



Fluorine-modified sialyl-Tn-CRM197 vaccine elicits a robust immune response

Chengcheng Song^{1,2} · Xiu-Jing Zheng² · Haili Guo¹ · Yafei Cao² · Fan Zhang¹ · Qin Li² · Xin-Shan Ye² · Yifa Zhou¹

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Abstract

Even though a vaccine that targets tumor-associated carbohydrate antigens on epithelial carcinoma cells presents an attractive therapeutic approach, relatively poor immunogenicity limits its development. In this study, we investigated the immunological activity of a fluoro-substituted Sialyl-Tn (F-STn) analogue coupled to the non-toxic cross-reactive material of diphtheria toxin-197 (CRM197). Our results indicate that F-STn-CRM197 promotes a greater immunogenicity than non-fluorinated STn-CRM197. In the presence or absence of adjuvant, F-STn-CRM197 remarkably enhances both cellular and humoral immunity against STn by increasing antigen-specific lymphocyte proliferation and inducing a mixed Th1/Th2 response leading to production of IFN- γ and IL-4 cytokines, as well as STn-specific antibodies. Furthermore, antisera produced from F-STn-CRM197 immunization significantly recognizes STn-positive tumor cells and increases cancer cell lysis induced by antibody-dependent cell-mediated cytotoxicity (ADCC) or complement-dependent cytotoxicity (CDC) pathways. Our data suggest that this F-STn vaccine may be useful for cancer immunotherapy and possibly for prophylactic prevention of cancer.

Keywords TACAs · STn · Glycoconjugate vaccine · Cancer immunotherapy

Introduction

Abnormal glycosylation (*e.g.* sugar chain truncation and sequence alteration) is correlated with the development and progression of cancer [1]. These glyco-antigens are specifically and/or excessively expressed on the surface of tumor cells (known as tumor-associated carbohydrate antigens, TACAs) and as such are potential targets for the treatment and prevention of tumors [2]. However, TACAs are only weakly immunogenic, because they are expressed at specific developmental stages in normal tissues and their structures are similar to

normal antigens. This makes them more likely to be recognized as ‘self’ by the immune system and consequently tolerated immunologically [3]. Various efforts to overcome their weak immunogenicity have been made, for example with the use of: 1) adjuvants to modify and prolong an immune response [4], 2) immunogenic carriers, like proteins [5], zwitterionic polysaccharides [6], or lipopeptides [7] to induce a T cell-dependent immune response, and 3) clustered antigens to mimic the configuration of carbohydrate antigens on tumor cells to promote BCR cross-linking [8, 9]. Although progress has been made in this field, no TACA-based vaccine has so far been approved by the FDA. Thus, there remains a great need to improve the immunogenicity of carbohydrate antigens.

The concept of modifying carbohydrate antigen structures (MCAS) to improve vaccine immunogenicity was first proposed by Jennings in 1986 [10]. One of the main factors affecting the MCAS strategy is the affinity of the antibody for the structurally-modified carbohydrate antigen to that of the natural antigen. Therefore, it is necessary to control the extent of antigen modifications, so that the antigen has sufficient immunogenicity to break the tolerance and induce cross-reactive antibodies at the same time [11]. In recent years, the MCAS strategy has been applied to cancer prevention and treatment, as well as for antibacterial [12] and HIV-targeting

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✉ Xin-Shan Ye
xinshan@bjmu.edu.cn

✉ Yifa Zhou
zhouyf383@nenu.edu.cn

¹ School of Life Sciences, Northeast Normal University, Changchun 130024, China

² State Key Laboratory of Natural and Biomimetic Drugs, School of Pharmaceutical Sciences, Peking University, Beijing 100191, China

agents [13]. GD3- and GD2-lactone in the treatment of melanoma [14, 15] or N-propyl polysialic acid in the treatment of small cell lung cancer [16] had promising results in clinical trials. In preclinical studies, some structurally modified-TACAs, such as N-propionyl GM3 [17], N-fluoroacetyl Thomsen-nouveau (Tn) [18], N-acyl-modified Thomsen-Friedenreich (TF) [19], N₃-Globo H [20], N-propionyl STn [21], induced strong cross-reactive immune responses in animals, thereby enhancing the immunogenicity of the antigen and attenuating immune tolerance.

STn antigen is a sialylated disaccharide that is O-linked to a serine or threonine residue of mucins [Neu5Ac α 2-6GalNAc α -O-Ser/Thr]. This is one of the most common TACAs. The STn antigen has been widely used as an immunotherapeutic target for carcinomas expressing STn, such as colorectal, lung, ovarian, pancreas, prostate, breast cancer [22]. The natural STn antigen was conjugated to the carrier protein keyhole limpet hemocyanin (KLH) by Biomira, Inc., to construct the vaccine STn-KLH (Theratope®) for the prevention of colorectal and breast cancer metastasis [23]. However, results from Phase III clinical trials demonstrated that Theratope® failed to decrease disease progression time and increase overall survival [24]. Only modest clinical efficacy was achieved when patients were treated in conjugation with hormone therapy [25]. One possible reason for this could have been due to insufficient enhancement of STn immunogenicity. Therefore, researchers made numerous efforts to structurally modify the STn antigen to improve immunogenicity. Chun-Cheng Lin and coworkers modified the STn antigen by acyl-substitution of the 5-NHAc group of sialic acid. Their results showed that N-propionyl STn was the most immunogenic derivative, evoking two times more antibodies than STn conjugated to KLH [21]. Koichi Fukase and coworkers synthesized two fully synthetic N-acetyl and N-propionyl STn trimer (triSTn) vaccines. The N-propionyl triSTn vaccine induced anti-triSTn IgG antibodies that effectively recognized cancer cells expressing clustered STn [26]. Linhardt *et al.* synthesized several C-linked STn analogues [27, 28], but results of their immunological activity have not been reported. Our group systematically studied modifications of the STn antigen, by synthesizing more than 50 STn analogues, including S-glycoside-linked STn [29] and O-glycoside-linked STn [30]. Three O-linked fluorine-containing STn analogue glycoconjugates displayed excellent results with 3 to 5 times greater anti-STn antibody titers than those of natural STn-KLH [30].

