A EUROPEAN JOURNAL OF CHEMICAL BIOLOGY

CHEMBIOCHEM

SYNTHETIC BIOLOGY & BIO-NANOTECHNOLOGY

Accepted Article

Title: Chemical Synthesis of GM2 Glycans, Bioconjugation with Bacteriophage Qβ and the Induction of Anti-Cancer Antibodies

Authors: Zhaojun Yin; Steven Dulaney; Craig McKay; Claire Baniel; Katarzyna Kaczanowska; Sherif Ramadan; M.G. Finn; Xuefei Huang

To be cited as: ChemBioChem 10.1002/cbic.201500499

Link to VoR: http://dx.doi.org/10.1002/cbic.201500499

A Journal of

ChemPubSoc Europe

www.chembiochem.org

WILEY-VCH

Chemical Synthesis of GM2 Glycans, Bioconjugation with Bacteriophage Qβ and the Induction of Anti-Cancer Antibodies

Zhaojun Yin,[1a] Steven Dulaney,[1a] Craig S. McKay,[2] Claire Baniel,[1] Katarzyna Kaczanowska,[2] Sherif Ramadan,[1] M.G. Finn,[2] Xuefei Huang[1]

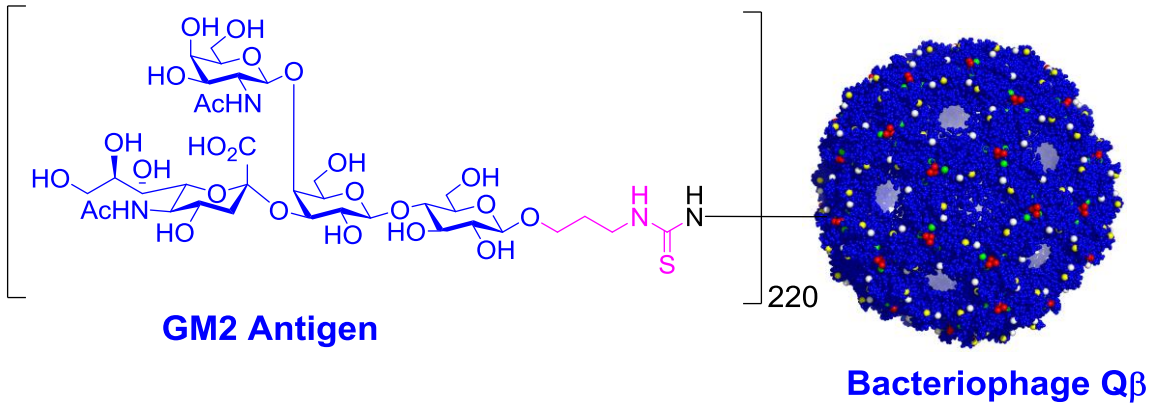
Authors' Affiliations: [1]Department of Chemistry, Michigan State University, East Lansing, Michigan; [2]School of Chemistry & Biochemistry, Georgia Institute of Technology, Atlanta, Georgia.

[a]These authors have contributed equally.

Corresponding Author: Xuefei Huang, Department of Chemistry, Michigan State University, 578 S. Shaw Lane, Room 426, East Lansing, MI 48824-1322, USA. Phone: 517-355-9715, ext 329; Email: xuefei@chemistry.msu.edu

Dedication: This manuscript is dedicated to Prof. Koji Nakanishi for his 90th birthday.

Graphical abstract



Abstract

The development of carbohydrate based anti-tumor vaccine is an attractive approach towards tumor prevention and treatment. Herein, we focused on the GM2 tumor associated carbohydrate antigen (TACA), which was overexpressed on a wide range of tumor cells. GM2 was synthesized chemically and conjugated with a virus like particle derived from bacteriophage Qβ. While the copper catalyzed azide-alkyne

cycloaddition reaction efficiently introduced 237 copies of GM2 per Qβ, this construct failed to induce significant amounts of anti-GM2 antibodies compared to Qβ control. In contrast, GM2 immobilized on Qβ through a thiourea linker elicited high titers of IgG antibodies, which recognized GM2 positive tumor cells and effectively induced cell lysis through complement-mediated cytotoxicity. Thus, bacteriophage Qβ is a suitable platform to boost the antibody responses towards GM2, a representative member of an important class of TACA, i.e., the ganglioside.

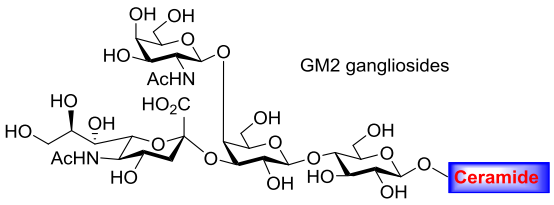
Keywords:
Antibodies, Carbohydrates, Immunology, Synthesis, Vaccines

Introduction

Aberrant glycosylation is a hallmark of many human cancers.[1-4] Tumor associated carbohydrate antigens (TACAs) are attractive targets for anti-tumor vaccines due to their high levels of expression on tumor cells.[5-8] However, the development of an effective carbohydrate based anti-tumor vaccine is extremely challenging. In nature, TACAs are often expressed as a heterogeneous mixture. As a result, it is difficult to obtain sufficient quantities of TACAs in conjugatable forms through isolation. In addition, there are concerns of highly active trace contaminants present in isolated samples. Thus, synthesis becomes critical to produce these complex molecules.[9-10]

Besides the challenge in accessing TACAs, the immunological obstacle is that TACAs are T cell independent B cell antigens.[5-8] When administered alone, they generally produce low titers of low affinity IgM antibodies, which do not persist for a long time. To induce high affinity IgG antibodies, a typical approach is to conjugate TACAs with carriers containing helper T (Th) cell epitopes, which include immunogenic proteins,[10-11] peptides,[6, 12-13] multiple antigenic glycopeptides,[14-15] nanoparticles,[16-18] polymers[18-20] and polysaccharides.[21] Recently, we have demonstrated that self-assembled virus-like particles (VLPs) could be used to deliver a TACA, the Tn antigen to the immune system and generate powerful antibody responses.[22-25] The antibodies induced bound strongly with Tn expressing tumor cells resulting in tumor cell death and protection of immunized mice from tumor development.[22]

