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Boosting Immunity to Small Tumor-Associated Carbohydrates with Bacteriophage Qβ Capsids

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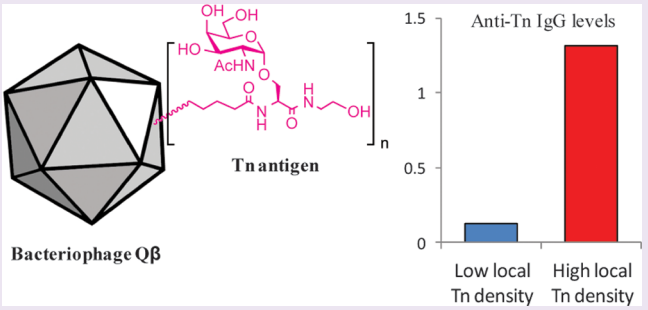
Supporting Information

ABSTRACT: The development of an effective immunotherapy is an attractive strategy toward cancer treatment. Tumor associated carbohydrate antigens (TACAs) are overexpressed on a variety of cancer cell surfaces, which present tempting targets for anticancer vaccine development. However, such carbohydrates are often poorly immunogenic. To overcome this challenge, we show here that the display of a very weak TACA, the monomeric Tn antigen, on bacteriophage Qβ virus-like particles elicits powerful humoral responses to the carbohydrate. The effects of adjuvants, antigen display pattern, and vaccine dose on the strength and subclasses of antibody responses were established. The local density of antigen rather than the total amount of antigen administered was found to be crucial for induction of high Tn-specific IgG titers. The ability to display antigens in an organized and high density manner is a key advantage of virus-like particles such as Qβ as vaccine carriers. Glycan microarray analysis showed that the antibodies generated were highly selective toward Tn antigens. Furthermore, Qβ elicited much higher levels of IgG antibodies than other types of virus-like particles, and the IgG antibodies produced reacted strongly with the native Tn antigens on human leukemia cells. Thus, Qβ presents a highly attractive platform for the development of carbohydrate-based anticancer vaccines.



Vaccine strategies aiming to stimulate the immune system to eradicate tumor cells are highly attractive for tumor prevention and therapy.[1] One such approach is to target tumor associated carbohydrate antigens (TACAs), which are overexpressed on the surfaces of many types of malignant cells. Some cancer patients can naturally develop antibodies against TACAs and high levels of anti-TACA antibodies have been correlated with improved prognosis and survival.[2] However, the induction of highly potent immune responses against TACAs is challenging.

The major difficulty in generating effective anti-TACA immunity is that TACAs are typically T cell independent B cell antigens.[3] TACAs can directly interact with B cells leading to the secretion of low titers of IgM antibodies, which generally last only a short time. To elicit strong and long lasting immunity, B cell stimulation by T helper cells is required, leading to a switch of antibody isotype from IgM to IgG and the induction of immunological memory.[4] In this way, when transformed cells bearing TACAs emerge in immunized hosts, the immune system can recognize the disease-related carbohydrate epitopes, quickly reactivate and remove the malignant cells.

Innovative studies have been carried out to develop TACA-based anticancer vaccines,[5−7] which include glycoengineering to improve immunogenicity,[7−10] synthesis of novel adjuvants,[11] enhancement of immune responses through pre-existing antibodies,[12,13] as well as the development of novel carriers.[14−20] We focus on the last of these approaches, since carriers can provide the necessary epitopes to promote T helper cell activation in a general way, applicable to a variety of antigens.

Immunogenic proteins such as keyhole limpet hemocyanin (KLH) and tetanus toxoid (TT) are popular choices as carriers, having been conjugated with TACAs to induce strong IgM and

