JACS ARTICLES

Published on Web 11/16/2010

Synthesis of a Single-Molecule L-Rhamnose-Containing Three-Component Vaccine and Evaluation of Antigenicity in the Presence of Anti-L-Rhamnose Antibodies

Sourav Sarkar,[†] Steven A. Lombardo,[‡] Danielle N. Herner,[‡] Rommel S. Talan,[†] Katherine A. Wall,*[,‡] and Steven J. Sucheck*[,†]

Department of Chemistry and Department of Medicinal and Biological Chemistry, The University of Toledo, 2801 West Bancroft Street, Toledo, Ohio 43606, United States

Received August 5, 2010; E-mail: kwall@utnet.utoledo.edu; steve.sucheck@utoledo.edu

Abstract: Carbohydrates are generally considered to be poorly immunogenic. Therefore, new approaches for enhancing their immunogenicity are important for the development of carbohydrates as vaccine components. We hypothesized that conjugation of an L-rhamnose (Rha) moiety to a carbohydrate antigen would enhance the antigenicity of the antigen in mice possessing anti-Rha antibodies via an antibody-dependent antigen uptake mechanism. To explore this hypothesis, we synthesized a single-molecule three-component vaccine containing the GalNAc-*O*-Thr (Tn) tumor-specific antigen, a 20 amino acid helper T-cell epitope (YAF) derived from an outer-membrane protein of *Neisseria meningitides*, and a Rha moiety. The vaccine was synthesized by automated Fmoc-based solid-phase peptide synthesis and deacetylated by brief treatment with NaOMe. Groups of female BALB/c mice were immunized and boosted with Rha−ovalbumin (Rha−OVA) formulated with either TiterMax Gold or Sigma Adjuvant System for a period of 35 days in order to determine optimal conditions for generating anti-Rha titers in mice. Anti-Rha antibody titers were >100 fold higher in groups of mice immunized with Rha−OVA than in the control groups. Mice producing anti-Rha were challenged with Rha−YAF−Tn or YAF−Tn. Sera collected from the groups initially immunized with Rha−OVA and later challenged with Rha−YAF−Tn showed a 2-fold increase in anti-Tn titer at 1/100 serum dilution relative to mice not immunized with Rha−OVA. An in vitro T-cell proliferation study using cells primed with either Rha−YAF−Tn or YAF−Tn was done to examine possible differences in antigen uptake and presentation due to anti-Rha antibody and chemical modification. Proliferation of T cells was stimulated by a 10-fold lower antigen concentration in the presence of Rha antibodies. The results strongly suggest that T cells present in the spleen were presented with higher concentrations of Rha−YAF−Tn as a result of the presence of the anti-Rha antibodies.

Introduction

The glycoconjugates on the surface of cancer cells are often expressed in abnormal quantities and show unique structural modifications in their carbohydrate moieties in comparison with the glycoconjugates found on normal cells.[1−3] These aberrant carbohydrate epitopes, also known as tumor-associated cancer antigens (TACAs), have been widely used as important markers for cancer detection and disease progression.[3−7] Studies have shown prolonged survival of cancer patients by virtue of antibodies generated against these cancer antigens.[8] This observation suggests that the immune system can be enhanced by vaccination with TACAs, possibly leading to improved treatment outcomes for cancer patients.[8,9]

An obstacle to the use of TACAs as cancer vaccines is their inherently weak immunogenicity.[9] The generation of high-affinity immunoglobulin G (IgG) antibodies against cancer depends upon the combined interaction of B cells and helper T cells, which requires the antigen to be preferentially displayed on major histocompatibility complex (MHC) class-II molecules that occur on the surface of antigen-presenting cells (APCs) such as dendritic cells.[10] In general, carbohydrate epitopes alone do not activate the helper T cells, resulting in the production of low-affinity IgG and IgM antibodies against cancer antigens. Earlier efforts to break this immunological tolerance have focused on the conjugation of cancer antigens to large immunogenic carrier proteins such as keyhole limpet hemocyanin

[†] Department of Chemistry.
[‡] Department of Medicinal and Biological Chemistry.

(1) Patton, S.; Gendler, S. J.; Spicer, A. P. *Biochim. Biophys. Acta* **1995**, *1241*, 407–423.
(2) Springer, G. F.; Desai, P. R.; Banatwala, I. *J. Natl. Cancer Inst.* **1975**, *54*, 335–339.
(3) Takahashi, H. K.; Bigbee, W. L.; Kim, Y. S. *Cancer Res.* **1989**, *49*, 197–204.
(4) Springer, G. F. *Science* **1984**, *224*, 1198–1206.
(5) Springer, G. F. *J. Mol. Med.* **1997**, *75*, 594–602.
(6) Kim, Y. S.; Gum, J.; Brockhausen, I. *Glycoconjugate J.* **1996**, *13*, 693–707.
(7) Springer, G. F. *Crit. Rev. Oncogenesis* **1995**, *6*, 57–85.
(8) Livingston, P. O.; Wong, G. Y.; Adluri, S. *J. Clin. Oncol.* **1994**, *12*, 1036–1044.
(9) Solvin, S. F.; Keding, S. J.; Raghupati, G. *Immunol. Cell Biol.* **2005**, *83*, 418–428.
(10) Jennings, H. J.; Snood, R. K. In *Neoglycoconjugates: Preparation and Applications*; Lee, Y. C., Lee, R. T., Eds.; Academic Press: San Diego, CA, 1994; p 325.

 10.1021/ja107029z

(KLH),[11−18] bovine serum albumin (BSA),[17,19,20] Toll-like receptor (TLR) agonists,[21] and zwitterionic polysaccharides.[22] A number of drawbacks to the carrier protein conjugation strategy have been noted.[21] For example, the conjugation reaction can often be uncontrollable, resulting in the formation of conjugates with unspecified epitope ratios, which leads to issues of reproducibility in the immunological experiments.[23] Furthermore, the carrier protein itself can induce a strong immune response, leading to the suppression of antibody production against the cancer antigen.[21] In addition, the linkers used for conjugating the carrier proteins to the carbohydrate epitopes can themselves be immunogenic.[24] More recently, smaller antigenic peptides have been conjugated to TACAs and studied as vaccines in which these issues may be mitigated.[12,20,25−28] As an example, Boons and co-workers[21] conjugated YAF, a 20 amino acid peptide derived from the outer membrane of *Neisseria meningitides* and an MHC class-II restricted site for human and mouse T cells, to the cancer antigen α-*N*-acetylgalactosamine-*O*-threonine (Tn) and the lipopeptide *S*-[(*R*)-2,3-dipalmitoyloxypropyl]-*N*-palmitoyl-(*R*)-cysteine (Pam_3Cys), which has strong immunoadjuvant properties and interacts with TLR-2, resulting in the production of pro-inflammatory cytokines and chemokines and thereby stimulating the APCs. The production of high IgG antibody titers against Tn provided strong evidence that small peptides can successfully be used to stimulate helper T cells.

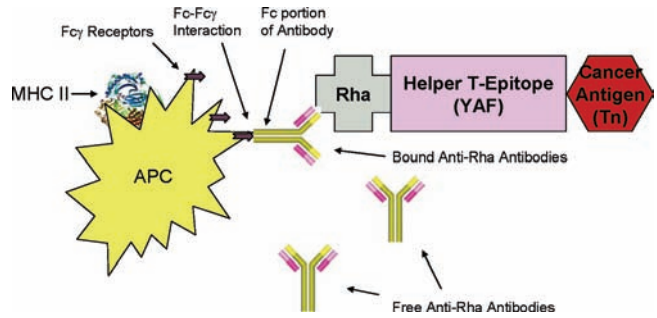


Figure 1. Diagramatic representation of cancer antigen uptake mediated by the presence of anti-Rha antibodies (APC = antigen-presenting cell, e.g., a dendritic cell).

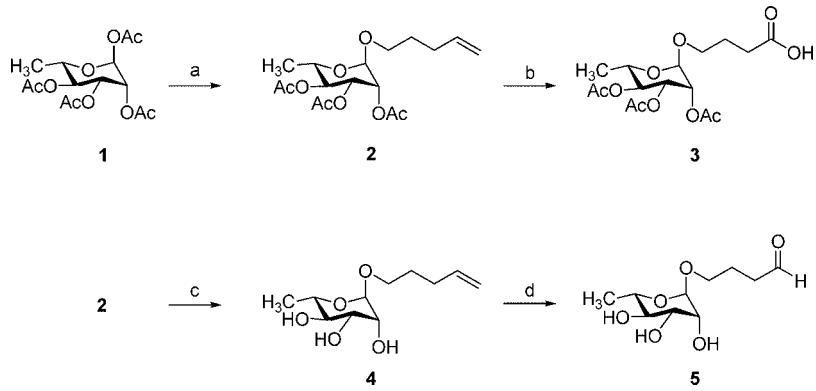
Another avenue for boosting the immune response to tumor antigens involves targeting the antigens of interest to APCs.[29,30] Recent studies by Galili and co-workers[31,32] explored the in vivo interaction of Galα1−3Galβ1−4GlcNAc−R (α-Gal) epitopes in model vaccines against HIV gp120 and flu virus with naturally occurring anti-Gal antibodies in α-1,3-galactosyltransferase knockout mice by an antibody-dependent antigen uptake mechanism that showed a greater than 100-fold increase in immunogenicity. Human serum has been reported to contain large amounts of naturally occurring anti-rhamnose antibodies. These antibodies are highly antigen-specific, and reproducibly high titer results have been reported for a large collection of serum samples from various individuals of different age groups.[33,34] In the present work, we hypothesized that the effectiveness of a cancer vaccine could be increased by conjugation of a helper T-cell peptide and B-cell antigen with an L-rhamnose (Rha) carbohydrate epitope by the same antibody-mediated antigen uptake mechanism described by Galili and co-workers (Figure 1). Our study focused on the idea that the Fc portion of the in vivo-generated immune complex between an anti-Rha IgG or IgM antibody and the Rha-containing conjugate vaccine can be recognized by the Fcγ receptors or other receptors on APCs such as dendritic cells.[16] This results in an overall internalization of the vaccine and better presentation on the human MHC. The Rha epitopes on the N-terminus of the synthetic peptide are not expected to interfere with MHC class-II binding of the YAF peptide. This idea is supported by promising results reported by Galili and co-workers in which naturally occurring anti-Gal antibodies had been targeted using α-Gal epitopes on a model vaccine.[30] In this study, we synthesized a single-molecule three-component vaccine containing a Rha epitope, the 20 amino acid peptide YAF, and the cancer antigen Tn and explored the immunological effects of this vaccine using in vivo and in vitro experiments in mice.

