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Sialyl-Tn Polysaccharide A1 as an Entirely Carbohydrate Immunogen: Synthesis and Immunological Evaluation

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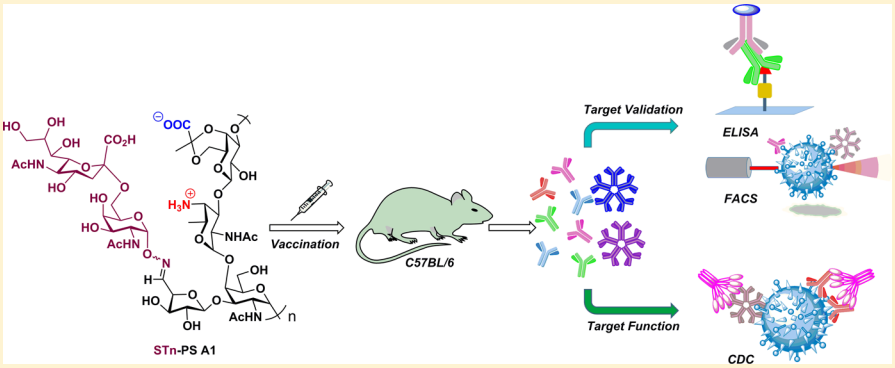
Supporting Information



ABSTRACT: Sialyl Thomsen-nouveau (STn) is a tumor-associated carbohydrate antigen (TACA) that is overexpressed in a variety of carcinomas such as breast, ovarian, and colon cancer. In normal tissue, STn is not detectable, which is critical for opportunities in developing cancer immunotherapies. A novel, entirely carbohydrate, semisynthetic STn-polysaccharide (PS) A1 conjugate was prepared and evaluated in C57BL/6 mice. STn-PS A1 was combined with commercially available monophosphoryl lipid A-based adjuvant, and after immunization, ELISA indicated a strong immune response for inducing anti-STn IgM/IgG antibodies. The specificity of these antibodies was concomitantly investigated using FACS analysis, and the results indicated excellent cell surface binding events to STn-expressing cancer cell lines MCF-7 and OVCAR-5. An INF-γ ELISpot assay was conducted to further confirm a robust cellular immunity invoked by STn-PS A1. Most importantly, the raised antibodies conferred complement-dependent cellular cytotoxicity against MCF-7 and OVCAR-5 cells.

INTRODUCTION

Aberrant glycosylation, affiliated with certain proteins and glycosyltransferases, is observed in the carcinogenesis of cells, which leads to truncated patterns of oligosaccharides on cancer cell surfaces. These "abnormal" oligosaccharides can serve as biomarkers to distinguish tumor cells from normal healthy cells and are known as tumor-associated carbohydrate antigens (TACAs).[1] The unique biological features of TACAs provides an opportunity for exploiting the immune system in the development of anti-TACA vaccines for cancer immunotherapy.[2,3] On the basis of the general theory of vaccination, if exogenous TACA conjugates can be processed and presented to effector cells of adaptive immunity, then an immune response can be stimulated to generate corresponding antibodies *and* immune memory.

One of the major hurdles in materializing this theory is the immunological nature of carbohydrate epitopes. It is well-known that TACAs cannot elicit strong T cell-dependent immune responses on their own and have failed to induce class switching that can lead to high affinity IgG antibodies and memory B cells.[4] For these deficiencies to be overcome, the introduction of immunological "carriers" is necessary.[5] A number of studies have indicated that antigen "carriers" play a vital role in cancer vaccine development;[6−9] applied "carriers" are immunogenic proteins, such as keyhole limpet hemocyanin (KLH),[10] diphtheria toxin (CRM197),[11] and tetanus toxoid (TT),[12] to name only a few. These protein-based vaccines have been widely studied since Coley attempted to eradicate erysipelas.[13] Although there has been promise with glycoprotein conjugates, two major drawbacks hinder further success in cancer therapy: (1) the immunogenicity of protein carriers may overwhelm that of TACAs, leading to an "epitope suppression" effect,[14] and (2) non-site specific coupling may cause heterogeneities and ambiguities of chemical composition.[15]

The Sialyl-Tn (STn) antigen (Neu5Acα2-6GalNAcα-*O*-Ser/Thr) is an *O*-linked mucin TACA that is overexpressed in human carcinomas and negligible in fetal and adult tissues.[16] In cancer cells, the biosynthesis of STn is catalyzed by sialyltransferase ST6GalNAc I, which outcompetes other *O*-glycan elongating glycosyltransferases and promotes the

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generation of truncated sialylated *O*-glycans on cancer cell surfaces.[17] Detection of STn is associated with various types of cancers, such as breast and ovarian, and high levels of STn correlate with a poor prognosis for patients. Therefore, STn is a relevant target for tumor immunotherapy.[18] In the past few decades, many synthetic chemists, immunologists, and vaccinologists alike have been dedicated to the development of effective cancer vaccines that target STn or STn-related mucins. Among all attempts, THERATOPE (STn-KLH) in clinical trials I and II, exhibited efficacy in inducing T cell-dependent responses, generating IgG antibodies in breast and ovarian cancer patients. However, in Phase III, THERATOPE failed to demonstrate improved overall survival.[19] Although deemed as a defeat, both preclinical and clinical studies of THERATOPE provided valuable information and insights for the development of new anti-STn vaccines; we believe activation of T cell-dependent immunity for the production of high affinity anti-STn IgGs is paramount.