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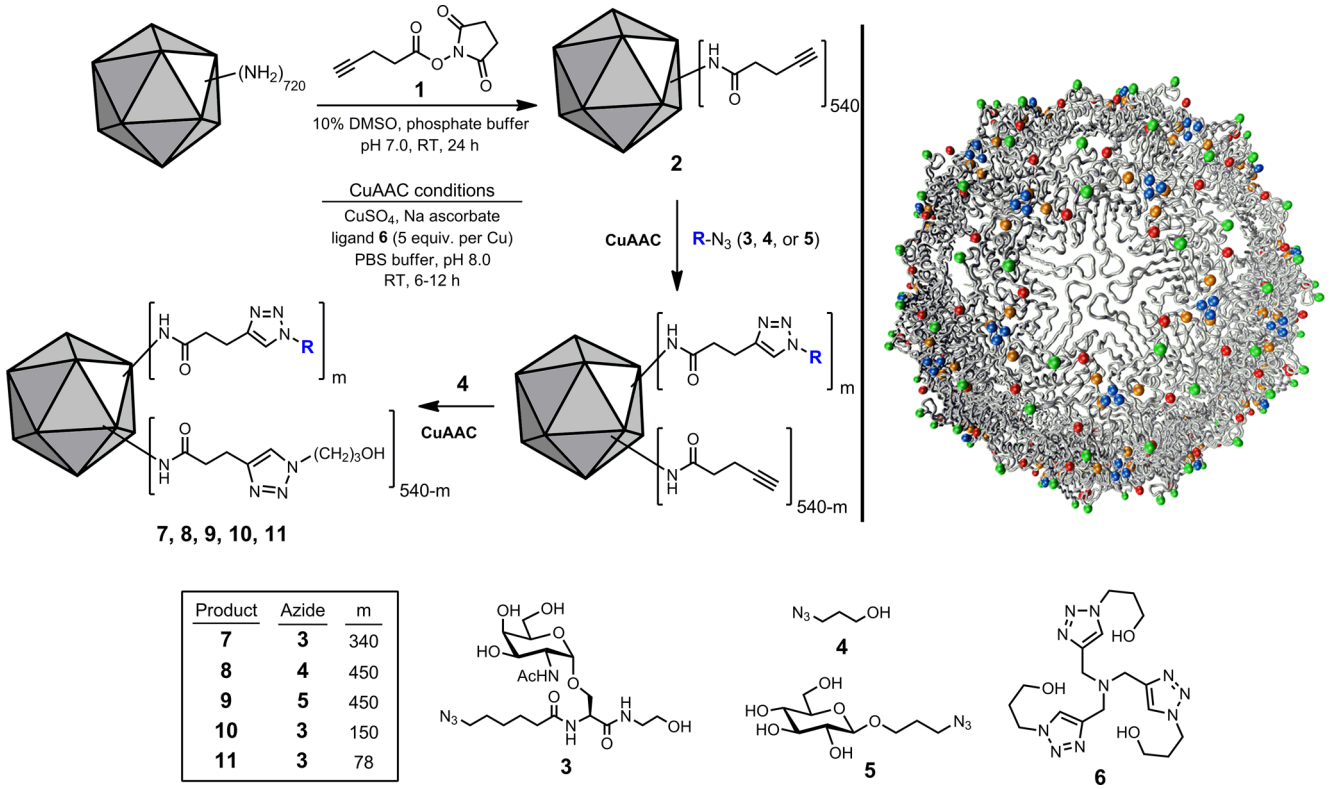

Figure 1. Syntheses of Qβ conjugates **7**−**11**. Upper right: structure of the Qβ capsid, with external amino groups marked in color. *N*-termini = orange, K2 = blue, K13 = red, K16 = green.

IgG responses in various preclinical and clinical evaluations.[15,21] These carrier proteins contain multiple T helper cell epitopes, which are important in the potentiation of antibody responses.[21] This mechanistic viewpoint has inspired several elegant reductionist approaches as well, featuring synthetic multicomponent vaccines bearing well-defined T helper cell epitopes, TACA antigens, and adjuvants with promising results.[22,23] In addition, a variety of nonprotein carriers have been investigated to improve the quality of anti-TACA immune responses, including dendrimers,[14,20] polysaccharides,[16] gold nanoparticles,[17,18] and very small proteoliposomes.[19]

We have become interested in exploring virus-like particles (VLPs) as TACA delivery platforms.[24−26] VLPs, which are composed of subunit proteins that self-assemble in a highly ordered manner, emerge as a promising direction in vaccine development.[27] Noninfectious to humans or animal hosts, VLPs are safe and highly immunogenic because of several properties,[28] including their sizes (<100 nm diameter, promoting both uptake by antigen presenting cells and trafficking to lymph nodes[29]), repetitive structure promoting B cell recognition,[30] ability to cross-link B cell receptors,[31] and presentation of immunostimulatory motifs such as RNA ligands of Toll-like receptors.[32]