In a previous study, we demonstrated that the glycoconjugate F-STn-KLH, in which the two N-acetyl groups of STn were substituted with N-fluoroacetyl groups, could effectively inhibit the growth of tumors and remarkably prolong the survival time of tumor-bearing mice compared with STn-KLH. The greater anti-tumor activity of the F-STn-KLH vaccine was reflected primarily in increased

cellular and humoral immune responses [31]. The carrier protein KLH is a large heterogeneous protein complex with high glycosylation. The complexity of the KLH protein leads to poor reproducibility in its preparation, and thus limits its clinical application [32]. Therefore, in order to facilitate the clinical usefulness of the structurally modified STn vaccine, we selected the protein CRM197 whose structure is relatively simple and widely used in clinical practice as a carrier, and studied its immunological activity of F-STn-CRM197 in combination with different adjuvants. The immunological properties of the glycoconjugate were assessed, including antigen-specific lymphocyte proliferation, IFN- γ /IL-4 cytokine secretion by spleen lymphocytes, and STn-specific antibody responses. Additionally, the ability of immunized F-STn-CRM197-produced antisera to recognize STn-positive tumor cells and to lyse tumor cells by the ADCC or CDC pathways was also assessed.

Materials and methods

Compounds and reagents

STn and fluorine-modified derivatives (F-STn with the two N-acetyl groups of STn substituted by two N-fluoroacetyl groups) were synthesized in our laboratory [30]. C34 adjuvant was synthesized in our laboratory according to the literature [33]. CRM197 was purchased from Sinovac Research & Development Co., Ltd. (China). Rabbit anti-STn IgG polyclonal antibody was prepared by our laboratory. FITC-conjugated goat anti-mouse IgG antibody was purchased from Jackson (America). Horseradish peroxidase-conjugated goat anti-mouse IgG (γ -chain specific), IgG1, IgG2a, IgG2b and IgG3 were purchased from Southern Biotechnology Associates, Inc. (America). Fetal bovine serum (FBS) and DMEM medium were purchased from Hyclone (America). IL-4 and IFN- γ Mouse ELISPOT Kits were purchased from Mabtech (Sweden). LDH Cytotoxicity Assay Kit was purchased from Promega (America). Freund's complete adjuvant (CFA) and Freund's incomplete adjuvant (IFA) were purchased from Sigma (America).

Cell lines and culture

Human colon carcinoma cells LS-C expressing STn antigen were kindly provided by Dr. Steven H. Itzkowitz. LS-C cells were cultured in DMEM medium containing 1% (v/v) streptomycin-penicillin and 10% (v/v) fetal bovine serum.

Glycoconjugates

The method of coupling carbohydrate to CRM197 or BSA (bovine serum albumin) was done by previously established

procedures with minor modifications [18]. The molar equivalent ratio of carbohydrate, CRM197 and NaBH₃CN was 4:2:3. Other reaction conditions were consistent with those reported in the literature [18]. The epitope ratios for STn-BSA and F-STn-BSA were determined by estimating protein content using the BCA assay [34], and sialic acid content was determined by using the resorcinol method [35]. Epitope ratios of STn-CRM197 and F-STn-CRM197 were determined by MALDI-TOF MS analysis.

Animal immunization

Female BALB/c mice (age 6–8 weeks, No. SCXKjing2012–0001, SPF/VAF, purchased from Peking University Health Science Center) were randomly divided into six groups ($n = 6$). On days 0, 14, 28 and 42, the mice were intramuscularly or subcutaneously immunized with STn-CRM197 or F-STn-CRM197 glycoconjugates (each containing 2 μ g of carbohydrate in PBS) in the presence or absence of adjuvants. On days 41 (13 days after the third vaccination) and 56 (14 days after the fourth vaccination), mice were bled via their tail veins, and blood was clotted to obtain sera for serological assay. On day 56, the mice were euthanized, and their spleens were harvested for analysis described below. A detailed immunization schedule is shown in Fig. 2a.

Lymphocyte proliferation assay

Two weeks after final immunization, the mice were euthanized and their spleens were harvested for the preparation of splenocytes following lysis of the red blood cells with 0.84% ammonium chloride. These splenocytes (5×10^5 cells/well) were cultured in RPMI-1640 complete medium containing STn-BSA (each well contained 0.2 μ g carbohydrate) at 37 °C and 5% CO₂ for 48 h. CCK-8 was added to each well, and the plates were further incubated for 3 h at 37 °C. The optical density was measured by using a Microplate Reader (Tecan) at 450 nm. Splenocytes from BALB/c mice stimulated with Concanavalin A were used as a positive control (data not shown). These experiments were repeated in triplicate.

