody titers of pooled sera induced by MPLA-Tn-KRN7000 with different dosages, asterisks with brackets indicate significant difference using unpaired two-tailed Student's *t* test,  $***P < 0.001$ . (B, C) Titers of IgM (B) and IgG (C) antibodies in pooled sera collected from mice on Days 21, 27, and 38 after immunization with Tn-CRM197/Al, Tn-MPLA, Tn-KRN7000, Tn-MPLA/Tn-KRN7000 or MPLA-Tn-KRN7000 conjugates. (D–H) Titers of Kappa, IgM and IgG antibody isotypes in individual Day 38 mouse antiserum induced by conjugates Tn-CRM197/Al (D), Tn-MPLA (E), Tn-KRN7000 (F), Tn-MPLA/Tn-KRN7000 (G) and MPLA-Tn-KRN7000 (H). The dot represents the antibody titers of each mouse serum, and the horizontal bar represents the average antibody titers of each group, data are indicated as the average  $\pm$  SD ( $n = 6$ ), a Student's *t*-test.

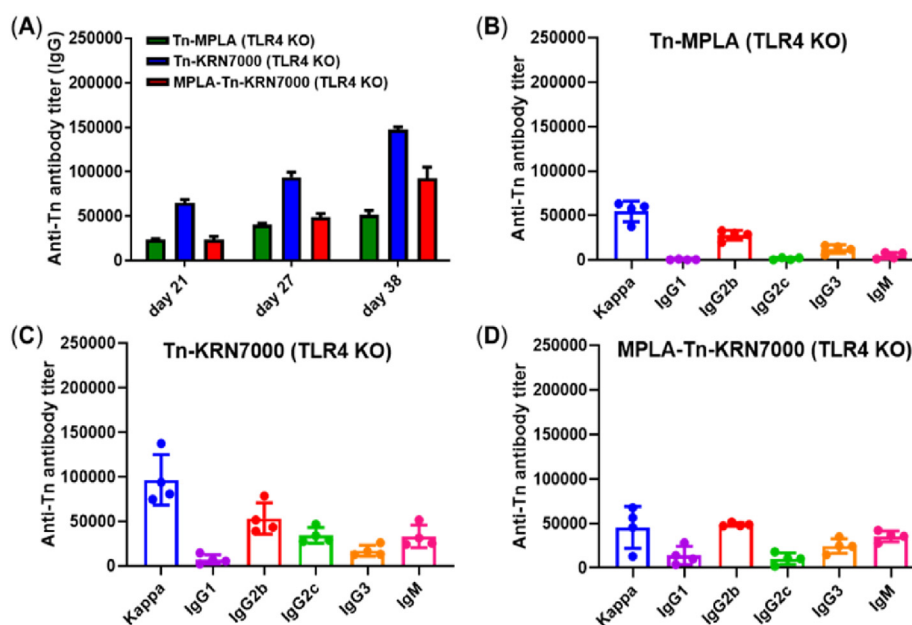
immunostimulant may synergistically activate TLR4 and iNKT cell signal paths, more effectively improving anti-Tn immune response than MPLA and KRN7000 alone. Interestingly, the IgG antibody titer induced by the mixture of Tn-MPLA and Tn-KRN7000 (102,063) was slightly lower than that of Tn-MPLA (113,550) or Tn-KRN7000 (125,076) alone and about 50% of that elicited by MPLA-Tn-KRN7000 (214,631). These results reveal that the simply combining of Tn-MPLA and Tn-KRN7000 did not have synergies.

The titers of kappa, IgM and IgG subclass antibodies in each Day 38 mouse antisera are depicted in Fig. 3D–H. High titers of kappa and IgG antibodies were observed, suggesting a robust Tn-specific and T-cell dependent immune response that is in demand for cancer treatment was elicited by all our synthetic conjugates. The isotypes of IgG antibody present mainly IgG2b, IgG2c, IgG3 and a low level of IgG1 antibodies, revealing that all fully synthetic conjugates could induce mixed Th1/Th2 immune responses and that Th1-type might predominate. All average titers of kappa, IgG1 and IgG2b antibodies elicited by MPLA-Tn-KRN7000 were significantly higher than those of Tn-CRM197/Al, Tn-MPLA and Tn-KRN7000, indicating that covalently linked MPLA-KRN7000 as a built-in adjuvant is a more effective stimulant than CRM197, MPLA and KRN7000. The average levels of kappa, IgG1 and IgG2b antibody titers induced by Tn-MPLA/Tn-KRN7000 were lower than Tn-MPLA and Tn-KRN7000, further indicating that the promoting effect of the mixture of Tn-MPLA and Tn-KRN7000 is little. Collectively, ELISA results suggest that the covalently linked three-component conjugate MPLA-Tn-KRN7000 can induce a much stronger Tn-specified immune response than the corresponding two-component conjugate Tn-CRM197, Tn-MPLA, Tn-KRN7000 and the mixture of Tn-MPLA and Tn-KRN7000 at equimolar concentrations, preliminarily demonstrating the feasibility of our strategy for self-adjuncting cancer vaccine design.

