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L-Rhamnose Antigen: A Promising Alternative to α-Gal for Cancer Immunotherapies

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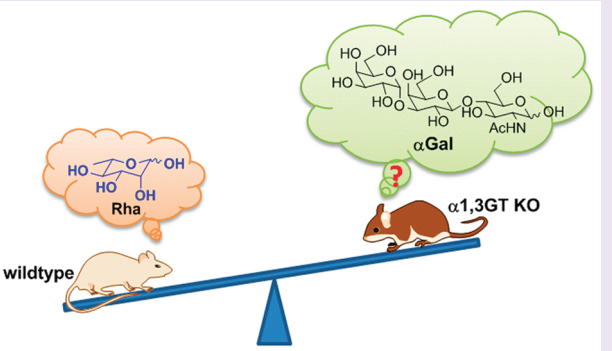
Supporting Information

ABSTRACT: The targeting of autologous vaccines toward antigen presenting cells (APCs) *via* the *in vivo* complexation between anti α-Gal (anti-Gal) antibodies and α-Gal antigens presents a promising cancer immunotherapy with enhanced immunogenicity. This strategy takes advantage of the ubiquitous anti-Gal antibody in human serum. In contrast to the α-Gal epitope, the recent identification of high titers of anti-L-rhamnose (anti-Rha) antibodies in humans reveals a new approach toward immunotherapy employing L-rhamnose (Rha) monosaccharides. In order to evaluate this simple antigen in preclinical applications, we have synthesized Rha-conjugated immunogens and successfully induced high titers of anti-Rha antibodies in wildtype mice. Moreover, our studies demonstrate for the first time that wildtype mice could replace α1,3galactosyltransferase knockout (α1,3GT KO) mice in such antigen/antibody-mediated vaccine design when developing cancer immunotherapies.



Gal-α(1,3)-Gal-β(1,4)-GlcNAc/Glc, termed the α-Gal epitope, represents one of the most well-known carbohydrate antigens, playing a crucial role in organ xenotransplantation. The significance of this unique antigen originates from the fact that anti-α-Gal antibodies (anti-Gal) are naturally present in large amounts in humans, constituting about 1% of serum IgG.[1,2] This aspect of the α-Gal epitope makes it an important target in potential clinical treatment. Besides its importance in xenotransplantation, the α-Gal epitope has been applied to enhance immunogenicity of vaccines by forming *in vivo* complexes with natural anti-Gal antibodies. Specifically, the injection of an α-Gal conjugated vaccine could result in *in situ* complexes of anti-Gal/α-Gal, thus effectively targeting the vaccine to antigen presenting cells (APCs) by the interaction between the Fc portion of the anti-Gal antibody and Fcγ receptors (FcγR) on APCs (Figure 1a).[3,4] This promising feature of α-Gal in clinical medicine implies the similar potential of other carbohydrate antigens with naturally high antibody levels, if identified in humans. Several advanced high-throughput carbohydrate arrays have been successfully developed for evaluating carbohydrate−protein interactions,[5−7] More recently, the Gildersleeve group[8] and Bovin group[9] developed comprehensive carbohydrate antigen arrays to profile human serum. Consequently, it was discovered that, other than anti-Gal antibodies, a number of unexpected anticarbohydrate antibodies exist at relatively high levels. More specifically, both of their studies confirmed high levels of antibodies against mono-L-rhamnose (Rha) and GalNAc-α(1,3)-GalNAc (Forssman disaccharide) across all individuals (Figure 1b). This striking discovery suggests that Rha or the Forssman disaccharide could be attractive alternatives to the α-Gal epitope for cancer vaccine development.

Early studies have immunologically characterized Rha bearing *O*-antigens from a variety of bacteria.[10−13] Rha oligosaccharides are constituent carbohydrate units of microbial, immunogenic heteroglycans and lipopolysaccharides, in which they often function as the immuno-determinant groups of these immunogens.[14] Rha was also isolated from Buckthorn (Rhamnus)[15] and poison sumac. In addition, Rha is a component of the outer cell membrane of acid-fast bacteria in the *Mycobacterium* genus,[16] which includes the bacterium that causes tuberculosis. Unfortunently, the complexity of these identified Rha oligosaccharides restrained them from further study and application. The recent discovery of high levels of anti-Rha antibodies could greatly impact the recognition of the Rha antigen in potential therapeutics,[17] as it appears to be a better alternative to α-Gal as a result of its natural abundance and structural simplicity.