One potential limitation of using immunogenic carrier proteins is the possible diminution of the desired immune response due to pre-existing anticarrier immunity established by prior exposure to the carrier. Such carrier-induced epitopic suppression has been reported for both KLH and TT.[33,34] To overcome this challenge, structurally distinct vaccine carriers are needed. This prompted us to investigate the utility of a VLP, i.e., bacteriophage Qβ as a new TACA carrier. This particle is composed of 180 copies of a 14.3-kD subunit, arranged in an icosahedral structure approximately 28 nm in diameter,[35] and is very stable toward chemical manipulation. Therapeutic vaccine candidates based on Qβ have demonstrated preclinical or clinical efficacy in a variety of diseases. Herein we report Qβ as a powerful antigen delivery platform for anticancer vaccine development by boosting the immune response to a TACA, the Tn antigen.

The Tn antigen (GalNAc-α-O-Ser/Thr), is overexpressed on the surface of a variety of cancer cells including breast, colon, and prostate cancer[36,37] and is involved in aggressive growth and lymphatic metastasis of cancers.[38] The expression level of Tn was found to correlate strongly with overall survival, nodal status and tumor size in breast cancer patients.[36] However, the development of anti-Tn vaccines has been quite challenging. Presumably because of its small size, the monomeric Tn antigen did not generate strong responses in immunized mice, even after conjugation to an immunogenic carrier.[20,39,40] To overcome this, a common approach is to cluster multiple consecutive Tn together rendering it more prominent to the immunological system.[20,40] This can be synthetically challenging and time-consuming. Rather than constructing Tn clusters, we attached multiple copies of the monomeric glycan to the icosahedral Qβ scaffold, a one-step operation that can conveniently provide high-density organized displays of the desired epitope. As a weak TACA, Tn serves as a useful model, and the results obtained here can provide critical insights for the rational design of effective TACA-based anticancer vaccines.

RESULTS AND DISCUSSION

Syntheses of Qβ−Tn Conjugates and Control Particles. Tn ligation onto the Qβ VLP was performed with the copper-catalyzed azide−alkyne cycloaddition (CuAAC) reac-