Results

Synthesis of Conjugable L-Rhamnopyranosides. To test our hypothesis, we synthesized conjugable L-rhamnopyranosides **3**

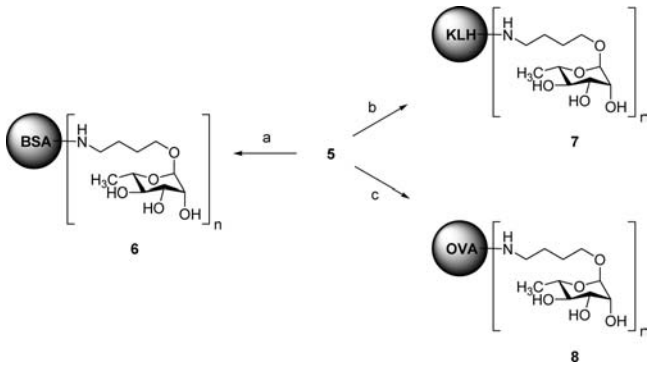
(11) Kuberan, B.; Linhardt, R. J. *Curr. Org. Chem.* **2000**, *4*, 653–677.
(12) Buskas, T.; Ingale, S.; Boons, G. J. *Angew. Chem., Int. Ed.* **2005**, *44*, 5985–5988.
(13) Ragupathi, G. *Cancer Immunol. Immunother.* **1996**, *43*, 152–157.
(14) Deshpande, P.; Kim, H.; Zatorski, A.; Park, T.; Ragupati, G.; Livingston, P.; Live, D.; Danishefsky, S. *J. Am. Chem. Soc.* **1998**, *120*, 1600–1614.
(15) Allen, J. R.; Allen, J. G.; Zhang, X. F.; Williams, L. J.; Zatorski, A.; Ragupathi, G.; Livingston, P. O.; Danishefsky, S. J. *Chem.—Eur. J.* **2000**, *6*, 1366–1375.
(16) Ragupathi, G.; Howard, L.; Cappello, S.; Koganty, R. R.; Qiu, D.; Longenecker, B. M.; Reddish, M. A.; Lloyd, K. O.; Livingston, P. O. *Cancer Immunol. Immunother.* **1999**, *48*, 1–8.
(17) Kuduk, S.; Schwarz, J.; Chen, X.; Glunz, P.; Sames, D.; Ragupathi, G.; Livingston, P.; Danishefsky, S. J. *J. Am. Chem. Soc.* **1998**, *120*, 12474–12485.
(18) Reddish, M.; Jackson, L.; Koganty, R. R.; Qiu, D.; Hong, W.; Longenecker, B. M. *Glycoconjugate J.* **1997**, *14*, 549–560.
(19) Kunz, H.; Birnbach, S. *Angew. Chem.* **1986**, *98*, 354–355.
(20) Toyokuni, T.; Hakomori, S.; Singhal, A. *Bioorg. Med. Chem.* **1994**, *2*, 1119–1132.
(21) Bukas, T.; Ingale, S.; Boons, G. *Angew. Chem., Int. Ed.* **2005**, *44*, 5985–5988.
(22) (a) Tzianabos, A. O.; Onderdonk, A. B.; Rosner, B.; Cisneros, R. L.; Kasper, D. L. *Science* **1993**, *262*, 416–419. (b) Cobb, B. A.; Wang, Q.; Tzianabos, A. O.; Kasper, D. L. *Cell* **2004**, *117*, 677–687. (c) Mazmanian, S. K.; Kasper, D. L. *Nat. Rev. Immunol.* **2006**, *6*, 849–858. (d) Duan, J.; Avci, F. Y.; Kasper, D. L. *Proc. Natl. Acad. Sci. U.S.A.* **2008**, *105*, 5183–5188. (e) De Silva, R. A.; Wang, Q.; Chidley, T.; Appulage, D. K.; Andreana, P. R. *J. Am. Chem. Soc.* **2009**, *131*, 9622–9623.
(23) Anderson, P.; Pichichero, M.; Stein, E.; Porcelli, S.; Betts, R.; Connuck, D.; Korones, D.; Insel, R.; Zahradnik, J.; Eby, R. *J. Immunol.* **1989**, *142*, 2464–2468.
(24) Bukas, T.; Li, Y.; Boons, G. *Chem.—Eur. J.* **2004**, *10*, 3517–3523.
(25) Lo-Man, R.; Vichier-Guerre, S.; Perraut, R.; Deriaud, E.; Huteau, V.; BenMohamed, L.; Diop, O. M.; Livingston, P. O.; Bay, S.; Leclerc, C. *Cancer Res.* **2004**, *64*, 4987–4994.
(26) Kagan, E.; Ragupathi, G.; Yi, S.; Reis, C.; Gildersleeve, J.; Kahne, D.; Clausen, H.; Danishefsky, S.; Livingston, P. *Cancer Immunol. Immunother.* **2005**, *54*, 424–430.
(27) Reichel, F.; Ashton, P.; Boons, G. *Chem. Commun.* **1997**, 2087–2088.
(28) Keil, S.; Claus, C.; Dippold, W.; Kunz, H. *Angew. Chem., Int. Ed.* **2001**, *40*, 366–369.
(29) Macher, B.; Galili, U. *Biochim. Biophys. Acta* **2008**, *1780*, 75–88.
(30) Abdel-Motal, U. M.; Wigglesworth, K.; Galili, U. *Vaccine* **2009**, *27*, 3072–3082.
(31) Abdel-Motal, U.; Wang, S.; Lu, S.; Wigglesworth, K.; Galili, U. *J. Virol.* **2006**, *80*, 6943–6951.
(32) Abdel-Motal, U.; Guay, H.; Wigglesworth, K.; Welsh, R.; Galili, U. *J. Virol.* **2007**, *81*, 9131–9141.
(33) Oyelaran, O.; McShane, L. M.; Dodd, L.; Gildersleeve, J. C. *J. Proteome Res.* **2009**, *8*, 4301–4310.
(34) Schwarz, M.; Spector, L.; Gargir, A.; Shtevi, A.; Gortler, M.; Altstock, R.; Dukler, A.; Dotan, N. *Glycobiology* **2003**, *13*, 749–754.

Scheme 1. Synthesis of Conjugable L-Rhamnopyranosides[a]



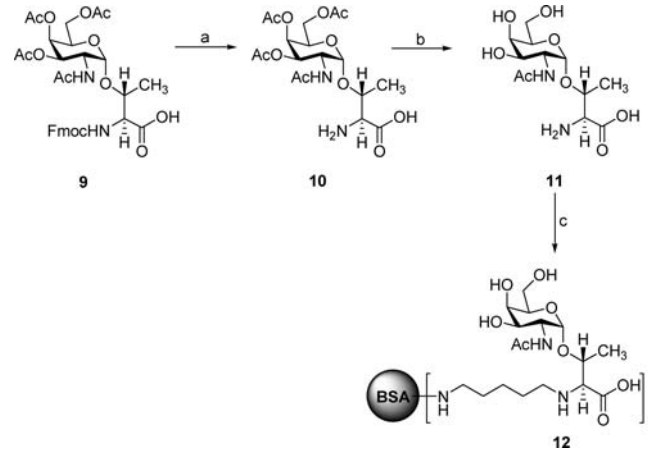
[a] Reagents and conditions: (a) 4-penten-1-ol, $BF_3 \cdot Et_2O$, CH_2Cl_2, 0 °C → r.t., 12 h, 69%; (b) $NaIO_4$, $RuCl_3$, 2:2:3 $CH_2Cl_2/CH_3CN/H_2O$, r.t., 18 h, 98%; (c) Na, MeOH, r.t., 1 h, quantitative; (d) O_3, 1:4 $MeOH/CH_2Cl_2$, Me_2S, −70 °C → r.t., 87.5%.

Scheme 2. Synthesis of L-Rhamnopyranoside Conjugates with BSA, OVA, and KLH[a]



[a] Reagents and conditions: (a) BSA, PBS (pH 7.2), $NaBH_3CN$, 37 °C, 24 h; (b) KLH, PBS (pH 7.2), $NaBH_3CN$, 37 °C, 24 h; (c) OVA, PBS (pH 7.2), $NaBH_3CN$, 37 °C, 24 h. n = Rha/protein molecule.

Scheme 3. Synthesis of the Tn−BSA Conjugate[a]