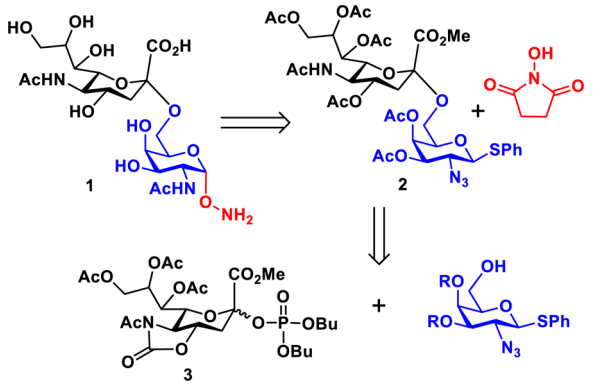
In this context, our group has a continuing effort aimed at bypassing the noted immune challenges with protein−carbohydrate cancer vaccines.[7] In the course of seeking nonprotein "carriers" for cancer vaccine design and development, we have demonstrated the potential of utilizing zwitterionic polysaccharide (ZPS) PS A1 as a "carrier" for a Thomsen-nouveau (Tn)-PS A1 entirely carbohydrate immunogen. This construct invoked a T cell-dependent immune response capable of binding the Tn antigen and less concern of possible epitope suppression to other carbohydrate antigens.[7] Biological evaluation from our previous studies has driven our pursuit of more synthetically challenged STn for conjugation, leading to semisynthetic STn-PS A1 and more detailed immunological studies of STn-PS A1 as an entirely carbohydrate vaccine construct in combatting breast and ovarian tumors.

RESULTS AND DISCUSSION

Synthesis of Aminooxy STn and α-Selective Sialylation. Challenges in the chemical syntheses of sialyl-containing oligosaccharides are stereoselective sialylation and rate enhancement. To improve reactivity and selectivity of α-sialylation,[20−24] attempts have focused on the development of activating groups at the anomeric position,[25−31] installation of auxiliary groups at C-1[32] and C-3,[33,34] incorporation of strong electron-withdrawing groups on the nitrogen atom at C-5,[35−37] and the use of stereodirecting nitrile solvents.[38,39] Recent progress in utilizing a 4*N* 5*O* *trans*-fused oxazolidinone moiety at the C-4 and C-5 positions has led to excellent α selectivity[40−43] because of the strong electron-withdrawing nature and *trans* orientation of the oxazolidinone group. These effects created a dipolar moment that greatly diminished the anomeric effect, which subsequently led to a new equilibrium favoring the formation of α-sialyl glycosides.[44,45] Phosphate esters have been used as anomeric-leaving groups in many glycosylation reactions,[30,46] and benefits include augmented reactivity as well as facile activation, especially when compared to the widely applied thiol-leaving group. The combination of oxazolidinones and phosphates in sialylation reactions leads to highly α-selective and highly reactive sialyl donors, which have been proven to be an optimized solution for *O*-, *S*-, and *C*-sialylation.[47−50]

On the basis of the information presented above, our strategy for the synthesis of α-aminooxy STn (**1**) is shown in Scheme 1 and includes a key stereochemical transformation that is highly

Scheme 1. Retrosynthetic Analysis of Aminooxy STn Antigen



α-selective between sialyl donor **3** and a suitably protected 2-azido-galactose acceptor. The resulting disaccharide can undergo a simple protecting group manipulation that can readily yield compound **2**. Introduction of the *N*-hydroxysuccinimide at the reducing end of **2** allows for the desired aminooxy disaccharide. The sialyl carboxylic methyl ester can be easily and selectively removed prior to the removal of the *N*-succinimidyl and acetyl groups, which results in our desired compound **1**.

On the basis of this glycosylation strategy, our early stage synthetic efforts focused on investigation of the optimized acceptors for sialylation as shown in Table 1. We designed and tested three thiol-galactopyranoside acceptors, **4**, **6**, and **8**, that included free hydroxyls at the 4 and 6 positions. The reactions led to excellent yields and quantitative α-selectivity as observed in ^{1}H NMR analysis of the unpurified disaccharide.

As noted in entries 1 and 2 in Table 1, the α-/β-acceptors led to good yields and α-selectivity of compounds **8** and **9**. In entry 3, an interesting result was observed. After 30 min, TLC analysis indicated complete consumption of the sialyl donor and formation of two products. We speculated that this was not an α/β-mixture but rather a product of partial deprotection of the acid-sensitive *p*-methoxybenzyl (PMB) protecting group at the 3 position.[51] Instead of quenching the reaction, we elected to raise the temperature to 0 °C and pursue complete in situ deprotection of the PMB group. After 45 min, quantitative removal of the PMB group was noted from TLC. Full characterization and analysis of isolated product **9** proved that our initial speculations were correct, and future work on sialyl glycoside synthesis will proceed in a manner that allows for multiple reaction processes to occur in a single pot. In entry 4, 2,3-protected allyl-D-galactopyranoside **7** was tested, and we again observed only α-glycosidic bond formation in 86% yield.