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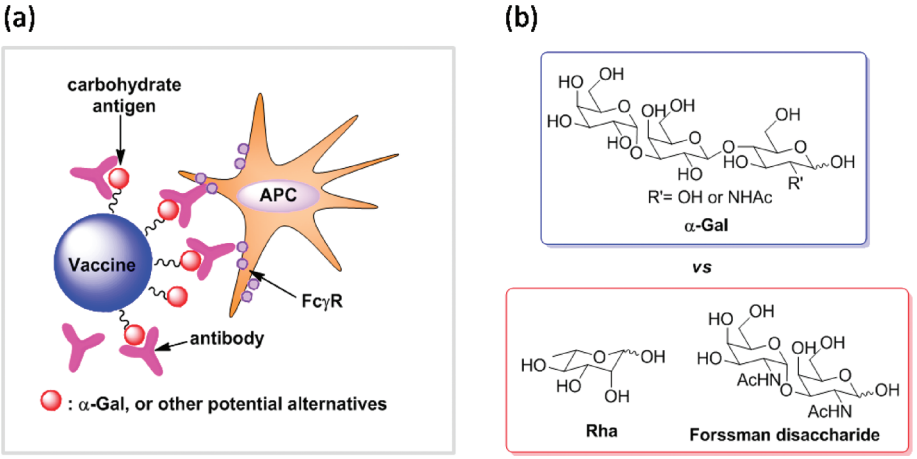

Figure 1. (a) Carbohydrate antigen/antibody-mediated vaccine with enhanced immunogenicity. (b) Structures of α-Gal, Rha and Forssman disaccharide

On the basis of the recent discovery of the ubiquitous presence of anti-Rha antibodies in humans, we initiated a new approach toward cancer immunotherapy by employing the Rha antigen as a replacement for the α-Gal counterpart. Our studies reveal that wildtype mice do not have high levels of anti-Rha antibodies. In order to establish an animal model for preclinical evaluations of the Rha antigen, we report here a well-established method for immunizing mice by synthetic Rha antigens for production of significant amounts of anti-Rha antibody titers, comparable to those of humans.

RESULTS AND DISCUSSION

Pre-existing high anti-Gal titers in human serum have distinguished the α-Gal from other carbohydrate antigens in potential clinical applications over recent decades. Similarly, the Rha monosaccharide could become an attractive alternative for the same reasons discussed previously. In order to confirm the comparably high level of natural anti-Rha antibodies in human serum, we performed ELISA experiments to evaluate titers of both anti-Rha antibodies and anti-Gal antibodies. As a result, expected high levels of anti-Gal IgG (1:1600) and IgM (1:3200) were confirmed in pooled normal human serum (Figure 2a). Encouragingly, high titers of both anti-Rha IgG (1:6400) and IgM (1:6400) were also identified, in which anti-Rha IgG titers are four times higher than those of anti-Gal. These observations were consistent with recent carbohydrate antigen microarray results. In addition to these verifications, our free monosaccharides competitive ELISA further validated high levels of anti-Rha titers against Rha in human serum (Figure 2c). In this experiment, only the free Rha pulled down the antibodies in the human serum, whereas the other seven common monosaccharides did not. This indirectly indicates the existence of the antibodies specific to Rha. On the other hand, we examined the levels of anti-Gal and anti-Rha in wildtype mice serum (Figure 2b). In contrast to humans, natural anti-Rha antibodies were observed at low pre-existing levels (1:800). Additionally, there was no evidence of anti-Gal existing naturally in mice. This suggested that wildtype mice could be utilized for evaluation of Rha-associated cancer immunotherapies, whereas using α-Gal requires α1,3galactosyltransferase knockout (α1,3GT KO) mice. All of these results suggested that Rha may be a promising replacement for α-Gal in preclinical animal experiments and clinical applications for humans.

With successful validation of high titers of anti-Rha antibodies across human but not in wildtype mice, we accordingly designed and synthesized Rha conjugated protein antigens for immunization to establish an optimal mouse model with high anti-Rha antibody titers for future evaluation. The synthesis of a Rha antigen was divided into two steps: (1) installation and activation of a linker on Rha and (2) chemical ligation between the Rha-linker and carrier protein. In our design, Rha was furnished with two different spacers bearing an *N*-hydroxysuccinimide (NHS) ester, which could readily conjugate with multiple lysine residues on carrier proteins under mild physiological condition.[18] Herein, syntheses of Rha-linker-1 (**R1**) and Rha-linker-2 (**R2**) were illustrated as follows (Scheme 1), which furnished Rha with two different spacers in order to avoid cross-interaction effects of linkers between immunization and ELISA assays. The synthesis of **R1** started from the free L-rhamnose (Scheme 1a). Peracetylation of the starting material gave pure intermediate **1** with α configuration in quantitative yield without purification. The following glycosylation between peracetate donor **1** and azido linker **2** promoted by BF_3-Et_2O led to compound **3** with predominant α selectivity. Deacetylation of compound **3** by NaOMe resulted in azido linker **4**. Subsequent installation of the carboxylic acid function group was accomplished by a copper-catalyzed Huisgen 1,3-dipolar cycloaddition[19] between compound **4** and the 5-hexynoic acid **5**. Final conversion of the carboxylic acid to an NHS activated ester was initially performed through traditional method, by which NHS and *N*,*N*′-diisopropylcarbodiimide (DIC) were used. However, these attempts provided unsatisfactory activation results. Conversely, utilizing *N*,*N*,*N*′,*N*′-tetramethyl-*O*-(*N*-succinimidyl)-uronium tetrafluoroborate (TSTU),[20,21] an activated form of NHS, offered a much better activation of the acid **6** to furnish Rha-Linker-1 (**R1**) in anhydrous DMF solvent in the presence of Et_3N. By applying a minimal amount of each reagent during the activation step, the crude product, following solvent removal, was directly used for conjugation with carrier proteins or stored in a freezer for at least 1 year with intact reactivity. This activation strategy allowed for convenient preparation and storage of NHS activated linkers on a relative large scale.