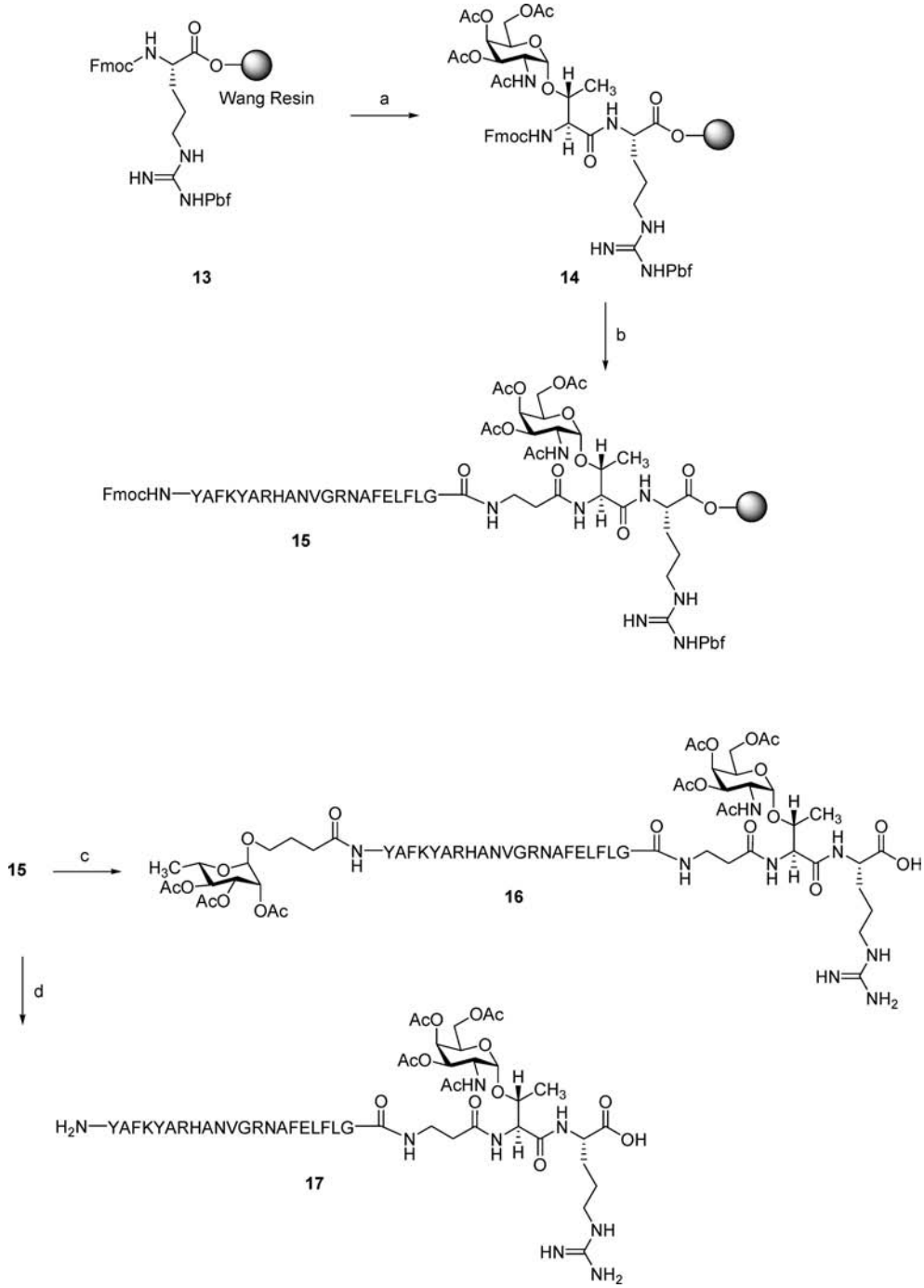
[a] Reagents and conditions: (a) MeOH, AcOH, 10% Pd/C, H_2, 3 h; (b) NaOMe/MeOH, 2 h; (c) BSA, 0.2% glutaraldehyde, $NaBH_3CN$, 20 min.

and **5** (Scheme 1). Tetraacetyl rhamnopyranoside **1** was glycosylated in presence of boron trifluoride etherate with 4-pentene-1-ol to generate the rhamnose pentenyl glycoside **2**[35] (69%). Sodium periodate-catalyzed oxidation of **2** in the presence of ruthenium trichloride afforded acid **3**[36] (98%). Deacetylation using Zemplén conditions followed by ozonolysis of **2** afforded the L-rhamnose aldehyde **5** (87.5%).[37,38]

Synthesis of Rha−BSA, Rha−KLH, Rha−OVA, and Tn−BSA Conjugates. Compound **5** was conjugated with BSA, KLH, and ovalbumin (OVA) by incubation in the presence of sodium cyanoborohydride in 0.1 M phosphate-buffered saline (PBS, pH 7.2) to produce the conjugates Rha−BSA (**6**), Rha−KLH (**7**), and Rha−OVA (**8**), respectively (Scheme 2).[39] The conjugates were purified by filtration through dialysis tubing with a molecular weight cutoff value of 10 000 Da, with six or seven changes of PBS. The protein concentrations of the conjugates were determined by the Bradford method using standard curves for BSA, KLH, and OVA as references.[40] The carbohydrate concentrations of **6**, **7**, and **8** were determined by the phenol−sulfuric acid method using a standard curve for **5**.[41] The epitope ratios of **6**, **7**, and **8** were calculated to be 11.6, 259, and 4.5 Rha/protein molecule, respectively. A Tn−BSA conjugate was prepared by deprotection of the Fmoc group in glycosyl amino acid **9**[42] using catalytic hydrogenolysis conditions in the presence of AcOH/MeOH followed by deacetylation with sodium methoxide to generate compound **11** (Scheme 3).[43−45] Compound **11** was conjugated to BSA by incubation in the presence of 0.2% glutaraldehyde and sodium cyanoborohydride in 0.1 M PBS (pH 7.2) to furnish **12**.[46] The protein concentration of **12** was determined by the Bradford method

(35) Yu, C. S.; Wang, H. Y.; Chiang, L. W.; Pei, K. *Synthesis* **2007**, *9*, 1412–1420.
(36) Kantchev, E. A. B.; Chang, C. C.; Chang, D. K. *Biopolymers* **2006**, *84*, 232–240.
(37) Chen, L.; Li, J.; Luo, C.; Liu, H.; Xu, W.; Chen, G.; Liew, O. W.; Zhu, W.; Puah, C. M.; Shen, X.; Jiang, H. *Bioorg. Med. Chem.* **2006**, *14*, 8295–8306.
(38) Tietze, L. F.; Bratz, M.; Kaino, M.; Yamamoto, H. *Org. Synth.* **1993**, *71*, 314–319.
(39) Raghupati, G.; Koganty, R. R.; Qiu, D.; Lloyd, K. O.; Livingston, P. O. *Glycoconjugate J.* **1998**, *15*, 217–221.
(40) Bio-Rad manufacturer's manual.
(41) Dubois, M.; Gilles, K. A.; Hamilton, J. K.; Rebers, P. A.; Smith, F. *Anal. Chem.* **1956**, *28*, 350–356.
(42) (a) Lacombe, J. M.; Pavia, A. A. *J. Org. Chem.* **1983**, *48*, 2557–2563. (b) Winans, K. A.; King, D. S.; Rao, V. R.; Bertozzi, C. R. *Biochemistry* **1999**, *38*, 11700–11710. (c) Bukowski, R.; Morris, L. M.; Woods, R. J.; Weimar, T. *Eur. J. Org. Chem.* **2001**, 2697–2705.
(43) Campo, V. L.; Carvalho, I.; Allman, S.; Davis, B. G.; Field, R. A. *Org. Biomol. Chem.* **2007**, *5*, 2645–2657.
(44) Sjolin, P.; Elofsson, M.; Kihlberg, J. *J. Org. Chem.* **1996**, *61*, 560–565.
(45) Paulsen, H.; Hölck, J.-P. *Carbohydr. Res.* **1982**, *109*, 89–107.
(46) *The Protein Protocols Handbook*; Walker, J. M., Ed.; Humana Press: Totowa, NJ, 1995.

Scheme 4. Synthesis of Acetylated Rha−YAF−Tn and YAF−Tn[a]



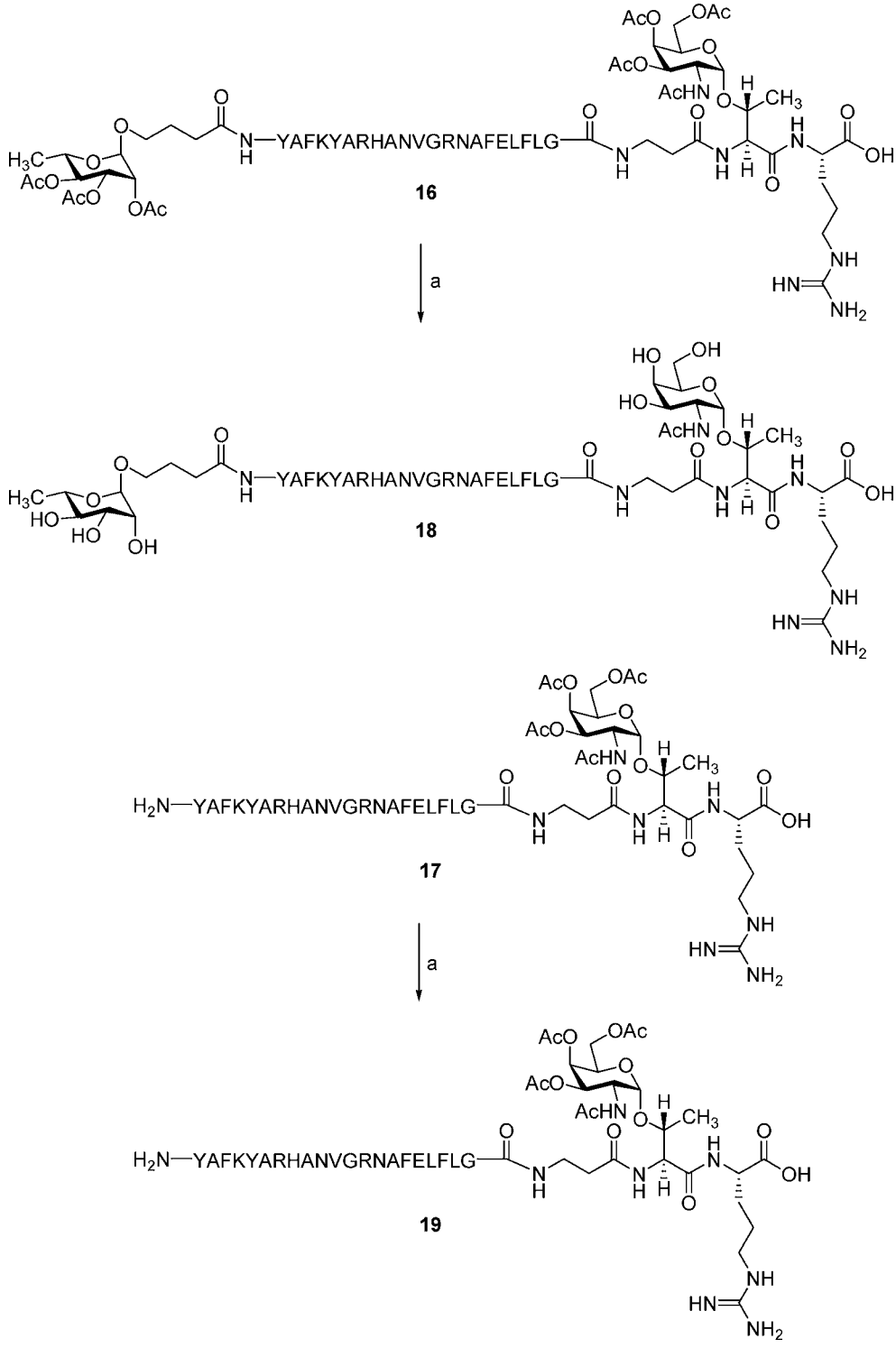
[a] Reagents and conditions: (a) (i) 25% piperidine/DMF, (ii) HOBt ester of **9**, DIC/NMP; (b) Repeat step (a) with G, L, F, L, E, F, A, N, R, G, V, N, A, H, R, A, Y, K, F, A, Y in sequence; (c) (i) 25% piperidine/DMF, (ii) HOBt ester of **3**, DIC/NMP; (iii) 88% TFA, 3% thioanisole, 5% ethanedithiol, 2% water, 2% phenol; (d) (i) 25% piperidine/DMF, (ii) 88% TFA, 3% thioanisole, 5% ethanedithiol, 2% water, 2% phenol. Pbf = 2,2,4,6,7-pentamethyldihydrobenzofuran-6-sulfonyl.