As shown in Scheme 2, the synthesis of α-aminooxy STn commenced from α-selective sialylation. Compound **4** was then used as an acceptor for the subsequent glycosylation reaction with sialyl phosphate donor **3**. The reaction, which proceeded smoothly in the presence of TMSOTf in DCM at −45 °C, resulted in exclusive α-configured disaccharide **5** in excellent yield. The oxazolidinone-protecting group and acetyl groups were removed using the Zemplén method, and the free hydroxyls were protected using acetic anhydride in pyridine and DMAP to afford dissaccharide **2**. The thiol-donor sugar **2** was then activated using the NIS/TfOH reagent system[52] followed by addition of *N*-hydroxysuccinimide to obtain our key intermediate **13**. Compound **13** was obtained with exclusive

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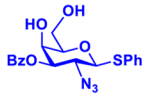
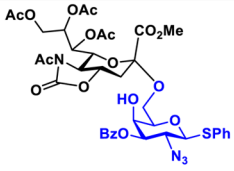
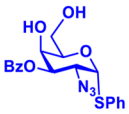
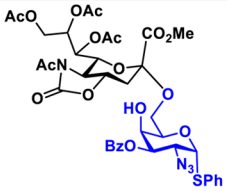
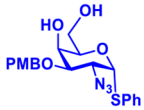
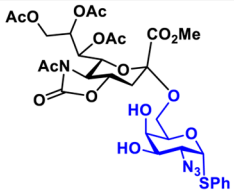
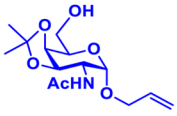
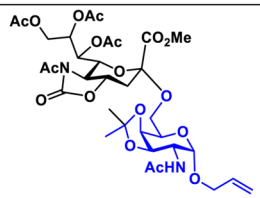
Table 1. Results of Sialylation Using Different D-Galactopyranose Acceptors and Compound 3 as the Donor[a]

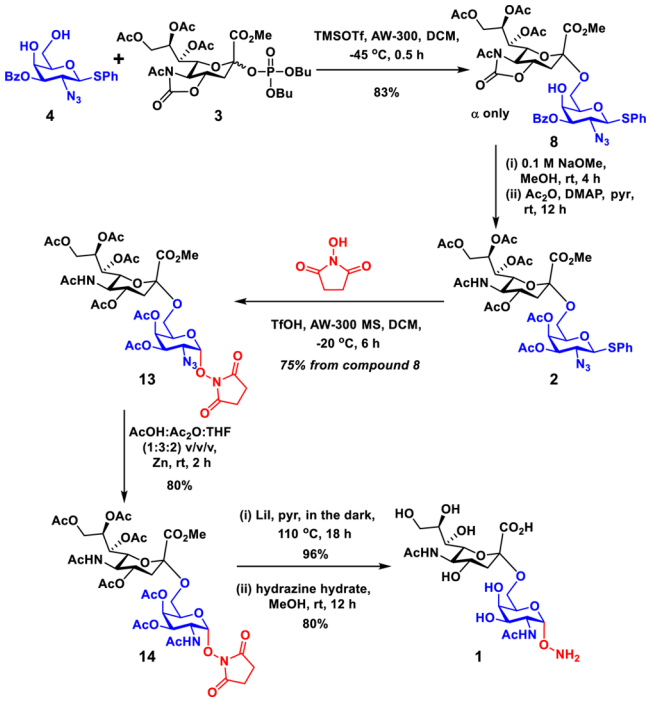
entry	acceptor	product: yield[b] (α/β[c])
1	 4	 **8** - 83% (α only)
2	 5	 **9** - 81% (α only)
3	 6	 **10** - 78%[d] (α only)
4	 7	 **11** - 86% (α only)

[a]Typical conditions: 1.2 equiv of donor **3**, 1.3 equiv of TMSOTf, dry DCM, and −45 °C for 30 min. [b]Isolated yield. [c]Determined by ^{1}H NMR spectroscopic analysis of the unpurified reaction mixture. [d]Reaction mixture stirred at −45 °C for 30 min, gradually warmed to 0 °C, and finally stirred for another 45 min to obtain the product.

α-selectivity and in 75% isolated yield from **8**. Utilizing a nonparticipating azido group at the C-2 position of D-galactose was crucial for α-selectivity.[53] Compound **14** was then afforded by a facile transformation that commenced from the concomitant reduction and acetylation of the 2-azido group using zinc powder and acetic anhydride under acidic conditions. This one-pot reduction/protection reaction was followed by a chemoselective Krapcho demethylation of the sialyl methyl ester by treating **14** with lithium iodide and pyridine under refluxing conditions.[54] Finally, global deprotection of the sugar oxysuccinimide was carried out using hydrazine hydrate yielding aminooxy STn (**1**) as the desired final product.

It is important to note that the deprotection of the oxazolidinone (**8** → **13**) was performed before installation of the oxysuccinimide group. The purpose for this sequence was to avoid any possible conflicts between the oxysuccinimide and oxazolidinone in later-stage deprotection steps (see Supporting Information (SI) S7).[55] For selective removal of the oxazolidinone to be achieved, the use of sodium methoxide was required.[41,42] However, because the oxysuccinimide group is base label, we were cautious of the embedded imide bond, which is known to immediately cleave and convert to an amide

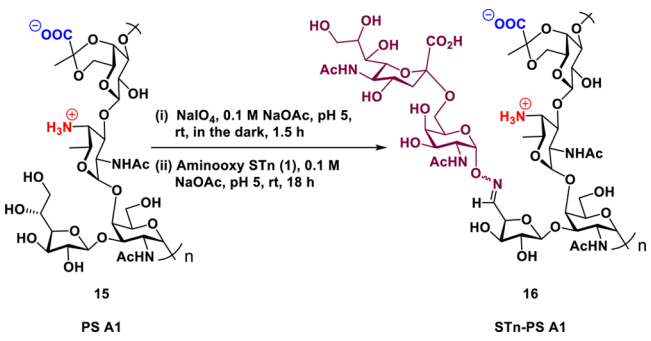
Scheme 2. Synthesis of α-Aminooxy STn Antigen (1)



plus a methyl ester under conditions of sodium methoxide.[56] Moreover, removal of the amide bond can be very challenging in such circumstances because strong acidity and heat are required,[57] which can compromise the stereointegrity of the disaccharide itself. Furthermore, the Krapcho demethylation of sialyl methyl ester should be conducted prior to that of oxysuccinimide installation. There are two predominant reasons for this sequence of reaction conditions: (1) the nucleophilic hydrazine can attack the sialyl methyl ester and convert it to the carboxamide[58] and (2) the Krapcho reaction is highly specific to methyl esters. Therefore, the succinimide group will stay intact.[36,54] For future studies, this strategy will be adapted to other carbohydrate syntheses, especially for those containing both sialyl and aminooxy moieties.