Building on the success of the VLP-Tn studies, we became interested in testing whether the VLP platform can potently induce antibody responses against another important family of TACAs, i.e., the gangliosides[3] as represented by GM2. GM2 contains a sialic acid terminated branched tetrasaccharide linked to a ceramide chain. GM2 is expressed on the surfaces of a wide range of human cancers, which include cancer cells of neuroectodermal origin (melanoma, sarcoma and neuroblastoma) as well as epithelial cancers such as breast and prostate cancers.[7, 26-27] The wide expression of GM2 on multiple types of cancer renders it an intriguing target for developing a potentially "universal" anti-cancer vaccine. In addition, clinical studies have shown that elevated levels of anti-GM2 IgM antibodies are strongly associated with prolonged survival of melanoma patients.[28-29] Both passive administration of anti-GM2 monoclonal antibodies[30] and active immunity gained through vaccination[28, 31] could lead to favorable prognosis such as tumor regression or longer disease-free interval. These clinical outcomes have inspired the drive towards GM2 based anti-cancer vaccines.[28, 32-34]

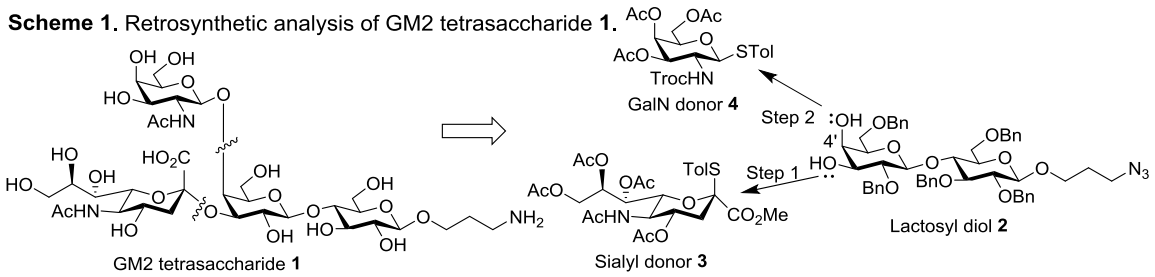


The generation of antibodies is a highly complex process. Many structural features of the construct can significantly impact the results of antibody responses. Livingston and coworkers showed that the anti-GM2 antibody titers were highly dependent upon the carrier moiety of the vaccine construct.[35] The Lo-Man group demonstrated that GM2 coupled with a Th epitope through the copper catalyzed azide-alkyne cycloaddition (CuAAC) reaction gave good titers of anti-GM2 antibodies.[34] Yet, when the same Th cell peptide was conjugated with two GM2 molecules, despite the higher valency, it failed to elicit detectable levels of IgM or IgG antibodies in mice even after repeated immunizations. Thus, the structure of a vaccine construct needs to be carefully designed and evaluated. Herein, we report our results on using synthetic GM2 antigens arrayed over the surface of the VLP bacteriophage Qβ capsid for the induction of anti-tumor antibodies.

Results and Discussion

Prior anti-GM2 vaccine studies have primarily utilized GM2 glycan extracted from mammalian tissues[28, 32] or enzymatically synthesized.[33-34, 36] Chemical synthesis can bestow flexibility in functionalizing the antigen for immunological investigations. Although GM2 glycans have been chemically synthesized previously,[37-39] with the need for stereoselective sialylation and formation of branched glycans, its preparation in a conjugatable form is not a trivial task. Our synthetic target was the GM2 tetrasaccharide **1** bearing a reducing end free amine, which was prepared by regioselective sialylation of the lactosyl diol acceptor **2** by sialyl donor **3** followed by glycosylation of the 4'-OH by galactosamine (GalN) donor **4** (**Scheme 1**).

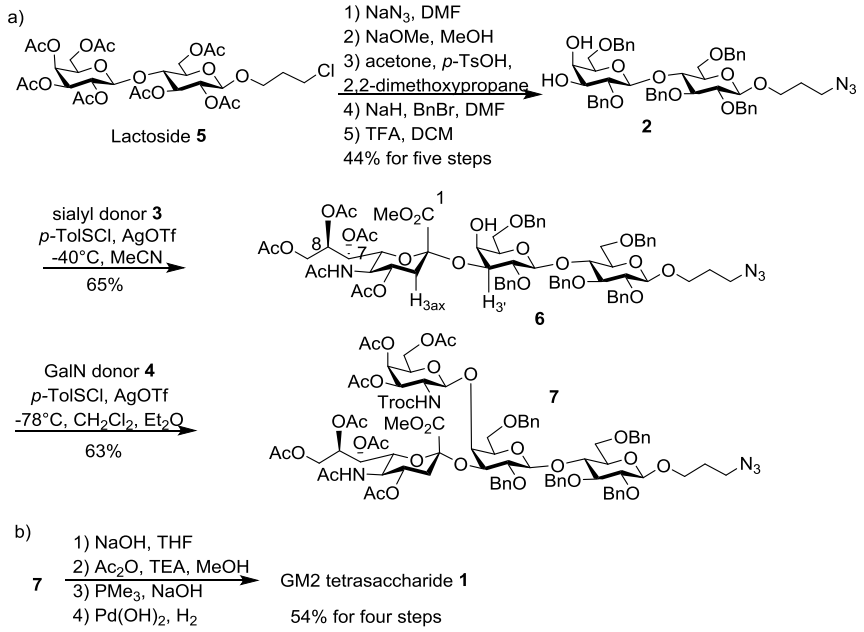
Scheme 1. Retrosynthetic analysis of GM2 tetrasaccharide **1**.



