ACS Chemical Biology

Articles

pubs.acs.org/acschemicalbiology

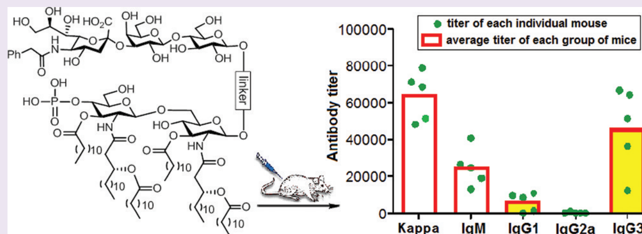
Carbohydrate-Monophosphoryl Lipid A Conjugates Are Fully Synthetic Self-Adjuvanting Cancer Vaccines Eliciting Robust Immune Responses in the Mouse

Qianli Wang, Zhifang Zhou, Shouchu Tang, and Zhongwu Guo*

Department of Chemistry, Wayne State University, 5101 Cass Avenue, Detroit, Michigan 48202, United States

S Supporting Information

ABSTRACT: Tumor-associated carbohydrate antigens (TACAs) are useful targets in the development of therapeutic cancer vaccines. However, a serious problem with them is the poor immunogenicity. To overcome the problem, a monophosphorylated derivative of *Neisseria meningitidis* lipid A was explored as a potential carrier molecule and built-in adjuvant for the construction of structurally defined fully synthetic glycoconjugate vaccines. Some paradigm-shifting discoveries about the monophosphoryl lipid A (MPLA)-TACA conjugates were that they elicited robust IgG antibody responses, indicating T cell-mediated immunity, without an external adjuvant and that an external adjuvant, *e.g.*, Titermax Gold, actually reduced rather than promoted the immunological activity of the conjugates. The induced antibodies were proved to bind selectively to target tumor cells. MPLA was therefore demonstrated to be a powerful built-in immunostimulant and adjuvant for an all new design of fully synthetic glycoconjugate cancer vaccines.



The abundant occurrence and availability of tumor-associated carbohydrate antigens (TACAs) on the cancer cell surface make them easily recognizable targets for the human immune system. This combined with the substantial structural conservativeness of cell surface carbohydrates and the important roles they play in various oncological processes renders TACAs ideal templates for the design and development of therapeutic cancer vaccines or cancer immunotherapies.[1,2] However, carbohydrates are usually poorly immunogenic, and therefore TACAs alone typically cannot elicit robust immune responses, in particular T cell-mediated immune responses, necessary for effective cancer immunotherapy.[3] To create TACA-based cancer vaccines that can elicit T cell-mediated immunity, which is linked to antibody isotype switching from IgM to IgG, antibody affinity maturation, and immunological memorization, semisynthetic glycoconjugate vaccines generated by covalent coupling of TACAs to carrier proteins have been extensively explored.[4,5] Despite the fact that in many cases coupling carbohydrates to a protein can indeed convert them from T cell-independent to T cell-dependent antigens and that significant progress has been achieved in developing carbohydrate-based bacterial vaccines by this strategy,[6] there is still no clinically functional carbohydrate-based cancer vaccine established yet. As a result, new vaccine strategies are desirable. In this context, glycoconjugate vaccines derived from chemically modified TACAs were explored in combination with metabolic engineering of cancer cell surface glycans.[7−9] Recently, fully synthetic glycoconjugate vaccines with TACAs coupled to small molecule carriers, including immunostimulants and other immunologically active epitopes, have attracted significant attention.[10−13] In contrast to glycoproteins, these glycoconjugates possess homogeneous and well-defined structures, which will facilitate their characterization and quality control. Moreover, fully synthetic glycoconjugates have definite and easily reproducible structures and chemical and biological properties to enable detailed structure−activity relationship (SAR) analysis and various immunological and clinical studies.

The goal of this work is to establish a novel strategy for the development of fully synthetic cancer vaccines. For this purpose, we are interested in glycoconjugates having TACAs coupled to a monophosphoryl lipid A (MPLA). MPLA is the 1-*O*-dephosphorylated form of lipid A, which is the most immunologically active portion of lipopolysaccharide (LPS), one of the most abundant and biologically active constituents of Gram-negative bacteria cell walls.[14] MPLAs are strong immunostimulants and vaccine adjuvants.[15−17] The immunostimulatory activity of MPLAs is attributed to their ability to provoke cytokine cascades through interacting with toll-like receptor 4 (TLR4),[18] which can stimulate antigen presentation,[19,20] activate T-helper (Th) cells, and thereby elicit T cell-mediated immunity. We envisioned that MPLA may be used as both a carrier molecule and a built-in adjuvant to form conjugate vaccines that can have multiple advantages.