ELISPOT assay

IFN- γ - and IL-4-producing splenocytes were detected by ELISPOT mouse IFN- γ and IL-4 kits according to procedures previously reported with minor modifications [18]. Splenocytes (5×10^5 cells/well) isolated from differently treated mice two weeks after the last vaccination were co-cultured with STn-BSA (each well contained 0.2 μ g carbohydrate). Splenocytes from BALB/c mice stimulated with Concanavalin A were used as a positive control (data not shown). These experiments were repeated in triplicate.

Serological assay

Sera were tested as described previously [30] for anti-STn and anti-modified-STn (F-STn) antibodies by ELISA using BSA-STn or F-STn-BSA. The antibody titer was defined as the highest dilution showing an absorbance of 0.1, after subtracting background.

Flow cytometry

Sera were examined by flow cytometry on STn-bearing tumor LS-C cells according to our previously established protocol [30]. Briefly, cells (5×10^5 cells/tube) were washed three times in PBS with 3% FBS and incubated with test sera (diluted 1/20) for 30 min on ice. Cells were washed three times to remove unbound antibodies and then incubated with FITC-linked goat anti-mouse IgG antibody (diluted 1/25) for 30 min on ice. Finally, cells were washed and suspended in PBS. Mean fluorescence intensity (MFI) and percent of positively stained cells were analyzed using a FACScan (Becton Dickinson).

ADCC assay

LS-C cells (5×10^3 cells/well) were seeded in U bottom 96-well plates as target cells and incubated with test serum (diluted 1/10 in RPMI-1640) at 37 °C for 2 h. Then, the cells were washed twice with PBS to remove unbound antibodies. Peritoneal macrophages were then added as effectors with an effector/target cell ratio of 10:1 and incubated at 37 °C for 18 h. Finally, cell supernatants were isolated and used to detect cell lysis by the LDH assay according to the manufacturer's protocol [31].

CDC assay

LS-C cells (1×10^4 cells/well) were seeded in U bottom 96-well plates and incubated with test sera (diluted 1/10 in RPMI-1640) at 37 °C. Following 2 h incubation, cells were washed twice with PBS to remove unbound antibodies, and rabbit complement serum (1:10 in RPMI-1640) was added and incubated at 37 °C for another 4 h. Cell supernatants were then isolated and used to detect cell lysis by the LDH assay according to the manufacturer's protocol [31].

Statistical analysis

Unpaired *t*-tests were used to analyze the lymphocyte proliferation, ELISPOT and antibody titers. One-way ANOVA analyses were performed to evaluate the data of ADCC assay and CDC assay experiments. $P < 0.05$ is considered as statistically significant. We used SPSS 13.0 for statistical analysis and GraphPad Prism 5 for graphics.

Results

Glycoconjugate synthesis and immunization

To prepare the glycoconjugates (Fig. 1), allyl alcohol-modified STn and F-STn were synthesized as previously reported [30]. The carbon-carbon double bond of the anomeric *O*-allyl group was then oxidized to form the aldehyde intermediate for coupling (reductive amination) with carrier proteins CRM197 and BSA. Carbohydrate loading levels for the glycoconjugates were calculated from molecular weights of glycoconjugates and carrier proteins determined by MALDI-TOF MS (Supplementary Table S1, Fig. S1-S3).

To evaluate effects from different adjuvants on the immune response, we used C34 and Freund's adjuvant, administered via intramuscular and subcutaneous injection, respectively. Adjuvant-free glycoconjugates were used as negative controls. The detailed immunization schedule is shown in Fig. 2a. The cytotoxicity of F-STn-CRM197 and STn-CRM197 on lymphocytes showed that no significant toxicity was observed relative to the control group when the concentration of the glycoconjugate was 10 $\mu\text{g/mL}$ and 50 $\mu\text{g/mL}$ (Supplementary, Fig. S4).

Evaluation of the T cell response to glycoconjugates in mice

To evaluate the cellular immune response from glycoconjugates, we first examined the proliferation of spleen lymphocytes, the number of IFN- γ -producing splenocytes, and IL-4-producing splenocytes following the fourth immunization.

Splenocytes from each mouse were stimulated *in vitro* with the corresponding glycoconjugates, and the magnitude of the proliferative response was expressed as the optical density. The ranking of the lymphocyte proliferation response from strong to weak was Freund's adjuvant, C34 adjuvant, and no adjuvant. In the presence or absence of adjuvant, mice immunized with F-STn-CRM197 showed a higher lymphocyte proliferative response than those immunized with STn-CRM197. The difference between the two glycoconjugates was significant in the Freund's adjuvant-assisted group (Fig. 2b). These data suggest that F-STn-CRM197 conjugates induce a greater

lymphocyte proliferative response to antigen specificity than STn-CRM197, an effect that should benefit tumor immunotherapy.