### 2.5. Immunological studies of Tn-MPLA, Tn-KRN7000, and MPLA-Tn-KRN7000 in TLR4 knockout (KO) C57BL/6 mice

To determine whether the covalently linked MPLA and KRN7000 immunostimulant enhances Tn-specified immune response through synergistically activating TLR4 and iNKT cell, immunological evaluation of Tn-MPLA, Tn-KRN7000 and MPLA-Tn-KRN7000 was then performed with TLR4 knockout (KO) C57BL/6 mice. Each group containing four mice was immunized with Tn-MPLA, Tn-KRN7000 and MPLA-Tn-KRN7000 in the same protocol and schedule to wild-type mice. The titers of IgG antibodies in pooled antisera collected from immunized mice on Days 21, 27 and 38, and the titers of kappa, IgM and IgG subclass antibodies in Day 38 antisera were examined.

As shown in Fig. 4A, a degree number of IgG antibody titers were observed in all Day 21 antisera and increased further after booster immunizations. Compared to wild-type mice, all titers of IgG antibodies in Days 21, 27 and 38 antisera from TLR4 KO mice that immunized with Tn-MPLA and MPLA-Tn-KRN7000 were distinct decreased. The reduction ratios of Tn-MPLA and MPLA-Tn-KRN7000 of Day 38 IgG antibody titers were 54.9% (titers: 51,179 vs. 113,550) and 57.0% (titers: 92,305 vs. 214,631), respectively. These results reveal that the knockout of TLR4 seriously down-regulated the immunocompetence of Tn-MPLA and MPLA-Tn-KRN7000. Differently, the knockout of TLR4 almost did not significantly affect the antibody titer elicited by Tn-KRN7000. Similar to IgG antibodies, the titers of kappa, IgM and IgG antibody isotypes in Tn-MPLA and MPLA-Tn-KRN7000 groups also decreased significantly, while the Tn-KRN7000 group showed little change (Fig. 4B–D). These results reveal that Tn-MPLA and MPLA-Tn-KRN7000 can activate TLR4 to improve Tn-specified immune response, while Tn-KRN7000 is not. After the fourth immunization, the level of IgG antibody titers of MPLA-Tn-KRN7000 was 1.8-fold that of Tn-MPLA. This result suggests that the KRN7000 molecule in



**Figure 4** (A) Titers of IgG antibodies in pooled sera collected from TLR4 KO mice on Days 21, 27, and 38 after immunization with Tn-MPLA, Tn-KRN7000, MPLA-Tn-KRN7000 conjugates (B–D) Titers of kappa, IgM and IgG antibody isotypes in individual Day 38 TLR4 KO mouse antiserum induced by Tn-MPLA (B), Tn-KRN7000 (C), MPLA-Tn-KRN7000 (D). The dot represents the antibody titers of each mouse serum, and the horizontal bar represents the average antibody titers of each group, data are represented as the average  $\pm$  SD ( $n = 4$ ), a Student's *t*-test.



MPLA-Tn-KRN7000 may enhance the Tn-specified immune response through activating the iNKT cells, as KRN7000 is an agonist of them. Combining the comparison of the immunological effect of the three-component conjugate to the two-component constructs alone and when mixed in wild-type mice, we preliminary concluded that the covalently linked MPLA and KRN7000 immunostimulant in MPLA-Tn-KRN7000 can synergistically activate TLR4 and iNKT cell to improve Tn-specified immune response.

## 2.6. Binding affinity of Tn-MPLA, Tn-KRN7000, and MPLA-Tn-KRN7000 to CD1d protein

The mechanism that KRN7000 activates iNKT cells is as follows: KRN7000 binds to the CD1d protein, a nonpolymorphic antigen-presenting protein responsible for the regulation of NKT cell activation, to form a complex<sup>47</sup>. This complex is recognized by NKT cells through T cell receptors, leading to the activation of NKT cells<sup>48</sup>. To further investigate whether the three-component vaccine MPLA-Tn-KRN7000 could activate iNKT cell directly, the binding affinity of it to mouse and Human CD1d-His proteins, respectively, were determined and compared with Tn-MPLA and Tn-KRN7000.

As shown in Fig. 5, compared to the control, Tn-MPLA did not show significant binding to mouse CD1d-His or Human CD1d-His protein. This result revealed that MPLA could not activate iNKT cell directly. In contrast, Tn-KRN7000 and MPLA-Tn-KRN7000 could recognize and bind to both mouse CD1d-His and Human CD1d-His proteins. These results revealed that similar to KRN7000, the covalently linked MPLA and KRN7000 could also directly activate iNKT cells through binding to CD1d protein. The binding affinity of MPLA-Tn-KRN7000 to mouse CD1d-His or Human CD1d-His proteins was both lower than Tn-KRN7000 at the same concentration, this may be the reason that Tn-KRN7000 elicited higher IgG antibody titers than MPLA-Tn-KRN7000 in TLR4KO C57BL/6 mice. These results indicated that the covalently linked MPLA and KRN7000 in MPLA-Tn-KRN7000 can indeed play a synergistic role in the enhancement of Tn-specified immune response.