RESULTS AND DISCUSSION

Immunological Studies of the GM3-MPLA Conjugate. To probe the above concept, *Neisseria meningitidis*

Received: February 7, 2011
Accepted: October 20, 2011
Published: October 20, 2011

 © 2011 American Chemical Society

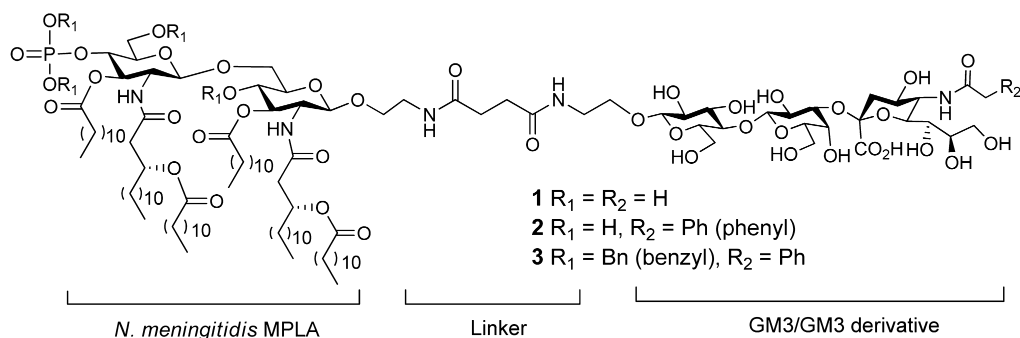


Figure 1. Structures of the synthetic glycoconjugate vaccines **1**−**3**.

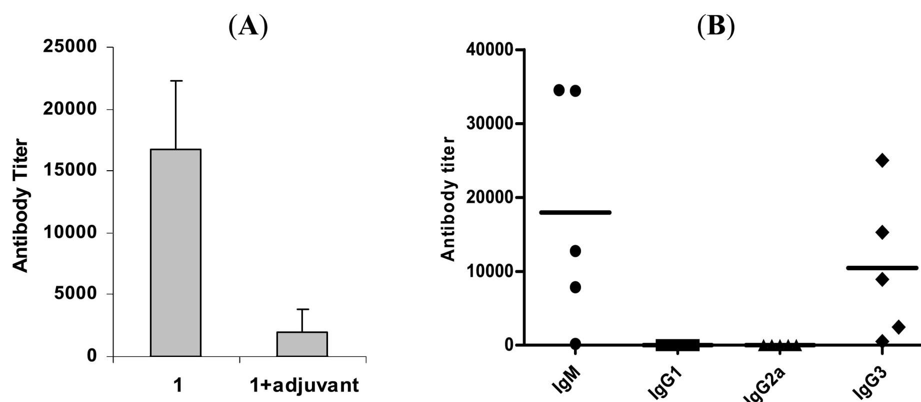


Figure 2. ELISA results of GM3-specific antibodies in the antisera of mice immunized with **1**. (A) GM3-specific total antibodies in the day 38 antisera of mice immunized with **1** alone or with **1** plus the external adjuvant Titermax Gold. Each column represents the antibody level in the serum pooled from a group of five mice. (B) Titers of different isotypes of GM3-specific antibodies in the day 38 antisera of mice immunized with **1**. Each dot shows the result of an individual mouse, and the black bar represents the average antibody level of each group of five mice.

MPLA was synthesized and coupled to GM3,[21] a TACA richly expressed by human melanoma and other tumors.[22,23] The immunological properties of the resulting glycoconjugate **1** (Figure 1) were assessed in the C57BL/6 mouse. For immunological studies, **1** was incorporated into a phospholipid-based liposome, since liposomes were expected to improve the solubility and the immunogenicity of glycolipids.[10,24] The liposome of **1** was prepared by sonication of a mixture of 1,2-distearoyl-*sn*-glycero-3-phosphocholine, cholesterol, and **1**. Then, the liposome of **1** was used to inoculate a group of five C57BL/6 mice by subcutaneous injection, and each mouse was inoculated four times on days 1, 14, 21, and 28 using 0.1 mL of liposome containing 15 μg of GM3. To study the influence of an external adjuvant on the activity of **1**, an emulsion of the liposome of **1** and Titermax Gold was administered to another group of five mice using the same vaccination scheme. The elicited immune responses in mice were assessed by enzyme-linked immunosorbent assay (ELISA) for GM3-specific antibodies, including total antibody and various antibody isotypes such as IgM, IgG1, IgG2a, and IgG3, in the day 27 and day 38 antisera. The human serum albumin (HSA) conjugate of GM3[7] was employed as the capture antigen in ELISA. Since the linkers and carriers in **1** were different from those in the GM3-HSA conjugate, ELISA experiments detected only antibodies specific for GM3. Antibody titers were determined by linear regression analysis of plots of the optical density (OD) values against the serum dilution numbers and defined as the dilution number yielding an OD value of 0.2.

Using the pooled antisera of all five immunized mice, we revealed that **1** alone provoked a strong GM3-specific immune response (Figure 2A). Furthermore, the antibody titers of the day 38 antisera were significantly higher than that of the day 27 antisera (see Supporting Information), reflecting the general trend of enhanced immune responses to recurring exposure to the same antigen. Further analysis of the antibody isotypes of the day 38 antiserum of individual mouse showed that **1** induced not only GM3-specific IgM antibody but also a high level of IgG3 antibody (Figure 2B), which is a typical anticarbohydrate response[25,26] and is consistent with a T cell-mediated immunity.[27,28] Since GM3 itself, including its protein conjugates,[4] is poorly immunogenic and elicits almost exclusively IgM antibodies, the immunological results of **1** suggest that MPLA as a carrier molecule can effectively improve the immunogenicity of GM3 and help elicit T cell-dependent immunity. In addition, because effective vaccination typically requires a vaccine adjuvant[29] but no external adjuvant was utilized in the immunization with **1**, MPLA can also act as a built-in adjuvant.