IFN- γ is a hallmark cytokine of Th1 cells. Antigen-specific IFN- γ -producing T cells were examined by using an enzyme-linked immunosorbent spot (ELISPOT) assay. As shown in Fig. 2c, there was a significant increase in the number of IFN- γ -releasing splenocytes after F-STn-CRM197/FA immunization compared with that of mice vaccinated with STn-CRM197/FA. However the number of IFN- γ -releasing splenocytes following immunization with F-STn-CRM197 was not significantly different from that of mice immunized with STn-CRM197 and assisted with C34 or without adjuvant. IL-4 is a hallmark cytokine of Th2 cells. As shown in Fig. 2d, there was a significant increase in the quantity of IL-4-releasing splenocytes following F-STn-CRM197/FA immunization compared to mice vaccinated with STn-CRM197/FA. The IL-4-producing frequency of splenocytes in the mice that were treated only with F-STn-CRM197, increased slightly without significant differences compared with STn-CRM197. In the presence of C34, the number of spleen lymphocytes secreting IL-4 after immunization with STn-CRM197 was higher than those immunized with F-STn-CRM197. The ranking in the number of lymphocytes secreting cytokines from high to low was Freund's adjuvant, C34 adjuvant, and no adjuvant. These results showed that in the presence of Freund's adjuvant, immunization with F-STn-CRM197 could establish a strong Th1/Th2 response that is critical for effective cancer immunotherapy.

Antibody response to glycoconjugates

To assess antibody production in mice following the third and fourth immunizations, pooled antisera from each group were first screened by ELISA (enzyme-linked immunosorbent assay). STn-BSA and F-STn-BSA were used as coating antigens for detecting anti-STn antibody titers and anti-modified-STn antibody titers (Table 1). Immunization with F-STn-CRM197 (in the presence or absence of adjuvant) provoked a strong STn-specific immune response and elicited higher titers of anti-STn IgG antibodies than STn-CRM197. The IgG from F-STn-CRM197-, F-STn-CRM197/C34- and F-STn-CRM197/FA-immunized mice showed a strong binding to

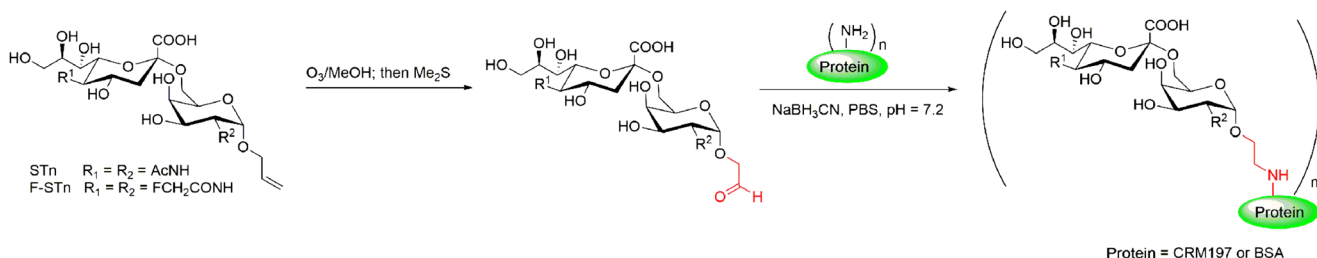


Fig. 1 Synthesis of glycoconjugates. Reagents and conditions: 1) O_3 , MeOH, -72°C ; 2) PBS, pH = 7.2, NaCNBH_3 , CRM197 or BSA, RT