The alternative synthesis of Rha linker employed the intermediate **1** (Scheme 1b). The installation of phathalimide protected amine linker was achieved by using linker acceptor **7**[22] through glycosylation with peracetate precursor **1**. Then all

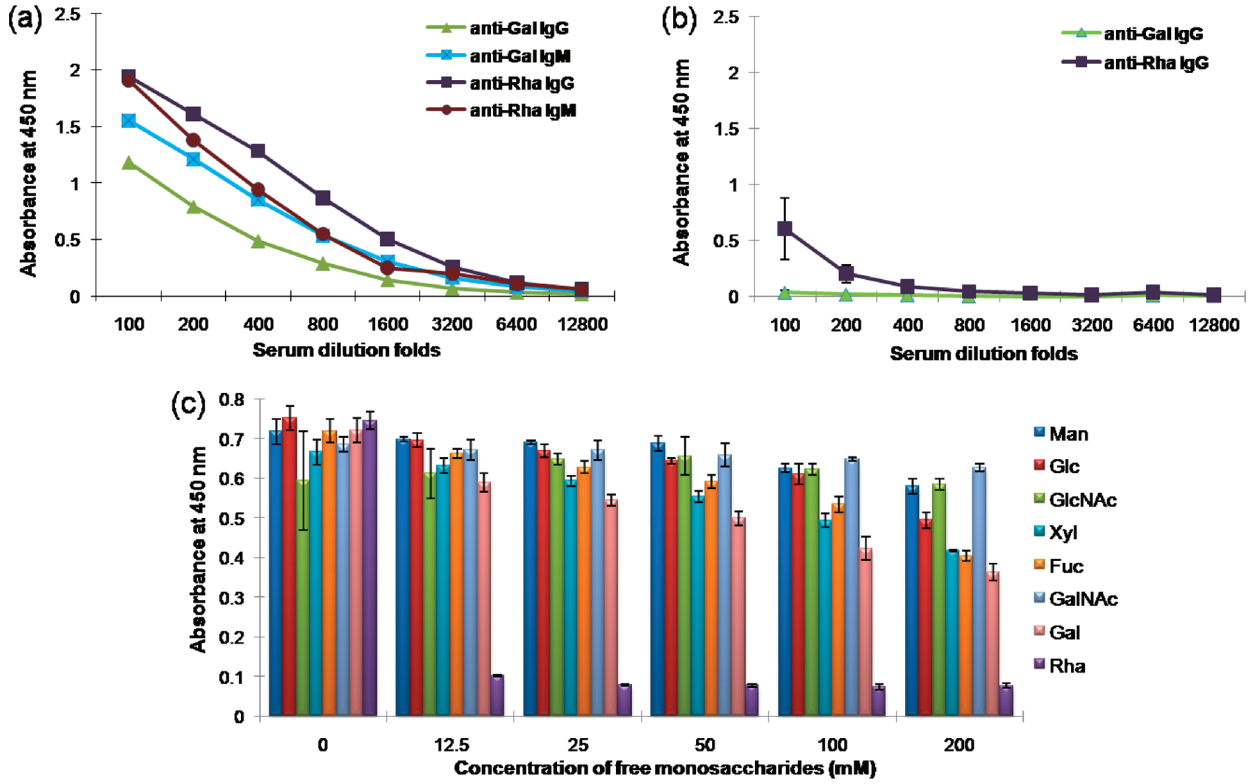

Figure 2. Evaluations of anti-Gal and anti-Rha antibodies. (a) ELISA assays of antibodies in pooled complement normal human serum. (b) ELISA assays of antibodies in wildtype mice serum. (c) Competitive ELISA assays of 8 common monosaccharides performed with pooled complement normal human serum. Rha conjugated BSA was used as immobilizing antigen, and free D-mannose (Man), D-glucose (Glc), *N*-acetyl-D-glucosamine (GlcNAc), D-xylose (Xyl), L-fucose (Fuc), *N*-acetyl-D-galactosamine (GalNAc), D-galactose (Gal), and L-rhamnose (Rha) were used as competing antigens (2-fold dilutions from 200 to 12.5 mM).

