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COMMUNICATION

Rhamnose Modified Bovine Serum Albumin as Carrier Protein Promotes the Immune Response against sTn Antigen

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Rhamnose and sTn antigen were co-conjugated to bovine serum albumin (BSA), a weakly immunogenic carrier protein, for cancer vaccine development. The immune responses against sTn have been significantly augmented with the involvement of Rha-specific antibodies to enhance antigen uptake.

The aberrant glycans overexpressed on the cancer cell surface, known as tumor-associated carbohydrate antigens (TACAs), are attractive targets for the development of anti-cancer vaccines.[1-3] However, carbohydrate antigens are usually T cell- independent antigens which make them low immunogenicity and fail to elicit antibody affinity maturation and immunological memorization.[1, 2] To overcome this obstacle, the conventional strategy is to conjugate TACAs to a carrier protein that provides T cell-epitopes, allowing the TACAs to be taken up by antigen-presenting cells (APCs) and presented to T helper cells by MHCII complex together with the epitopes in the carrier.[4] Thus, the induced T cell help can prime an optimal B cell responses and the immunogenicity of carbohydrate antigens can be significantly enhanced.[2] For example, the sTn (Neu5Ac-α2,6-GalNAc-α-O-Ser/Thr) antigen has been conjugated with keyhole limpet hemocyanin (KLH) as a vaccine Theratope which induced predominantly humoral immune responses against breast cancer in a clinical trial.[5] Intensive studies based on MUC1 glycopeptide containing sTn and using tetanus toxoid or BSA as carriers were developed as well and showed promising clinical translational results.[6-8] Globo H has been coupled with KLH or diphtheria toxin mutant (CRM197) and exhibits efficacy in clinical trials, as well.[1]

Although the great success of TACAs based glycoconjugate vaccine has been achieved, it is often observed that a strong immune response against carrier protein, but not against the TACAs, is elicited. The large number of peptide epitopes presented in carrier proteins may compete with carbohydrate hapten for a limited number of helper T cells and B cells, leading to undesired activation or differentiation signals and factors that suppress the immune response against carbohydrate antigen. For example, in our previous study, we found that Globo H-KLH conjugate elicited a much higher KLH-specific antibody titer than that of Globo H-specific.[9] In addition, only a few carrier proteins are approved in clinical for vaccine industry and the same carriers are used in different vaccines which may cause vaccine interference and further lead to immune suppression against carbohydrate antigens.[10, 11] Therefore, new carriers for vaccine construct are needed.

To overcome this potential issue, novel strategies, employing immunostimulatory molecules, such as Pam3CSK4[12-14], MPLA[9], αGalCer[15, 16], and others, to conjugate with TACAs as fully synthetic vaccines have been developed and the immune responses against TACAs are improved. Other non-protein carriers, including dendrimers[17], polysaccharides[18], nanoparticles and virus-like particles[19], also have been explored to construct TACAs-based vaccines.

Endogenous antibodies are naturally pre-existing antibodies in human serum. Profiling studies have identified several abundant antibodies ranging from 1% to 3% in human blood, such as anti-galactose-α-(1,3)-galactose (α-Gal), anti-2,4-dinitrophenyl group (DNP) and anti-rhamnose (Rha) antibodies etc.[20] Recruiting these antibodies to cancer cells could trigger antibody-dependent cell-mediated cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) to destruct target cells.[21] Notably, it has been demonstrated that vaccine constructs containing one of the above mentioned haptens could form an *in situ* immune complexes *in vivo* and selectively deliver antigen to APCs by the binding between the endogenous antibody Fc portion and the Fc receptors on APCs, leading to effective internalization and enhanced cellular and humoral immune response.[20, 22, 23] For example, the studies initiated by Galili and