Our synthesis commenced with lactoside **5**,[40] which was derived from D-lactose and subsequently transformed to diol **2** through protective group manipulations (**Scheme 2a**). Sialylation of acceptor **2** was performed with thio-sialoside donor **3**. Initial coupling of **2** and **3** was mediated using *N*-iodosuccinimide (NIS) and triflic acid as the promoter, which gave the desired α-sialoside **6** in 42% yield along with 8% of the β-anomer. The stereochemistry of the newly formed glycosyl linkage of **6** was assigned based on the 3-bond coupling constant between C_1 and H_{3ax} of sialic acid ($^3J_{C1,H3ax}$ = 8 Hz) as well as that between H-7 and H-8 of sialic acid ($^3J_{H\text{-}7,H\text{-}8}$ = 7.9 Hz).[41-42] The regioselectivity was confirmed by the correlation between C_2 of sialic acid with $H_{3'}$ of the lactose unit in the HMBC NMR spectrum. In order to improve the sialylation yield, various reaction conditions were examined. While changing the solvent, reaction time, or temperature did not lead to significant enhancement, the combination of *p*-TolSCl/AgOTf[43-44] as the promoter system improved the yield of **6** to 65%. Recently, modified sialyl donors with groups such as 4-*O*,5-*N* oxazolidinone, and 5-*N*-trifluoroacetyl have been shown to give high yields and stereoselectivities in sialylation reactions.[45-49] Donor **3** has the advantage that no additional synthetic steps were needed to adjust the protective groups on C-5 of sialic acid, while achieving good yield and stereoselectivity. With

trisaccharide **6** in hand, its glycosylation by the GalN donor **4** was carried out using the *p*-TolSCl/AgOTf promoter system producing the protected GM_2 **7** in 63% yield with the new glycosidic bond being exclusively β ($^1J_{H1,C1\ of\ GalN}$ = 161.4 Hz,[50] $^3J_{H1,H2\ of\ GalN}$ = 8.8 Hz).

Scheme 2. Synthesis of GM2 tetrasaccharide **1**.

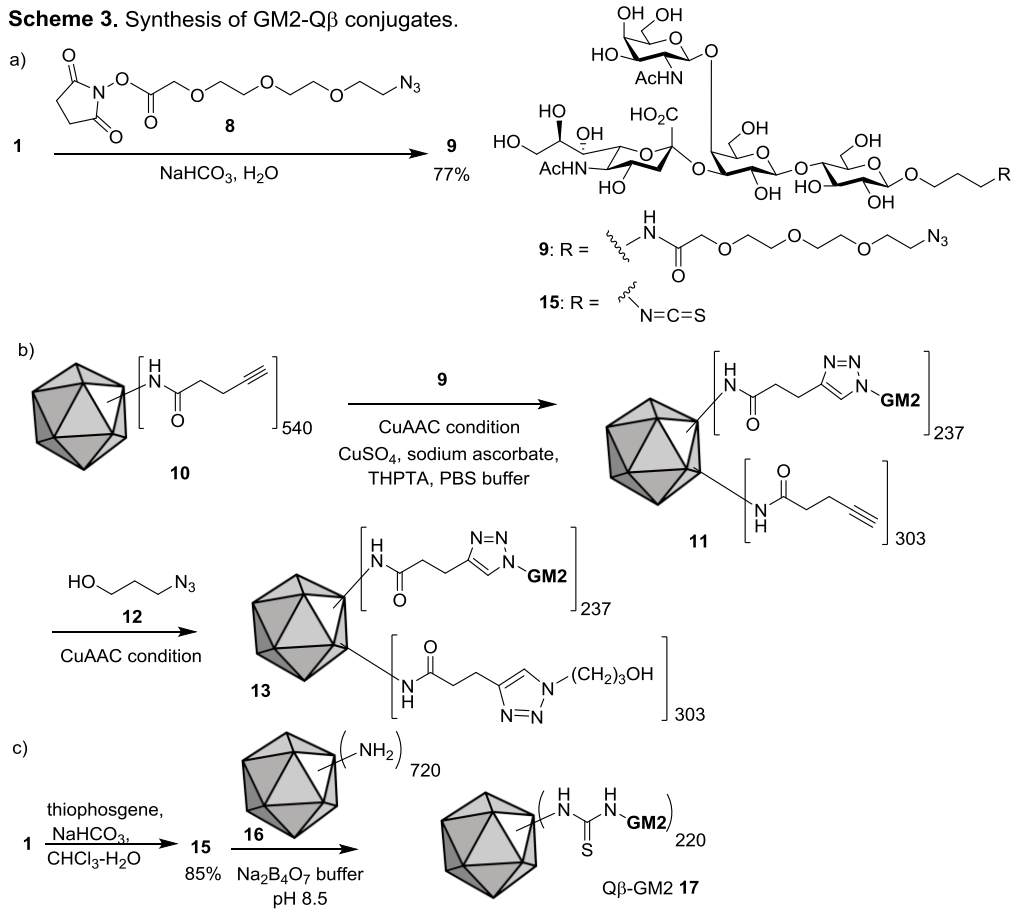


Deprotection of **7** was performed in four steps starting from the hydrolysis of *O*-acetyl groups concomitant with Troc removal (**Scheme 2b**). The newly freed amino group on GalN was selectively acetylated with acetic anhydride in methanol. Finally Staudinger reduction of the azido group and global debenzylation with Pearlman's catalyst provided the fully deprotected GM2 tetrasaccharide **1** in 54% yield over the four deprotection steps.

With the GM2 glycan in hand, we prepared GM2 conjugate vaccine with the VLP bacteriophage Qβ as the carrier, as we have previously shown that Qβ is superior to several other VLP platforms in boosting anti-Tn immunity.[23] Our initial approach for bioconjugation utilized the CuAAC reaction, due to its high reaction rate, mild reaction condition, and bio-orthogonal nature.[51-52] GM2 **1** was treated with the activated ester **8** to attach an azide moiety to the reducing end (GM2 **9**, 77% yield) for bioconjugation (**Scheme 3a**). Subsequently, **9** was coupled with the alkyne functionalized Qβ **10** under CuAAC conditions, which introduced approximately 237 copies of GM2 antigen to each

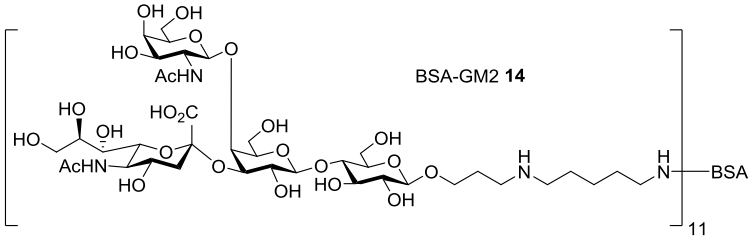
Qβ capsid (**Scheme 3b**). The remaining free alkyne groups on Qβ were capped with 3-azido-1-propanol **12** to afford Qβ-GM2 **13**.

Scheme 3. Synthesis of GM2-Qβ conjugates.