More interestingly, we found that mice immunized with **1** plus an external adjuvant, Titermax Gold, showed only very weak immune response (Figure 2A), indicating an inhibitory impact of the external adjuvant on the immunological activity of **1**. This result is contradictory to the general concept that immunological adjuvants enhance immune response to vaccines.

Immunological Studies of *N*-Phenylacetyl GM3 (GM3NPhAc)-MPLA Conjugate. To further explore MPLA-based glycoconjugate vaccines, we examined the

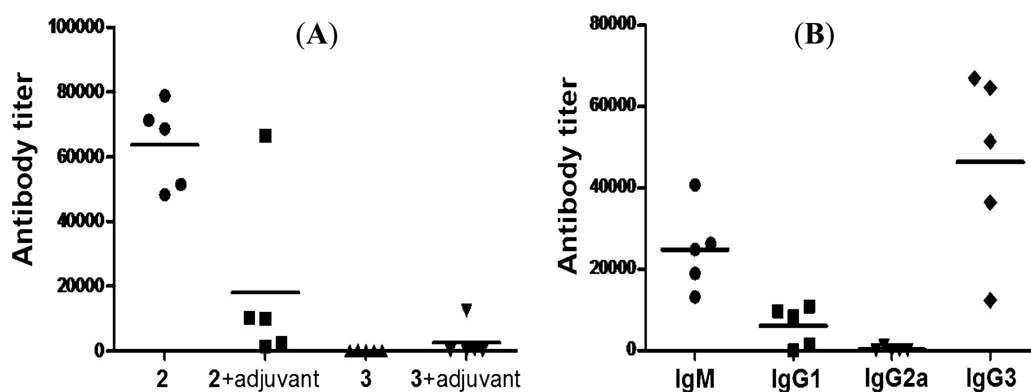


Figure 3. ELISA results of GM3NPhAc-specific antibodies in the antisera of mice immunized with **2** and **3**. (A) GM3NPhAc-specific total antibodies in the day 38 antisera of mice immunized with **2** alone, **2** + Titermax Gold, **3** alone, or **3** + Titermax Gold. (B) Titers of different isotypes of GM3NPhAc-specific antibodies in the day 38 antisera of mice immunized with **2** alone. Each dot shows the result of an individual mouse, and the black bar represents the average antibody titer of each group of five mice.

immunological properties of **2** (Figure 1), a conjugate having MPLA coupled to a synthetic GM3 derivative, GM3NPhAc, which was examined as a potential vaccine candidate for glycoengineered cancer immunotherapy.[7,9] Immunization of mice with **2** or **2** plus Titermax Gold followed the same protocol as described above, and the antiserum of each mouse was individually evaluated. The results (Figure 3) showed that **2** elicited a strong immune response, with the total antibody titer (Figure 3A) 3.8 times higher than that of **1** (63710 *vs* 16780), which is consistent with our previous discovery that GM3NPhAc was more immunogenic than GM3.[7] ELISA analysis of antibody isotypes of the day 38 antisera (Figure 3B) revealed that the increase of total antibody titer was mainly owing to the increase of IgG3 antibody titer (from 10435 to 46373), while the IgM antibody level remained similar to that of **1**. Meanwhile, a significant level of IgG1 antibodies was also produced. These results further demonstrated that MPLA is a powerful vaccine carrier molecule and a functional built-in adjuvant to augment the immunogenicity of carbohydrate antigens.

As discussed above, because the linkers and carriers in **2** and GM3NPhAc-HSA were different, the ELISA experiments should detect only antibodies specific for GM3NPhAc. To verify that the observed antibody titers were indeed generated by GM3NPhAc-specific antibodies, we performed a competitive ELISA study, for which the experimental protocols were the same as for regular ELISA except for the use of free GM3NPhAc to compete with GM3NPhAc-HSA attached to the ELISA plate. The results (shown in Figure 4) clearly indicated that high concentrations (>133 μM) of free GM3NPhAc could nearly completely inhibit antibody binding to GM3NPhAc-HSA on the ELISA plate, thereby proving that the antibody titers shown in Figure 3 did reflect the levels of GM3NPhAc-specific antibodies in the mouse antisera.

Again, we found that immunization of mice with **2** plus Titermax Gold elicited a remarkably weaker antigen-specific immune response (Figure 2A) than **2** alone, which suggested an inhibitory impact of the external adjuvant on the immunological activity of **2**. Literature results about the structure–activity relationships of lipid A and MPLA disclosed that the phosphate group linked to the 4′-*O*-position of their nonreducing end GlcNAc residue is critical for their immunostimulant activity.[17,30] Accordingly, we propose that certain component(s) of the external adjuvant may interact