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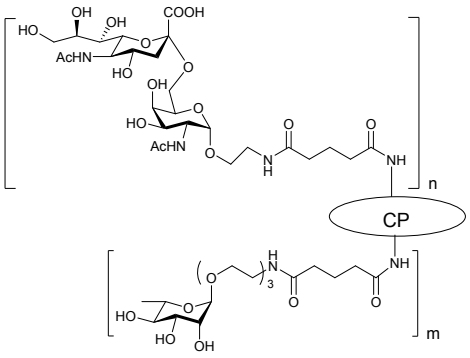
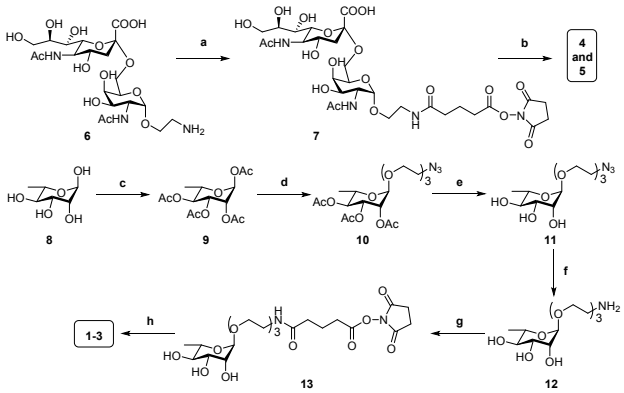


1 CP=BSA sTn-BSA-Rha-0.5 (sTn loading=7.8%, Rha loading=0.5%)
2 CP=BSA sTn-BSA-Rha-1 (sTn loading=7.8%, Rha loading=1%)
3 CP=BSA sTn-BSA-Rha-2.8 (sTn loading=7.8%, Rha loading=2.8%)
4 CP=BSA sTn-BSA (sTn loading=7.8%, Rha loading=0%)
5 CP=HSA sTn-HSA (sTn loading=8.3%, Rha loading=0%)

Figure 1. Design and structure of sTn-BSA-Rha vaccine construct.

colleagues demonstrated that α-Gal epitope could significantly augment the immunogenicity of tumor cells, HIV and influenza vaccine.[23] More recently, this strategy was utilized to improve the immunogenicity of drug molecule successfully, such as cocaine and fentany.[24, 25] Sucheck and co-workers proved that anchoring Rha hapten to a full synthetic anti-MUC1 cancer vaccine, either in covalent or liposome form, could dramatically enhance the immune responses in the presence of anti-Rha antibodies.[22]

In the present work, we explore a novel strategy using Rha-and sTn co-conjugated protein to construct TACAs-based vaccines by recruiting the endogenous antibodies to improve the immune efficacy. Instead of using conventional high immunogenicity carrier protein, low immunogenicity and non-toxic protein, BSA, is selected as a carrier protein. In this design, sTn, a promising TACA antigen in anti-cancer vaccine development, which is overexpressed in various carcinomas, such as breast, ovarian and colon cancer, is conjugated to BSA at first; then followed by conjugation with different loading of Rha to obtain sTn-BSA-Rha glycoconjugates for immunological studies. The purpose of



Scheme 1. (a) disuccinimidyl glutarate, DMF:PBS (4:1), rt; (b) BSA/HSA, PBS, rt; (c) Ac_2O, pyridine, rt; (d) azido-triethylene glycol, $BF_3{\cdot}Et_2O$, CH_2Cl_2, rt, 68%; (e) MeONa, MeOH; (f) 10% Pd/C, H_2, MeOH, rt; (g) disuccinimidyl glutarate, DMF:PBS (4:1), rt; (h) **4**, PBS, rt.

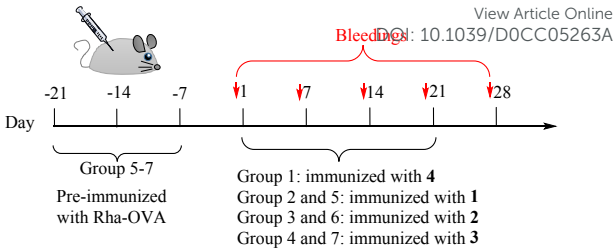


Figure 2. Immunization strategy.

this study is to augment the immunogenicity of sTn while reducing the potential adverse immune response caused by a carrier protein.