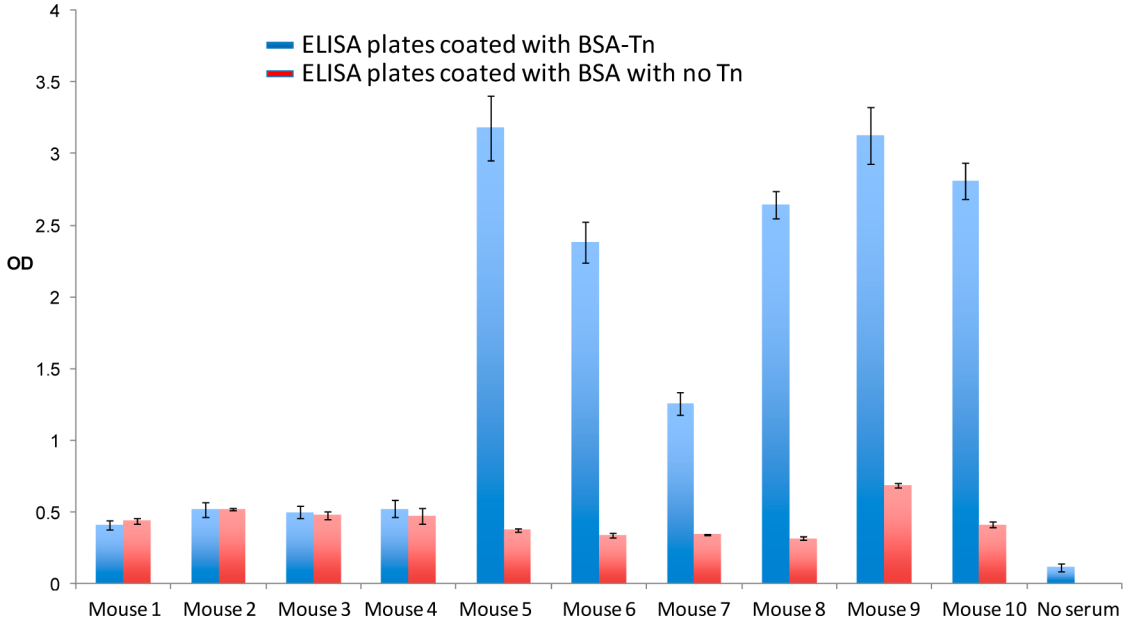

Figure 2. ELISA analysis of the binding of sera from mice inoculated with antigens **7**–**9** against immobilized BSA–Tn conjugate **12** and BSA alone. Antigens: mice 1, 2 = Qβ–glucose **9**; mice 3, 4 = Qβ–propanol **8**; mice 5, 6 = Qβ–Tn **7** (1 μg Tn); mice 7, 8 = Qβ–Tn **7** (4 μg Tn); mice 9, 10 = Qβ–Tn **7** (20 μg Tn). Each serum was added to four separate wells on the ELISA plate. The error bars were the standard deviations of OD values of wells incubated with a specific serum.

tion, optimized for bioconjugative operations.[41] This process allows high densities of decoration to be achieved on the desired biomolecular scaffold with a minimum of high-value reactants.[42] Qβ capsid was first treated with a large excess of the pent-4-ynoic acid *N*-hydroxysuccinimide ester **1**, to place alkyne groups at 540 amino groups (lysine side chains and subunit *N*-termini) accessible on the outer surface of the capsid (Figure 1). Following removal of the excess reagent, azide modified Tn **3** was added to the alkyne Qβ **2** in the presence of the copper catalyst using ligand **6** (THPTA).[41] Depending on the reaction time and reagent concentrations, the number of Tn units attached could be varied. A "high loading" of 340 copies of Tn per particle was produced using only 5 equiv of azide **3** per subunit (1.5–2 equiv per alkyne), and the excess Tn could be recovered from the reaction mixture. The remaining unreacted alkyne groups on Qβ virion were capped in a second CuAAC reaction using a large excess of 3-azido 1-propanol **4** producing Qβ–Tn **7**. Control particles bearing *n*-propanol (Qβ–propanol **8**) and glucose (Qβ–glucose **9**) were prepared similarly, each displaying an average of 450 ± 50 attached units per capsid (Figure 1). These controls were designed to probe the effects of capsid and the carbohydrate structural specificity of immune responses.

The reactive amino groups on the Qβ exterior are generally well dispersed over the protein surface (Figure 1). While K16 (green residue in Figure 1) is most exposed, there are no great differences in accessibility to solution-phase species among the amine groups, except when extensive substitution is desired at the K2 residues clustered tightly around the 3-fold symmetry axis (blue residue). Thus, acylation and subsequent triazole formation should occur evenly at the exterior amine positions.[43] (Interior-surface amine groups are blocked by the bacterial and plasmid RNA that is sequestered inside the particle.[44]) When the overall number of attached Tn moieties per particle is increased, they are expected to be arrayed in larger numbers of repetitive clustered patterns over the icosahedral capsid.

Establishment of Enzyme-Linked Immunosorbent Assay (ELISA) Protocol. With Qβ–Tn and the control particles in hand, we examined the humoral responses to these particles in the presence of three different adjuvants: complete Freund's adjuvant (CFA), alum, or TiterMax Gold. Three groups of five C57BL/6 mice were vaccinated three times at two-week intervals with Qβ–Tn **7**, Qβ–propanol **8**, or Qβ–glucose **9** in the presence of the respective adjuvant. An additional group of mice was inoculated using Qβ–Tn **7** without any exogenous adjuvant (PBS group). One week after the final boost, serum samples were taken and analyzed for anti-Tn antibody responses.