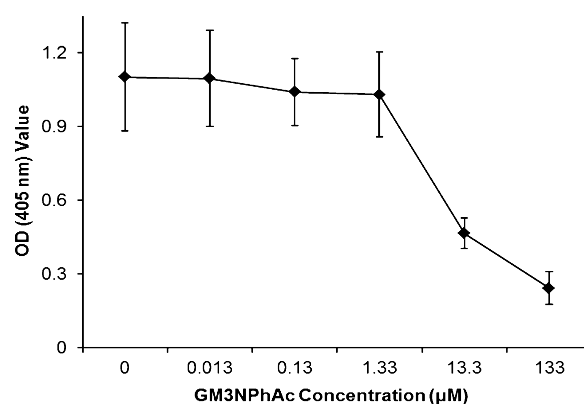


Figure 4. Results of the competitive ELISA experiment of pooled mouse antiserum derived from glycoconjugate **2** alone. The experimental protocols were the same as for regular ELISA except for the use of various concentrations of free GM3NPhAc (0, 0.013, 0.13, 1.33, 13.3, and 133 μM) to compete with GM3NPhAc-HSA on the ELISA plate for antibody binding. The secondary antibody used in this study was alkaline phosphatase linked goat anti-mouse kappa antibody. Thus, OD (405 nm) values reflect the amounts of total antibodies bond to the GM3NPhAc-HSA-coated ELISA plate in the presence of various concentrations of GM3NPhAc.

with the free phosphate, and potentially the hydroxyl groups as well, of the MPLA moiety in **1** and **2** to affect MPLA binding to TLR4 and the elicitation of immune responses.

To study this hypothesis, we examined the immunological properties of a glycoconjugate **3** with the phosphate and the hydroxyl groups of MPLA protected by benzyl (Bn) groups. Neither **3** alone nor **3** plus Titermax Gold could elicit any significant immune response in the mouse (Figure 3A). These results unambiguously proved that the increased immunogenicity of TACAs in the MPLA conjugates was indeed attributed to the free form of MPLA. Therefore, the free phosphate moiety and potentially the free hydroxyl groups of MPLA are essential for the immunostimulant activity of MPLA and the high immunogenicity of the resultant glycoconjugates.

Studies of Linker Impact on the Immunological Properties of MPLA Conjugates. Because it was reported that linkers in synthetic glycoconjugate vaccines may have a major impact on the immunological activities,[31] we prepared conjugate **4** (Figure 5A), having GM3NPhAc coupled to MPLA through a triazolyl moiety,[32] and compared it with **2** for

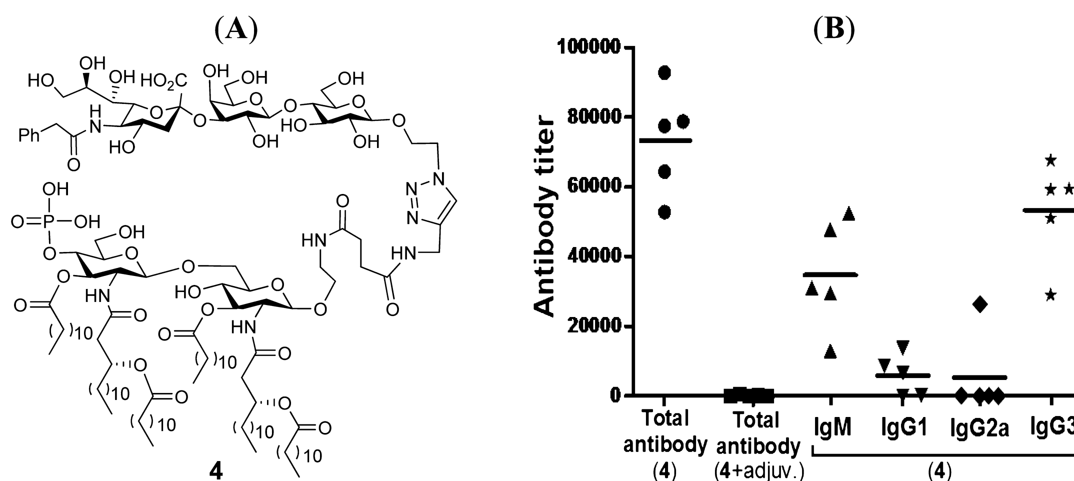


Figure 5. Structure of conjugate **4** and its immunological results. (A) The structure of glycoconjugate **4**. (B) ELISA results of GM3NPhAc-specific total antibody titers in the day 38 antisera of mice immunized with **4** or **4** + Titermax Gold and the titers of GM3NPhAc-specific IgM, IgG1, IgG2a, and IgG3 antibodies in the antisera of mice immunized with **4** alone. Each dot shows the result of an individual mouse, and the black bar represents the average antibody level of each group of five mice.

investigation of the influence of linkers on the immunological properties of MPLA conjugates. The only structural difference between **2** and **4** is their linker region. Similar to **2**, glycoconjugate **4** alone elicited a high titer of GM3NPhAc-specific IgG3 antibody (Figure 5B), in addition to IgM antibody, which suggested that probably neither the amide linker in **2** nor the triazolyl linker in **4** had a substantial influence on the immunological properties of MPLA conjugates. This agrees with our previous report.[33] Therefore, both linkers may be safely utilized for the construction of fully synthetic MPLA-based conjugate vaccines. The results also support our conclusion that the increased immunogenicity of GM3NPhAc in MPLA conjugates is due to the immunostimulatory property of MPLA. In addition, we have verified again that Titermax Gold had a suppressive effect on the immunological activity of MPLA conjugate **4** (Figure 5B).