The study was initiated by the construction of sTn-BSA-Rha glycoconjugates (**1-5**) (**Figure 1**). The synthesis of sTn and Rha derivatives, as well as their conjugation with BSA, is described in **Scheme 1**. The sTn antigen **6** with an ethylamine at the reducing end was synthesized according to a reported procedure (Scheme S1 in SI).[26] The sTn antigens were then conjugated with protein BSA *via* the bifunctional glutaryl ester method which was a well-established method used in our previous projects and did not affect the immunological properties of conjugates.[9, 27] Compound **6** reacted with a large excess (15 eq.) of disuccinimidyl glutarate (DSG) in DMF to afford corresponding monoesters **7**, which was briefly purified by repeated precipitation to remove excess DSG. Then, **7** was mixed with BSA or HSA in PBS buffer to generate conjugate sTn-BSA **4** or sTn-HSA **5** that were purified by centrifugal filter devices (MW cut off: 10 kDa) to remove excess **7** and other small molecules. The loading of sTn antigen in conjugates **4** and **5** was determined by MALDI-TOF-MS (Table S1 and Figure S6 and S7), which was 7.8% and 8.3%, respectively, suggesting that the conjugating reactions were successful and the antigen loading levels were suitable for biological studies.

The Rha antigen was synthesized starting from L-rhamnose **8** (**Scheme 1**). Firstly, rhamnose **8** was peracetylated to give **9**, which were following by a glycosylation reaction with azido-triethylene glycol in the presence of $BF_3{\bullet}Et_2O$ to produce compound **10**. After removing the acetyl group in Zemplén transesterification condition and subsequent hydrogenation to reduce the azide group, **12** with an amine group at the reducing end was obtained in good yield, which was transformed to monoester **13** using DSG following the previous procedure. Finally, sTn-BSA conjugate **4** was incubated with different amounts of monoester **13** in PBS buffer (pH = 7.4), respectively, to generate sTn-BSA-Rha glycoconjugates **1**, **2** and **3**. The loading of Rha hapten in conjugate **1-3** was determined by MALDI-TOF-MS (Table S1 and Figure S8-S10 in SI), which were 0.5%, 1% and 2.8%, respectively. The low to high loading of Rha in conjugates sTn-BSA-Rha **1-3** with a fixed amount of sTn could help to reveal the capability of anti-Rha antibody-mediated vaccination against sTn.

To evaluate the immunological activities of our vaccine conjugates, we set seven groups of mice (groups 1-7) for these studies (**Figure 2**). Earlier studies have proved that laboratory

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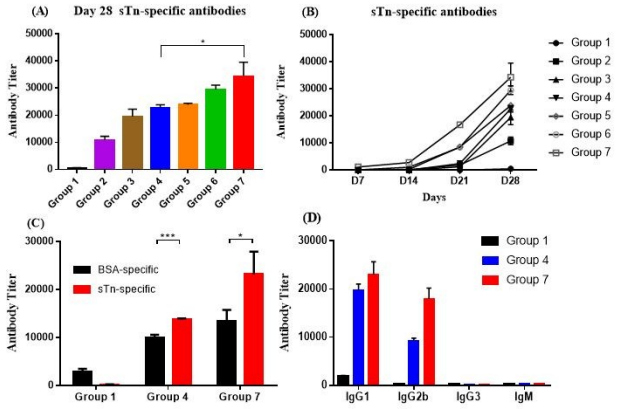

Figure 3 Immunological evaluation of the synthetic vaccine: (A) Antibody titers of different groups at Day 28. Conjugate **5** as coating antigens to detect sTn-specific antibodies. (B) The average titers of sTn-specific total antibodies in pooled antisera collected on day 0, 14, 21 and 28 from mice of group 1-7. (C) Evaluations of BSA-specific and sTn-specific antibodies in pooled antisera of group 1, 4, and 7. (D) Antibody isotypes and subtypes of the conjugate vaccine. The mean of antibody titers of three parallel experiments is shown for each sample, and the error bar shows the standard error of three replicate experiments. (* represents $p < 0.05$, ** represents $P< 0.01$, *** represents $P<0.005$.)

mice do not contain significant titer of naturally occurring anti-Rha antibodies.[28] Therefore, we pre-immunized mice in groups 5-7 with Rha-OVA to establish high levels of anti-Rha antibodies which were considered as endogenous antibodies. Figure S1 in the supporting information shows that after immunization with Rha-OVA, high titers of Rha-specific antibody (about 200000) were detected in the mice sera of groups 5-7. After that, all the groups of mice were immunized with sTn-BSA-Rha conjugate **1**-**4** according to **Figure 2** on day 1 and boosts on day 7, 14 and 21. Mouse blood samples were collected on day 7, 14, 21 and 28 after the first immunization. The antibodies were determined by enzyme-linked immunosorbent assays (ELISA). The antibody titer is set as the dilution factor at the optical density (OD) 415 nm reaches 0.2 from the regression analysis of the curves of the OD value against serum dilution number.