Aminooxy STn Links to PS A1 via Oxime. As shown in Scheme 3, aldehyde groups were introduced to PS A1 (**15**) by selectively oxidizing the terminal vicinal diols of the embedded D-galactofuranose residues with sodium periodate.[14,47] Although there is a *trans*-diol present on D-galactofuranose, it is much less labile to periodate oxidation,[59] thus, only vicinal dial oxidation was observed. Aldehyde-functionalized PS A1

Scheme 3. Preparation of STn-PS A1 (16) Immunogen from PS A1 (15) and Aminooxy STn Antigen (1)



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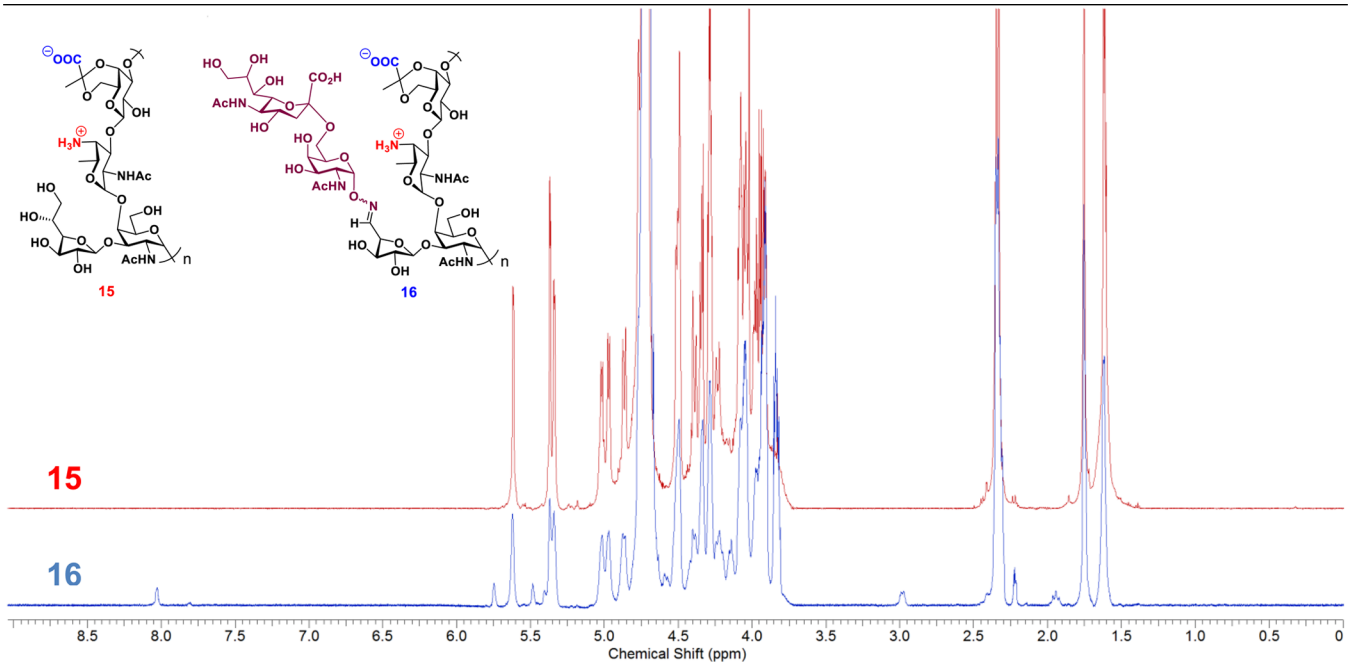

Figure 1. Comparison of ^{1}H NMR of PS A1 (**15**) and STn-PS A1 (**16**).

was then conjugated with aminooxy STn (**1**) under slightly acidic conditions, giving rise to the STn-PS A1 construct (**16**). The structure of STn-PS A1 (**16**) was confirmed with 1D and 2D NMR analyses. As a means to improve the resolution of spectra, all of the NMR experiments were performed at 60 °C as shown in Figure 1 and then compared to naturally occurring PS A1.[60,61] The peak at 8.02 ppm indicates the formation of an oxime bond, and anomeric protons on oxidized D-galactofuranose moieties appear at 5.48 ppm. COSY and 1D TOCSY experiments further confirmed the selectivity of periodate oxidation on the vicinal diol as well as the structure of the oxime-bearing-D-galactofuranose spin system. In Figure 1, characteristic signals of the STn antigen were also identified, as the anomeric proton of the D-GalNAc sugar was observed at 5.74 ppm. The equatorial and axial protons at C-3 of sialic acid were located at 2.99 and 1.96 ppm, respectively. With the assistance of COSY and 1D TOCSY, D-GalNAc and the D-Neu5Ac spin systems of conjugated STn were delineated, and their structural features were noted for highly resembling that of monomeric aminooxy STn (see SI). Finally, the loading of STn to oxidized PS A1 was determined using two methods: (1) ^{1}H NMR integration that allowed us to determine the loading at 11% and (2) use of the Svennerholm method,[62] through which we determined the loading to be at 10%.