using a standard curve for BSA as the reference.[40] The estimated Tn/BSA epitiope ratio was 28.[47]

Synthesis of Rha−YAF−Tn and YAF−Tn Vaccines. The TACA-containing vaccines were synthesized by the Fmoc strategy using solid-phase chemistry. Preloaded Fmoc-L-Arg Wang resin was used for the peptide synthesis (Scheme 4). An Arg residue was used at the C-terminus because a preloaded Tn Wang resin was not available and we were concerned about the potential for poor loading on the Wang resin using the bulky Fmoc-Thr-(GalNAc(Ac)$_3$-α-D)-OH (**9**) building block. The Fmoc groups were deprotected using 25% piperidine in *N*,*N*-dimethylformamide (DMF). The amino acid esters of 1-hydroxybenzotriazole (HOBt) were coupled sequentially using 1,3-diisopropylcarbodiimide (DIC) as the

(47) Calculated value based on the 28 surface-exposed lysines of BSA: Xie, H.; Tkachenko, A. G.; Glomm, W. R.; Ryan, J. A.; Brennaman, M. K.; Papanikolas, J. M.; Franzen, S.; Feldheim, D. L. *Anal. Chem.* **2003**, *75*, 5797–5805.

Scheme 5. Deacetylation of Rha−YAF−Tn and YAF−Tn[a]



[a] Reagents and conditions: (a) NaOMe/MeOH, r.t., 2 h, 85%.

coupling agent. Finally, the peptides were cleaved from the resin using a modified reagent K cocktail [88% trifluoroacetic acid (TFA), 3% thioanisole, 5% ethanedithiol, 2% water, 2% phenol], filtered, precipitated, and centrifuged. The identities of peptides **16** and **17** were confirmed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI−TOF MS) analysis (for compound **16**, [M + H] *m*/*z* calcd for $C_{158}H_{231}N_{39}O_{49}$ = 3459.6783, found 3459.6902; for compound **17**, [M + H] *m*/*z* calcd for $C_{142}H_{209}N_{39}O_{40}$ = 3101.5519, found 3101.5452).

The acetyl groups in compounds **16** and **17** were deprotected by treatment with 6 mmol NaOMe for 2 h[27] (Scheme 5). Purification was accomplished by size-exclusion chromatography (SEC) on Bio-Gel (P-2, 45−90 μM). The vaccines were lyophilized to obtain compounds Rha−YAF−Tn (**18**) and YAF−Tn (**19**) as white powders (85%). The identities of the

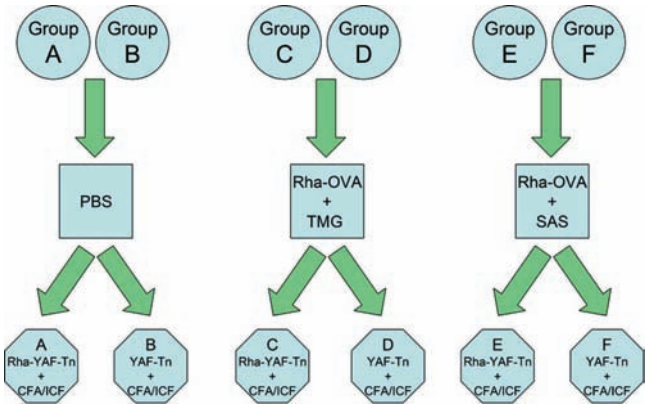


Figure 2. Stepwise immunization plan. A−F represent six groups of female BALB/c mice. Stage I: groups A and B were immunized with PBS, groups C and D with Rha−OVA/TMG, and groups E and F with Rha−OVA/SAS. Stage II: vaccine challenge was performed on groups A, C, and E with Rha−YAF−Tn/CFA (boosted with Rha−YAF−Tn/ICF) and on groups B, D, and F with YAF−Tn/CFA (boosted with YAF−Tn/ICF). [Rha−YAF−Tn = rhamnose vaccine, YAF−Tn = non-rhamnose vaccine, TMG = TiterMax Gold adjuvant, SAS = Sigma Adjuvant System, CFA = Complete Freund's Adjuvant, ICF = Incomplete Freund's Adjuvant.]

vaccines were determined by high-resolution (HR) MALDI−TOF analysis (for compound **18**, [M + H] *m*/*z* calcd for $C_{146}H_{220}N_{39}O_{43}$ = 3207.614, found 3207.165; for compound **19**, [M + H] *m*/*z* calcd for $C_{136}H_{204}N_{39}O_{37}$ = 2975.520, found 2975.562).

Immunological Results and Discussion

Anti-Rhamnose Antibody Generation. The first goal of the immunological study was to determine whether mice contain naturally occurring anti-rhamnose antibodies in their serum. Serum samples from two different nonimmunized mice (C57BL/6 and Swiss Webster) were screened against Rha−BSA conjugate **6** using an enzyme-linked immunosorbent assay (ELISA) (Figures S1 and S2 in the Supporting Information). Competition with free rhamnose and galactose added to the serum was also performed to test the competitive binding of the naturally occurring anti-rhamnose antibodies in the serum (if any) with the bound rhamnose on the plates. Absorbances at 620 nm showed that the anti-rhamnose antibody concentrations in nonvaccinated mice were similar to the background at all serum dilutions. All of the absorbances decreased comparably with serial dilutions of the serum samples from both mouse types. From this study, we concluded that laboratory mice do not contain significant titers of naturally occurring anti-rhamnose antibodies.

Six groups of mice (groups A−F) were then immunized on day 0 with either PBS (control, groups A and B), Rha−OVA/TiterMax Gold (TMG) adjuvant (groups C and D), or Rha−OVA/Sigma Adjuvant System (SAS) (groups E and F) and boosted on day 25 (Figure 2). An ELISA on the serum collected from the mice showed that the antibodies against Rha−BSA in groups C−F were 100-fold higher than in the control groups A and B (Figures 3). Antibody titers for individual mice within groups A−F after the second Rha−OVA boost are shown in Figure S3 in the Supporting Information. This immunization step confirmed that anti-rhamnose antibodies were artificially generated in the experimental groups of mice (C−F).

We also determined the anti-rhamnose antibody titers from groups A−F against Rha−KLH conjugate **7** (data not shown). These titers were compared with those determined using

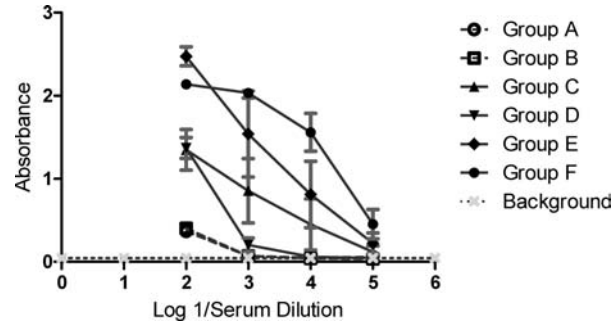


Figure 3. Group average anti-Rha−BSA antibody titers after the second Rha−OVA boost. Groups A and F represent averages of groups of 3 mice, whereas groups B−E represent averages of groups of 4 mice.

Rha−BSA conjugate **6** to evaluate whether there were any measurable differences when Rha conjugates with different epitope ratios were used to detect anti-rhamnose antibody titer. It was observed that the anti-rhamnose antibody titers were comparable at every serum dilution when either conjugate **6** or **7** was used. As an example, the absorbances at 620 nm for group E at 1/100 serum dilution were 1.629 and 1.663 for conjugates **6** and **7** respectively. These results show that the anti-rhamnose antibody titer determined using the Rha−BSA conjugate did not differ markedly from the titer determined using the Rha−KLH conjugate. Therefore, we used Rha−BSA conjugate in subsequent assays.

Vaccination with YAF−Tn and Rha−YAF−Tn Conjugates. After the successful generation of anti-rhamnose antibodies in our experimental groups of mice, the next step was to challenge the mice with either Rha−YAF−Tn **18** or YAF−Tn **19** and compare the anti-Tn antibodies in the titers. Therefore, mice groups A, C, and E were challenged in vivo on day 52 with 80 μg of **18** per mouse in 50 μL emulsions containing equal volumes of 0.01 M PBS (pH 7.2) and Complete Freund's Adjuvant (CFA), whereas mice groups B, D, and F were challenged in vivo with 80 μg of **19** per mouse in 50 μL emulsions of equal volumes of 0.01 M PBS (pH 7.2) and CFA.[21,26,39,48] The mice were then boosted in vivo on day 81 with 50 μL equal-volume emulsions of 0.01 M PBS (pH 7.2) and Incomplete Freund's Adjuvant (ICF) containing either 80 μg per mouse of Rha−YAF−Tn (groups A, C, and E) or YAF−Tn (groups B, D, and F). The serum was pooled on day 91, and the anti-Tn antibody titers were determined using an ELISA. A plot of absorbance (at 620 nm) versus $\log_{10}$[1/serum dilution] showed that anti-Tn antibody titers fell close to the background for all of the groups beyond 1/100 serum dilution (Figure S4 in the Supporting Information). A careful investigation at 1/100 serum dilution revealed that the anti-Tn antibody ELISA absorbance recorded at 620 nm for groups C (0.900) and E (0.678) were 2- and 1.5-fold greater, respectively, than the anti-Tn antibody titer of group A (0.400) (Figure 4). The absorbances at 620 nm corresponding to the anti-Tn antibody titers for groups B (0.690), D (0.704), and F (0.601), which received the vaccine challenge without the rhamnose epitope, were comparable. The data show that groups C and E, which had previously been immunized with Rha−OVA, generated more anti-Tn antibodies after vaccination with the Rha conjugate vaccine Rha−YAF−Tn than did mice vaccinated with the vaccine lacking the Rha moiety. The increased anti-Tn antibody

(48) Lo-Man, R.; Bay, S.; Vichier-Guerre, S.; Deriaud, E.; Cantacuzene, D.; Leclerc, C. *Cancer Res.* **1999**, *59*, 1520–1524.