protecting groups on intermediate **8** were removed by treatment with hydrazine in anhydrous MeOH to give amine **9**. The reaction between **9** and succinic anhydride in MeOH yielded regioselective amination product **10** with a terminal carboxylic acid group. Finally, the acid was successfully activated by TSTU to generate the linker Rha-Linker-2 (**R2**).

To specifically characterize the production of antibodies against the Rha epitope during the follow-up bioassay, we accordingly prepared two NHS linker counterparts, which were later conjugated with coating protein for ELISA assays. For this purpose, **Linker-1** and **Linker-2** were synthesized *via* intermediates **11**[23] and **12**,[24] followed by the same activation procedures as for the two Rha linkers (Scheme 2). This "linker-only" design allowed us to address any additional immunogenic effect from any component other than the Rha moiety on the synthetic glycoprotein antigens.

Following the synthesis of these NHS activated linkers, conjugations with carrier protein under different conditions were investigated in order to obtain optimal Rha immunogens. Our initial model reactions employed bovine serum albumin (BSA) as a carrier protein in order for convenient characterization by SDS-PAGE and mass spectrometry (Supplementary Figure S1). It was suggested that 3x PBS was the best medium for such conjugation presumably because it provides better buffer capacity and more accessible sites for ligation. With this conclusion, all the following ligations were carried out in 3x PBS for 1 h and quenched by ultrafiltration to remove the excess linkers. The conjugation results were characterized by both SDS-PAGE and MALDI to estimate the number of linkers per protein molecule. This strategy successfully led to Rha/Linker conjugated proteins in good yields (Figure 3 and Supplementary Figure S2).

With these synthetic Rha-antigens ready, our first goal was to establish a protocol to produce high titers of anti-Rha antibodies in wildtype mice. The initial mice immunization and assay procedure used **R2** conjugated BSA (BSA-**R2**) as an immunogen, and **R1** conjugated ovalbumin (OVA-**R1**) was employed as coating antigen for ELISA assays. Unfortunately, this method failed to show unambiguously anti-Rha antibody production (Supplementary Figure S3). This result imposed a challenge for our original protocol. After deliberated consideration of all possible factors, it appeared that OVA might not be a suitable coating protein for ELISA assays, in that the OVA is a glycoprotein. Consequently, its native glycan moiety might bind with non-Rha related antibodies in mouse serum, thus interfering with the ELISA assay. In order to address this concern, an alternative modified protocol using **R2** conjugated OVA (OVA-**R2**) as immunogen and **R1** conjugated BSA (BSA-**R1**) as coating protein was carried out. This modification was then able to show the distinctly different amounts of antibody IgG titers against two different coating antigens (BSA-**R1** and BSA-**Linker-1**), which explicitly indicated the successful production of antibody specifically against the Rha monosaccharide in the immunized mice (Figure 4). These dramatically different results also confirmed our early assumption of OVA interference during the assay. Some nonspecific antibodies produced by Freund's complete adjuvant (FCA) (since the FCA contains heat-killed *Mycobacterium tuberculosis*, antibacteria carbohydrate antibodies may be also induced) might have bound to the carbohydrate moieties on