The ability of Qβ-GM2 **13** to generate anti-GM2 antibodies was evaluated next. C57BL/6 mice were immunized subcutaneously with three biweekly injections of Qβ-GM2 **13**, and sera from these mice were collected one week after the final boost injection. The control group of mice received the unconjugated Qβ only. For enzyme linked immunosorbent assay (ELISA) analysis of serum antibodies, a bovine serum albumin (BSA) conjugate of GM2 (BSA-GM2 **14**) was prepared through reductive amination with glutaraldehyde,[53] with an average of 11 GM2 glycans coupled to BSA. ELISA showed no significant binding to BSA-GM2 **14** by any post-immune sera compared to the control sera from mice immunized with Qβ only. To test serum binding with GM2 expressed in its native environment, i.e., on tumor cell surface, flow cytometry

analysis of all sera were performed. None of the sera was able to bind with GM2-positive human lymphoma Jurkat cells even at a relatively high concentration (1:10 dilution). These results demonstrated that Qβ-GM2 **13** was unable to elicit high titers of anti-GM2 antibodies *in vivo*.



To better understand Qβ-GM2 **13** vaccine, the epitope profiles of antibodies generated were screened by ELISA. BSA conjugates to structural components of GM2, i.e., *N*-acetyl galactosamine (GalNAc),[24] lactose, GM3, as well as BSA-triazole[22] were synthesized and immobilized onto ELISA plates. While there were some IgG bindings to BSA-GalNAc, BSA-GM3 and BSA-GM2, the binding to BSA-triazole was significantly stronger (**Figure 1**). This suggests that the triazole linker is the dominant epitope among the components analyzed.

10.1002/cbic.201500499

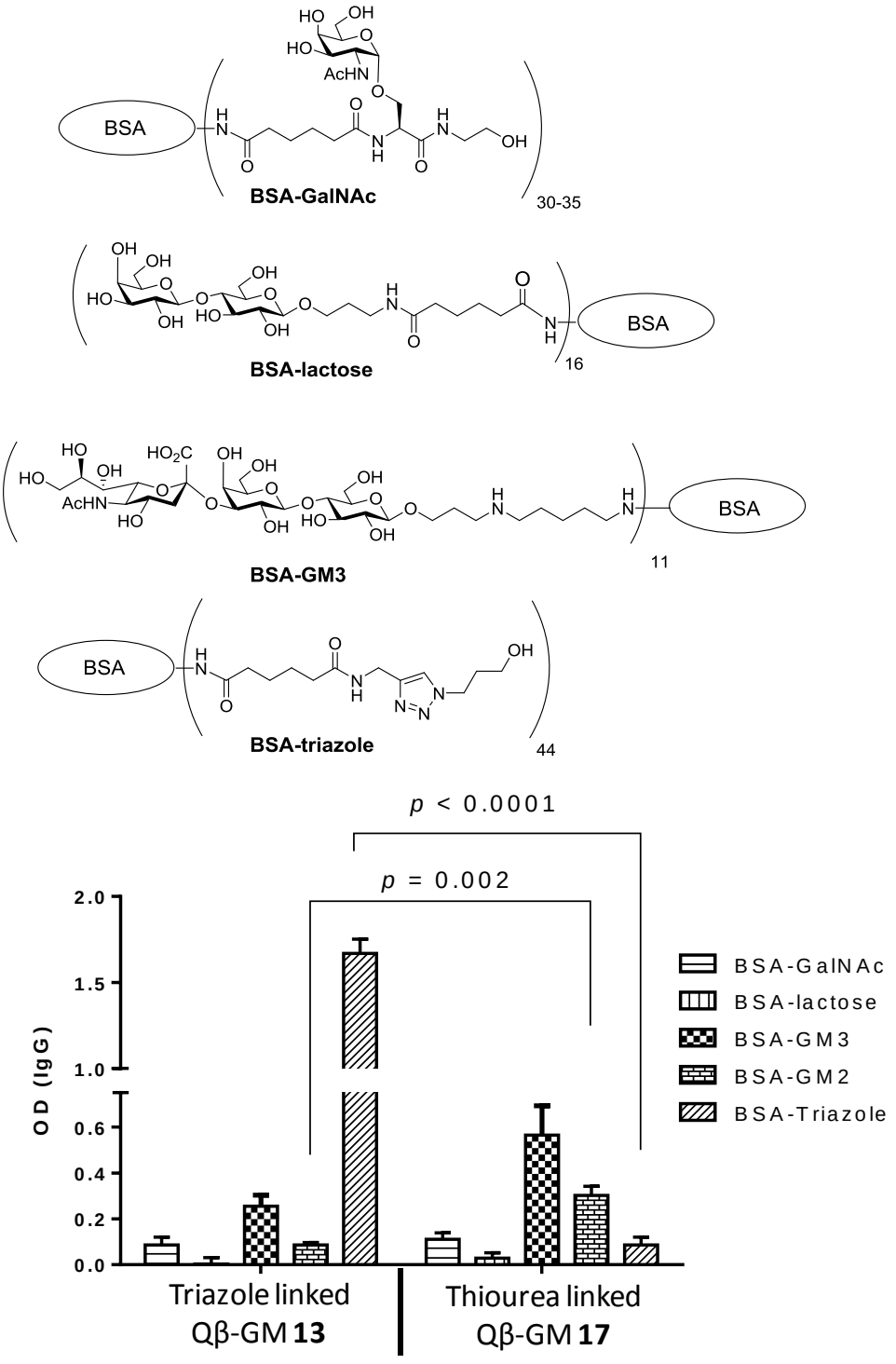


Figure 1. ELISA analysis of the epitope profiles of post-immune sera from mice immunized with triazole linked Qβ-GM2 conjugate **13** and thiourea linked Qβ-GM2 **17** respectively. For Qβ-GM2 **13**, anti-triazole antibody is significantly higher than other type of antibodies such as anti-GM2 or anti-GM3 antibodies ($p < 0.0001$). Qβ-GM2 **17**

induced significantly higher anti-GM2 antibodies (p = 0.002) but much less anti-triazole antibodies (p < 0.0001) than Qβ-GM2 **13**. The sera from each group were analyzed at 1600 fold dilution. The average of optical density value and SEM were shown. Statistics was performed through student's t-test.