Immunological Studies: Antibody Response(s) Against the STn Antigen. Previous clinical studies of THERATOPE have indicated that the utilization of natural STn-expressing mucins for serological assays could lead to more clinically relevant data compared to that of synthetic STn glycoprotein conjugates.[63−66] It is well-known that the linkers used in synthetic conjugates will exhibit a certain level of influence on antibody−antigen recognition events. Both ovine submaxillary mucin (OSM) and bovine submaxillary mucin (BSM) predominantly contain STn moieties[18,67] and have become the preferred choices for serological assays. For the specificity of the antibody induced by STn-PS A1 (**16**) to be determined, sera from Jax C57BL/6 mice were collected and tested on BSM as shown in Figure 2. Sera obtained from mice immunized with STn-PS A1 plus Sigma adjuvant system (SAS) showed prominent binding events against BSM, whereas sera from a group of mice injected with STn-PS A1 plus TiterMax Gold (TMG) adjuvant produced moderate binding events of antibodies against BSM. The group of mice that was treated

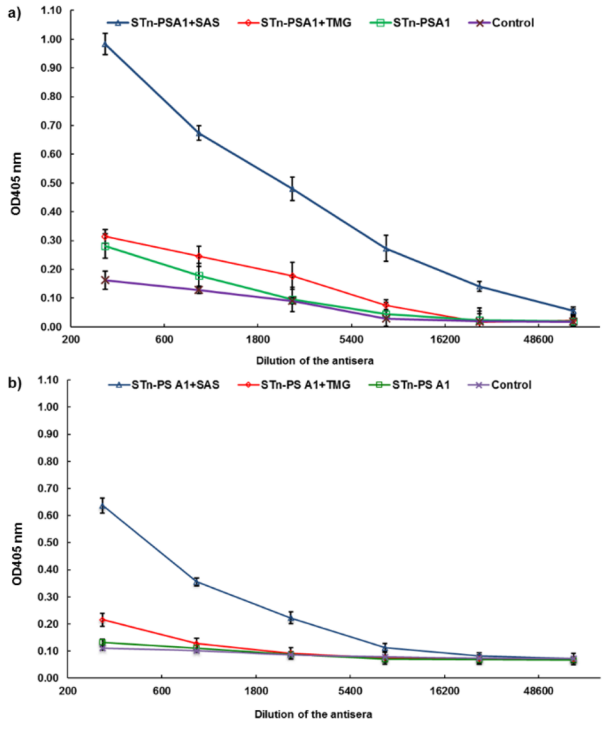

Figure 2. ELISA analysis of antisera induced by STn-PS A1 + SAS, STn-PS A1 + TMG, and STn-PS A1 against BSM: (a) group average IgG and (b) group average IgM. Control sera obtained from nonimmunized mice. The error bars represent standard deviation (SD) of two triplicate tests.

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with only the STn-PS A1 (**16**) construct gave a moderate response to the natural STn antigen. Under these conditions, we observed negligible IgG and IgM binding toward BSM.

On the basis of the IgG and IgM ELISA results, the benefit of utilizing suitable adjuvants becomes obvious. First, "adjuvant effects" can be beneficial for antigen−antibody binding events.[68] The antibody titers of both adjuvanting groups, SAS and TMG, are multiple folds greater as observed in Figure 2. Furthermore, the choice of adjuvant can affect the outcome of antibody production. Previous studies have confirmed that monophosphoryl lipid A (MPL), which is the major component of SAS, preserved most of the immunostimulatory activity of lipid A with a significant decrease in toxicity.[69,70] MPL is an agonist for TLR-4, which can increase the cellular immune response and is recommended in many types of mice immunizations.[71−73] TiterMax Gold (TMG), known as a "depot" adjuvant, is less toxic compared to SAS; however, several studies have reported that use of TMG can lead to inferior antibody production compare to MPL-containing vaccines.[67,68] This is most likely a direct result of TMGs ability to protect the antigen from both dilution and rapid degradation and elimination by the host rather than target a specific receptor. Although covalently incorporating specific receptor-based adjuvants directly on vaccine constructs has been done before,[74] the field has yet to completely adopt this strategy due to the lack of a clear understanding. Although adjuvants remain an essential component in numerous ongoing studies, the released clinical and preclinical data of THERATOPE indicated an immunological benefit when MPL adjuvant was used.[19,75,76]

The safety of KLH protein has been proven in THERATOPE Phase I−III trials, however, KLH is a very potent carrier protein. A very plausible concern when utilizing STn-KLH vaccine is epitope suppression, which is a result of overwhelming carrier-specific T cell responses over that of the target antigens. Increased exposure of STn-KLH may lead to increased antibody responses to KLH and diminished responses to conjugated STn antigens.[77,78] For properly evaluating the immunity of the STn-PS A1 conjugate, it is necessary to determine the carrier response, especially anti-PS A1 antibody levels after immunization. On the basis of the primary ELISA analysis (Figure 2), we choose the STn-PS A1 + SAS sera to investigate carrier response by using an ELISA plate coating construct of PS A1-poly-L-lysine (PS A1-PLL) (see SI). As shown in Figure 3, both anti-PS A1 IgGs and IgMs were detected on PS A1-PLL coated plates, and the response levels were relatively stronger than those of anti-STn IgGs and IgMs. A stronger immune response of PS A1 should be expected because the dosage of PS A1 content (18 μg) in each injection is 9× greater than that of STn moieties (2 μg); thus, the dose ratio is 9:1. However, both IgG and IgM antibody ratios of anti-PS A1/anti-STn are smaller than the dose ratio, particularly for IgG. The anti-PS A1/anti-STn equals 2.3:1 (IgM ratio is 8.2:1, see SI). The IgG ratio was a very positive signal, indicating that there was a relatively balanced T cell response between PS A1 and STn. Thus, PS A1 is very unlikely to cause epitope suppression in this case. In contrast, the IgG ratio of KLH/STn obtained from the official THERATOPE Phase III report is greater than 60:1.[79]