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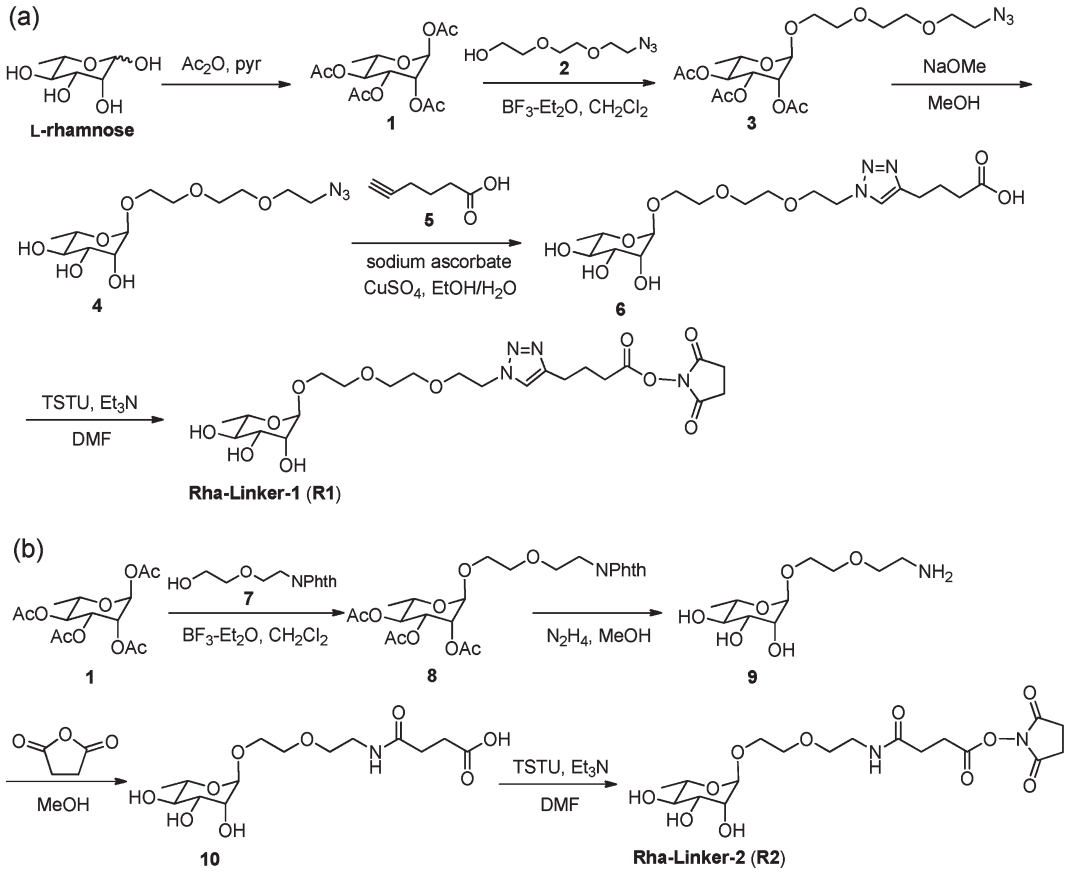
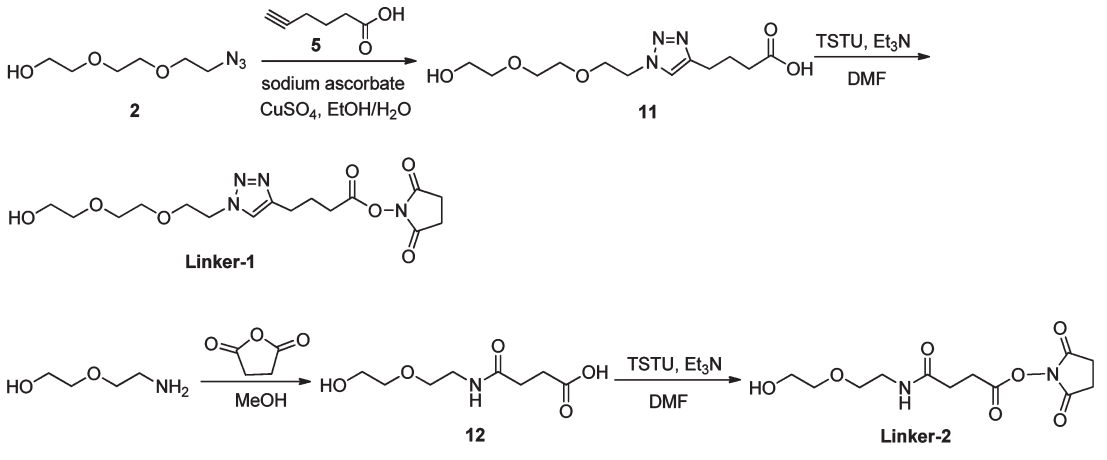
Scheme 1. Syntheses of two different NHS activated Rha linkers R1 and R2.



Scheme 2. Syntheses of corresponding NHS activated linkers without an Rha moiety.



OVA when it was used for coating and thus disturbed the previous ELISA assay.