## 2.7. Evaluation of cytokine levels provoked by conjugates

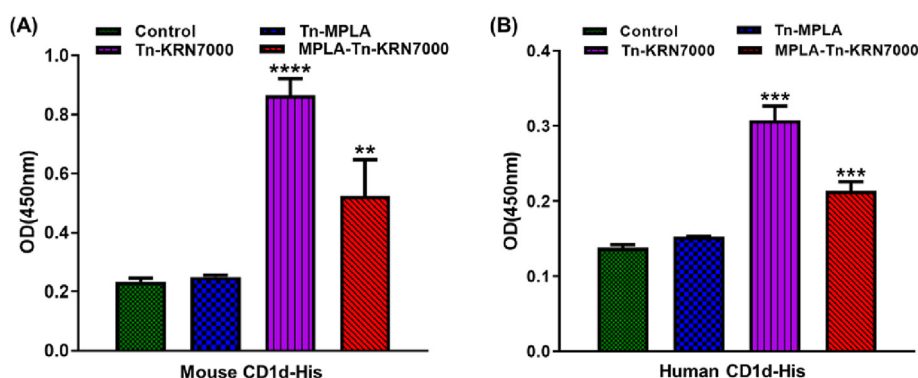
To investigate whether the synthetic conjugates can provoke cytokine production, the gamma interferon (IFN- $\gamma$ ) and

interleukin 4 (IL-4) levels in pooled antisera were evaluated. As depicted in Fig. 6, all conjugates elicited high expression of IFN- $\gamma$  and IL-4 as compared to normal mouse sera (NS). Among them, MPLA-Tn-KRN7000 exhibited the highest levels of IFN- $\gamma$  and IL-4 (821.3 and 110.4 pg/mL, respectively), suggesting that the three-component vaccine MPLA-Tn-KRN7000 has the strongest immunogenicity. The high induction of IFN- $\gamma$  suggests the activation of Th1 cells, which can induce macrophages activation and the IgG antibody conversion. The high production of IL-4 suggests the activation of Th2 cells, contributing to the enhancement of B cell immunity and the formation of IgG1 antibodies. These results indicate that all fully synthetic conjugates induce Th1/Th2 mixed immune responses, consistent with ELISA results. The IFN- $\gamma$ /IL-4 ratios (5.17 for Tn-CRM197/Al, 8.77 for Tn-MPLA, 8.83 for Tn-KRN7000, 4.27 for Tn-MPLA/Tn-KRN7000 and 7.44 for MPLA-Tn-KRN7000) also indicate that all conjugates elicited a predominately cellular immune response, which is desirable for cancer immunotherapy.

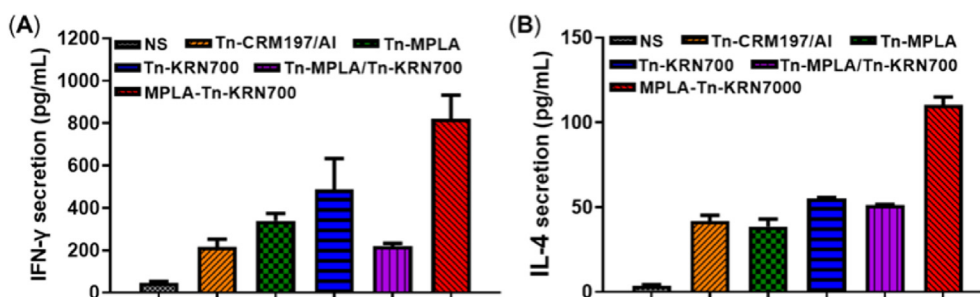
## 2.8. Abilities of antiserum binding to cancer cells

We utilized fluorescence-activated cell sorting (FACS) assay to evaluate the ability of antisera to recognize and bind to Tn antigens expressed on tumor cell surfaces. Murine breast cancer cell line TA3Ha and human breast cancer cell line MCF-7, both of them exhibit high levels of Tn antigen on their surfaces, were used in this study. The Tn-negative cancer cell line MDA231 was selected as a control. Each cancer cell line was treated with normal mouse serum or antisera induced by conjugates, followed by incubating with fluorescein isothiocyanate (FITC)-labeled goat anti-mouse IgG antibody and analyzed by flow cytometry.