Binding Studies of the Mouse Antisera to Target Cancer Cells. The capability of antibodies induced by **2** to recognize and bind to target cancer cells was studied by fluorescence-activated cell sorting (FACS) technology. The cancer cell line employed in these studies was SKMEL-28, a human melanoma cell line, which was shown to express GM3 as a major TACA[34] and which could be effectively metabolically glycoengineered to express GM3NPhAc on the treatment with *N*-phenylacetyl-D-mannosamine (ManNPhAc).[35] In these studies, SKMEL-28 cells were first treated with 0 or 100 μg/mL of ManNPhAc for 3 days and then incubated with either normal mouse sera or antisera obtained from mice immunized with **2**. Thereafter, the tumor cells were cultured with fluorescein isothiocyanate (FITC)-labeled goat anti-mouse kappa antibody, followed by FACS analysis. Figure 6A shows the FACS results concerning the binding of normal mouse sera and antisera obtained from **2**-immunized mice to ManNPhAc-treated cancer cells. Clearly, the antisera, but not the normal mouse sera, had strong binding to the metabolically glycoengineered cancer cells. Figure 6B shows the results about the binding of antisera obtained from **2**-immunized mice to wild-type and ManNPhAc-treated cancer cells. It was evident that the antisera had strong binding to the metabolically glycoengineered cancer cells but not to the wild-type of cancer cells. These results have demonstrated that antibodies induced by **2** were specific to GM3NPhAc and that the GM3NPhAc-specific antibodies could selectively recognize, target, and interact with cancer cells that were metabolically glycoengineered to express GM3NPhAc. In the meanwhile, we have also observed that the mouse antisera obtained with glycoconjugate **1** could selectively bind to SKMEL-28 (see Supporting Information for details).

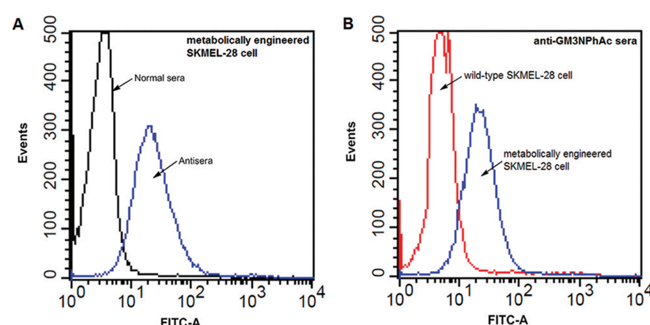


Figure 6. FACS assays of the binding between SKMEL-28 cancer cells and mouse sera. (A) Binding of normal mouse sera (black) and anti-GM3NPhAc mouse sera derived from conjugate **2** (blue) to metabolically glycoengineered SKMEL-28 cancer cells, *i.e.*, cells treated with 100 μg/mL of ManNPhAc. (B) Binding of anti-GM3NPhAc mouse sera to wild-type SKMEL-28 cancer cells (red) and metabolically glycoengineered SKMEL-28 cancer cells (blue).

Conclusion. In summary, *N. meningitidis* MPLA has been shown to possess compelling immunostimulant and adjuvant activities as a carrier molecule for the creation of potent, fully synthetic TACA-based cancer vaccines. An important discovery about the MPLA-TACA conjugates is that they elicited strong IgG antibody responses without the use of an external adjuvant. Moreover, MPLA-TACA conjugates elicited mainly IgG3 antibody, which is a carbohydrate-specific immune response,[25,26] whereas glycoproteins typically elicit IgG1 antibodies. Consequently, MPLA conjugates and glycoproteins may function *via* different mechanisms, which can be an interesting research topic in immunology. It was established with glycoprotein vaccines that, following antigen processing, the carrier protein can provide peptides that would interact noncovalently with class II MHC molecules for the antigen

presentation to Th cells and provoke the switch of antibody production from IgM to IgG1. For MPLA conjugates, however, GM3 may generate some highly multivalent forms through lipid−lipid interactions, which tend to elicit IgG3 antibodies. In general, because of positive cooperativity, self-associating IgG3 antibodies can bind more effectively to multivalent forms of carbohydrate antigens than variable domain-identical IgG1 antibodies, which may have a positive impact on the selectivity of the antibodies for cancer cells. Another important discovery is that Titermax Gold, an adjuvant that is commonly utilized to potentiate glycoprotein vaccines, reduced the immunological activity of MPLA conjugates. We proposed and had demonstrated in part using a glycoconjugate **3** containing a protected form of MPLA that Titermax Gold might interfere with the free phosphate group, and potentially the free hydroxyl groups as well, of the MPLA moiety to affect its interaction with the immune system. However, Titermax Gold may interact with glycoproteins as well, albeit these interactions typically result in positive adjuvant effects. Therefore, MPLA and protein conjugates must involve different immunological pathways. We have also examined the immunization of mice using pure glycoconjugate **2**, instead of its liposomes. However, these experiments yielded inconsistent results, because the glycoconjugate was nearly insoluble in buffer and it was difficult to get homogeneous preparations and control the administered doses. Therefore, liposomes seem to be an ideal carrier for glycolipid conjugate vaccines. It is noteworthy that this work has verified an all new construct of fully synthetic glycoconjugate cancer vaccines that possess interesting and beneficial properties. The MPLA conjugates described herewith can also be useful tools for the investigation of adjuvant effects and other related immunological issues.