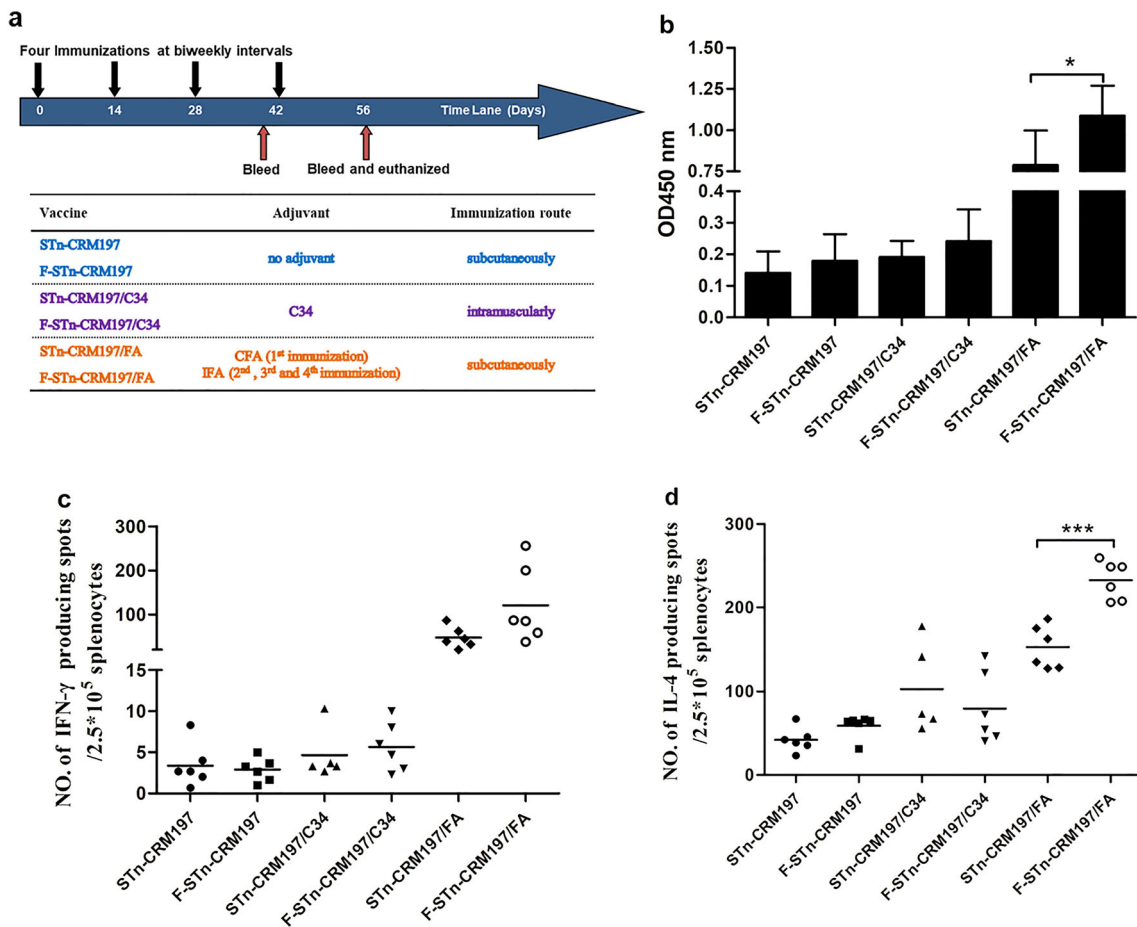


Fig. 2 Effect of glycoconjugates on the spleen lymphocytes from immunized mice. **a** Immunization schedule. Two weeks after the fourth immunized, animals were euthanized and splenocytes were separated from each mouse for immunological evaluation. **b** Lymphocyte Proliferation assays: 5×10^5 splenocytes derived from each mouse and were cultured with STn-BSA (0.2 μ g of carbohydrate/well). **c-d**

ELISPOT assays of the number of IFN- γ -releasing splenocytes (**c**) and IL-4-releasing splenocytes (**d**) among 2.5×10^5 splenocytes derived from each mouse. Results are presented as median values for groups of 5–6 mice. The statistical analysis was performed by the unpaired t-tests. * $P < 0.05$ and *** $P < 0.001$

STn, with Freund’s adjuvant being the most effective at inducing a high IgG response. For the IgM response, no anti-STn antibody was detected in sera at a dilution of 1:100. For tumor immunotherapy, the IgG response is more desirable than IgM due to immunological memory and affinity maturation [36]. Lastly, we examined the anti-STn IgG titer from

individual mice. The anti-STn IgG level for F-STn-CRM197 was obviously increased compared with STn-CRM197, when Freund’s or C34 adjuvant was used (Fig. 3a-b, Supplementary Table S2-S3). In the absence of adjuvant, the IgG level for F-STn-CRM197 was increased compared with STn-CRM197, but the difference was not significant. In addition, F-STn-

Table 1 Immunological results after vaccination with synthetic glycoconjugates

Vaccine	ELISA titer anti-STn				ELISA titer anti-F-STn	
	After Third		After Fourth		After Third	After Fourth
	IgG	IgM	IgG	IgM	IgG	IgG
STn-CRM197	377	<100	8540	<100	–	–
F-STn-CRM197	25,472	<100	152,523	<100	98,012	229,272
STn-CRM197/C34	316	<100	3299	<100	–	–
F-STn-CRM197/C34	164,764	<100	557,897	<100	236,506	1,251,092
STn-CRM197/FA	357,479	<100	582,095	<100	–	–
F-STn-CRM197/FA	1,323,659	<100	1,845,925	<100	1,346,824	2,610,534