All antisera obtained from immunized mice exhibited a stronger binding ability to TA3Ha and MCF-7 cancer cells when compared to normal serum (Fig. 7A and B). In contrast, the binding abilities of normal serum and antisera to MDA231 cancer cells were not significantly different (Fig. 7C). These results reveal that all antisera could recognize and exhibit a strong binding ability to Tn-positive cells, while the binding to Tn-negative cells was little. The binding affinity to TA3Ha follows the order: MPLA-Tn-KRN7000 > Tn-MPLA Tn-KRN7000 ~ Tn-MPLA/Tn-KRN7000 > Tn-CRM197/Al. The binding activity to MCF-7 follows the order: MPLA-Tn-KRN7000 > Tn-MPLA > Tn-KRN7000 > Tn-CRM197/Al > Tn-MPLA/Tn-



**Figure 5** The binding affinity of Tn-MPLA, Tn-KRN7000 and MPLA-Tn-KRN7000 to mouse CD1d-His or Human CD1d-His proteins. Plates were coated with MeOH (control), Tn-MPLA, Tn-KRN7000 or MPLA-Tn-KRN7000 (1.1 nmol/well) and incubated with mouse CD1d-His or human CD1d-His. Data are represented as the mean  $\pm$  SD in each group ( $n = 3$ ), asterisks indicate significant differences to control using one-way ANOVA followed by Dunnett's multiple comparison test, \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , \*\*\*\* $P < 0.0001$ , Student's  $t$ -test.



**Figure 6** IFN- $\gamma$  (A) and IL-4 (B) concentrations of Day 38 antisera provoked by all conjugates.

KRN7000. These results indicated that antibodies in MPLA-Tn-KRN7000 antiserum showed strongest binding activity, which was consistent with ELISA results and provided another piece of evidence that the three-component conjugate MPLA-Tn-KRN7000 has significantly stronger immunological activity than the corresponding glycoprotein vaccine Tn-CRM197 with an alum adjuvant, the two-component conjugates (Tn-MPLA and Tn-KRN7000) and the mixture of Tn-MPLA and Tn-KRN7000.

### 2.9. Antibody-mediated CDC to cancer cells

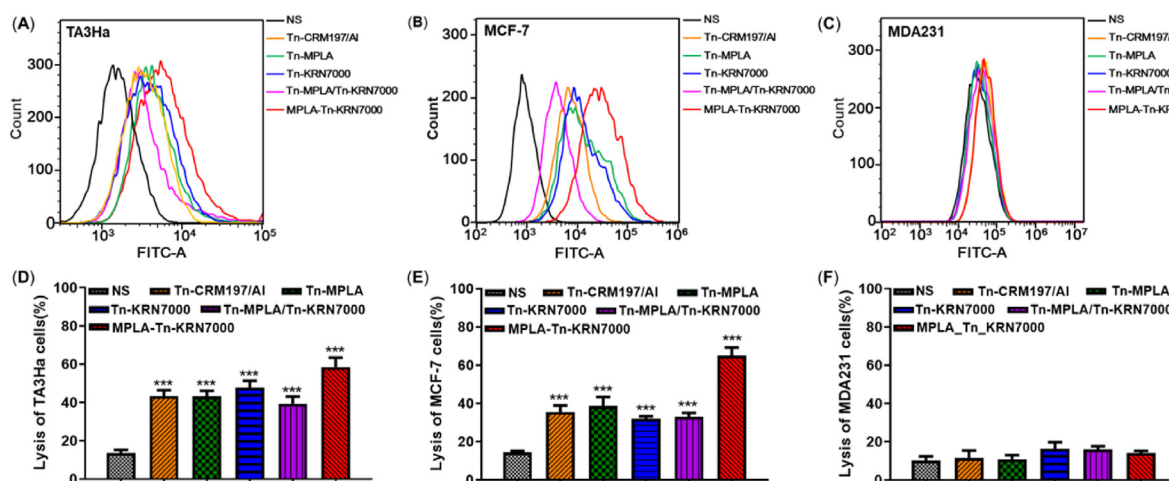
Given the antibodies induced by all conjugates naturally exhibited strong binding affinity to the Tn antigen, complement-dependent cytotoxicity (CDC) was then measured. CDC was also tested against TA3Ha, MCF-7 and MDA231 cancer cells. Each cell line was incubated with normal mouse serum or antisera and then treated with rabbit complement serum. The lysis rates were measured using the lactate dehydrogenase (LDH) assay.

The results of cell lysis revealed that antisera induced by all conjugates effectively activated the complement system and exhibited significant cytotoxicity against Tn positive TA3Ha and MCF-7 cells as compared to normal mouse sera (Fig. 7D and E). For TA3Ha cells, the order of relative effects of antisera were: MPLA-Tn-KRN7000 > Tn-KRN7000 > Tn-CRM197/Al ~ Tn-