■ METHODS

Materials, Reagents, and Animals. Glycoconjugates **1**, **2**, **3**,[21] and **4**,[32] HSA-GM3 and GM3NPhAc conjugates,[7] and ManNPhAc[35] were synthesized according to the procedures previously reported by our laboratory (for more details see Supporting Information). Titermax Gold adjuvant, 1,2-distearoyl-*sn*-glycero-3-phosphocholine, and cholesterol were purchased from Sigma-Aldrich. The SKMEL-28 cancer cell line, Dulbecco's Modified Eagle's Medium (DMEM) for cell culture, and fetal bovine serum (FBS) were purchased from American Type Culture Collection. Penicillin-streptomycin and trypsin-EDTA were purchased from Invitrogen. Alkaline phosphatase linked goat anti-mouse kappa, IgM, IgG1, IgG2a, and IgG3 antibodies and FITC-labeled goat anti-mouse kappa antibody were purchased from Southern Biotechnology. Female C57BL/6 mice of 6−8 weeks age used for immunological studies were purchased from The Jackson Laboratory.

General Procedure for the Preparation of Liposomes of Glycoconjugates 1−4. The mixture of a specific MPLA conjugate **1−4** (0.474 μmol), 1,2-distearoyl-*sn*-glycero-3-phosphocholine (2.44 mg, 3.08 μmol), and cholesterol (0.92 mg, 2.37 μmol) (a 10:65:50 molar ratio) was dissolved in CH_2Cl_2 and MeOH (1:1, v/v, 2 mL) in a 10 mL round-bottomed flask. Then, the solvents were removed in vacuum to form a thin lipid film on the flask wall, which was hydrated by adding 2.0 mL of HEPES buffer (20 mM, pH 7.5) containing NaCl (150 mM) and shaking the mixture under an argon atmosphere at 40 °C for 1 h. The milky suspension was finally sonicated for 1 min to obtain the desired liposomes.

Immunization of Mouse. Each group of five female C57BL/6 mice were immunized on day 1 by subcutaneous (sc) injection of 0.1 mL of the liposomal solution of a specific glycoconjugate **1−4** containing 15 μg of the carbohydrate antigen or by injection of an emulsion of the liposomal solution of a specific glycoconjugate vaccine and Titermax Gold adjuvant prepared according to the manufacturer's protocol (each 2 mL of the liposomal solution mixed with 1 mL of Titermax Gold adjuvant). Following the initial immunization, mice were boosted 3 times on day 14, day 21 and day 28 by sc injection of the same conjugate and by the same immunization protocol. Blood samples of each mouse were collected through the tail veins prior to the initial immunization on day 1 and after immunization on day 27 and day 38 and were clotted to obtain antisera that were stored at −80 °C before use.

Protocols for ELISA. ELISA plates were treated with 100 μL of a solution of GM3-HSA or GM3NPhAc-HSA conjugate (2 μg/mL) dissolved in coating buffer (0.1 M bicarbonate, pH 9.6) at 37 °C for 1 h, which was followed by treatment with blocking buffer and washing 3 times with phosphate-buffered saline (PBS) containing 0.05% Tween-20 (PBST). Thereafter, a pooled or an individual mouse antiserum with serial half-log dilutions from 1:300 to 1:656100 in PBS was added to the coated ELISA plates (100 μL/well), which was followed by incubation at 37 °C for 2 h. The plates were then washed with PBS and incubated at rt for another 1 h with a 1:1000 diluted solution of alkaline phosphatase linked goat anti-mouse kappa, IgM or IgG2a antibody or with a 1:2000 diluted solution of alkaline phosphatase linked goat anti-mouse IgG1 or IgG3 antibody (100 μL/well), respectively. Finally, these plates were washed with PBS and developed with 100 μL of *p*-nitrophenylphosphate (PNPP) solution (1.67 mg/mL in buffer) for 30 min at rt, followed by colorimetric readout using a BioRad 550 plate reader at 405 nm wavelength. For titer analysis, optical density (OD) values were plotted against antiserum dilution values, and a best-fit line was obtained. The equation of this line was employed to calculate the dilution value at which an OD of 0.2 was achieved, and the antibody titer was calculated at the inverse of the dilution value.

Protocols for Competitive ELISA. The protocols for this experiment were the same as for regular ELISA described above except that free GM3NPhAc was utilized to compete with plate-bound GM3NPhAc-HSA for antibody binding. Therefore, after an ELISA plate was treated with GM3NPhAc-HSA and the blocking solution sequentially and then washed with PBS, 100 μL of the pooled anti-GM3NPhAc serum (1:300 dilution) in PBS containing 0, 0.013, 0.13, 1.33, 13.3, and 133 μM of free GM3NPhAc was added to each well of the ELISA plate. Thereafter, the plate was incubated, washed, treated with alkaline phosphatase linked goat anti-mouse kappa antibody (1:1000 dilution), washed, developed with PNPP, and finally subjected to colorimetric readout at 405 nm wavelength, as described above.