To avoid antibody responses to the triazole linker, alternative strategies were explored. Previously, we have shown that reducing the number of triazoles on the Qβ by removing the triazole used for capping the unreacted alkynes did not lead to enhanced anti-glycan responses.[22] Therefore, we utilized another bioconjugation approach to ligate GM2 to Qβ. Treatment of GM2 **1** with thiophosgene converted the amine group to isothiocyanate[54] in 85% yield (**Scheme 3c**). The resulting GM2 **15** was incubated with the wild type Qβ particle **16** at pH = 8.5 to afford the Qβ-GM2 conjugate **17**. This reaction proceeded smoothly introducing an average of 220 copies of GM2 per Qβ particle (**Scheme 3c**).

With Qβ-GM2 **17** in hand, mice were immunized. In contrast to Qβ-GM2 **13**, ELISA analysis of post-immune sera showed good anti-GM2 IgG and IgM antibody responses with IgG as the main antibody type (**Figure 2a**). The subclass of IgG antibodies were also determined. The levels of IgG2 antibodies (IgG2b and IgG2) were much higher than those of IgG1 and IgG3, suggesting a more Th1-weighted immune response (**Figure 2b**).[55-56] This is likely due to the ability of Qβ to encapsulate single stranded *E. coli* RNA in the interior, which are potent agonists of Toll like receptors 7 and 8 for immune-potentiation favoring a Th1 response.[57] The antibodies elicited by Qβ-GM2 **17** could bind with multiple types of GM2 positive tumor cells as determined by flow cytometry (**Figures 2c,d**), while sera from the control mice receiving Qβ or the pre-immunized mice did not show any tumor cell recognition.

The epitope profiles of antibodies induced by Qβ-GM2 **17** were analyzed by ELISA (**Figure 1**). The antibodies exhibited strongest binding to BSA-GM3, while the recognition of BSA-GalNAc and BSA-lactose was much weaker. This suggests that the sialic acid motif contains the major recognition sites of GM2. This observation is consistent with a literature report, where the removal of sialic acid from GM2 abrogated the binding by anti-GM2 polyclonal antibodies.[34]

To assess the therapeutic potential of anti-GM2 antibodies, we evaluated the complement-dependent cytotoxicity against tumor cells. The classical pathway of complement activation is triggered by multivalent binding between C1 complex and Fc region of antibodies.[58] Compared to other IgG subclasses, the IgG2 antibodies in mice have the strongest abilities to initiate the complement cascade.[59] As shown in **Figure 2E**, the antibodies induced by Qβ-GM2 **17** were able to efficiently kill GM2 positive Jurkat cells by the complement mechanism.

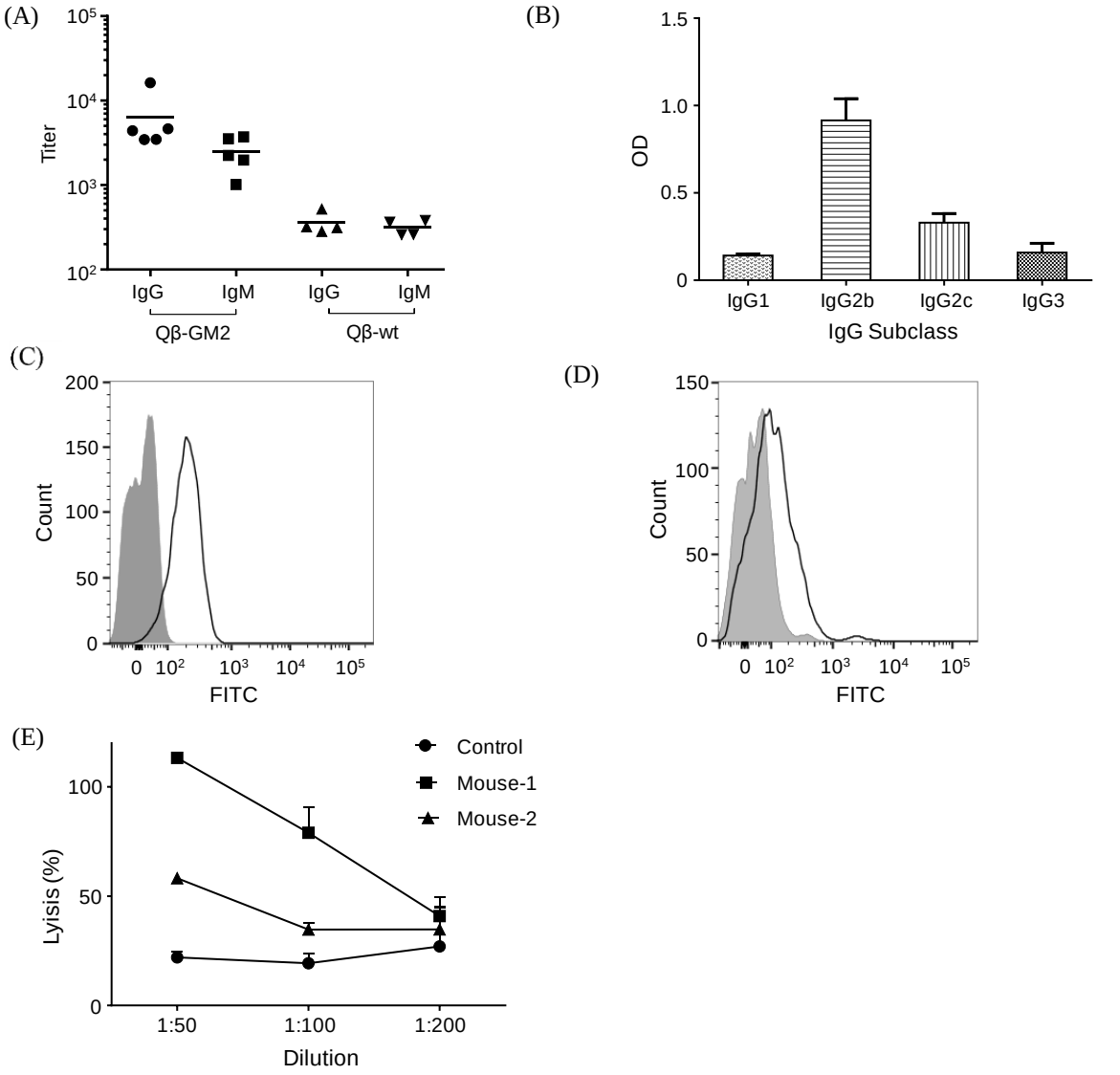


Figure 2. Immunological evaluation of Qβ-GM2 conjugate vaccine **17**. (A) IgM and IgG titer of anti-GM2 antibodies tested by ELISA. Sera from mice immunized with wild type Qβ particle were tested as control; (B) the levels of anti-GM2 IgG subclasses as

determined by ELISA. The sera were diluted at 1:1000 dilution; Binding of GM2-expressing (C) Jurkat cells and (D) MCF-7 cells with representative mouse sera diluted at 1:20. Grey filled: pre-immune sera and sera from mice immunized with Qβ only; solid line: day 35 sera from a mouse immunized with Qβ-GM2 **17**; (E) complement-dependent toxicity against Jurkat cells measured by LDH assay. Sera from two mice immunized with Qβ-GM2 **17** were shown. The control shown was with pre-immune serum. Sera from mice immunized with Qβ gave similar results as the pre-immune sera.