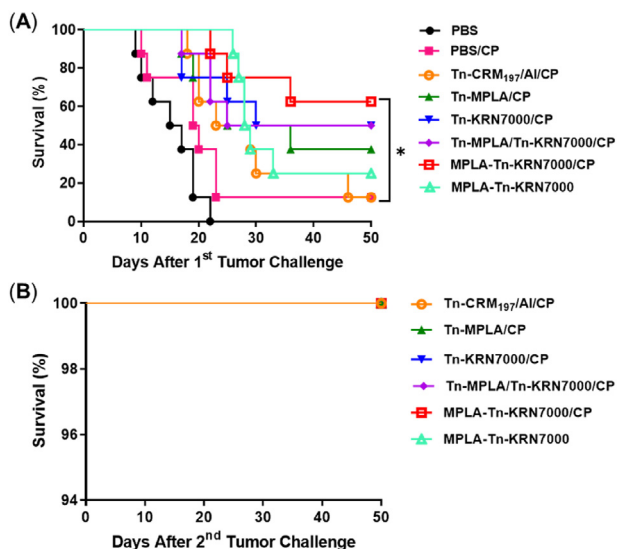
MPLA > Tn-MPLA/Tn-KRN7000 (with lysis rates 58.5%, 47.7%, 43.3%, 43.1% and 39.4%). For MCF-7 cells, the relative effects of antisera followed the order: MPLA-Tn-KRN7000 > Tn-MPLA > Tn-CRM197/Al > Tn-MPLA/Tn-KRN7000 ~ Tn-KRN7000 (with lysis rates 65.0%, 38.7%, 35.6%, 33.2% and 32.1%). Different from TA3Ha and MCF-7 cells, almost no cytotoxicity was observed for Tn-negative MDA231 cells for any group. Thus, all antisera exhibited moderate to good cytotoxicity to tumor cells that overexpressed Tn antigens and no cytotoxicity to cells without Tn antigens. Meanwhile, MPLA-Tn-KRN7000 antisera showed stronger CDC than others, demonstrating the three-component conjugate of Tn, MPLA and KRN7000 is a better vaccine candidate for cancer immunotherapy.

### 2.10. Tumor challenge studies

To further validate the potential of the MPLA-Tn-KRN7000 conjugate as a vaccine candidate, the ability of all conjugates to protect tumor-challenged mice was evaluated and compared. Eight groups of C57BL/6 mice (6–8 week-old) were given TA3Ha cancer cells ( $9.0 \times 10^5$  cells) through intraperitoneal injection on Day 0 of the experiment. On one day before vaccination, six groups of them intraperitoneally administrated cyclophosphamide (CP) with a dose of 50 mg/kg, which could reduce T-regulatory cells and enhance the immune response. Then, each of the six



**Figure 7** (A–C) Flow cytometry analysis of antisera binding to Tn-positive TA3Ha (A), MCF-7 (B) or Tn-negative MDA231 (C) cells. Normal mouse serum (black), pooled antisera induced by glycoprotein Tn-CRM197/Al (orange), Tn-MPLA (green), Tn-KRN7000 (blue), the mixture of Tn-MPLA/Tn-KRN7000 (pink) or three-component conjugate MPLA-Tn-KRN7000 (red). (D–F) CDC for Tn positive TA3Ha (D), MCF-7 (E) or negative MDA231 (F) cancer cell line mediated by normal mouse serum or pooled antisera. The same volume of sera was used for the assay. Data are represented as the mean  $\pm$  SD in each group ( $n = 6$ ), asterisks indicate a significant difference to control NS using one-way ANOVA followed by Dunnett's multiple comparison test, \*\*\* $P < 0.001$ , Student's  $t$ -test.



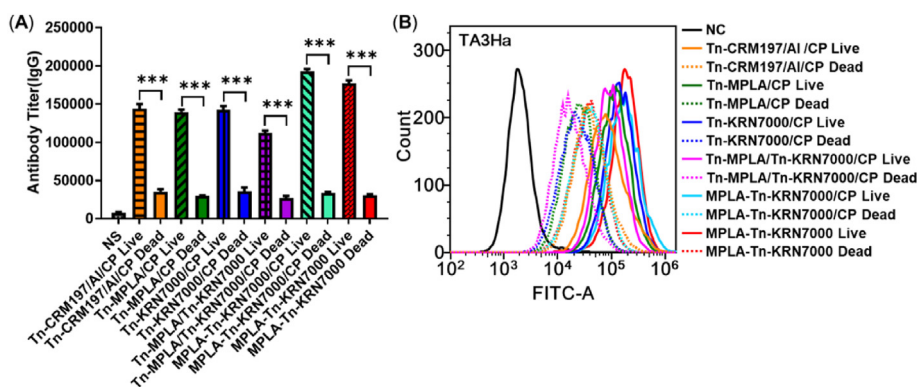
**Figure 8** The abilities of all conjugates protected mice from tumor development. (A) The survival rate of mice immunized with PBS, Tn-CRM197/Al, Tn-MPLA, Tn-KRN7000, Tn-MPLA/Tn-KRN7000 and MPLA-Tn-KRN7000 with or without the administration of CP. Eight mice in each group received intraperitoneally with  $9.0 \times 10^5$  TA3Ha cells on Day 0. The survival time of mice was followed up for 50 days. Asterisk with bracket indicates significant difference using unpaired two-tailed Student's *t*-test,  $*P < 0.05$ ,  $n = 8$ . (B) The survival rate of surviving mice rechallenged with another  $9.0 \times 10^5$  TA3Ha cells.

groups was subcutaneously immunized with PBS, Tn-MPLA, Tn-KRN7000, Tn-MPLA/Tn-KRN7000, MPLA-Tn-KRN7000 (6  $\mu$ g of Tn in 0.1 mL liposome) or Tn-CRM197/Al (1.7  $\mu$ g of Tn in 0.1 mL emulsion) on Day 1 of the experiment. One of the remaining two groups only received MPLA-Tn-KRN7000, and another one only administered PBS as the control. Each group received booster immunization on Days 4 and 8 with the same protocols, for a total of three vaccinations per animal.

