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IgG Antibody Response Elicited by a Fully Synthetic Two-Component Carbohydrate-Based Cancer Vaccine Candidate with α-Galactosylceramide as Built-in Adjuvant

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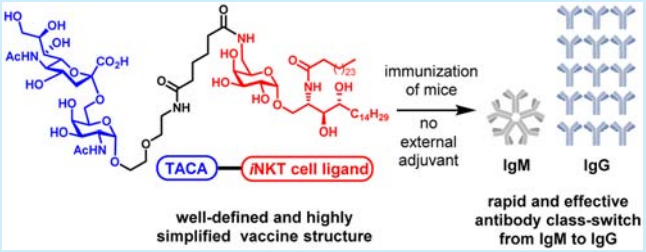
Supporting Information

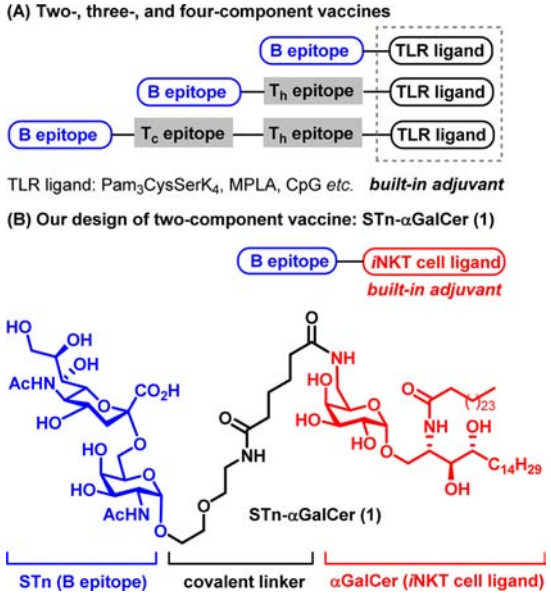
ABSTRACT: A fully synthetic self-adjuvanting cancer vaccine candidate was constructed through covalent conjugation of invariant natural killer T (*i*NKT) cell ligand α-galactosylceramide (αGalCer) with sialyl Tn (STn), a representative tumor-associated carbohydrate antigen (TACA). This two-component vaccine STn-αGalCer is devoid of antigenic peptide, featuring the well-defined structure with high simplicity. STn-αGalCer showed remarkable efficacy in inducing antibody class switching from IgM to STn-specific IgG. Subtypes of IgG antibody were primarily IgG1 and IgG3.



High titers of antigen specific IgG antibodies have been referred to as the "holey grail" in carbohydrate-based vaccinology.[1] In order to avoid "epitope suppression" effect[2] in semisynthetic hapten-protein vaccines and only retain the essential elements for evoking a desired high-affinity IgG antibody response, fully synthetic self-adjuvanting vaccines with a well-defined structure have been pursued,[3] especially in the development of cancer vaccine targeting weakly immunogenic tumor-associated carbohydrate antigens (TACAs).[4,5]

The built-in adjuvant is an essential component in the synthetic vaccines (Scheme 1A).[6] In general, these adjuvants predominantly include lipopeptide or lipoamino acid based Toll-like receptor (TLR) 2 ligands[7] (such as $Pam_3CysSer$). Upon conjugation to carbohydrate, the amphiphilic molecules self-assemble antigens in the outer leaflet of a liposome that provide the necessary multivalency for B cell stimulation. Toyokuni et al. reported the first fully synthetic carbohydrate vaccine that covalently linked a Tn dimer to $Pam_3CysSer$, but it showed limited isotype switching from IgM to IgG.[8] By incorporating a peptide (helper T (Th) cell epitope), Boons and co-workers demonstrated that the three-component vaccine elicits exceptionally higher titers of IgG antibodies.[9] Dumy, BenMohamed, and co-workers extended the vaccine construct to four components, introducing an additional cytotoxic T (Tc) cell epitope. Immunological studies on mice revealed both tumoral regression and a significant increase of survival.[10] In spite of the key role of the Th epitope, the synthetic construct usually contains only one Th epitope.