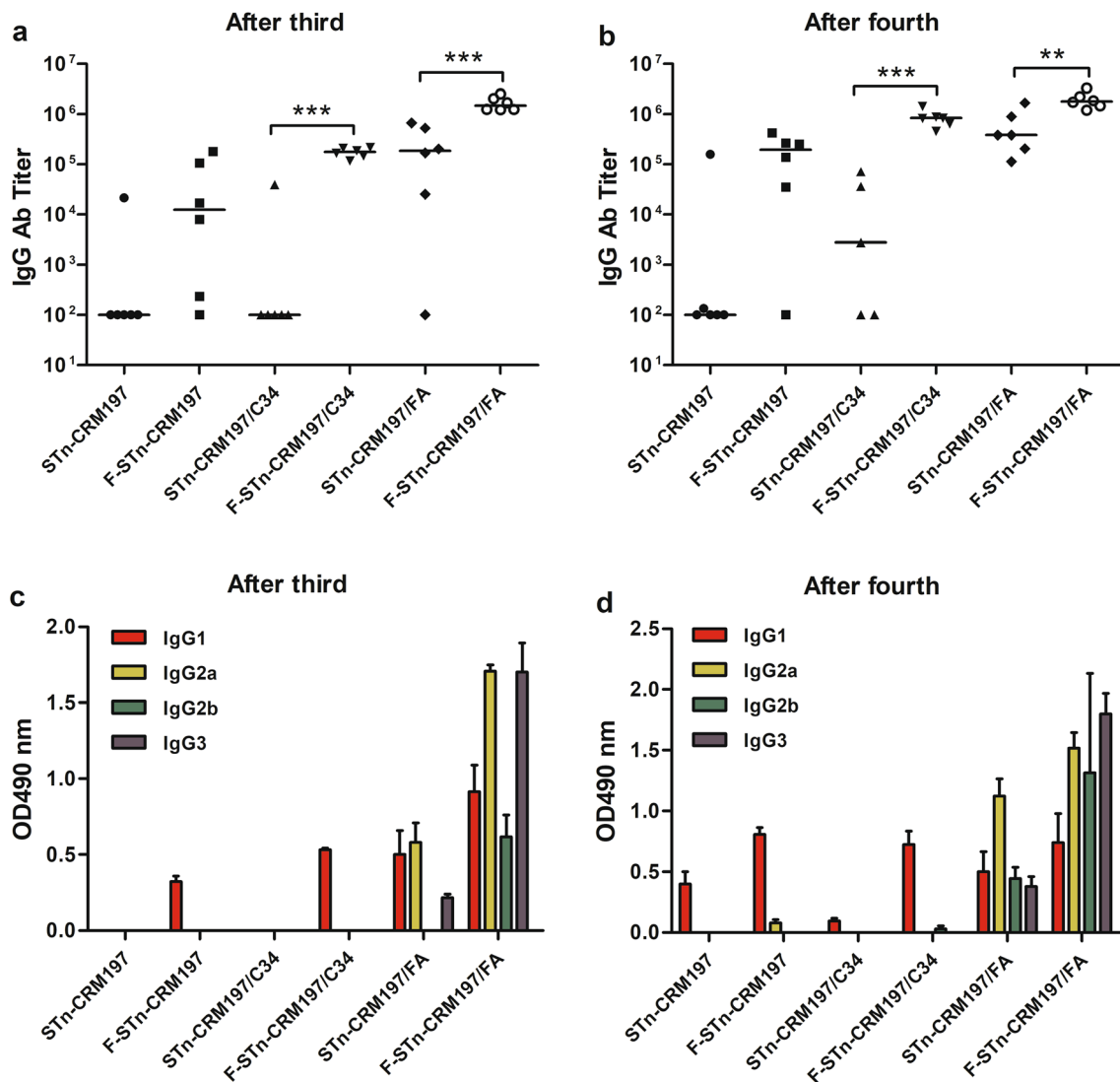


Fig. 3 Effect of glycoconjugates on STn-specific antibody production of IgG and IgG isotypes in mouse serum. IgG antibody titer against STn-BSA of individual mouse after the 3rd (a) and the 4th (b) vaccination. Each dot represents the ELISA result of an individual mouse, and each black line represents the median antibody level of a group of six mice.

IgG subtypes were tested by ELISA with a 1:1000 dilution of pooled sera. The pooled sera obtained from 13 days after the 3rd vaccination (c) and obtained from 14 days after the 4th vaccination (d). OD490 nm = optical density at 490 nm. The statistical analysis was performed by the unpaired t-tests. ** $P < 0.01$ and *** $P < 0.001$

CRM197 produced a high anti-F-STn antibody titer, relating to improved immunogenicity from the modified-STn. The strong cross-recognition efficiency results in the increase of anti-STn IgG antibodies.

IgG2a and IgG1 antibodies are usually associated with Th1 and Th2 responses, respectively. In the presence of Freund's adjuvant, pooled antisera of our immunized mice revealed that F-STn-CRM197 produced a much stronger Th1 response than STn-CRM197 (Fig. 3c-d). IgG2a from F-STn-CRM197-, F-STn-CRM197/C34-immunized mice showed a weaker binding to STn, and no binding of IgG2a to STn from STn-CRM197-, STn-CRM197/C34-immunized mice was detected in pooled sera at a dilution of 1:1000. Immunization with F-STn-CRM197/FA caused a stronger IgG3 antibody response

than that of STn-CRM197/FA, which is typical for an anti-carbohydrate response. These results indicate that the modified-STn glycoconjugate can enhance immunogenicity and promote a Th1 type response.

Recognition and killing of tumor cells by antibodies from vaccinated mice

To evaluate the immunotherapeutic potential of antibodies from immunized mice, we used flow cytometry to analyze their ability to specifically recognize tumor cells. Human colon carcinoma LS-C cells expressing STn were used as test cells, and pre-immune sera from mice were used as a negative control. These results are shown in Fig. 4 and in

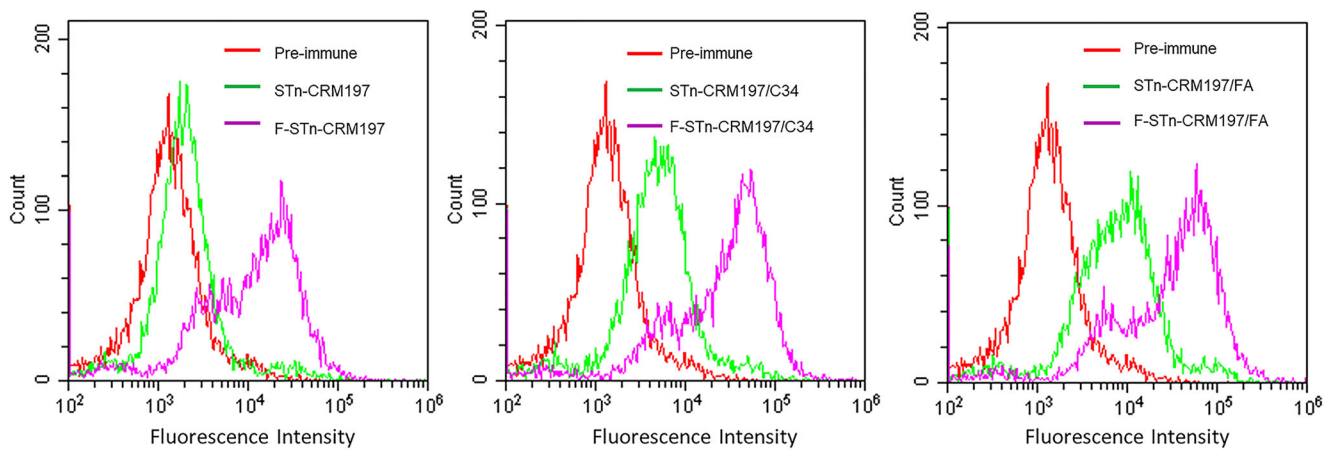


Fig. 4 Serological IgG analysis results on LS-C cells after the fourth immunization with vaccine by flow cytometry. The results are representative of at least two independent experiments