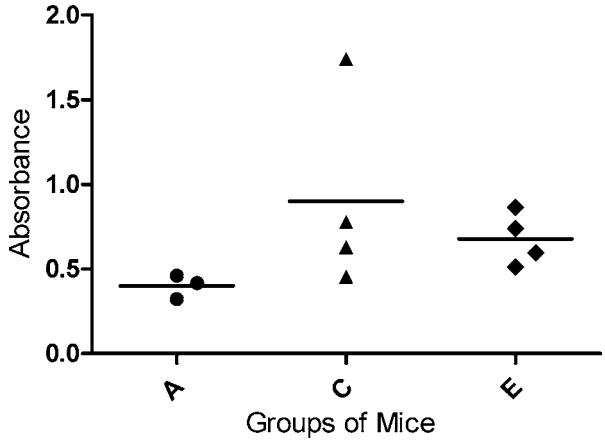


Figure 4. Individual anti-Tn−BSA antibody titers for groups A, C, and E after the second Rha−YAF−Tn boost at 1/100 serum dilution. A *t* test confirmed that groups A and E differed with a *P* value of <0.05.

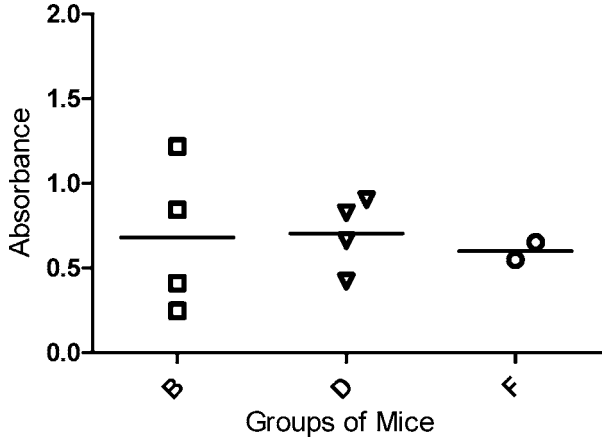


Figure 5. Individual anti-Tn−BSA antibody titers for groups B, D, and F after the second YAF−Tn boost at 1/100 serum dilution.

titer is presumably due to mediation of antigen uptake by anti-rhamnose antibodies.[30,31] This was evident from the low anti-Tn antibody generation for group A, in which the mice did not generate anti-rhamnose antibodies because of the prior lack of immunization with Rha−OVA conjugate **8**. The comparable anti-Tn antibody production for groups B, D, and F was also justified, as the challenge vaccine did not contain the rhamnose epitope and therefore would not have been expected to be influenced by anti-rhamnose antibodies (Figure 5). We also measured the anti-rhamnose antibody titers in the serum after the boost with Rha−YAF−Tn or YAF−Tn (Figure S5 in the Supporting Information) to confirm the presence of anti-rhamnose antibodies and therefore prove that the increase in the production of anti-Tn antibodies was a result of an anti-rhamnose antibody-mediated antigen uptake mechanism. The cumulative absorbances recorded at 620 nm for control groups A and B were 1.385 and 0.681, respectively. These results indicated that the anti-rhamnose antibodies in titers were 2 times greater in group A, which had been challenged with Rha−YAF−Tn, than in group B, where the mice had not been vaccinated with a rhamnose epitope. The cumulative absorbances for groups C−F, all of which had been immunized with Rha−OVA conjugate **8**, were 1.536, 1.029, 1.730, and 1.602, respectively; all of these were considerably higher than that for group B, confirming that the mice in these groups had considerable amounts of anti-rhamnose antibodies in their blood serum to form an in vivo immune complex with the rhamnose epitope in the vaccine. Also, mice that received Rha conjugates twice (groups C and E) had anti-Rha titers similar to those of mice that had received them only once (groups A, D, and F), indicating that the presence of Rha did not direct the immune response to expand the anti-Rha response at the expense of the anti-Tn response.

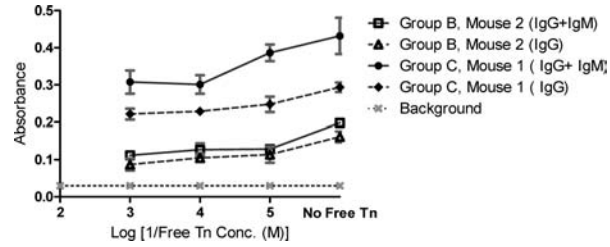


Figure 6. Competitive binding of anti-Tn antibodies with Tn−BSA in the presence of free Tn. Each point represents an average of a duplicate.

The specificity of the antibodies reactive against Tn−BSA was evaluated using a competitive binding assay by screening serum samples after the boost with Rha−YAF−Tn or YAF−Tn with or without prior incubation with free Tn **11** against BSA−Tn conjugate **12**. Serum dilutions of 1/100 from two mice in groups B and C were incubated with free Tn **11** at concentrations of 0, 10^{-5}, 10^{-4}, and 10^{-3} M in 0.1 M PBS (pH 7.2) prior to addition of the serum dilutions in the 96-well ELISA plates. Also, here we used both horseradish peroxidase (HPR) goat anti-mouse IgG + IgM as well as only IgG recognizing secondary antibodies to confirm the presence of both IgG and IgM against Tn in the mice sera. The absorbances decreased uniformly with increasing free Tn concentration in the serum dilutions. As an example, the absorbances at 620 nm for the serum dilution of a group C mouse at free Tn concentrations of 0, 10^{-5}, 10^{-4}, and 10^{-3} M using HPR goat anti-mouse IgG + IgM as a secondary antibody were 0.432, 0.386, 0.301, and 0.307, respectively. This trend was followed for all other serum dilutions (Figure 6). These results confirmed that the antibodies produced after vaccinations were specific toward Tn. The results also showed that there was a considerable amount of IgG antibody generation against Tn.

T-Cell Proliferation Study. T-cell proliferation assays were performed to further assess whether or not Rha conjugate vaccines are more efficiently presented by APCs in the presence of anti-rhamnose antibodies. Two female BALB/c mice were used for this experiment. Mouse 1 was immunized (day 0) by subcutaneous injection of a 100 μL emulsion of YAF−Tn/CFA (12.8 μg), and mouse 2 was injected subcutaneously with a 100 μL emulsion of Rha−YAF−Tn/CFA (12.8 μg). The mice were sacrificed on day 7; the spleens were removed, and separate single cell suspensions were prepared and combined with normal serum antibodies or anti-Rha serum antibodies. Vaccine solutions (Rha−YAF−Tn or YAF−Tn) were then added to the cell preparations. In the absence of added antibodies, the proliferative responses of spleen T cells from cells primed with YAF−Tn and challenged with either YAF−Tn [6008, 2932, 1844, and 1476 counts per minute (cpm) at antigen concentrations of 10^{-5}, 10^{-6}, 10^{-7}, and 10^{-8} M, respectively] or Rha−YAF−Tn (7220, 3412, 2475, and 2295 cpm at antigen concentrations of 10^{-5}, 10^{-6}, 10^{-7}, and 10^{-8} M, respectively) were comparable, indicating that Rha conjugation did not interfere with the presentation of the YAF epitope (Figure 7). Spleen T cells primed with Rha−YAF−Tn in the absence of added antibodies responded slightly better to Rha−YAF−Tn (4959, 2314, 1031, and 522

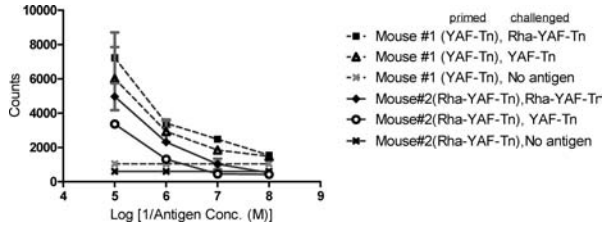


Figure 7. T-cell proliferation measured by [^{3}H]thymidine incorporation in cells from mice primed with YAF−Tn or Rha−YAF−Tn with no added serum antibodies.

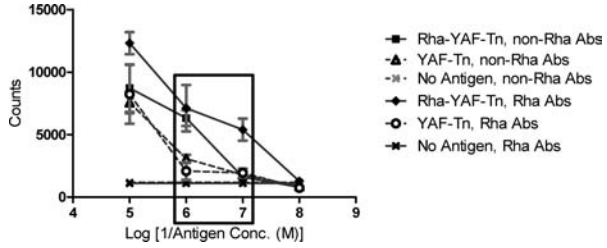


Figure 8. T-cell proliferation measured by [^{3}H]thymidine incorporation in cells from mice primed with Rha−YAF−Tn with in vitro-added Rha and non-Rha serum antibodies. The boxed region highlights the incorporation at antigen concentrations of 10^{-6} and 10^{-7} M.

cpm at antigen concentrations of 10^{-5}, 10^{-6}, 10^{-7}, and 10^{-8} M, respectively) in vitro than to YAF−Tn (3363, 1314, 454, and 425 cpm at antigen concentrations of 10^{-5}, 10^{-6}, 10^{-7}, and 10^{-8} M, respectively). When anti-rhamnose antibodies were added at the initiation of the culture, Rha−YAF−Tn-primed T cells showed similar stimulation at a 10-fold lower concentration when the antigen contained rhamnose. For example, cells reached ~9000 cpm using 10^{-7} M Rha−YAF−Tn with anti-Rha antibodies and 10^{-6} M Rha−YAF−Tn with non-Rha antibodies (Figure 8). When the YAF−Tn peptide was used to challenge the cells, no difference between the addition of anti-rhamnose and non-rhamnose antibodies was observed in the proliferation of the T cells at all antigen concentrations. The effect of anti-rhamnose antibodies was more pronounced in the mice primed with Rha−YAF−Tn as opposed to YAF−Tn for reasons that are under further investigation. This gives us insight into the role of the anti-rhamnose antibodies in allowing better uptake of the YAF peptide by the APCs, which in turn provides overall internalization of the vaccine and better presentation on the MHC.