Scheme 1. Fully Synthetic Self-Adjuvanting Vaccines



Because major histocompatibility complex (MHC) class II molecules are highly polymorphic, in order to guarantee

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consistent immunological efficiency in different individuals, multiple Th epitopes may be needed in a construct, which might be a synthetic challenge in term of structural complexity.[11]

Invariant natural killer T (*i*NKT) cells are a distinct subset of T lymphocytes with phenotypic properties of both T and natural killer (NK) cells and a wide range of immune effector properties.[12] Recently it became clear that *i*NKT cells are able to initiate a so-called "T dependent (TD) type II response",[13] which provides the basis for B-cell vaccines[14] directed against "T helper-less antigens"[15] (such as carbohydrate). Distinguishing from the conventional TD responses that recruit peptide-specific CD4+ T cells and result in substantial germinal center (GC) formation, affinity maturation, and memory response, TD type II response needs no participation of CD4+ T cells.[16]

Remarkably, the activation of *i*NKT cells involves not peptide but glycolipid presentation in CD1d (a MHC class I-related glycoprotein in antigen-presenting cells) to the T cell receptor of *i*NKT cells. The first glycolipid of *i*NKT cell activator was α-galactosylceramide (αGalCer, also known as KRN7000), a synthetic compound discovered through SAR studies on a class of glycolipid originally isolated from a marine sponge.[17] Recently, αGalCer was used as built-in adjuvant in vaccines against different B epitopes (capsular polysaccharides[15c] and peptide[18]). These vaccine do not require CD4+ T help but rather rely on stimulation of *i*NKT cells to provide the key signals in evoking robust IgG antibody class-switch.

As a part of study on the *i*NKT cell agonist,[19] we report herein a fully synthetic two-component cancer vaccine candidate against sialyl-Tn (STn), a typical TACA associated with the MUC1 mucin on a number of human cancer cells. The vaccine was constructed through covalent conjugation of STn with αGalCer (Scheme 1B). Notably, because TACAs feature T-independence and self-tolerance in mammalians, it is typically more challenging to induce immune response to TACAs than to the antigens of either peptide/protein[18] or nonmammalian carbohydrate.[15c]

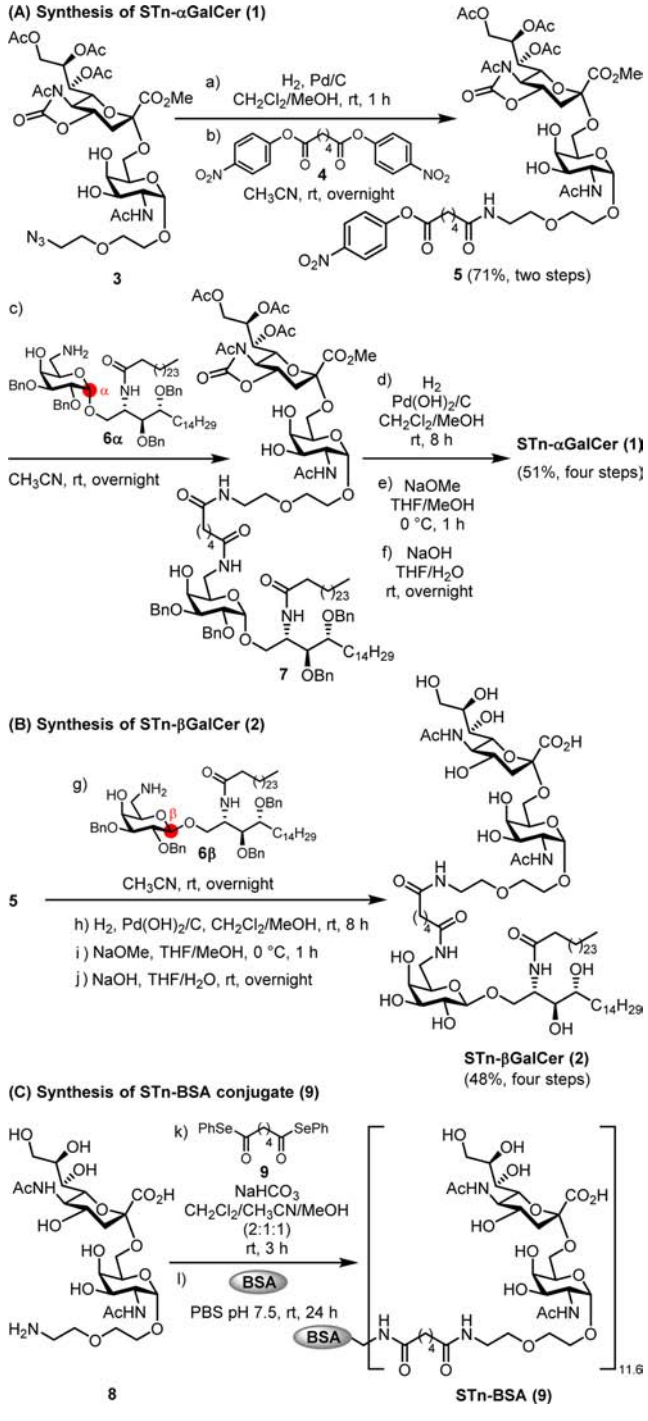
αGalCer plays multiple functions as one component: liposomal carrier vehicle, activation of antigen-presenting cells (APCs), and Th epitope-like function. This molecular construct showcases the well-defined structure with high simplicity. Importantly, in contrast to MHC class II, CD1d is a nonpolymorphic antigen-presenting molecule, so it is unnecessary to incorporate "multiple *i*NKT epitopes" to stimulate the consistent immune response of all vaccinated individuals, thus lowering the synthetic hurdle.