As shown in Fig. 8A, mice in the control group (PBS only) died out within 22 days. The treatment of CP alone slightly

prolonged the mean survival time of mice, however, all mice except one had died before Day 23. Typically, CP can not significantly affect the proliferation of TA3Ha cells. One possible reason for the small effect we observed is that CP potentially induces the abolition of immunosuppression triggered by myeloid-derived suppressor cells and the activation of effector cells, generating cytotoxicity to tumor cells<sup>49,50</sup>. Compared to PBS/CP groups, the combination of CP and conjugates protected against tumor development, significantly prolonging average survival time and improving survival rates. The average survival times and survival ratios of immunized groups had the rank order: MPLA-Tn-KRN7000/CP (survival time: 41.6 d; survival mice number: 5; survival ratio: 62.5%) > Tn-KRN7000/CP (36.1 d; 4; 50%) ~ Tn-MPLA/Tn-KRN7000/CP (35.8 d; 4; 50%) > Tn-MPLA/CP (34.0 d; 3; 37.5%) > Tn-CRM197/Al/CP (29.5 d; 1; 12.5%). The treatment of MPLA-Tn-KRN7000 alone also effectively inhibited tumor development, however, its effectiveness was lower than MPLA-Tn-KRN7000 plus CP (25% vs. 62.5% survival ratio). These results suggest that MPLA-Tn-KRN7000 provided significantly higher protection to mice with tumor challenge than that of Tn-CRM197/Al, Tn-MPLA, Tn-KRN7000 and the mixture of Tn-MPLA and Tn-KRN7000, further demonstrating the strong potential of the conjugate based on covalently linked TLR4 and iNKT cell agonists as a vaccine candidate. Meanwhile, a better effect can be achieved when MPLA-Tn-KRN7000 combines with CP. Then, all surviving mice in the first tumor-challenge experiment received a second dose of  $9.0 \times 10^5$  TA3Ha cells on Day 50. These mice all rejected the tumor without any additional treatment, indicating long-lasting immunity against TA3Ha cells was formed in these mice (Fig. 8B).

To understand the immune protection, blood samples were collected from surviving and dead mice in the tumor challenge. For surviving mice, sera were collected on Day 30. For dead mice, sera were collected right after the mouse was sacrificed due to the heavy tumor burden. The sera were prepared with standard methods. Total IgG antibody titers were examined using ELISA. Compared to normal mouse serum, the sera from surviving mice exhibited much higher levels of IgG antibodies (Fig. 9A). The MPLA-Tn-KRN7000 plus CP group showed the highest IgG antibody titers, which is



**Figure 9** (A) Analysis of IgG antibody titers of sera from live or dead mice immunized with Tn-CRM197/Al, Tn-MPLA, Tn-KRN7000, Tn-MPLA/Tn-KRN7000, MPLA-Tn-KRN7000 with or without CP. Asterisks with brackets indicate significant difference using unpaired two-tailed Student's *t*-test,  $***P < 0.001$ ,  $n = 3$ . (B) FACS analysis of sera from live (solid line) or dead (dotted line) mice immunized with Tn-CRM197/Al/CP (orange), Tn-MPLA/CP (green), Tn-KRN7000/CP (blue), Tn-MPLA/Tn-KRN7000/CP (pink), MPLA-Tn-KRN7000/CP (cyan) or MPLA-Tn-KRN7000 (red) against Tn positive TA3Ha tumor cells. For surviving mice, sera were collected on Day 30. For dead mice, sera were collected right after the mouse was sacrificed due to the heavy tumor burden.

presumably why it had the greatest effect. The unprotected mice sera also had positive IgG antibody titers; however, it was less than a quarter of that observed from surviving mice sera. The ability of antisera to recognize and bind to TA3Ha cells was also tested using FACS technology. Similar to the ELISA results, sera from surviving mice bound to more TA3Ha cells than that from dead mice (Fig. 9B). These results indicate that the production of high levels of Tn-specific IgG antibodies is the primary reason that surviving mice resist the tumor attack.