Conclusion

A fully synthetic L-rhamnose-containing three-component vaccine has been prepared. Laboratory mice do not produce naturally occurring anti-rhamnose antibodies, and therefore, anti-rhamnose antibodies were generated in the experimental groups of mice via immunization with Rha−OVA conjugate **8**. Vaccine challenge with Rha−YAF−Tn in groups A, C, and E (Figure 4) showed that anti-Tn antibody generation was 2 and 1.5 times greater in groups C and E, respectively, than in the control group, A. However the anti-Tn antibody titers were comparable for groups B, D, and F, in which YAF−Tn was used for the vaccine challenge. This observation has been attributed to the fact that the YAF peptide is better displayed on the MHC II as a result of the in vivo formation of an immune complex between the rhamnose epitope in the vaccine and the anti-rhamnose antibodies in the serum. This hypothesis has been further justified by a T-cell proliferation assay, which showed a 10-fold decrease in the amount of antigen needed to stimulate the T cells in the presence of anti-rhamnose antibodies. The vaccine did not induce an unwanted dominant response to rhamnose itself, in agreement with the predicted properties of natural antibodies to antigens such as rhamnose.[49] Overall, the results suggest that rhamnose conjugates can be used to harness endogenous anti-rhamnose antibodies for enhancing the immune response.

Experimental Section

General Methods. All fine chemicals such as L-rhamnose, 4-penten-1-ol, sodium periodate, ruthenium(III) chloride, and dimethyl sulfide as well as anhydrous solvents such as anhydrous methanol were purchased from Acros Organics. Boron trifluoride etherate was obtained from Aldrich. The chemicals were used without further purification. All of the other solvents were obtained from Fisher and used as received, except for dichloromethane, which was dried and distilled following standard procedures.[50] Silica (230−400 mesh) for flash column chromatography was obtained from Sorbent Technologies; precoated thin-layer chromatography (TLC) plates were purchased from EMD. Thin-layer chromatograms (silica gel 60, f_{254}) were visualized under UV light or by charring (5% H_2SO_4 in MeOH). Flash column chromatography was performed on silica gel (230−400 mesh) using solvents as received. ^{1}H NMR spectra were recorded on either a Varian VXRS 400 MHz or INOVA 600 MHz spectrometer in $CDCl_3$ or CD_3OD using residual $CHCl_3$ and CHD_2OH as internal references, respectively. ^{13}C NMR spectra were recorded on a Varian VXRS 100 MHz spectrometer in $CDCl_3$ using the triplet centered at δ 77.3 or CD_3OD using the septet centered at δ 49.0 as an internal reference. High-resolution mass spectrometry (HRMS) was performed on a time of flight (TOF) mass spectrometer. The peptides were synthesized on an Omega 396 synthesizer (Advanced ChemTech, Louisville, KY). Preloaded Fmoc-L-Arg (pbf)-Wang resin and all other Fmoc-L-amino acids were procured from Anaspec (San Jose, CA). Secondary antibodies were obtained from Jackson Immunoresearch Laboratories (West Grove, PA). All mice were obtained from the Jackson Laboratory (Bar Harbor, ME).

Pentenyl 2,3,4-Tri-*O*-acetyl-α-L-rhamnopyranoside (2). To a solution of 1,2,3,4-tetra-*O*-acetylrhamnopyranose (**1**) (1.41 g, 4.07 mmol) in CH_2Cl_2 (2.78 mL) were added 4-penten-1-ol (0.61 mL, 6.11 mmol) and $BF_3 \cdot Et_2O$ (1.54 mL, 12.22 mmol) at 0 °C, and the resulting solution was stirred at ambient temperature under a N_2 atmosphere.[35] The reaction was monitored by TLC and appeared to be complete after 12 h. The reaction mixture was diluted with CH_2Cl_2 (20 mL) and washed with saturated $NaHCO_3$ (20 mL), 10% NaCl (10 mL), and water (25 mL), after which the organic layer was dried (anhydrous Na_2SO_4). Excess solvent was removed under reduced pressure, and the crude material was purified by silica gel flash column chromatography (10.3 × 5.1 cm). Elution with 1:4 EtOAc/hexanes produced **2** as colorless glassy solid with a yield of 0.995 g (69%). R_f = 0.41 (1:4 EtOAc/hexanes). ^{1}H NMR (600 MHz, $CDCl_3$): δ 1.22 (d, 3H, J = 6 Hz, C-5 CH_3), 1.71 (m, 2H, O−CH_2−CH_2), 1.99 (s, 3H, $COCH_3$), 2.05 (s, 3H, $COCH_3$), 2.14 (m, 2H, O−CH_2−CH_2−CH_2), 2.16 (s, 3H, $COCH_3$), 3.44 (m, 1H, O−CH), 3.70 (m, 1H, O−CH), 3.88 (m, 1H, H-5), 4.71 (d, 1H, J = 1.2 Hz, H-1), 4.99 (dq, 1H, J = 1.8, 10.2 Hz, terminal olefinic CH), 5.06 (dq, 1H, J = 1.8, J = 16.5, terminal olefinic CH), 5.06 (t, 1H, J = 10.2 Hz, H-4), 5.24 (dd, 1H, J = 1.8, 3.6 Hz, H-2), 5.31 (dd, 1H, J = 3.6, 6.6 Hz, H-3), 5.81 (m, 1H, olefinic CH). ^{13}C NMR (100 MHz, $CDCl_3$): δ 17.56 (CH_3), 20.92, 20.99, 21.12, 28.61, 30.35, 66.38, 67.55, 69.31, 70.11, 71.31, 97.57 (C-1), 115.25, 137.98, 170.14 (C═O), 170.17 (C═O), 170.33 (C═O). HRMS [M + Na] m/z: calcd for $C_{17}H_{26}NaO_8$, 381.1525; found, 381.1495.

(49) Baumgarth, N.; Tung, J. W.; Herzenberg, L. A. *Springer Semin. Immunopathol.* **2005**, *26*, 347–362.
(50) Armarego, W. L. F.; Chai, C. L. L. *Purification of Laboratory Chemicals*, 5th ed.; Butterworth-Heinemann: Oxford, U.K., 2003.

4-*O*-(2,3,4-Tri-*O*-acetyl-α-L-rhamnopyranosyl)butanoic acid (3). To a solution of **2** (0.1 g, 0.279 mmol) in a 2:2:3 $CH_2Cl_2/CH_3CN/H_2O$ mixture were added $NaIO_4$ (0.24 g, 1.12 mmol) in a single portion and anhydrous $RuCl_3$ (0.001 g, 0.005 mmol), and the resulting solution was stirred at ambient temperature under a N_2 atmosphere for 2 h. Additional $NaIO_4$ (0.24 g, 1.12 mmol) was added, and the resulting solution was stirred at ambient temperature under a N_2 atmosphere.[36] The reaction was monitored by TLC and appeared to be complete after 18 h. The reaction mixture was diluted with CH_2Cl_2 (2.5 mL) and H_2O (3.8 mL), and the product was extracted from the aqueous layer by washing with CH_2Cl_2 (0.5 mL × 4). The organic layers were combined and dried (anhydrous Na_2SO_4). Excess solvent was removed under reduced pressure, and the crude material was purified by silica gel flash column chromatography (13.5 × 3.2 cm). Elution with 1:9:60:130 AcOH/MeOH/EtOAc/hexanes produced **3** as a colorless glassy solid with a yield of 0.103 g (98%). R_f = 0.57 (1:1:9:9 AcOH/MeOH/EtOAc/hexanes). ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.23 (d, 3H, J = 6.4 Hz, C-5 CH_3), 1.98 (t, 2H, J = 6 Hz, O−CH_2−CH_2), 2.00 (s, 3H, $COCH_3$), 2.07 (s, 3H, $COCH_3$), 2.16 (s, 3H, $COCH_3$), 2.46 (t, 2H, J = 7.2 Hz, O−CH_2−CH−CH_2), 3.49 (m, 1H, O−CH), 3.79 (m, 1H, O−CH), 3.87 (m, 1H, H-5), 4.73 (d, 1H, J = 1.6 Hz, H-1), 5.08 (t, 1H, J = 9.6 Hz, H-4), 5.26 (dd, 1H, J = 3.6, 6.4 Hz, H-3). ^{13}C NMR (100 MHz, $CDCl_3$): δ 17.52 (CH_3), 20.86, 20.93, 21.03, 24.59, 24.71, 66.58, 67.09, 69.38, 69.93, 71.14, 97.52 (C-1), 170.22 (C═O), 170.32 (C═O), 178.45 (COOH). HRMS [M + Na] *m*/*z*: calcd for $C_{16}H_{24}NaO_{10}$, 399.1267; found, 399.1248.

Pentenyl α-L-Rhamnopyranoside (4). To a solution of **2** (0.188 g, 0.33 mmol) in MeOH (5 mL) was added metallic Na (0.015 g), and the resulting solution was stirred at ambient temperature under a N_2 atmosphere.[37] The reaction was monitored by TLC and appeared to be complete after 1 h. Excess solvent was removed under reduced pressure, and the crude material was purified by silica gel flash column chromatography (9 × 3 cm). Elution with 2:23 MeOH/CH_2Cl_2 produced **4** as a colorless solid with a yield of 0.118 g (quantitative). R_f = 0.14 (1:19 MeOH/CH_2Cl_2). ^{1}H NMR (600 MHz, $CDCl_3$): δ 1.30 (d, 3H, J = 6.6 Hz, C-5 CH_3), 1.66 (m, 2H, O−CH_2−CH_2), 2.10 (m, 2H, O−CH_2−CH_2−CH_2), 3.40 (m, 1H, O−CH), 3.46 (t, 1H, J = 9.6 Hz, H-4), 3.62 (m, 1H, O−CH), 3.66 (m, 1H, H-5), 3.75 (d, 1H, J = 7.8 Hz, H-3), 3.92 (s, 1H, H-2), 4.46 (br s, 1H, O−H), 4.54 (br s, 1H, O−H), 4.74 (s, 1H, H-1), 4.86 (br s, 1H, O−H), 4.97 (dd, 1H, J = 1.2, 10.2 Hz, terminal olefinic C−H), 5.02 (dd, 1H, J = 1.2, 17.1 Hz, terminal olefinic C−H), 5.79 (m, 1H, olefinic C−H). ^{13}C NMR (100.57 MHz, $CDCl_3$): δ 17.72 (CH_3), 28.73, 30.40, 67.19, 68.26, 71.28, 71.98, 72.95, 99.92 (C-1), 115.25, 138.07. HRMS [M + Na] *m*/*z*: calcd for $C_{11}H_{20}NaO_5$, 255.1208; found, 255.1205.