The 6-position of galactosyl moiety in αGalCer was chosen to be the site of conjugation via an amide, because a PEGylated amide of αGalCer retains the specificity for the CD1d receptor and ability to activate *i*NKT cells.[20] Another synthetic target is a control molecule, STn-βGalCer (**2**). βGalCer possesses a β anomeric linkage between ceramide and galactose as a weak *i*NKT cell ligand.[21] An aliphatic straight-chain linker[22] between STn and α or βGalCer was chosen to avoid immunological suppression.[23]

The preparation of STn-αGalCer (**1**) (shown in Scheme 2A) began with hydrogenolysis of azide **3**[24] by H_2 in the presence of Pd/C followed by amidation of the resulting amine via an adipic acid *p*-nitrophenyl diester[22] (**4**). The activated ester **5** was then subjected to the coupling with **6α** (see the synthesis of **6α** and **6β** in the Supporting Information) to afford the protected STn-αGalCer conjugate **7**. All of the benzyl groups in **7** were deprotected through hydrogenolysis under an H_2 atmosphere using Pearlman's catalyst. Finally, removal of the *O*-acetyl groups under Zemplén conditions and subsequent hydrolysis of the methylester moiety led to totally deprotected target **1**, which was purified by size exclusion column chromatography (LH-20). Analysis of **1** by MALDI-TOF mass spectrometry revealed a signal at m/z = 1611.1 corresponding to $[M + 2Na - H]^+$. The control molecule STn-βGalCer (**2**) using **6β** was synthesized in an identical manner to **1** (Scheme 2 B).

STn-BSA (**9**), the coating antigen in enzyme-linked immunosorbent assay (ELISA) analysis, was prepared by the

Scheme 2. Synthesis of STn-αGalCer (1), STn-βGalCer (2), and STn-BSA Conjugate (9)[a]



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coupling of **8** with bovine serum albumin (BSA) via diselenoester linker **9** (Scheme 2 C). When a hapten/protein ratio of 30:1 was employed, the average number of STn hapten loaded per BSA was 11.6. It is noteworthy that under same conditions the use of linker **4** led to a lower loading level of STn hapten (4 STn per BSA).[24]

Next, the liposomal formulation of **1** and **2** was prepared by sonication of a mixture of 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC), cholesterol, and **1** or **2** (molar ratios 5:4:1) in a 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (20 mM, pH 7.5) containing NaCl (150 mM). The resulting liposomal vaccine **1** and **2** were used to immunize groups of five female BALB/c mice (6−8 weeks old) via intraperitoneal injection, without an external adjuvant, on days 1, 15, and 29 (Figure 1). The negative control group was

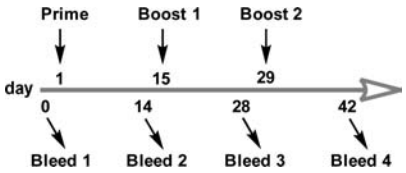

Figure 1. Mouse vaccination and bleed schedule.

administered phosphate-buffered saline (PBS) only. The mice were bled on day 0 before initial immunization and on day 14, day 28, and day 42 after boost immunizations.