Supplementary Table S4. No background reactivity was observed in the negative control group. In the absence of adjuvant (compared with pre-immune sera), antisera elicited by F-STn-CRM197 reacted strongly with LS-C cells, but the antisera elicited by STn-CRM197 reacted weakly with LS-C cells. This indicated that F-STn-CRM197 effectively improved immunogenicity. In the presence of adjuvant, antisera elicited by F-STn-CRM197 and STn-CRM197 reacted strongly with LS-C cells, and post-immunization antisera from F-STn-CRM197 showed an increase in reactivity with LS-C cells compared to that following immunization with STn-CRM197. Overall, antisera produced in the presence or absence of adjuvant in F-STn-CRM197 immunized mice significantly recognized STn-positive tumor cells compared with antisera produced by STn-CRM197. This indicates that the structurally-modified STn vaccine improves antigen immunogenicity, a finding that is highly relevant for tumor immunotherapy of carbohydrate vaccines.

To investigate the function of antibodies elicited by mice after immunization, we evaluated their capacity to lyse tumor cells expressing STn via CDC and ADCC pathways. Antisera obtained from pre-immune mice were used as a negative control. Antisera elicited by F-STn-CRM197 and STn-CRM197 significantly increased cancer cell lysis by ADCC and CDC

pathways compared to pre-immune sera. In the presence or absence of C34 adjuvant, antisera generated by immunization with F-STn-CRM197 increased cancer cell lysis by the ADCC pathway compared with that elicited by STn-CRM197 (Fig. 5a). In the presence of C34 or Freund's adjuvant, antibodies elicited by F-STn-CRM197 were more effective in lysing cells against STn-expressing cancer cells by the CDC pathway compared with STn-CRM197 (Fig. 5b). This highlights the importance of antigen modification for relevant antigenic responses.

Discussion

The CRM197 protein is an analog of diphtheria toxin that has reduced toxicity while maintaining immunostimulatory properties as the parent toxin. As a carrier protein, CRM197 is ideal for vaccine conjugation due to the absence of toxicity [37], immunogenicity, non-interference with carbohydrate antigens [38], *in vivo* safety, and absence of complex glycoprotein issues. CRM197 is currently used as a carrier protein for clinically licensed antibacterial conjugate vaccines, such as *Meningococcus*, *Pneumococci*, *Haemophilus influenza* and *Neisseria meningitides* [5, 39]. CRM197 is also often used

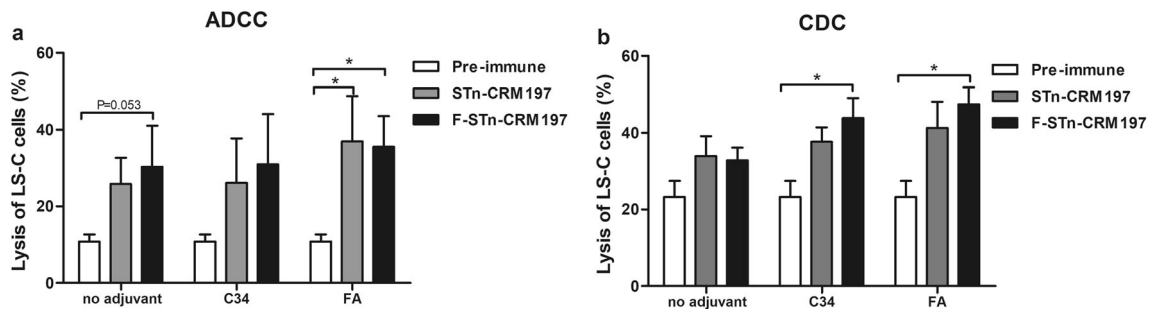


Fig. 5 The antisera elicited by glycoconjugates can kill cancer cells expressing the native STn antigen by ADCC (a) and CDC (b). The results represent the mean \pm standard error of measurement (SEM) of at

least three experiments. The statistical analysis was performed by the one-way ANOVA. * $P < 0.05$

as a carrier protein for anti-tumor conjugate vaccines in the preclinical setting. It has been reported that tumor-associated carbohydrate antigens Tn [18], TF [19], and Globo-H [20], have been coupled to CRM197 as antitumor vaccines for some epithelial tumors like colon and ovarian cancer. In this study, we used CRM197 as a carrier protein to investigate the immunological activity of both structurally-modified STn glycoconjugates F-STn-CRM197 and STn-CRM197 in the presence and absence of adjuvant. We showed that F-STn-CRM197 has a higher immunogenicity compared to STn-CRM197 with or without adjuvant. Noticeably, F-STn-CRM197 exhibited an even better immunoregulatory activity compared to STn-CRM197 on antigen-specific lymphocyte proliferation, cytokine secretion, and antibody response. Moreover, mouse antisera produced by immunization with F-STn-CRM197 significantly recognized STn-positive tumor cells and increased cancer cell lysis by ADCC and CDC pathways, indicating that it has excellent potential for anti-tumor immunotherapy.