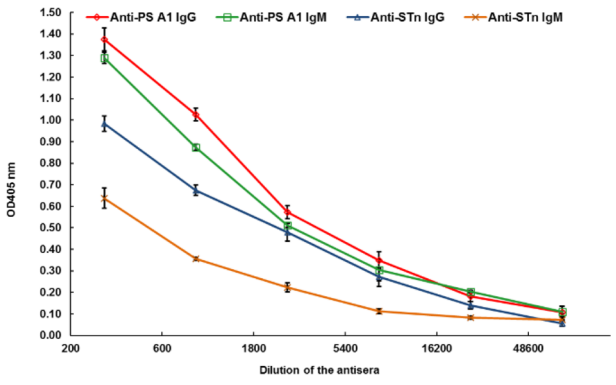

Figure 3. ELISA analysis of anti-PS A1 antibody induced by STn-PS A1 + SAS; anti-STn response was determined using BSM coating, and anti-PS A1 response was determined using PS A1-PPL coating. The error bars represent standard deviation (SD) of two triplicate tests.

Analysis of IgG Subclasses. IgGs are high affinity and long-term antibodies that target many pathogens. Their subclasses exhibit slightly different immunological functions but remain essential for complement recruitment. The subclasses of IgG induced by STn-PS A1 (**16**) vaccine were analyzed by a serological assay with BSM coating the ELISA plate (Figure 4). In the group of mice immunized with STn-PS A1 + SAS, a substantial amount of IgG2b against BSM was observed, followed by a moderate level of IgG1, and finally, a low level of IgG3 was observed when the anti STn-PS A1 sera was used. In the mouse study of STn-PS A1 + TMG, a moderate level of IgG2b was detected, and relatively low IgG1 and IgG3 binding events were noted. In the group of mice that were injected with only STn-PS A1 (**16**), negligible binding of IgG1, IgG2b, and IgG3 were detected. It is important to note that we did not test IgG2a activity due to the absence of the corresponding gene in C57BL/6 mice.[80] These data provide us with directionality to further understand the immunological contributions of STn-PS A1 conjugate.

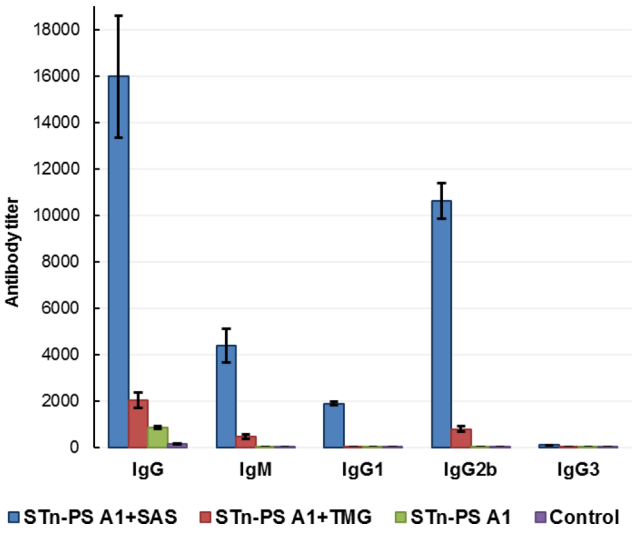

Figure 4. Determination of isotypes and subclasses of antibodies induced by STn-PS A1 + SAS, STn-PS A1 + TMG, and STn-PS A1. The error bars represent the standard deviation (SD) of two triplicate tests.

The high IgG2b/IgG1 ratio in both STn-PS A1 + SAS and STn-PS A1 + TMG groups is a strong indication that a Th1-type dominated immune response was being activated.[81] Furthermore, the enhanced IgG2b production in the STn-PS A1 SAS murine group can be attributed to MPL as an additive adjuvant. The recognition of MPL by TLR4 on antigen

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presenting cells is a key event in the activation of those cells and initiation of adaptive immunity.[82] MPL is known as a Th1-favored adjuvant and therefore can promote a Th1 response that leads to an increase in IgG2 subclass production. Released clinical trial data of THERATOPE has also provided evidence that the STn-KLH + Detox-B adjuvant (MPL as the major adjuvant component) developed a Th1 immune response toward the STn epitope in patients.[66] Because STn-PS A1 is an entirely carbohydrate construct void of proteins, peptides, or lipids, the ELISA data fit into the expected immunological profile of STn-PS A1. Consequently, it is very possible that these IgG2b antibodies are specifically targeting the disaccharide moiety (D-Neu5Acα2-6-D-GalNAcα) on BSM.