The ELISA results of seven groups on day 28 antisera were summarized in **Figure 3A**. As expected, group 1 immunized with sTn-BSA conjugate **4** without Rha produced the lowest sTn-specific antibody (about 534). For groups 2-4 immunized with sTn-BSA-Rha **1**-**3**, significant high titers of anti-sTn specific antibodies were elicited, which were 10809, 19491 and 22685, respectively. The elicited antibodies were improved approximately 20- to 42-fold compared with group 1. For groups 5-7 that was pre-immunized with Rha-OVA and then immunized with sTn-BSA-Rha **1**-**3**, notably higher anti-sTn specific antibodies were generated that were 23901, 29501 and 34306. Compared with group 1, the production of anti-sTn antibody was enhanced by 45, 55 and 64 fold, respectively. These results suggested that the endogenous anti-Rha antibodies pre-existing in mice serum could target delivery of glycoconjugate **1**-**3** to APC cells and result in more efficient transportation and internalization as well as the overall immune response. It was also interesting to observe that the anti-sTn titer was closely relevant to the loading level of Rha in glycoconjugate (group 2-4 and group 5-7). Conjugation of Rha hapten on the vaccine does not suppress the immunogenicity of sTn, whereas higher loading of Rha in vaccine generally lead to better uptake of the sTn antigen in the presence of anti-Rha antibodies and enable more anti-sTn specific antibody production.

We also analyzed the anti-sTn and anti-Rha specific immune response progress in groups 2-4 and 5-7. As presented in **Figure 3B** and **Figure S2-A**, mice in group 2-4 generated low titers of anti-sTn specific antibody on day 21 and relative higher anti-Rha antibody. However, both the anti-sTn and anti-Rha specific antibody was increased dramatically on day 28. This interesting finding indicated that sTn-BSA-Rha vaccine, especially for high loading Rha conjugate **3**, might have a self-adjuvanting effect at the late stage of immunization, which was attributed to anti-Rha antibody-mediated antigen uptake mechanism. While for group 5-7, high titers of anti-Rha antibody were maintained in the vaccination period (**Figure S2-B**). Consequently, a faster anti-sTn immune response was provoked with the help of endogenous anti-Rha antibodies (**Figure 3B**). For example, the elicited anti-sTn specific antibody on day 21 in group 5-7 was comparable to group 2-4 on day 28.

The elicited anti-BSA and anti-sTn specific antibodies in groups 1, 4 and 7 were analysed by ELISA. It was found that the produced anti-sTn-specific antibodies were significantly higher than that of BSA-specific in group 4 and 7 (**Figure 3C**). This result suggested that immune response against carrier protein was dramatically reduced because of the weak immunogenicity characters of BSA, which was different from other commonly used TACAs based vaccine design, where strong immunogenic carrier protein, such as KLH, was applied and anti-carrier protein immune response was dominated. Moreover, it is worth noting that with the help of endogenous Rha antibodies, the immune response of anti-sTn was enhanced without suppressing, even weak immunogenic carrier protein was used. This result inspired us that with hapten modification strategy, carrier proteins with low immune-stimulating abilities could be promisingly applied in carbohydrate-based vaccines in the future.

The antibody isotypes and subtypes are detected in groups 1, 4 and 7. IgG1 and IgG2b are the major subtypes in groups 4 and 7 which indicated that T cell immunity is successfully provoked by the Rha-modified BSA carrier protein (**Figure 3D**). In cancer immunotherapy, IgG1 usually mediated potent effector functions, such as ADCC and CDC, to destruct cancer cells. The preferred IgG1 was abundantly generated in this strategy, indicating that the vaccine may have a promising therapeutic effect.