Achieving the successful production of the anti-Rha antibodies in wildtype mice, we moved forward to immunize an appreciable group of mice, which was divided into four subgroups with a control. By using the established procedure, remarkable differences in anti-Rha antibody productions were observed as expected among these subgroups after three immunization periods. The ELISA assays showed that only the mice in OVA-**R2** immunization group produced significantly high titers of anti-Rha IgG antibody, while the other three control groups (corresponding to "OVA-Linker-2+adjuvant", "PBS+adjuvant", and "none" treatment groups) maintained low levels of corresponding antibody (Figure 5). It was very clear that ELISA assay by BSA-**R1** coating could competently reflect the specific binding between its Rha moiety and the induced anti-Rha antibody (Figure 4). Therefore,

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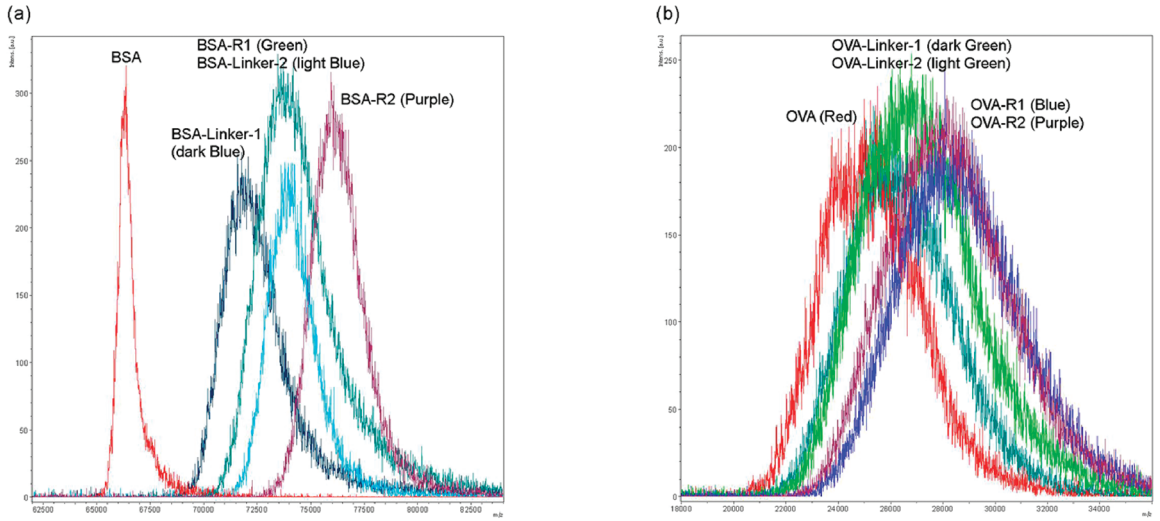

Linker	R1	R2	Linker-1	Linker-2
# of linkers/BSA	20	31	21	40

Linker	R1	R2	Linker-1	Linker-2
# of linkers/OVA	16	18	9	20

Figure 3. MALDI spectra of Rha and Linkers conjugated proteins. (a) Conjugations with BSA (BSA, red; BSA-R1, green; BSA-R2, purple, BSA-Linker-1, dark blue; BSA-Linker-2, light blue). (b) Conjugations with OVA (OVA^{2+} was observed as major peaks from MALDI; OVA, red; OVA-R1, blue; OVA-R2, purple, OVA-Linker-1, dark green; OVA-Linker-2, light green).

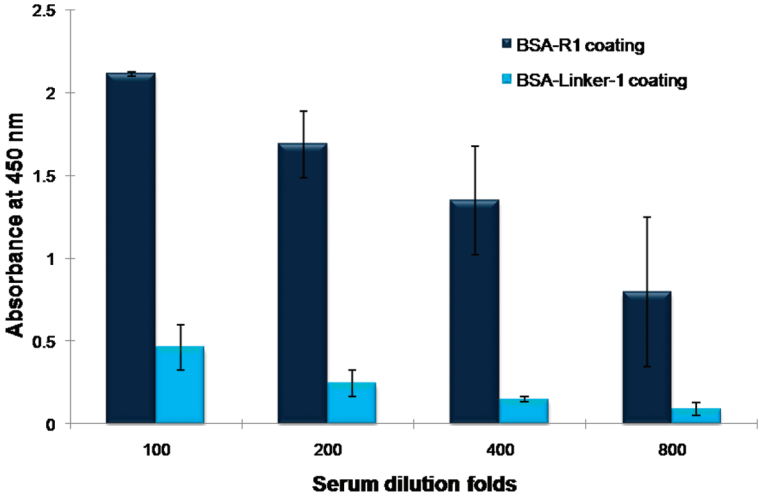

Figure 4. Evaluation of anti-Rha IgG antibody after immunization by ELISA assay. Five mice were immunized by OVA-**R2** for 3 immunization periods (2 weeks/period). Comparison between BSA-**R1** coating (with Rha) and BSA-**Linker-1** coating (without Rha) specifically illustrated the anti-Rha antibody by excluding other factors from both protein and linker portions.

the titers of anti-Rha IgG could be directly calculated from the absorbance reading. Our results indicated that the titers of anti-Rha IgG after Rha-immunization reached 1:6400, while those of the other three groups were 1:1600, 1:1600, and 1:800, respectively. It is worth mentioning that the natural anti-Rha antibody in wildtype mice gradually increased by aging during the whole immunization period but could never reach the same level as that in the immunized group. In addition, a few individual mice exhibited extreme sensitive or inert responses to the Rha immunization (Supplementary Figure S4). Nevertheless, the overall trend of antibody level after immunization demonstrated the successful production of a wildtype mice model with high anti-Rha titers, which is as high as that in natural human serum. The established anti-Rha mouse model, as well as our immunization procedure, should provide valuable knowledge for future cancer immunotherapies involving the Rha antigen.