### 3. Conclusions

Fully synthetic self-adjuncting TACA-based antitumor vaccines show promising prospects. At present, the construction of such vaccines typically uses a single immunostimulant as a carrier molecule. To further increase the immunogenicity of TACA, we propose a new strategy using a covalently linked TLR4 ligand and iNKT cell agonist as the immunostimulant. Using Tn antigen as the model, a three-component vaccine (MPLA-Tn-KRN7000) containing the TLR4 ligand MPLA and the iNKT cell agonist KRN7000 was designed and synthesized through an efficient method. The construction of MPLA-Tn-KRN7000 expanded fully synthetic self-adjuncting vaccine studies from a single molecule carrier to that with two different types of carriers. In addition, the corresponding two-component vaccines Tn-MPLA, Tn-KRN7000 and semisynthetic glycoprotein Tn-CRM197 were designed and synthesized as controls.

The immunological evaluation demonstrated that all conjugate vaccines could effectively elicit robust and consistent Tn-specific immune responses in mice. The assessment of antibody isotypes and the secretion of IFN- $\gamma$  and IL-4 suggest that all conjugates elicited a mixture of humoral and cellular immune responses, and the cellular immune response is predominant. Compared to glycoprotein Tn-CRM197/Al and two-component vaccines (Tn-MPLA and Tn-KRN7000), the three-component conjugate MPLA-Tn-KRN7000 elicited a stronger immune response. IgG titers of sera from the MPLA-Tn-KRN7000 group were more than three times greater than that of Tn-CRM197/Al, and twice that of Tn-MPLA and Tn-KRN7000 groups. In addition, we observed that a simple physical mixture of Tn-MPLA and Tn-KRN7000 did not provide a better immune response than Tn-MPLA or Tn-KRN7000 alone at equimolar concentrations. The comparison of the immunological effect of the three-component conjugate to the two-component constructs alone, along with the immunological studies in TLR4 knockout mice and the test of binding affinity to CD1d protein demonstrate the covalently linked MPLA-KRN7000 immunostimulant produces a synergistic activation of TLR4 and iNKT cell that improves the immunogenicity of Tn.

FACS analysis and CDC studies revealed that all conjugates induced antibodies can specifically recognize, bind to and exhibit effective complement-dependent cytotoxicity to murine and human Tn-positive cancer cells, resulting in tumor cell lysis. While almost no cytotoxicity to Tn-negative cancer cells. Meanwhile, all conjugates improved the survival rate and prolonged survival time of tumor-challenged mice through elicitation of high levels of IgG antibodies, and the surviving mice reject further tumor attacks without any additional treatment. Notably, the three-component conjugate MPLA-Tn-KRN7000 combated cancer the best, both *in vitro* and *in vivo*. In addition, the combination of MPLA-Tn-KRN7000 with CP can achieve better protection than MPLA-Tn-KRN7000 alone. Our results suggest that MPLA-Tn-

KRN7000 has a strong potential to be an antitumor vaccine candidate. Moreover, using two different immunostimulants covalently linked as a carrier is a powerful strategy for the future development of TACA-based vaccines, and the strategy merits additional investigation and development.

In conclusion, a new strategy using a TLR4 ligand and iNKT cell agonist as a build-in adjuvant to construct synthetic self-adjuncting cancer vaccines was established. Compared to typical glycoprotein conjugate vaccines and two-component fully synthetic vaccines, this three-component vaccine elicited a higher immune response without any external adjuvants. This work provides a new direction toward the construction of fully synthetic vaccines.

### 4. Experimental

#### 4.1. Mouse immunization

Each group of six female C57BL/6 wild-type or four TLR4 knockout mice (6–8 week-old) was immunized *via s.c.* injection with a liposomal of fully synthetic conjugates (6  $\mu$ g of Tn in 0.1 mL liposome) or an emulsion preparation of glycoprotein vaccine (1.7  $\mu$ g of Tn in 0.1 mL liposome) on Days 1, 14, 21 and 28 with the same protocols. Each mouse was bled on Days 0, 21, 27 and 38. Antisera were prepared and stored at  $-80^{\circ}\text{C}$  before immunological analysis.

#### 4.2. ELISA protocol

96-Wells plates were pre-coated by 100  $\mu$ L Tn-HSA conjugate (2  $\mu$ g Tn in 1 mL solution) dissolved in 0.1 mol/L carbonate buffer (pH = 9.6) at  $37^{\circ}\text{C}$  for 1 h. After washing with PBST buffer (0.1% Tween-20 in PBS) three times, blocking buffer (PBS containing 1% BSA) was added and the plates were incubated at rt for 1 h. After washing again, serial half-log diluted (from 1:300 to 1:656,100) pooled or individual antisera were added (3 wells were set in each group) and followed incubation at  $37^{\circ}\text{C}$  for 2 h. The plates were washed with PBST three times, and then 1:1000 diluted HRP-linked goat anti-mouse kappa, IgM or 1:2000 diluted IgG, IgG1, IgG2b, IgG2c and IgG3 antibodies solution was individually added. The plates were incubated at rt for 1 h, followed by shaking and washing with PBS. Then, 100  $\mu$ L of colorimetric substrate TMB was added. After incubating in the darkness for 20 min, the chromogenic reaction was quenched by 0.5 mol/L  $\text{H}_2\text{SO}_4$  solution (100  $\mu$ L). The optical density (OD) value was measured at 450 nm wavelength. The OD values were plotted against the antiserum dilution numbers after deduction of background, affording the best fit line utilized to calculate the antibody titer, which was defined as the dilution number when the OD value is 0.2.