For ELISA analysis of the resulting sera, a bovine serum albumin (BSA) amide-linked conjugate of Tn was prepared with 30–35 copies of Tn units attached per protein (BSA–Tn **12**). The amide connection between Tn and BSA in **12** was used to focus the assay on the Tn moiety rather than to the triazole present in the linker of the administered antigens. Sera from mice receiving Qβ–Tn **7** bound substantially better to BSA–Tn **12** than sera from mice receiving controls **8** or **9**, and very little binding was observed to unmodified BSA, indicating that anti-Tn responses were obtained using this protocol (Figure 2). In contrast, no selective binding was observed to a biotinated Tn antigen (**13**) immobilized onto neutravidin-coated plates, which is a commonly used ELISA protocol (Figure S1, Supporting Information).[24,25,45] These divergent ELISA results suggest that polyvalent antibody binding is required for detection since neutravidin-based display (four biotin-binding sites per neutravidin) is likely to be of substantially lower density than the BSA–Tn conjugate resulting in weak interactions and low avidities with anti-Tn antibodies.[46]

Effects of Immunoadjuvants and the Nature of the Particle on Anti-Tn Antibody Responses. Although some intragroup variation was observed, Qβ–Tn immunized mice receiving the CFA adjuvant produced significantly more IgG

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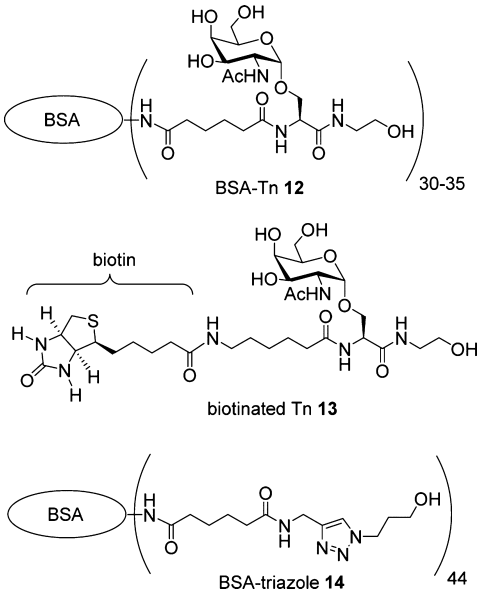

antibodies than those receiving TiterMax Gold or alum (Figure 3a). Interestingly, mice immunized with Qβ−Tn in PBS (without external adjuvant) also responded. There were no statistically significant differences in IgG levels between the PBS groups and CFA groups, although the CFA groups gave higher mean values. In contrast to the IgG antibodies, the IgM responses from all the groups were similar (Figure 3b). On the basis of these results, CFA was selected as the adjuvant for further studies.

For CFA immunization, CFA was only applied in the primary injection. In the two boost injections, incomplete Freund's adjuvant (IFA), which is just mineral oil without mycobacteria components, was used to avoid overstimulation of the immune system and toxicity of CFA. When IFA was applied as the adjuvant in both prime and boost injections with Qβ−Tn 7, a much poorer IgG response than the CFA protocol was obtained (Figure S2, Supporting Information), suggesting the bacterial components in CFA were important in eliciting high IgG responses. The success of Qβ−Tn 7 in PBS without exogenous adjuvant to generate IgG responses is consistent with the expectation that Qβ has favorable characteristics of size, structure, and costimulatory abilities.

Besides Qβ, other VLPs such as cowpea mosaic virus capsid (CPMV) and tobacco mosaic virus capsid (TMV) could be used as Tn carriers as well.[24,25] Tn was linked onto CPMV and TMV with on average 120 copies of Tn on CPMV (Supporting Information, Figures S5 and S6) and greater than 2000 copies of Tn installed on TMV.[24] The abilities of CPMV-Tn and TMV-Tn to potentiate anti-Tn IgG responses were compared with that of Qβ−Tn 7 under the identical immunization protocol (CFA adjuvant, 4 μg Tn). As shown in Figure S3 (Supporting Information), the IgG titers induced in mice following Qβ−Tn immunization were significantly higher compared to the levels induced by either CPMV−Tn or TMV−Tn. The underlying mechanisms behind the superior immunogenicity of Qβ remain to be fully elucidated, which is the subject of further investigation.