The CuAAC reaction and the triazole linker have been commonly used in carbohydrate based vaccines.[23-24, 60-65] In our recent studies on Qβ-Tn conjugates, we have observed that the triazole linked Qβ-Tn failed to induce antibodies capable of recognizing Tn expressed on tumor cells, which was attributed to the possible hindrance of Tn specific B cell binding to the vaccine construct by anti-triazole antibodies.[22] The inability of the triazole containing Qβ-GM2 **13** to generate anti-GM2 antibodies was consistent with the Qβ-Tn results, suggesting that the detrimental effect of triazole on anti-TACA immunity was not restricted to a small antigen such as Tn, which contains only a monosaccharide *N*-acetyl galactosamine linked with serine or threonine. While the exact reasons for the suppressive effect of triazole on anti-GM2 antibody responses need further investigations, these results indicate that cautions need to be taken in applying the CuAAC chemistry in future glycan based vaccine design.

Compared to GM2 vaccine candidates reported to date,[28, 32-34] the Qβ-GM2 **17** elicited similar total titers of anti-GM2 IgG antibodies and bindings to GM2 positive tumor cells. Conjugates such as KLH-GM2 produced more IgG1 and IgG3 in human patients.[35] Qβ-GM2 **17** elicited higher titers of IgG2, which can be potentially advantageous for future clinical applications as mouse IgG2s have been recognized as the most efficient IgG subclass to induce effector functions against tumor cells.[66]

In conclusion, we have established an efficient chemical synthesis of GM2 glycans. The synthetic approach can bestow flexibilities to prepare GM2 derivatives such as GM2 lactones[67-68] in the future to further enhance the immunogenecity of the antigen. In order to develop a GM2 based vaccine, our first generation approach utilized the CuAAC reaction linking 237 copies of GM2 onto a VLP carrier protein-

bacteriophage Qβ. However, no significant anti-GM2 antibodies were generated over control. To overcome this obstacle, isothiocyanate chemistry was employed introducing GM2 glycan onto Qβ. The resulting Qβ-GM2 conjugate **17** was able to induce high titers of anti-GM2 antibodies, in particular IgG2 antibodies. The antibodies produced were capable of binding GM2 expressing tumor cells and exhibited complement-dependent cytotoxicity lysing the tumor cells. Therefore, these results demonstrate that bacteriophage Qβ can be an effective vaccine platform for a GM2 based vaccine. Studies are ongoing to optimize the GM2 antigen structure as well as the vaccine construct to further enhance the vaccine efficacy.

Experimental Section

Immunizations of mice

Pathogen-free C57BL/6 female mice age 6-10 weeks were obtained from Charles River and maintained in the University Laboratory Animal Resources facility of Michigan State University. All animal care procedures and experimental protocols have been approved by the Institutional Animal Care and Use Committee (IACUC) of Michigan State University. Groups of five C57BL/6 mice were injected subcutaneously under the scruff on day 0 with 0.1 mL various Qβ constructs as emulsions in complete Freund's adjuvant (Sigma Aldrich, F5881), and boosters were given subcutaneously under the scruff on days 14 and 28 with 0.1 mL various Qβ constructs as emulsions in incomplete Freund's adjuvant (Sigma Aldrich, F5506). All GM2 vaccine constructs administered have the same amounts of GM2 antigen (4 μg). Serum samples were collected on day 0 (before immunization), 7 and 35. The final bleeding was done by cardiac bleed. Statistical analysis of immune responses of various groups were performed using student's t-test.

Antibody detection by ELISA and flow cytometry

Sera were tested as described previously for anti-Tn and anti-triazole antibodies by ELISA. The titer was determined by regression analysis with log10 dilution plotted with optical density.

Sera were tested by flow cytometry on GM2-bearing tumor cell lines Jurkat (kindly provided by Profs. Barbara Kaplan and Norbert Kaminski, Michigan State

University) and MCF-7 (kindly provided by Prof. Olivera J. Finn, University of Pittsburgh). Cells were incubated with 1:20 diluted mice sera on ice for 30 min, and then labeled with goat anti-mouse IgG conjugated with FITC (BioLegend, 405305) for 30 min. Acquisition of cells was performed with LSR II (BD), and data was analyzed with FlowJo software (Tree Star Inc.).

Complement dependent cytotoxicity

Mice sera was diluted with DMEM medium (10% FBS, without phenol red), mixed with 10^5 Jurkat cells and incubated in ice for 45 min. Then the 96 well plate was centrifuged and the supernatant was discarded. A final concentration of 10% baby rabbit complement (Cedarlane, CL3441-S) in DMEM medium was added and incubated at 37 °C for 4 hours. After centrifugation, 50 µl of the supernatant was transferred to a new 96-well plate, mixed with 50 µl of a lactose dehydrogenase substrate (CytoTox 96® non-radioactive cytotoxicity kit, G1780, Promega) and incubated at room temperature for 15 min, followed by addition of 50 µl stopping buffer. The plate was then read at 490 nm. The percentage of specific cell lysis was calculated as following: [(A-C)/(B-C)] x 100, where A represents absorbance obtained from mouse sera, B represents maximal lysis obtained by treating Jurkat cells with lysis buffer from kit, and C represents spontaneous lysis by treating Jurkat cells with complement only.

Acknowledgement

We are grateful to the National Cancer Institute (R01CA149451-01A1) for financial support of our work.

Supporting information available: Synthetic procedures and characterization data of GM2 and Qβ-GM2 conjugates. NMR and MS spectra for key compounds. Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/cbic.#####

Notes: The authors declare no competing financial interest.