Protocols for FACS Assay. After SKMEL-28 cells were incubated in DMEM containing 0 or 100 μg/mL of ManNPhAc at 37 °C for 3 d, these cells were treated with trypsin-EDTA and then harvested and counted. Cells ($\sim 1.0 \times 10^6$) were washed with FACS buffer (PBS containing 1% FBS and 0.1% NaN_3) twice and incubated with 100 μL of normal mouse sera (mouse sera collected at day 1) or anti-GM3PhAc mouse sera (1:10 dilution in FACS buffer) at 4 °C for 30 min. Cells were washed again with 1000 μL of FACS buffer twice and then incubated with FITC-linked goat anti-mouse kappa antibody (2.5 μg in 50 μL FACS buffer) at 4 °C for 30 min. Finally, cells were washed twice with 1000 μL of FACS buffer, suspended in 0.5 mL of FACS buffer, and then subjected to FACS analysis on a Becton Dickinson FACS scan flow cytometer.

■ ASSOCIATED CONTENT

Supporting Information

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

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: zwguo@chem.wayne.edu.

dx.doi.org/10.1021/cb200358r | ACS Chem. Biol. 2012, 7, 235−240

■ ACKNOWLEDGMENTS

This work was supported by a research grant from NIH/NCI (R01 CA95142). We thank N. Greenspan and C. Harding for helpful discussions about the manuscript.

■ REFERENCES

(1) Hakomori, S. (2001) Tumor-associated carbohydrate antigens defining tumor malignancy: Basis for development of anti-cancer vaccines. *Adv. Exp. Med. Biol. 491*, 369–402.

(2) Bitton, R. J., Guthmann, M. D., Babri, M. R., Carnero, A. J. L., Alonso, D. F., Fainboim, L., and Gomez, D. E. (2002) Cancer vaccines: An update with special focus on ganglioside antigens (Review). *Oncol. Rep. 9*, 267–276.

(3) Kuberan, B., and Lindhardt, R. J. (2000) Carbohydrate based vaccines. *Curr. Org. Chem. 4*, 653–677.

(4) Livingston, P. O. (1995) Approaches to augmenting the immunogenicity of melanoma gangliosides: from the whole melanoma cells to ganglioside-KLH conjugate vaccines. *Immunol. Rev. 145*, 147–156.

(5) Danishefsky, S. J., and Allen, J. R. (2000) From the laboratory to the clinic: A retrospective on fully synthetic carbohydrate-based anticancer vaccines. *Angew. Chem., Int. Ed. 39*, 837–863.

(6) Pon, R. A., Jennings, H. J. (2009) Carbohydrate-based antibacterial vaccines, In *Carbohydrate-Based Vaccines and Immunotherapies* (Guo, Z., Boons, G.-J., Eds.), pp 117–166, John Wiley & Sons, Inc., Hoboken.

(7) Pan, Y. B., Chefalo, P., Nagy, N., Harding, C. V., and Guo, Z. (2005) Synthesis and immunological properties of N-modified GM3 antigens as therapeutic cancer vaccines. *J. Med. Chem. 48*, 875–883.

(8) Wu, J., and Guo, Z. (2006) Improving the antigenicity of sTn antigen by modification of its sialic acid residue for development of glycoconjugate cancer vaccines. *Bioconjugate Chem. 17*, 1537–1544.

(9) Wang, Q., Zhang, J., and Guo, Z. (2007) Efficient glycoengineering of GM3 on melanoma cell and monoclonal antibody-mediated selective killing of the glycoengineered cancer cell. *Bioorg. Med. Chem. 15*, 7561–7567.

(10) Buskas, T., Ingale, S., and Boons, G. J. (2005) Towards a fully synthetic carbohydrate-based anticancer vaccine: Synthesis and immunological evaluation of a lipidated glycopeptide containing the tumor-associated Tn antigen. *Angew. Chem., Int. Ed. 44*, 5985–5988.

(11) Renaudet, O., BenMohamed, L., Dasgupta, G., Bettahi, I., and Dumy, P. (2008) Towards a self-adjuvanting multivalent B and T cell epitope containing synthetic glycolipopeptide cancer vaccine. *ChemMedChem 3*, 737–741.

(12) Ingale, S., Wolfert, M. A., Buskas, T., and Boons, G. J. (2009) Increasing the antigenicity of synthetic tumor-associated carbohydrate antigens by targeting Toll-like receptors. *ChemBioChem 10*, 455–463.

(13) Wilkinson, B. L., Day, S., Malins, L. R., Apostolopoulos, V., and Payne, R. J. (2011) Self-adjuvanting multicomponent cancer vaccine candidates combining per-glycosylated MUC1 glycopeptides and the Toll-like Receptor 2 agonist Pam3CysSer. *Angew. Chem., Int. Ed. 50*, 1635–1639.

(14) Erridge, C., Bennett-Guerrero, E., and Poxton, I. R. (2002) Structure and function of lipopolysaccharides. *Microbes Infect. 4*, 837–851.

(15) Casella, C. R., and Mitchell, T. C. (2008) Putting endotoxin to work for us: Monophosphoryl lipid A as a safe and effective vaccine adjuvant. *Cell. Mol. Life Sci. 65*, 3231–3240.

(16) Zhang, Y., Gaekwad, J., Wolfert, M. A., and Boons, G.-J. (2007) Modulation of innate immune responses with synthetic lipid A derivatives. *J. Am. Chem. Soc. 129*, 5200–5216.