Anti-STn antibody titers were determined by ELISA precoated with **9**. In comparison to control vaccine **2**, mice immunized with **1** elicited approximately equal titers of IgM antibody, but exceptionally higher titers of IgG antibodies than those of **2** (23-fold higher, Figure 2A). The remarkable IgG antibody response initiated by **1** suggests that αGalCer as a built-in adjuvant can effectively improve the immunogenicity of STn, which might be the result of activation of *i*NKT cells to provide B cell help. Guo and co-workers reported the two-component fully synthetic antitumor vaccines using mono-

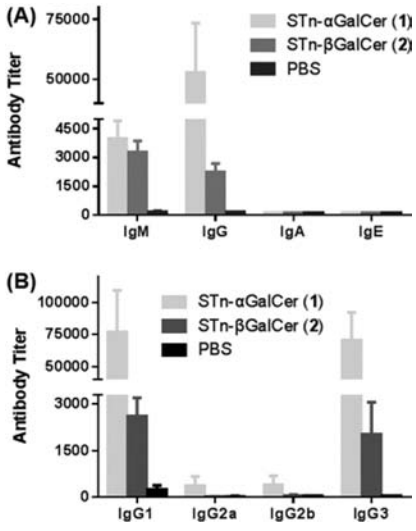

Figure 2. ELISA anti-STn antibody titers after three immunizations with **1**, **2**, and PBS. (A) IgM, IgG, IgA, and IgE; (B) IgG subclasses. ELISA plates were precoated with **9**. Each vertical bar reflects a group average (n = 5 female BABL/c mice per group). Error bars represent standard error of the mean (SEM).

phosphoryl lipid A (MPLA) as a built-in adjuvant, which are able to stimulate a powerful IgG response.[25]

Subtype analysis (Figure 2B) indicated the IgG antibodies were primarily IgG1 and IgG3, which were strong inducers of the complement-dependent cytotoxicity (CDC) and effective mediators of antibody-dependent cell-mediated cytotoxicity (ADCC).[26] Typically, the class switch to IgG2a/c and IgG3 is facilitated by IFN-γ, whereas IgG1 and IgG2b are facilitated by IL-4 and TGF-β, respectively.[27] The production of almost equal titers of IgG1 and IgG3 indicates a mixed Th1/Th2 response, which is consistent with the "Th0-like" profile of cytokine response initiated by KRN7000 through the activation of *i*NKT cells.

As shown in Figure 3, the day 14 serum obtained from mice inoculated with **1** only on day 1 already showed high IgG

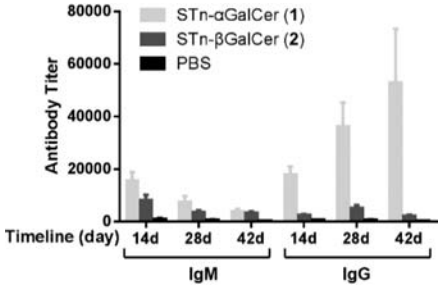

Figure 3. Total IgM and IgG antibody titers of pooled day 14, 28, and 42 sera derived from mice immunized with **1**, **2**, and PBS. ELISA plates were precoated with **9**. Each vertical bar reflects a group average (n = 5 female BABL/c mice per group). Error bars represent standard error of the mean (SEM).

antibody titers (7.5-fold higher than **2**), indicating that **1** could rapidly elicit robust immune responses. The IgG antibody titers of the day 28 and 42 antisera induced by **1** increased further after boost immunizations, reflecting the general trend of enhanced immune responses to recurring exposure to the same antigen.

In summary, we demonstrated that **1**, a well-defined and highly structural simplified two-component fully synthetic vaccine candidate using αGalCer as a built-in adjuvant, is capable of inducing a robust specific anti-STn IgG antibody response. Preliminary immunological studies reveal that **1** is a promising anticancer vaccine worthy of further investigation.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.orglett.6b03591.

Detailed experimental procedures, NMR spectra, MALDI-TOF MS spectra, biological assay methods (PDF)

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Author Contributions

[‖]X.-G.Y. and X.-Z.C. contributed equally.

Notes

The authors declare no competing financial interest.

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