To explore this possibility, the properties of antisera induced by these conjugates were further studies by a tumor cell binding assay detected by flow cytometry. The human breast cancer cell MCF-7 which has sTn expression was incubated with the pooled antisera collected from mice immunized with groups 1, 4 and 7, respectively. After the labelling by the fluorescent second antibody, the cell samples were measured by flow cytometry. **Figure 4A** revealed that the antisera induced by groups 4 and 7 could successfully recognize the sTn structure expressed on

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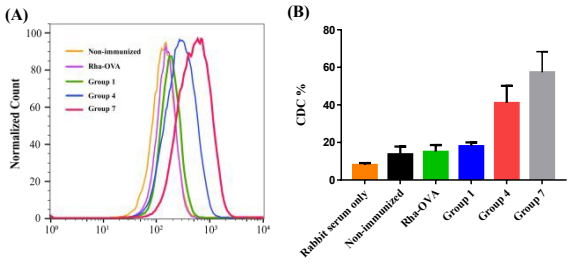

Figure 4 Immunological evaluation of the synthetic vaccine: (A) FACS analysis of the binding of antisera induced by groups 1, 4 and 7 to MCF-7 cells. (B) CDC activities of antisera from each group were measured using the CCK-8 assay. Error bars represent the SD of three parallel experiments.

MCF-7. Competitive FACS analysis by incubation group 7 sera with cancer cells in the presence of sTn antigen **6** verified that the surface sTn antigen on cancer cells is involved in the binding of anti-sTn antibodies to MCF-7 cells (SI, **Fig. S12**). We also examined the activities of antibody-mediated CDC. For this purpose, MCF-7 cells were first incubated with sera from groups 1, 4 and 7 at dilutions of 1/100 for 1 h, and then incubated with 1: 100 diluted rabbit serum (as recourses of complement) at 37℃ for 4 h. Thereafter, cell lysis was measured with a commercial CCK8 kit. As presented in **Figure 4B**, serum from groups 7 triggered the most potent CDC cytotoxicity to kill the cancer cells. This activity could be largely attributed to the fact that group 7 produced the highest anti-sTn antibodies.

In conclusion, we developed a new strategy for targeting vaccines to APCs based on endogenous antibodies. *In vivo* immunological studies demonstrated that in the presence of endogenous anti-Rha antibodies, sTn-BSA-Rha conjugates can form immune complexes by recruiting pre-generated anti-Rha antibodies to deliver the vaccine to APCs. The structure-activity study showed that the high loading of Rha hapten on the vaccine could mediate a better delivery to APCs, thereby triggering a stronger and faster immune response. This strategy provides a simple but efficient approach to augment the immunogenicity of carbohydrate antigen using weak and non-toxic carrier protein that otherwise is difficult to achieve. Considering the widely used carbohydrate antigen in vaccine research and industry, this strategy is potentially applicable to construct new glycoconjugates for vaccine development.