4-(*O*-α-L-Rhamnopyranosyl)butanal (5). A solution of **4** (0.04 g, 0.17 mmol) in 1:4 MeOH/CH_2Cl_2 (10 mL) was cooled to −70 °C. Oxygen gas was passed through the solution for 10 min. Ozone gas was then bubbled into the solution at −70 °C for 15 min to ensure complete conversion of the starting compound, and this was followed by the passage of O_2 gas for 2 min.[38] The reaction was monitored by TLC. Me_2S (0.2 mL) was added, and the reaction mixture was allowed to warm to ambient temperature. Excess solvent was removed under reduced pressure, and the crude material was purified by silica gel flash column chromatography (11.5 × 1.3 cm). Elution with 2:23 MeOH/CH_2Cl_2 produced **5** as a colorless glassy solid with a yield of 0.035 g (87.5%). R_f = 0.16 (1:9 MeOH/CH_2Cl_2). ^{1}H NMR (600 MHz, CD_3OD): δ 1.26 (d, 3H, J = 6 Hz, C-5 CH_3), 1.64 (m, 2H, O−CH_2−CH_2), 2.16 (m, 2H, O−CH_2−CH_2−CH_2), 3.31 (m, 1H, O−CH), 3.36 (t, 1H, J = 9.6 Hz, H-4), 3.41 (m, 1H, O−CH), 3.57 (m, 1H, H-5), 3.63 (dd, 1H, J = 9.6, 3 Hz, H-3), 3.78 (d, 1H, J = 1.2 Hz, H-2), 4.65 (d, 1H, J = 1.2 Hz, H-1), 9.71 (s, 1H, C*H*O). ^{13}C NMR (100 MHz, CD_3OD): 18.16 (CH_3), 26.12, 34.82, 68.42, 69.91, 72.47, 72.57, 74.12, 101.75 (C-1). HRMS [M + Na] *m*/*z*: calcd for $C_{10}H_{18}NaO_6$, 257.1001; found, 257.1000.

4-(*O*-α-L-Rhamnopyranosyl)butanal−BSA Conjugate (6). Compound **5** (0.005 g, 0.02 mmol) was dissolved in 1 mL of 0.1 M PBS (pH 7.2), and BSA (15 mg) followed by sodium cyanoborohydride (0.005 g, 0.080 mmol) was added, after which the mixture was incubated under gentle agitation at 37 °C for 24 h. After 16 h, additional sodium cyanoborohydride (0.002 g, 0.040 mmol) was added and the incubation continued. The mixture was filtered using dialysis tubing with a molecular weight cutoff value of 10 000 Da with six or seven changes of PBS at 4 °C.[39] The protein concentration in the conjugate was estimated by the Bradford method using BioRad dye reagent.[40] Absorbances were recorded at 595 nm. A standard curve was prepared with concentrations of BSA in the range 0.2−1 mg/mL prepared in 0.1 M PBS (pH 7.2). The absorbance of **6** was recorded following a 10-fold dilution, and the concentration of BSA in **6** was calculated to be 7.27 mg/mL using the standard curve. The concentration of Rha epitope in **6** was determined by the phenol−sulfuric acid method.[41] Absorbances were recorded at 480 nm. A standard curve was prepared with concentrations of **5** in the range 0.05−0.8 mg/mL in doubly distilled water. The absorbance of **6** was recorded after a 2-fold dilution, and the concentration of the Rha epitope in **6** was calculated to be 0.313 mg/mL using the standard curve. The epitope ratio in **6** was calculated to be 11.61.

4-(*O*-α-L-Rhamnopyranosyl)butanal−KLH Conjugate (7). Compound 5 (0.005 g, 0.02 mmol) was dissolved in 1 mL of 0.1 M PBS (pH 7.2), and KLH (15 mg) followed by sodium cyanoborohydride (0.005 g, 0.08 mmol) was added, after which the mixture was incubated under gentle agitation at 37 °C for 24 h. After 16 h, additional sodium cyanoborohydride (0.0025 g, 0.04 mmol) was added and the incubation continued. The mixture was filtered using dialysis tubing with a molecular weight cutoff value of 10 000 Da with six or seven changes of PBS at 4 °C.[39] The protein concentration in the conjugate was estimated by the Bradford method using BioRad dye reagent.[40] Absorbances were recorded at 595 nm. A standard curve was prepared with concentrations of KLH in the range 0.2−1 mg/mL prepared in 0.1 M PBS (pH 7.2). The absorbance of **7** was recorded following a 10-fold dilution, and the concentration of the KLH in **7** was calculated to be 5.51 mg/mL using the standard curve. The concentration of Rha epitope in **7** was determined by the phenol−sulfuric acid method.[41] Absorbances were recorded at 480 nm. A standard curve was prepared with concentrations of **5** in the range 0.05−0.4 mg/mL in doubly distilled water. The absorbance of **7** was recorded after a 2-fold dilution, and the concentration of the Rha epitope in **7** was calculated to be 0.728 mg/mL using the standard curve. The epitope ratio in **7** was calculated to be 259.

4-(*O*-α-L-Rhamnopyranosyl)butanal−OVA Conjugate (8). Compound **5** (0.005 g, 0.02 mmol) was dissolved in 1 mL of 0.1 M PBS (pH 7.2), and OVA (15 mg) followed by sodium cyanoborohydride (0.005 g, 0.08 mmol) was added, after which the mixture was incubated under gentle agitation at 37 °C for 24 h. After 16 h, additional sodium cyanoborohydride (0.0025 g, 0.04 mmol) was added and the incubation continued. The mixture was filtered using dialysis tubing with a molecular weight cutoff value of 10 000 Da with six or seven changes of PBS at 4 °C.[39] The protein concentration in the conjugate was estimated by the Bradford method using BioRad dye reagent.[40] Absorbances were recorded at 595 nm. A standard curve was prepared with concentrations of OVA in the range 0.2−1 mg/mL prepared in 0.1 M PBS (pH 7.2). The absorbance of **8** was recorded following a 10-fold dilution, and the concentration of the OVA in **7** was calculated to be 17.45 mg/mL using the standard curve. The concentration of Rha epitope in **8** was determined by the phenol−sulfuric acid method.[41] Absorbances were recorded at 480 nm. A standard curve was prepared with concentrations of **5** in the range 0.05−0.40 mg/mL in doubly distilled water. The absorbance of **8** was recorded after a 2-fold dilution, and the concentration of the Rha epitope in **8** was calculated to be 1.12 mg/mL using the standard curve. The epitope ratio in **8** was calculated to be 4.5.

3-*O*-(2-Acetamido-2-deoxy-α-D-galactopyranosyl)-L-threonine (11). To a solution of **9** (200 mg, 0.298 mmol) in dry MeOH (10 mL) was added 10% activated Pd/C (133 mg) followed by glacial AcOH (1.0 mL). The mixture was stirred under a H_2 atmosphere for 3 h. The reaction mixture was filtered through Celite, concentrated, and codistilled with toluene to remove excess AcOH.[43] The byproduct was extracted with 1:1 diethyl ether/toluene (3 × 3 mL), and the residue was dried under vacuum overnight. The residue was dissolved in dry MeOH (2 mL), and NaOMe (1 M) was added until the solution became alkaline (pH 8−9). The reaction mixture was stirred at ambient temperature for 2 h and then neutralized with Amberlite H^+ exchange resin, filtered, and concentrated.[44] The residue was then dissolved in deionized water and lyophilized to afford **11** as a white solid with a yield of 0.05 g (52%). $R_f = 0.08$ (4:1:1 *n*-butanol/AcOH/H_2O). The ^{1}H NMR (600 MHz, D_2O) and ^{13}C NMR (100 MHz, D_2O) chemical shifts for **11** correlated with the reported literature values.[45] Electrospray ionization MS [M + Na] *m*/*z*: calcd for $C_{12}H_{22}N_2NaO_8$, 345.3; found, 345.2.

GalNAc-α-*O*-Thr−BSA Conjugate (12). Compound **11** (6 mg, 18.62 μmol) was dissolved in PBS (0.5 mL, pH 7.2). To the solution was added BSA (6 mg) in PBS (1 mL, pH 7.2). The solution was mixed thoroughly, and 0.2% aqueous glutaraldehyde solution (0.41 mL) was added; the mixture was then incubated under gentle agitation at ambient temperature for 1 h. $NaBH_3CN$ in PBS (0.4 mL, pH 7.2) was added to the protein solution, which was then incubated for another 20 min at ambient temperature. The mixture was filtered using dialysis tubing with a molecular weight cutoff value of 10 000 Da with six or seven changes of PBS at 4 °C.[46] The protein concentration in the conjugate was estimated by the Bradford method using BioRad dye reagent.[40] Absorbances were recorded at 595 nm. A standard curve was prepared with concentrations of BSA in the range 0.2−1 mg/mL prepared in 0.1 M PBS (pH 7.2). The absorbance of **12** was recorded following a 10-fold dilution, and the concentration of the BSA in **12** was calculated to be 2.15 mg/mL using the standard curve.

Glycopeptides 16 and 17. The glycopeptides were synthesized by the Fmoc strategy on an Omega 396 synthesizer (Advanced ChemTech, Louisville, KY) using solid-phase chemistry. The peptide synthesis was performed by coupling amino acid esters of HOBt using DIC as the coupling agent. A 6-fold excess of N^{α}-Fmoc amino acid esters of HOBt in *N*-methyl-2-pyrrolidone (NMP) was used in the synthesis. A 1:1 amino acid/DIC ratio was used in all of the coupling reactions. Deprotection of the N^{α}-Fmoc group was accomplished by two treatments with 25% piperidine in DMF, the first for 5 min and the second for 25 min. After the synthesis was completed, the peptides were cleaved from the solid support and deprotected using a modified reagent K cocktail consisting of 88% TFA, 3% thioanisole, 5% ethanedithiol, 2% water, and 2% phenol. The cleavage cocktail (4 mL) was added to the dried peptide resins in a 15 mL glass vial blanketed with nitrogen. Cleavage was carried out for 2.5 h with gentle magnetic stirring. At the end of the cleavage time, the cocktail mixture was filtered on a Quick-Snap column. The filtrate was collected in 20 mL of ice-cold butane ether. The peptides were allowed to precipitate for 1 h at −200 C, centrifuged, and washed twice with ice-cold methyl *tert*-butyl ether. The precipitate was dissolved in 25% acetonitrile and lyophilized to a completely dry powder. The quality of the peptides was analyzed by analytical reversed-phase HPLC and MALDI−TOF MS using a MALDI TOF/TOF mass spectrometer (model 4800 from Applied Biosystems). For compound **16**: [M + H] *m*/*z* calcd for $C_{158}H_{232}N_{39}O_{49}$, 3459.6783; found, 3459.6902. For compound **17**: [M + H] *m*/*z* calcd for $C_{142}H_{210}N_{39}O_{40}$, 3101.5519; found, 3101.5452.