Conclusions. In this study, we have confirmed the existence of high titers of anti-Rha antibodies in humans, even at higher levels than anti-Gal antibodies. In addition, identification of naturally low titers of anti-Rha antibodies demonstrated the absence of natural Rha synthase in this model. Based on these pre-evaluations, Rha-conjugated immunogens have been designed and synthesized. To our knowledge, our immunization results presented the first successful production of high titers of anti-Rha antibodies in wildtype mice, which reached levels similar to those observed in humans. Furthermore, this study provides significant evidence that a single monosaccharide antigen is able to elicit B cell immunity for antibody production.

The targeting the autologous vaccines to APCs through the *in vivo* complexation of antigen/antibody presents a promising cancer immunotherapy with enhanced immunogenicity. This

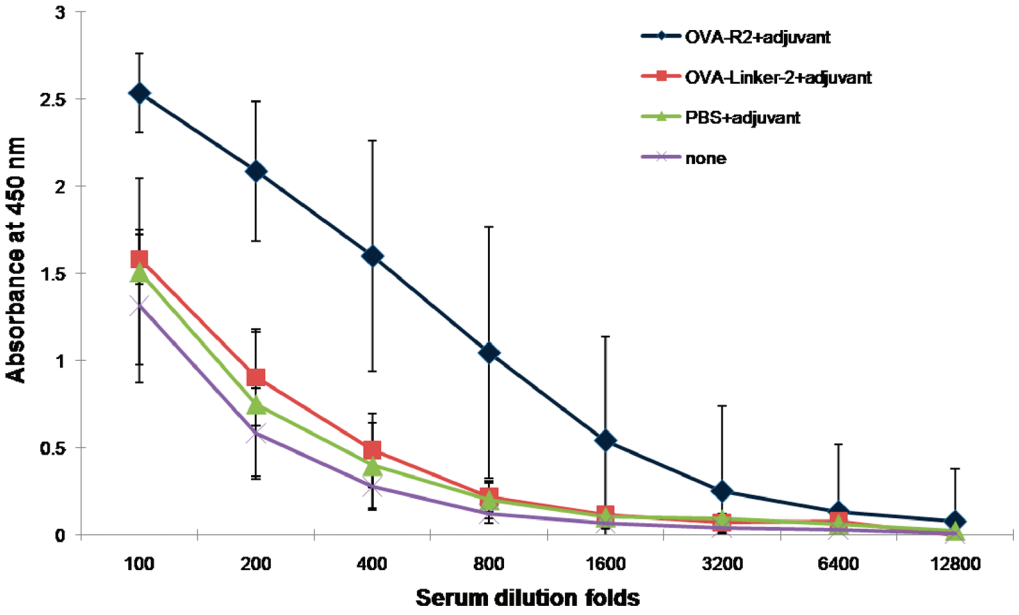

Figure 5. Evaluations of anti-Rha IgG titers in four different groups of mice (BSA-**R1** was used as coating protein). Group I: immunized with OVA-**R2** plus adjuvant (OVA-R2+adjuvant). Group II: immunized with OVA-**linker-2** plus adjuvant (OVA-Linker-2+adjuvant). Group III: immunized with PBS plus adjuvant (PBS+adjuvant). Group IV: no treatment (none).

strategy relies on the ubiquitous presence of certain antibodies in human serum. In contrast to the α-Gal epitope, our studies suggest that the monosaccharide Rha could become a promising alternative in the development of cancer immunotherapies, in that wildtype mice, as well as many of other nonprimate animals, could be directly used for preclinical evaluations.

METHODS

General Procedure for Linker Activation. TSTU (1.1 equiv) and Et_3N (1.5 equiv) were added to a solution of acid linker (1 equiv) in anhydrous DMF. The reaction was monitored by LC−MS. After stirring at RT for 1 h, the free acid completely disappeared. The reaction mixture was then concentrated and dried under vacuum to give crude NHS activated linkers, which were stored at −20 °C and directly used in the following conjugations without further purification.