To investigate the immunogenicity of the F-STn-CRM197 vaccine, mice were immunized with F-STn-CRM197 without adjuvant. The experiments for the lymphocyte proliferative response [40] and number of IFN- γ producing splenocytes [41] were used to evaluate the antigen specific cellular immune response. Here, we found that mice immunized with F-STn-CRM197 produced a slight cellular immunity but no significant difference compared with STn-CRM197. The Th2 immune response is important for anticancer immunotherapy [42], and antibodies against TACAs can eliminate circulating tumor cells and micrometastases [43]. In the present investigation, we found that the number of IL-4-releasing splenocytes after immunization with F-STn-CRM197 was slightly increased compared with STn-CRM197. Animals immunized with F-STn-CRM197 elicited greater anti-STn and anti-modified-STn IgG antibody titers than that of STn-CRM197, reflecting strong immunogenicity and powerful cross-recognition efficiency of the antibodies induced by F-STn-CRM197. Mice immunized with F-STn-CRM197 produced IgG2a following the fourth immunization, which is usually associated with Th1 responses. The therapeutic potential and functional studies of antibodies elicited by mice after immunization indicated that antisera obtained by immunization with F-STn-CRM197 had increased reactivity against LS-C cells compared with antisera after immunization with STn-CRM197. This antisera promoted increased lysis of STn positive tumor cells by the ADCC pathway, which may be related to higher levels of IgG and IgG1 antibodies produced upon immunization with F-STn-CRM197. Our results indicate that the F-STn-CRM197 vaccine can fundamentally improve the immune response and thus has the potential for use in tumor immunotherapy.

Adjuvants are widely used in the immunotherapy of various diseases, because they can improve the antigen-specific

immune response and determine the immunophenotype [44]. C34, an analogue of alpha-galactosyl ceramide (α -GalCer), can induce Th1-biased immunity, exhibiting a great anticancer effect in mice [45]. Freund's adjuvant (an oil-in-water (w/o) emulsion that induces mixed Th1 and Th2 cell responses) is widely used for immunization in animal models [44]. Therefore, we compared the effectiveness of the two immunological adjuvants at augmenting the immune response against these glycoconjugate vaccines. First of all, we found that using C34 as an adjuvant could help the glycoconjugate vaccines produce higher levels of cellular and humoral immune responses. The C34 adjuvant increases the level of cellular immune response of glycoconjugate vaccines in relation to its ability to induce Th1-biased immunity response. C34 adjuvant increases the level of antibody response to glycoconjugate vaccines, probably because C34 activates invariant natural killer T cells (iNKT) by binding to its surface TCR receptor. Activated iNKT cells can promote B cell secretion of antigen-specific antibodies in a non-cognate fashion [46, 47]. Comparing the immune effects of vaccines F-STn-CRM197 and STn-CRM197 in the presence of C34 adjuvant, we found that mice immunized with F-STn-CRM197 provoked a slightly higher cellular immune response and a significantly higher humoral immune response than mice immunized with STn-CRM197. Next, comparing the immune-assisted effects of both Freund's and C34 adjuvant, we found that Freund's adjuvant could help the glycoconjugate vaccines produce higher levels of cellular and humoral immune responses, including the level of antigen-specific lymphocyte proliferation, the number of lymphocytes secreting IFN- γ and IL-4 cytokines and the level of STn-specific antibodies. Freund's adjuvant enhances the cellular immune response of the glycoconjugate vaccine primarily depending on the heat-killed mycobacteria in the CFA, which enhances the Th1-type response through multiple pathways. In the absence of mycobacteria (also known as IFA adjuvant), T lymphocyte differentiation tends to assume a Th2 profile with strong antibody production only [48]. Comparing the immune effects of vaccines F-STn-CRM197 and STn-CRM197 in the presence of Freund's adjuvant, we found that mice immunized with F-STn-CRM197 provoked significantly higher cellular and humoral immune responses than mice immunized with STn-CRM197. Our results indicate that the F-STn vaccine combined with adjuvant can improve the immune response.

The incorporation of fluorine into STn could make the immune systems recognize the analogue as non-self, while the degree of modification is sufficient to permit vaccine-induced antibodies to cross-react with the native STn and thus breaking immune tolerance and improving the immune response. It has been reported that structural modification of tumor-associated antigens can improve the stability of the MHC-peptide-TCR complex [49], and fluorine could influence the reactivity of the adjacent

glycosidic bond [50]. It was because of this that we hypothesized that fluorination of STn may improve the immune response by increasing the stability of the MHC-peptide-TCR complex. However, the precise mechanism needs to be further investigated on the molecular and cellular levels.

In summary, we first covalently coupled STn to CRM197 and F-STn to CRM197, respectively, to study the immune effects of the two vaccines in the presence and absence of adjuvants. From our data, we conclude that: i) the vaccine F-STn-CRM197 can increase cellular and humoral immune responses to varying degrees compared with STn-CRM197 in the presence or absence of adjuvant, and ii) Freund's adjuvant-assisted vaccine response was better than that from the C34 adjuvant. Our research lays the foundation for the development and clinical application of carbohydrate-based anticancer vaccines.

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Compliance with ethical standards

Conflict of interest Xiu-Jing Zheng and Xin-Shan Ye have an ownership interest in the patent (China patent No. ZL 2010 10,202,388.5, Japan patent No. 5815687, Int. Appl. No. PCT/CN2011/000610). No potential conflicts of interest were disclosed by the other authors.

Ethical approval All procedures performed in studies involving animals were in accordance with the ethical standards of the Animal Ethical Experimentation Committee of Peking University (Beijing, China).

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