Antibody Binding to Cancer Cell Surfaces. Utilization of fluorescence-activated cell sorting (FACS) is a useful method when studying the immunological potential of STn-PS A1 as a vaccine designed to target the STn antigen on human tumor cells. On the basis of the serological assay and IgG subclass analyses as noted above, antisera induced by STn-PS A1 + SAS was chosen for a cell surface antigen binding experiment on several cancer cell lines. Cancer cells treated with anti-PS A1 serum were used as serological controls, and cancer cells treated with only secondary FITC-labeled anti-IgG or Alexa Fluor 647 labeled anti-IgM antibody were used as antibody isotype controls. The flow cytometry results are described in Figure 5.

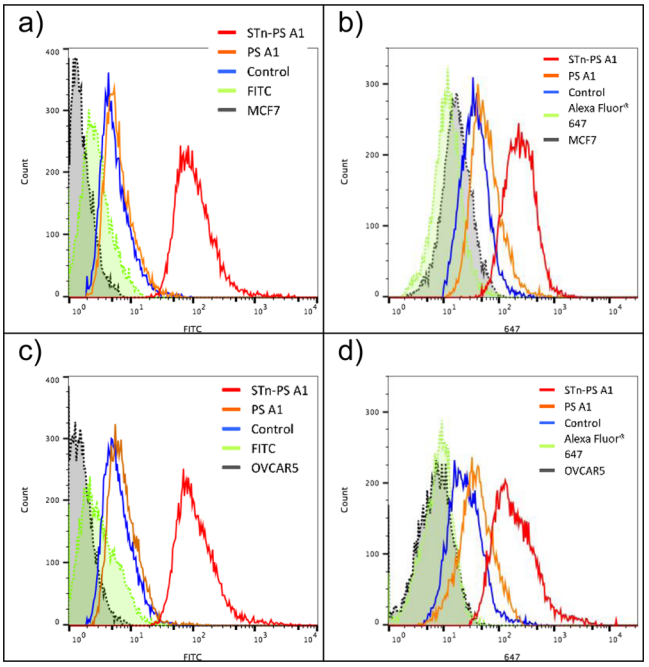

Figure 5. FACS analysis of IgG tumor cell binding for (a) MCF-7 and (c) OVCAR-5 cells. IgM tumor cell binding for (b) MCF-7 and (d) OVACR-5 cells.

We elected to utilize human breast cancer cell line MCF-7,[83] and ovarian cancer cell line OVCAR-5[66,84−86] because they have been proven to be STn positive cell lines. The antisera (immunizations using STn-PS A1 + SAS) clearly exhibited antibody binding against surface STn antigens as noted by flow cytometry (Figure 5a−d). The STn positive cell lines showed strong surface binding events with both IgG and IgM antibodies. The best results were observed in the IgG binding tests; the percentage of positive cells for MCF-7 was 71% with enhanced mean fluorescent intensity (MFI: 155; Figure 5a), and for OVCAR-5, the positive percentage was 61% (MFI: 100; Figure 5c). IgM antibodies exhibited relatively mild binding with 38% positive cells using the MCF-7 cell line (MFI: 286; Figure 5b) and 44% with OVCAR-5 cells (MFI: 340; Figure 5d). In contrast, antisera obtained from the control mice showed only negligible IgM or IgG binding to the STn-positive cancer cell lines (Figure 5b and d). Anti-PS A1 sera was used as a substance control to determine any possible "epitope suppression" effects of the PS A1 "carrier" to STn antigens and, as expected, only very low/negligible binding events were detected.

Antibody-Mediated Complement-Dependent Cytotoxicity (CDC). On the basis of our preliminary conclusions drawn from the FACS assay, that both IgM and IgG antibodies can be raised against the STn-PS A1 + SAS formulation and are very specific in targeting STn-positive cancer cells, we turned our attention to CDC studies. Antibody-mediated cytolysis studies are necessary to determine the potential therapeutic value of vaccine candidates, and in our case, the STn-PS A1 construct was in need of examination. One of the effector killing mechanisms is through complement-dependent cytotoxicity (CDC) of certain classes/subclasses of antibodies, which leads to compromised tumor cell membrane integrity.[87−89] The ELISA and FACS assays gave us positive data regarding target validation but did not provide us with an understanding of antibody function as a direct correlation to antibodies raised from STn-PS A1 + SAS immunizations. First, the anti-STn-PS A1 serum contains a moderate amount of anti-STn IgM, which can be particularly effective in CDC due to the pentameric nature of IgMs. Second, there was a substantial amount of IgG2b observed in our ELISA data, which, on the basis of a number of studies, has been demonstrated to be highly potent in activating CDC compared to that of other IgG subclasses.[90]

The results of the CDC employing MCF-7 and OVCAR-5 STn positive cell lines are summarized in Figure 6. Normal human mammary cell line MCF-10A was used as the control cell line due to the fact that it does not possess surface antigen

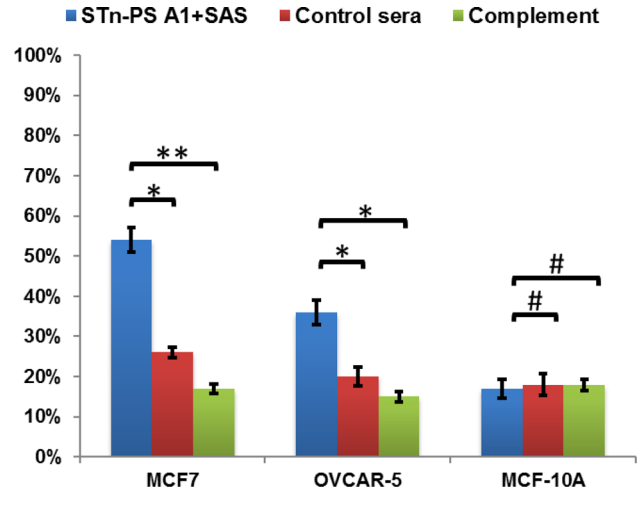

Figure 6. Antibodies raised against STn-PS A1 + SAS mediate complement-dependent cytotoxicity (CDC) to kill STn-containing tumor cells. Cytotoxicity was determined using the commercially available LDH assay. Data shown are mean values of two parallel triplicate tests where $*P < 0.01$ and $**P < 0.001$ were obtained using a Student's t test and where # is $P > 0.5$. The error bars represent the standard deviation (SD) of two triplicate tests.