#### 4.3. Mouse and human CD1d-His binding assay

Tn-MPLA, Tn-KRN7000 and MPLA-Tn-KRN7000 were dissolved in MeOH (1.1 nmol/mL) and added to 96-well plates (100  $\mu$ L per well, 3 wells per group were set). The solvents were evaporated at room temperature. The plates were incubated at  $4^{\circ}\text{C}$  overnight and followed at  $37^{\circ}\text{C}$  for 1 h. After washing with PBST 3 times, the plates were treated with blocking buffer and washed again. Then, the coated plates were incubated with 50  $\mu$ L mouse CD1d-His or human CD1d-His protein (10  $\mu$ g/mL in PBS) for 1.5 h at  $37^{\circ}\text{C}$ . After washing with PBST, HRP-anti His was

added, and the plates were incubated for another 1 h. Finally, a colorimetric substrate TMB was added and the OD value was measured at 450 nm.

#### 4.4. FACS assay protocol

The protocol for FACS analyses was similar to the reported literature<sup>28</sup>. Briefly, a suspension of  $5.0 \times 10^5$  target cells was incubated with 50  $\mu$ L of normal or anti-Tn serums (1:10 diluted by medium) at 4 °C for 1 h. Then, the plates were washed three times with FACS buffer, followed by treating with FITC-labeled goat anti-mouse IgG antibody (1:50 diluted) at 4 °C for 1 h. The plates were washed again with FACS buffer, and the cells were recorded by flow cytometer.

#### 4.5. CDC protocol

TA3Ha, MCF-7 or MDA231 cancer cells ( $1.0 \times 10^4$  cells/well, 6 wells per group were set in each group) were seeded in the 96-well plate and cultured overnight at 37 °C. The plates were washed with medium without FBS and then treated with 100  $\mu$ L of normal or Day 38 antisera (1:50 dilution) induced by the conjugates at 37 °C for 2 h. After washing twice with medium without FBS, rabbit complement serum (100  $\mu$ L, 1:10 dilution) was added. For control groups, the rabbit complement serum and the antisera were replaced with 100  $\mu$ L of the medium (low control) or 5% LDH release solution (high control). After incubation for 1 h, the supernatant of each well (20  $\mu$ L) was transferred to a new 96-well plate and diluted with 80  $\mu$ L of DPBS buffer. Thereafter, 100  $\mu$ L LDH was added to the plate and cultured in the darkness for 30 min. The OD value was measured at 490 nm wavelength. The cell lysis rates were calculated with Eq. (1):

$$\text{Cell lysis(\%)} = \frac{(\text{Experimental OD} - \text{Low control OD})}{(\text{High control OD} - \text{Low control OD})} \times 100 \quad (1)$$

#### 4.6. Tumor challenge experiments

Eight groups of 6–8 week-old C57BL/6 mice (each group containing 8 mice) were used to test against TA3Ha tumors. All mice received TA3Ha cancer cells ( $9.0 \times 10^5$  cells) through intraperitoneal injection on Day 0. Six groups of them intraperitoneally received cyclophosphamide (CP) with a dose of 50 mg/kg on one day before vaccination. On Day 1 of the experiment, each of the six groups received one of PBS, Tn-MPLA, Tn-KRN7000, Tn-MPLA/Tn-KRN7000, MPLA-Tn-KRN7000 (6  $\mu$ g of Tn in 0.1 mL liposome) or Tn-CRM197/Al (1.7  $\mu$ g of Tn in 0.1 mL emulsion) through s.c. injection. One of the remaining two groups only received MPLA-Tn-KRN7000, and another one only administered PBS as the control. Each group received booster immunization on Days 4 and 8 with the same protocols. Mouse survival time was monitored for 50 days after receiving tumor cells. The survival mice immunized with conjugates were injected with another  $9.0 \times 10^5$  TA3Ha cells, and the survival time was recorded for another 50 days.

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#### Author contributions

Guochao Liao conceived the project and designed the experiments. Deying Yang synthesized the self-adjuncting conjugates and glycoprotein conjugates. Lingqiang Gao and Chengxin Wang assisted with the synthesis of MPLA-Tn-KRN7000. Qinghai Lian performed the sample preparation, characterization and biological experiment. Xiaoxiao Qi assisted with the animal experiments. Xiang Luo and Guochao Liao wrote the manuscript and prepared the Supporting Information. Guochao Liao, Zhongqiu Liu, Rong Zhang and Xiang Luo contributed funding acquisition and supervision. All authors discussed the results and commented on the manuscript.

#### Conflict of interest

The authors declare no competing financial interest.

#### Appendix A. Supporting information

Supporting data to this article can be found online at <https://doi.org/10.1016/j.apsb.2022.05.028>.

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