Glycopeptide 18. Compound **16** (0.007 g, 2.02 μmol) was dissolved in 2 mL of dry methanol, and 12 μL of freshly prepared 1 M sodium methoxide was added. Next, the reaction mixture was stirred at ambient temperature under a N_2 atmosphere for 2 h and then neutralized with solid carbon dioxide. The reaction mixture was concentrated and purified by Bio-Gel (P-2, fine 45−90 μm) SEC using deionized water as the solvent. Lyophilization of the elutants afforded **18** as a white powder with a yield of 0.0055 g (85%). HR-MALDI-MS [M + H] *m*/*z*: calcd for $C_{146}H_{220}N_{39}O_{43}$, 3207.614; found, 3207.165.

Glycopeptide 19. Compound **17** (0.0055 g, 1.77 μmol) was dissolved in 2 mL of dry methanol, and 12 μL of freshly prepared 1 M sodium methoxide was added. Next, the reaction mixture was stirred at ambient temperature under a N_2 atmosphere for 2 h and then neutralized with solid carbon dioxide. The reaction mixture was concentrated and purified by Bio-Gel (P-2, fine 45−90 μm) SEC using deionized water as the solvent. Lyophilization of the elutants afforded **19** as a white powder with a yield of 0.0045 g (85%). HR-MALDI-MS [M + H] *m*/*z*: calcd for $C_{136}H_{204}N_{39}O_{37}$, 2975.520; found, 2975.562.

Immunizations. The 24 female BALB/c mice (6−8 weeks old, The Jackson Laboratory) used for the immunological study were divided into six groups A−F containing four mice each. Groups A and B were subcutaneously injected (day 0) with 100 μL of PBS (pH 7.2) and served as the control groups. Groups C and D were subcutaneously injected with a 100 μL equivolume emulsion of Rha−OVA conjugate **8** (prepared in PBS) and TMG, whereas groups E and F were injected subcutaneously with a 100 μL equivolume emulsion of Rha−OVA conjugate **8** (prepared in PBS) and SAS. Groups C and D received 100 μg and groups E and F 75 μg of Rha−OVA. Mice were boosted (day 25) by subcutaneous injections of 100 μL of PBS (groups A and B), a 100 μL emulsion of Rha−OVA/TMG (groups C and D; 50 μg of Rha−OVA per mouse), or 100 μL of Rha−OVA/ICF (groups E and F; 100 μg of Rha−OVA per mouse). The mice were bled on day 36, and the collected sera were tested for anti-Rha antibodies.

ELISA for Measuring Anti-Rha Antibody Titers. The 96-well plates (Immulon 4 HBX) were coated with Rha−BSA conjugate **6** (2 μg/mL) in 0.01 M PBS (pH 7.2) and incubated overnight at 4 °C. The plates were washed five times with PBS containing 0.1% Tween-20. Blocking was achieved by incubating the plates for 1 h at room temperature with BSA in 0.01 M PBS (1 mg/mL). The plates were then washed five times and incubated for 1 h with serum dilutions in PBS. Unconjugated antibody in the serum was removed by washing, and the plates were incubated for 1 h at room temperature with HPR goat anti-mouse IgG + IgM (Jackson Immunoresearch Laboratories) diluted 2000 times in PBS/BSA. The plates were washed, and 3,3′,5,5′-tetramethylbenzidine (TMB) one-component HRP microwell substrate (Bio FX, Owings Mills, MD) was added and allowed to react for 10 min. Absorbances were recorded at 620 nm and plotted against $\log_{10}$[1/serum dilution].

ELISA for Comparing the Anti-Rha Antibody Attachment Capacities of Rha−BSA and Rha−KLH. Half of a 96-well plate (Immulon 4 HBX) was coated with Rha−BSA conjugate **6** (2 μg/mL) in 0.01 M PBS (pH 7.2), and the other half was coated with Rha−KLH conjugate **7** (2 μg/mL) in 0.01 M PBS (pH 7.2), after which the plate was incubated overnight at 4 °C. The ELISA was then performed as described above.

Vaccinations. Vaccine challenge was performed on day 52. Two separate emulsions were prepared by mixing 1.1 mg of glycopeptide **18** (Rha−YAF−Tn) in 400 μL of 0.01 M PBS (pH 7.2) with 400 μL of CFA (Rha-Vacc) and 1.1 mg of glycopeptide **19** (YAF−Tn) in 400 μL of 0.01 M PBS (pH 7.2) with 400 μL of CFA (Vacc). Groups A, C, and E were challenged with Rha-Vacc (50 μL emulsion subcutaneous injections containing 80 μg of Rha−YAF−Tn per mouse). Groups B, D, and F were challenged with Vacc (50 μL emulsion injections containing 80 μg of YAF−Tn per mouse). Mice were boosted with the challenge on day 81. Groups A, C, and E were subcutaneously injected with 50 μL emulsions of Rha−YAF−Tn/ICF (80 μg of Rha−YAF−Tn per mouse), whereas groups B, D, and F were subcutaneously injected with 50 μL emulsions of YAF−Tn/ICF (80 μg YAF−Tn per mouse). Mice were bled on day 91, and the collected sera were tested for anti-Tn antibodies.

ELISA for Measuring Anti-Tn Antibody Titers. The 96-well plates (Immulon 4 HBX) were coated with Tn−BSA conjugate **12** (2 μg/mL) in 0.01 M PBS (pH 7.2) and incubated overnight at 4 °C. The ELISA was continued as described above.

ELISA for Competitive Binding with Free Tn. A 96-well plate (Immulon 4 HBX) was coated with Tn−BSA conjugate **12** (2 μg/mL) in 0.01 M PBS (pH 7.2) and incubated overnight at 4 °C. The plate was washed five times with PBS containing 0.1% Tween-20. Blocking was achieved by incubating the plate for 1 h at room temperature with BSA in 0.01 M PBS (1 mg/mL). The plate was then washed five times and incubated for 1 h with 1/100 serum dilutions in PBS without free Tn (compound **11**) or with prior mixing with **11** at a concentrations of 10^{-5}, 10^{-4}, or 10^{-3} M in 0.01 M PBS (pH 7.2). Unconjugated antibody in the serum was removed by washing, and the plate was incubated for 1 h at room temperature with HPR goat anti-mouse IgG + IgM (secondary antibody) diluted 2000 times in PBS/BSA or HPR goat anti-mouse IgG diluted 1000 times in PBS/BSA. The plate was washed, and TMB one-component HRP microwell substrate was added and allowed to react for 10 min. Absorbances were recorded at 620 nm and plotted against $\log_{10}$[1/free Tn concentration].

T-Cell Proliferation Study. Immunization. Two female BALB/c mice (The Jackson Laboratory) were used for this experiment. Mouse 1 was immunized (day 0) by a subcutaneous injection of a 100 μL emulsion of YAF−Tn/CFA (12.8 μg), and mouse 2 was injected subcutaneously with a 100 μL emulsion of Rha−YAF−Tn/CFA (12.8 μg).

Preparation of Anti-Rha Antibodies. The PBS and Rha−OVA-immunized mice were bled on day 36 of the previous experiment, and the sera were pooled. Antibody fractions from each pool were prepared by precipitation at 40% saturation of ammonium sulfate. The mixtures were incubated overnight, centrifuged at 10000*g* for 10 min, and resuspended in 0.5 mL of water. The antibody solutions were concentrated and the buffer was changed twice with PBS using an Ultrafree 0.5 centrifugal filter device (Millipore, Billerica, MA) having a molecular cutoff of 50 000 D. Absorbances of the antibody solutions were recorded at 280 nm for calculation of the concentrations, and the normal and anti-Rha antibody solutions were diluted to 0.5 mg/mL.

Preparation of Spleen Cell Suspensions and Assay Setup. On day 7, the mice were sacrificed and the spleens removed and placed separately into freshly prepared spleen cell culture medium (5 mL). Single cell suspensions were prepared using two separate sterile glass homogenizers. The cells were washed three times with culture medium and brought to a concentration of 2.5×10^6 cells/mL. Aliquots (100 μL) of the spleen cell suspensions were added to 96-well plates (2.5×10^5 cells per well), and then normal serum antibodies or anti-Rha serum antibodies (5 μg per well) were added. Control wells were deprived of the antibody treatment. Next, vaccine solutions (Rha−YAF−Tn or YAF−Tn) prepared in 0.01 M PBS (pH 7.2) were added to the wells in the concentration range 0.01−10 μM. The plates were incubated at 37 °C for 5 days. On day 5, the cells were pulsed with [^{3}H]thymidine (40 μCi/mL, 25 μL per well) and incubated overnight at 37 °C. The cells were harvested on glass-fiber filters, and incorporation was determined by measurements on a Top Count scintillation counter (Packard, Downers Grove, IL).

Acknowledgment. This work was supported in part by the University of Toledo deArce Memorial Endowment Fund (to S.J.S.), the Elsa U. Pardee Foundation (to S.J.S.), the Ohio Cancer Research Associates (to S.J.S.), and a University of Toledo Interdisciplinary Research Initiation Award (to S.J.S. and K.A.W.).

Supporting Information Available: Figures of anti-rhamnose antibody titers in nonimmunized C57BL/6 and Swiss Webster mouse serum; individual mouse anti-Rha−BSA antibody titers for groups A−F; copies of ^{1}H and ^{13}C NMR spectra of compounds **2**, **3**, **4**, **5**, and **11**; and MALDI−TOF spectra of glycopeptides **16**, **17**, **18**, and **19**. This material is available free of charge via the Internet at http://pubs.acs.org.

JA107029Z