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Notes and references

1. S. J. Danishefsky, Y. K. Shue, M. N. Chang and C. H. Wong, *Acc. Chem. Res.*, 2015, **48**, 643-652.
2. S. F. Slovin, S. J. Keding and G. Ragupathi, *Immunol. Cell Biol.*, 2005, **83**, 418-428.
3. S. Lang and X. Huang, *Front. Chem.*, 2020, **8**, 284.
4. F. Y. Avci, X. Li, M. Tsuji and D. L. Kasper, *Nat. Med.*, 2011, **17**, 1602-1609.
5. L. A. Holmberg, *Abstr. Pap. Am. Chem. S*, 2006, **232**.
6. A. Kaiser, N. Gaidzik, U. Westerlind, D. Kowalczyk, A. Hobel, E. Schmitt and H. Kunz, *Angew. Chem. Int. Ed. Engl.*, 2009, **48**, 7551-7555
7. N. Gaidzik, A. Kaiser, D. Kowalczyk, U. Westerlind, B. Gerlitzki, H. P. Sinn, E. Schmitt and H. Kunz, *Angew. Chem. Int. Ed. Engl.*, 2011, **50**, 9977-9981.
8. N. Stergiou, M. Glaffig, H. Jonuleit, E. Schmitt and H. Kunz, *ChemMedChem*, 2017, **12**, 1424-1428.
9. Z. Zhou, G. Liao, S. S. Mandal, S. Suryawanshi and Z. Guo, *Chem. Sci.*, 2015, **6**, 7112-7121.
10. R. Dagan, J. Poolman and C. A. Siegrist, *Vaccine*, 2010, **28**, 5513-5523.
11. K. Pobre, M. Tashani, I. Ridda, H. Rashid, M. Wong and R. Booy, *Vaccine*, 2014, **32**, 1423-1430.
12. S. Ingale, M. A. Wolfert, J. Gaekwad, T. Buskas and G. J. Boons, *Nat. Chem. Biol.*, 2007, **3**, 663-667.
13. H. Cai, Z. Y. Sun, M. S. Chen, Y. F. Zhao, H. Kunz and Y. M. Li, *Angew. Chem. Int. Ed. Engl.*, 2014, **53**, 1699-1703.
14. M. Li, Z. Wang, B. Yan, X. Yin, Y. Zhao, F. Yu, M. Meng, Y. Liu and W. Zhao, *Medchemcomm.*, 2019, **10**, 2073-2077.
15. R. J. Anderson, C. W. Tang, N. J. Daniels, B. J. Compton, C. M. Hayman, K. A. Johnston, D. A. Knight, O. Gasser, H. C. Poyntz, P. M. Ferguson, D. S. Larsen, F. Ronchese, G. F. Painter and I. F. Hermans, *Nat. Chem. Biol.*, 2014, **10**, 943-949.
16. J. J. Du, C. W. Wang, W. B. Xu, L. Zhang, Y. K. Tang, S. H. Zhou, X. F. Gao, G. F. Yang and J. Guo, *iScience*, 2020, **23**, 100935.
17. M. Skwarczynski, M. Zaman, C. N. Urbani, I. C. Lin, Z. Jia, M. R. Batzloff, M. F. Good, M. J. Monteiro and I. Toth, *Angew. Chem. Int. Ed. Engl.*, 2010, **49**, 5742-5745.
18. M. Shi, K. A. Kleski, K. R. Trabbic, J. P. Bourgault and P. R. Andreana, *J. Am. Chem. Soc.*, 2016, **138**, 14264-14272.
19. X. Wu, Z. Yin, C. McKay, C. Pett, J. Yu, M. Schorlemer, T. Gohl, S. Sungsuwan, S. Ramadan, C. Baniel, A. Allmon, R. Das, U. Westerlind, M. G. Finn and X. Huang, *J. Am. Chem. Soc.*, 2018, **140**, 16596-16609.
20. R. T. Sheridan, J. Hudon, J. A. Hank, P. M. Sondel and L. L. Kiessling, *Chembiochem*, 2014, **15**, 1393-1398.
21. H. Hong, Z. Zhou, K. Zhou, S. Liu, Z. Guo and Z. Wu, *Chem. Sci.*, 2019, **10**, 9331-9338.
22. P. Karmakar, K. Lee, S. Sarkar, K. A. Wall and S. J. Sucheck, *Bioconjug. Chem.*, 2016, **27**, 110-120.
23. U. Galili, *Immunology*, 2013, **140**, 1-11
24. N. T. Jacob, K. Anraku, A. Kimishima, B. Zhou, K. C. Collins, J. W. Lockner, B. A. Ellis and K. D. Janda, *Chem. Commun. (Camb.)*, 2017, **53**, 8156-8159
25. J. X. Wang, B. Ellis, B. Zhou, L. M. Eubanks, S. Blake and K. D. Janda, *Chem. Commun. (Camb.)*, 2020, **56**, 6551-6554.
26. Z. Zhou, M. Mondal, G. Liao and Z. Guo, *Org. Biomol. Chem.*, 2014, **12**, 3238-3245.
27. H. Kunz, S. Birnbach, *Angew. Chern. Int. Ed. Engl.*, 1986, **25**, 360-362.
28. S. Sarkar, S. A. Lombardo, D. N. Herner, R. S. Talan, K. A. Wall and S. J. Sucheck, *J. Am. Chem. Soc.*, 2010, **132**, 17236-17246.