Reference:

[1] A. Cazet, S. Julien, M. Bobowski, J. Burchell, P. Delannoy, *Breast Cancer Res.* **2010**, *12*, 204.

[2] S. Hakomori, *Adv. Exp. Med. Biol.* **2001**, *491*, 369-402.

[3] S. Hakomori, Y. Zhang, *Chem. Biol.* **1997**, *4*, 97-104.

[4] A. Vasconcelos-Dos-Santos, I. A. Oliveira, M. C. Lucena, N. R. Mantuano, S. A. Whelan, W. B. Dias, A. R. Todeschini, *Front. Oncol.* **2015**, *5*, doi:10.3389/fonc.2015.00138.

[5] Z. Guo, Q. Wang, *Curr. Opin. Chem. Biol.* **2009**, *13*, 608-617.

[6] T. Buskas, P. Thompson, G.-J. Boons, *Chem. Commun.* **2009**, 5335-5349.

[7] Z. Yin, X. Huang, *J. Carbohydr. Chem.* **2012**, *31*, 143-186 and references cited therein.

[8] C.-C. Liu, X.-S. Ye, *Glycoconjugate J.* **2012**, *29*, 259-271.

[9] D. P. Galonic, D. Y. Gin, *Nature* **2007**, *446*, 1000-1007.

[10] S. J. Danishefsky, J. R. Allen, *Angew. Chem. Int. Ed.* **2000**, *39*, 836-863.

[11] P. O. Livingston, G. Ragupathi, *Human Vaccines* **2006**, *2*, 137-143 and references cited therein.

[12] B. L. Wilkinson, S. Day, L. R. Malins, V. Apostolopoulos, R. J. Payne, *Angew. Chem. Int. Ed.* **2011**, *50*, 1635-1639.

[13] N. Gaidzik, U. Westerlind, H. Kunz, *Chem. Soc. Rev.* **2013**, *42*, 4421-4442 and references cited therein.

[14] R. Lo-Man, S. Vichier-Guerre, S. Bay, E. Deriaud, D. Cantacuzene, C. Leclerc, *J. Immunol.* **2001**, *166*, 2849-2854.

[15] R. Lo-Man, S. Vichier-Guerre, R. Perraut, E. Deriaud, V. Huteau, L. BenMohamed, O. M. Diop, P. O. Livingston, S. Bay, C. Leclerc, *Cancer Res.* **2004**, *64*, 4987-4994.

[16] R. P. Brinas, A. Sundgren, P. Sahoo, S. Morey, K. Rittenhouse-Olson, G. E. Wilding, W. Deng, J. J. Barchi, *Bioconjugate Chem.* **2012**, *23*, 1513-1523.

[17] S. Sungsuwan, Z. Yin, X. Huang, *ACS Appl. Mater. Interface* **2015**, *7*, 17535-17544.

[18] A. L. Parry, N. A. Clemson, J. Ellis, S. S. R. Bernhard, B. G. Davis, N. R. Cameron, *J. Am. Chem. Soc.* **2013**, *135*, 9362-9365.

[19] Q. Qin, Z. Yin, P. Bentley, X. Huang, *Med. Chem. Commun.* **2014**, *5*, 1126-1129.

[20] L. Nuhn, S. Hartmann, B. Palitzsch, B. Gerlitzki, E. Schmitt, R. Zentel, H. Kunz, *Angew. Chem. Int. Ed.* **2013**, *52*, 10652-10656.

[21] R. A. De Silva, Q. Wang, T. Chidley, D. K. Appulage, P. R. Andreana, *J. Am. Chem. Soc.* **2009**, *131*, 9622-9623.

[22] Z. Yin, W. S. Wright, C. McKay, C. Baniel, K. Kaczanowska, P. Bentley, J. C. Gildersleeve, M. G. Finn, L. BenMohamed, X. Huang, *ACS Chem. Biol.* **2015**, *10*, 2364-2372.

[23] Z. Yin, M. Comellas-Aragones, S. Chowdhury, P. Bentley, K. Kaczanowska, L. BenMohamed, J. C. Gildersleeve, M. G. Finn, X. Huang, *ACS Chem. Biol.* **2013**, *8*, 1253-1262.

[24] Z. Yin, H. G. Nguyen, S. Chowdhury, P. Bentley, M. A. Bruckman, A. Miermont, J. C. Gildersleeve, Q. Wang, X. Huang, *Bioconjugate Chem.* **2012**, *23*, 1694-1703.

[25] A. Miermont, H. Barnhill, E. Strable, X. Lu, K. A. Wall, Q. Wang, M. G. Finn, X. Huang, *Chem. Eur. J.* **2008**, *14*, 4939-4947.

[26] S. Zhang, C. Cordon-Cardo, H. S. Zhang, V. E. Reuter, S. Adluri, W. B. Hamilton, K. O. Lloyd, P. O. Livingston, *Int. J. Cancer* **1997**, *73*, 42-49.

[27] G. Ritter, P. O. Livingston, *Semin. Cancer Biol.* **1991**, *2*, 401-409.

[28] P. O. Livingston, G. Y. C. Wong, S. Adluri, Y. Tao, M. Padavan, R. Parente, C. Hanlon, M. J. Calves, F. Helling, G. Ritter, H. F. Oettgen, L. J. Old, *J. Clin. Oncol.* **1994**, *12*, 1036-1044.

[29] P. C. Jones, L. L. Sze, P. Y. Liu, D. L. Morton, R. F. Irie, *J. Nat. Cancer Inst.* **1981**, *66*, 249-254.

[30] R. Irie, T. Matsuki, D. Morton, *Lancet* **1989**, *333*, 786-787.

[31] P. O. Livingston, E. J. J. Natoli, M. J. Calves, E. Stockert, H. F. O. Oettgen, L. J. , *Proc. Natl. Acad. Sci. USA* **1987**, *84*, 2911-2915.

[32] P. B. Chapman, D. M. Morrissey, K. S. Panageas, W. B. Hamilton, C. Zhan, A. N. Destro, L. Williams, R. J. Israel, P. O. Livingston, *Clin. Cancer Res.* **2000**, *6*, 874-879.

[33] J. R. Rich, W. W. Wakarchuk, D. R. Bundle, *Chem. Eur. J.* **2006**, *12*, 845-858.