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STn. The percent of lysed cells was determined using a lactate dehydrogenase (LDH) assay (Roche Applied Science) without further optimization. The substance control was settled by treating cancer cells exclusively with rabbit complement. The antisera-mediated cell lysis rate for MCF-7 was determined to be 54%, and for OVCAR-5 was determined to be 36%. In comparison with the CDC of antisera collected from the control PS A1 group and substance control, the antisera of the STn-PS A1 + SAS group was capable of inducing significant cytotoxicity toward MCF-7 and OVCAR-5 cancer cells. There was no statistically relevant cytotoxicity observed in the MCF-10A cells due to the absence of STn antigen as noted above.

Cellular Immunity Induced by STn-PS A1. Because our results draw to the conclusion that STn-PS A1 can induce a Th1-dominant immune response, which is critical to enhance cellular immunity against tumor cells,[91] it would be of great importance to access antigen-specific T cell activities brought forth by STn-PS A1. To accomplish this, splenocytes were collected from mice immunized with STn-PS A1 + SAS pulsed with stimuli containing the STn moiety (STn-PS A1, BSM) and finally incubated on INF-γ ELISpot plates. None of the stimuli was added to the control wells. The results of ELISpot are shown in Figure 7, whereby STn-PS A1 was noted to be

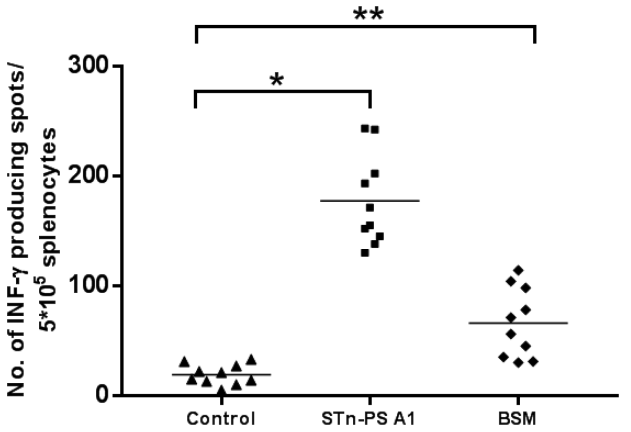

Figure 7. IFN-γ ELISpot splenocyte assay determining the number of IFN-γ-secreting splenocytes among 5×10^5 cells. Each point represents an individual mouse in which the data was generated in duplicate and, the horizontal bars represent the mean value of the group of tests where $^*P < 0.001$ and $^{**}P < 0.05$ were obtained using a Student's t test.

capable of eliciting significant INF-γ production compared to that of the control. T cells that secrete INF-γ play key roles in cellular immunity because INF-γ can upregulate MHC-I expression levels, which lead to cytotoxicity toward tumor cells and increased antibody-dependent cytotoxicity.[92] In our experiment, BSM was used as a natural source of STn because it can induce moderate levels of INF-γ production. It is worth noting that the chemical structure of STn residues on BSM highly resemble those found on cancer mucins. These results support our conclusion of a Th1-dominant cellular immune response based on the fact that the INF-γ ELISPOT data indicate that STn-PS A1 can induce antigen-specific cellular immune responses.[74]

SUMMARY

Herein, we have described the preparation and immunological evaluation of an entirely carbohydrate STn-PS A1 conjugate that mimics THERATOPE. First, a highly chemoselective and adaptive synthetic route for aminooxy-STn antigen was developed. The aminooxy sugar was conjugated to aldehyde-functionalized PS A1 through an oxime linker. The structure of STn-PS A1 was unambiguously characterized using NMR analysis. The combination of STn-PS A1 plus Sigma adjuvant system demonstrated the capability of inducing anti-STn antibodies in C57BL/6 mice as indicated by ELISA. We also employed FACS to study binding events on STn expressing MCF-7 and OVCAR-5 cancer cell lines. The results from both assays further confirmed the excellent specificity and selectivity of antibodies raised against the STn-PS A1 immunogen for binding the tumor cell surface STn antigen. Moreover, key data collected in an in vitro LDH tumor killing assay exhibited the promising therapeutic potential of anti-STn antibodies for inducing complement-dependent cytotoxicity. An INF-γ ELISpot assay clearly indicated that STn-PS A1 is capable of inducing robust cellular immune responses in mice, which can possibly enhance several mechanisms responsible for the eradication of tumor cells. Combined, the promising results from the documented experiments suggest a new approach for the development of a next generation cancer vaccine, however, further studies are currently underway to validate tumor killing function of STn-PS A1 utilizing in vivo murine model systems.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.6b05675.

Chemical syntheses; biological studies including ELISA, FACS, CDC, and ELISpot assays; and NMR spectra of related compounds (PDF)

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Notes

The authors declare no competing financial interest.

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