(17) Persing, D. H., Coler, R. N., Lacy, M. J., Johnson, D. A., Baldridge, J. R., Hershberg, R. M., and Reed, S. G. (2002) Taking toll: lipid A mimetics as adjuvants and immunomodulators. *Trends Microbiol. 10* (Suppl.), S32–S37.

(18) Dobrovolskaia, M. A., and Vogel, S. N. (2002) Toll receptors, CD14, and macrophage activation and deactivation by LPS. *Microbes Infect. 4*, 903–914.

(19) Jonuleit, H., Knop, J., and Enk, A. H. (1996) Cytokines and their effects on maturation, differentiation and migration of dendritic cells. *Arch. Dermatol. Res. 289*, 1–8.

(20) Masihi, K. N., Lange, W., Johnson, A. G., and Ribi, E. (1986) Enhancement of chemiluminescence and phagocytic activities by nontoxic and toxic forms of lipid A. *J. Biol. Response Mod. 5*, 462–469.

(21) Wang, Q., Xue, J., and Guo, Z. (2009) Synthesis of a monophosphoryl lipid A derivative and its conjugation to a modified form of a tumor-associated carbohydrate antigen GM3. *Chem. Commun. (Cambridge, U. K.)*, 5536–5537.

(22) Tsuchida, T., Saxton, R. E., Morton, D. L., and Irie, R. F. (1987) Gangliosides of human melanoma. *J. Natl. Cancer Inst. 78*, 45–54.

(23) Ollila, D. W., Kelley, M. C., Gammon, G., and Morton, D. L. (1998) Overview of melanoma vaccines: active specific immunotherapy for melanoma patients. *Semin. Surg. Oncol. 14*, 328–336.

(24) Estevez, F., Carr, A., Solorzano, L., Valiente, O., Mesa, C., Barroso, O., Sierra, G. V., and Fernandez, L. E. (1999) Enhancement of the immune response to poorly immunogenic gangliosides after incorporation into very small size proteoliposomes (VSSP). *Vaccine 18*, 190–197.

(25) Perlmutter, R. M., Hansburg, D., Briles, D. E., Nicolotti, R. A., and Davie, J. M. (1978) Subclass restriction of murine anti-carbohydrate antibodies. *J. Immunol. 121*, 566–572.

(26) Greenspan, N. S., and Cooper, L. J. (1992) Intermolecular cooperativity: a clue to why mice have IgG3? *Immunol. Today 13*, 164–168.

(27) Markham, R. B., Pier, G. B., and Schreiber, J. R. (1991) The roel of cytophilic IgG3 antibody in T cell-mediated resistance to infection with the extracellular bacterium *Pseudomonas aeruginosa*. *J. Immunol. 146*, 316–320.

(28) Gavin, A. L., Barnes, N., Dijstelbloem, H. M., and Hogarth, P. M. (1998) Cutting edge: Identification of the mouse IgG3 receptor: Implications for antibody effector function at the interface between innate and adaptive immunity. *J. Immunol. 160*, 20–23.

(29) Guy, B. (2009) Adjuvants for protein- and carbohydrate-based vaccines, In *Carbohydrate-Based Vaccines and Immunotherapies* (Guo, Z., Boons, G.-J., Eds.), pp 89–116, John Wiley & Sons, Inc., Hoboken.

(30) Kanegasaki, S., Tanamoto, K., Yasuda, T., Homma, J. Y., Matsuura, M., Nakatsujka, M., Kumazawa, Y., Yamamoto, A., Shiba, T., Kusumoto, S., Imoto, M., Yoshimura, H., and Shimamoto, T. (1986) Structure-activity relationship of lipid A: Comparison of biological activities of natural and synthetic lipid A's with different fatty acid compositions. *J. Biochem. 99*, 1203–1210.

(31) Buskas, T., Li, Y., and Boons, G. J. (2004) The immunogenicity of the tumor-associated antigen Lewis(y) may be suppressed by a bifunctional cross-linker required for coupling to a carrier protein. *Chemistry 10*, 3517–3524.

(32) Tang, S., Wang, Q., and Guo, Z. (2010) Synthesis of a monophosphoryl derivative of *Escherichia coli* lipid A and its efficient coupling to a tumor-associated carbohydrate antigen. *Chem.—Eur. J. 16*, 1319–1325.

(33) Wang, Q., Ekanayaka, S. A., Wu, J., Zhang, J., and Guo, Z. (2008) Synthetic and immunological studies of 5′-N-phenylacetyl sTn to develop carbohydrate-based cancer vaccines and to explore the impacts of linkage between carbohydrate antigens and carrier proteins. *Bioconjugate Chem. 19*, 2060–2068.

(34) Hirabayashi, Y., Hamaoka, A., Matsumoto, M., Matsubara, T., Tagawa, M., Wakabayashi, S., and Taniguchi, M. (1985) Syngeneic monoclonal antibody against melanoma antigen with interspecies cross-reactivity recognizes GM3, a prominent ganglioside of B16 melanoma. *J. Biol. Chem. 260*, 13328–13333.

(35) Chefalo, P., Pan, Y., Nagy, N., Harding, C., and Guo, Z. (2006) Effective metabolic engineering of GM3 on tumor cells by *N*-phenylacetyl-D-mannosamine. *Biochemistry 45*, 3733–3739.