General Procedure for Conjugation between Linkers and Proteins. The synthetic linkers in solution (10 mg mL^{-1} in 3x PBS) were added to the same volume of protein solution (10 mg mL^{-1} in 3x PBS) and was stirred at RT for 1 h. Then the resultant solution was ultrafiltrated and washed with 1x PBS using Amicon Centrifugal Filter Devices (Ultracel 10,000). The collected glycoprotein solution was quantitated by Pierce BCA Protein Assay Kit (Pierce) and stored at 4 °C for following immunological evaluation. The yields of the glycoproteins varied from 85% to 95% based on the colorimetric detection and quantification of total protein using this protocol. MALDI analysis of the glycoconjugates was performed by using Bruker Microflex TOF.

SDS-PAGE. Protein conjugates were suspended in 12 μL of sample buffer (5% (w/v) SDS, 10% (v/v) glycerol, 25 mM Tris-Cl, pH 6.8, 10 mM DTT, 0.01% (w/v) bromophenol blue), loaded on different lanes of a 1.5-mm-thick, 12% (w/v) SDS-PAGE gel, and visualized by Coomassie Brilliant Blue R-250 staining.

ELISA Assay for Detecting Anti-Rha Antibodies. ELISA plates (96-well) were coated at 4 °C overnight with coating protein (Rha conjugated protein) (10 μg mL^{-1}) in 1x PBS buffer (pH 7.4). The plates were washed twice with PBS buffer containing 0.2% (v/v) Tween 20 (PBST) and then blocked by 5% (w/v) nonfat milk in PBST at 4 °C overnight. The plates were washed and then incubated for 2.5 h at RT with human or mice sera in 2-fold dilution with PBST from 1:100. The plates were washed three times with PBST, followed by the incubation with anti-human IgG or IgM specific horse radish peroxidase-conjugated antibodies (Invitrogen, USA) for 1.5 h at RT. After the plates were washed, enzyme substrate tetramethylbenzidine (TMB) was added and allowed to react for 10−20 min before the enzymatic reaction was terminated by adding 1 N HCl and the absorbance was read at wavelength of 450 nm in FlexStation 3 Microplate Reader (Molecular devices). The titers were calculated to the highest dilution that gave an OD value beyond 0.1.

ELISA Assay for Detecting Anti-Gal Antibodies. ELISA plates (96-well) were coated at 4 °C overnight with coating protein (α-Gal conjugated BSA) (10 μg mL^{-1}) in 1x PBS buffer (pH 7.4). The remaining procedure followed the same as for previous anti-Rha antibody assays.

Competitive ELISA Assay. To further verify the specific antibody against Rha epitopes, inhibition ELISA was performed by immobilizing Rha conjugated BSA (BSA-R1) (10 μg mL^{-1}) on the 96-well plate, and free D-mannose (Man), D-glucose (Glc), *N*-acetyl-D-glucosamine (GlcNAc), D-xylose (Xyl), L-fucose (Fuc), *N*-acetyl-D-galactosamine (GalNAc), D-galactose (Gal), and L-rhamnose (Rha) were used as competing antigens (2-fold dilutions from 200 to 12.5 mM). After being coated with BSA-R1 at 4 °C overnight, the solution was depleted and the plate was washed with PBST three times (3 min each time). Then, the plate was blocked with 5% (w/v) nonfat milk (PBST) in RT for 1.5 h and rinsed with PBST once. Normal human serum diluted (1:2000) previously, containing different free monosaccharide at different concentration, was added into the 96-well plate with 0.1 mL per well. After 2 h of incubation, the plate was rinsed by PBST 3 times. Then 0.1 mL of horseradish peroxidase (HRP)-conjugated antihuman IgG antibody (1:3000) was added into each well, and the plate remained at RT for 1 h. Finally, after the plates were washed, enzyme substrate tetramethylbenzidine (TMB) was added and allowed to react for 10−20 min before the enzymatic reaction was terminated by adding 1 N HCl and the absorbance was read at wavelength of 450 nm in a FlexStation 3 Microplate Reader (Molecular devices).

Mice and Immunization Procedures. The mice (female, BALB/c, 6−8 weeks), obtained from The Jackson Laboratory, were

maintained at the animal facility of The Ohio State University. Groups of at least 5 mice were immunized subcutaneously (several different sites with a total of 150 μL) on days 0, 14, and 28 with 30 μg of Rha conjugates. Freund's complete adjuvant (FCA), incomplete adjuvant (FIA), and no adjuvant were used, respectively, in the above 3 times of immunizations. The mice were bled (tail vein) on the seventh day after the third immunization, and the sera were tested for the presence of anti-Rha antibodies. All experiments with mice were performed according to IACUC (Institutional Animal Care and Use Committee) guidelines.

■ ASSOCIATED CONTENT

Supporting Information. This material is available free of charge *via* the Internet at http://pubs.acs.org.

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