[34] S. Bay, S. Fort, L. Birikaki, C. Ganneau, E. Samain, Y. M. Coic, F. Bonhomme, E. Deriaud, C. Leclerc, R. Lo-Man, *ChemMedChem* **2009**, *4*, 582-587.

[35] F. Helling, S. Zhang, A. Shang, S. Adluri, M. Calves, R. Koganty, B. M. Longenecker, T. J. Yao, H. F. Oettgen, P. O. Livingston, *Cancer Res.* **1995**, *55*, 2783-2788.

[36] S. Jacques, J. R. Rich, C.-C. Ling, D. R. Bundle, *Org. Biomol. Chem.* **2006**, *4*, 142-154.

[37] J. C. Castro-Palomino, G. Ritter, S. R. Fortunato, S. Reinhardt, L. J. Old, R. R. Schmidt, *Angew. Chem. Int. Ed.* **1997**, *36*, 1998-2001.

[38] Y. S. Cho, Q. Wan, S. J. Danishefsky, *Bioorg. Med. Chem.* **2005**, *13*, 5259-5266.

[39] M. Sugimoto, M. Numata, K. Koike, Y. Nakahara, T. Ogawa, *Carbohydr. Res.* **1986**, *156*, C1-5.

[40] B. Sun, B. Yang, X. Huang, *Sci. China Chem.* **2012**, *55*, 31-35.

[41] H. Paulsen, H. Tietz, *Angew. Chem. Int. Ed.* **1982**, *21*, 927-928.

[42] G.-J. Boons, A. V. Demchenko, *Chem. Rev.* **2000**, *100*, 4539-4566.

[43] X. Huang, L. Huang, H. Wang, X.-S. Ye, *Angew. Chem. Int. Ed.* **2004**, *43*, 5221-5224.

[44] B. Sun, B. Srinivasan, X. Huang, *Chem. Eur. J.* **2008**, *14*, 7072-7081.

[45] P. K. Kancharla, C. Navuluri, D. Crich, *Angew. Chem. Int. Ed.* **2012**, *51*, 11105-11109.

[46] X.-T. Zhang, Z.-Y. Gu, G.-W. Xing, *Carbohydr. Res.* **2014**, *388*, 1-7 and references cited therein.

[47] H. Tanaka, Y. Nishiura, T. Takahashi, *J. Am. Chem. Soc.* **2006**, *128*, 7124-7125.

[48] C. De Meo, M. Farris, N. Ginder, B. Gulley, U. Priyadarshani, M. Woods, *Eur. J. Org. Chem.* **2008**, *2008*, 3673-3677.

[49] C.-C. Lin, K.-T. Huang, C.-C. Lin, *Org. Lett.* **2005**, *7*, 4169-4172.

[50] K. Bock, C. Pedersen, *J. Chem. Soc., Perkin Trans. 2* **1974**, 293-297.

[51] M. G. Finn, V. V. Fokin, *Chem. Soc. Rev.* **2010**, *39*, 1231-1232 and references cited therein.

[52] V. Hong, S. I. Presolski, C. Ma, M. G. Finn, *Angew. Chem. Int. Ed.* **2009**, *48*, 9879-9883.

[53] I. Migneault, C. Dartiguenave, M. J. Bertrand, K. C. Waldron, *Biotechniques* **2004**, *37*, 790-802.

[54] D. F. Smith, D. A. Zopf, V. Ginsburg, *Methods Enzymol.* **1978**, *50*, 169-171.

[55] D. J. Lefeber, B. Benaissa-Trouw, J. F. G. Vliegenthart, J. P. Kamerling, W. T. M. Jansen, K. Kraaijeveld, H. Snippe, *Infect. Immun.* **2003**, *71*, 6915-6920.

[56] T. Germann, M. Bongartz, H. Dlugonska, H. Hess, E. Schmitt, L. Kolbe, E. Kölsch, F. J. Podlaski, M. K. Gately, E. Rüde, *Eur. J. Immunol.* **1995**, *25*, 823-829.

[57] M. P. Schön, M. Schön, *Oncogene* **2008**, *27*, 190-199.

[58] V. N. Schumaker, P. Zavodszky, P. H. Poon, *Annu. Rev. Immunol.* **1987**, *5*, 21-42.

[59] J. L. Jansen, A. P. Gerard, J. Kamp, W. P. Tamboer, P. G. Wijdeveld, *J. Immunol.* **1975**, *115*, 387-391.

[60] E. Kaltgrad, S. Sen Gupta, S. Punna, C. Y. Huang, A. Chang, C. H. Wong, M. G. Finn, O. Blixt, *ChemBioChem* **2007**, *8*, 1455-1462.

[61] R. D. Astronomo, E. Kaltgrad, A. Udit, S.-K. Wang, K. J. Doores, C.-Y. Huang, R. Pantophlet, J. C. Paulson, C. H. Wong, M. G. Finn, D. R. Burton, *Chem. Biol.* **2010**, *17*, 357-370.

[62] T. Lipinski, T. Luu, P. I. Kitov, A. Szpacenko, D. R. Bundle, *Glycoconjugate J.* **2011**, *28*, 149-164.

[63] Q. Wang, Z. Zhou, S. Tang, Z. Guo, *ACS Chem. Biol.* **2012**, *7*, 235-240.

[64] Q. Y. Hu, M. Allan, R. Adamo, D. Quinn, H. L. Zhai, G. X. Wu, K. Clark, J. Zhou, S. Ortiz, B. Wang, E. Danieli, S. Crotti, M. Tontini, G. Brogioni, F. Berti, *Chem. Sci.* **2013**, *4*, 3827-3832.

[65] H. Cai, Z. Y. Sun, M. S. Chen, Y. F. Zhao, H. Kunz, Y. M. Li, *Angew. Chem. Int. Ed.* **2014**, *53*, 1699-1703.

[66] F. Nimmerjahn, J. V. Ravetch, *Science* **2005**, *310*, 1510-1512.

[67] G. Ragupathi, M. Meyers, S. Adluri, L. Howard, C. Musselli, P. O. Livingston, *Int. J. Cancer* **2000**, *85*, 659-666.

[68] G. Ritter, E. Boosfeld, R. Adluri, M. Calves, H. F. Oettgen, L. J. Old, P. Livingston, *Int. J. Cancer* **1991**, *48*, 379-385.