Effect of Dose on Anti-Tn Immune Response and Antibody Subtype. The dose of immunogen can significantly impact the immune responses. To establish the optimal dose, mice were immunized with Qβ−Tn 7 in amounts to provide 1, 4, and 20 μg Tn per injection following the aforementioned protocol. Determination of antibody titers by ELISA against BSA−Tn **12** showed high anti-Tn IgG titers for all Qβ−Tn immunized groups (Figure 4a). The highest average titer (263 000) was obtained from the 4 μg group, although the differences between groups did not reach statistical significance. The mean IgG titers of the 4 μg group were 2 orders of magnitude higher than those from the control groups (Qβ−propanol and Qβ−glucose, $p < 0.001$). Substantial anti-Tn IgM antibodies were elicited with highest average titer (13 400) from the 20 μg group (Figure 4b). As discussed in the Introduction, it has been challenging to elicit high titers of IgG antibodies using monomeric form of Tn as the antigen.[39,40] The high titers obtained with Qβ−Tn highlights the advantage of Qβ as a TACA carrier.

As discussed above, a prerequisite for potent IgG responses is the stimulation of B cells by T helper cells.[4] The high IgG titers obtained following Qβ−Tn immunization indicate that Qβ contains the necessary T helper epitopes for T cell potentiation, and Tn functionalization of Qβ does not significantly disrupt the functions of these epitopes. We suggest that the covalent linkage between Tn and Qβ ensures that Tn is processed and presented together with Qβ by the same B cells, thus presumably allowing potent B cell stimulation by matched

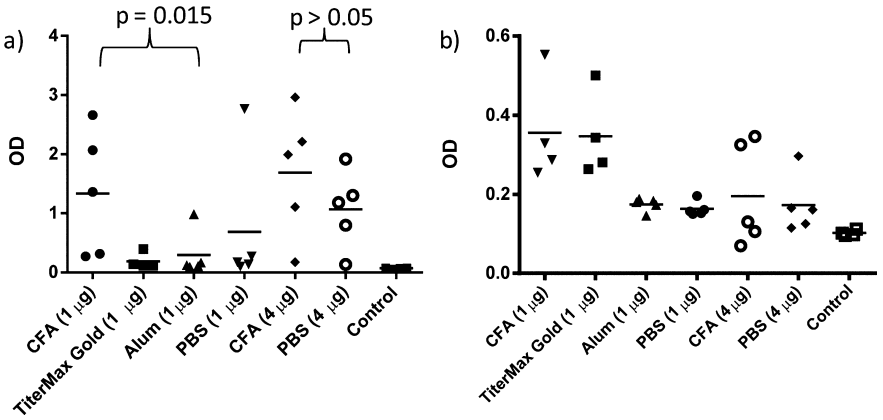

Figure 3. (a) IgG and (b) IgM responses against Tn antigen in immunized mice with or without exogenous adjuvants. The data shown here are measured at a serum dilution of 1:6400. Each data point represents the optical density at 450 nm measured from serum of individual mice. Lines represent the mean value for each group. Statistical analysis was performed using Student's *t*-test.

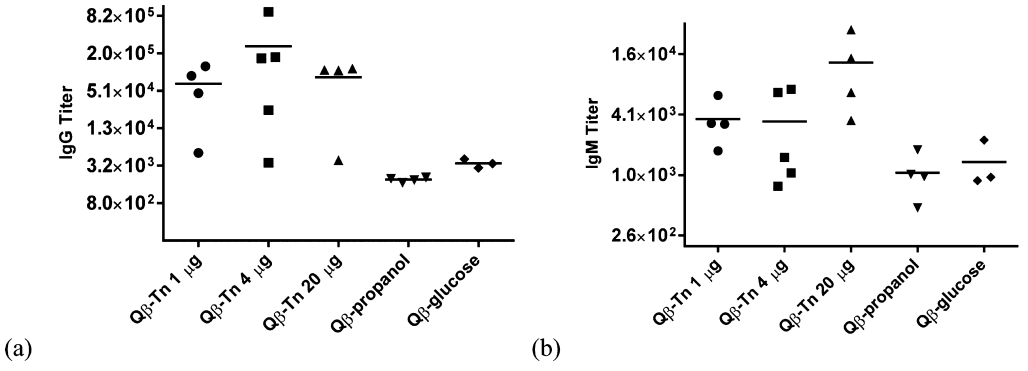

Figure 4. (a) IgG and (b) IgM titers against Tn in mice immunized with Qβ−Tn **7**, Qβ−propanol **8**, and Qβ−glucose **9**. The titer numbers were calculated as the highest folds of dilution that gave three times the absorbance of normal mouse sera at 1:1600 dilution.

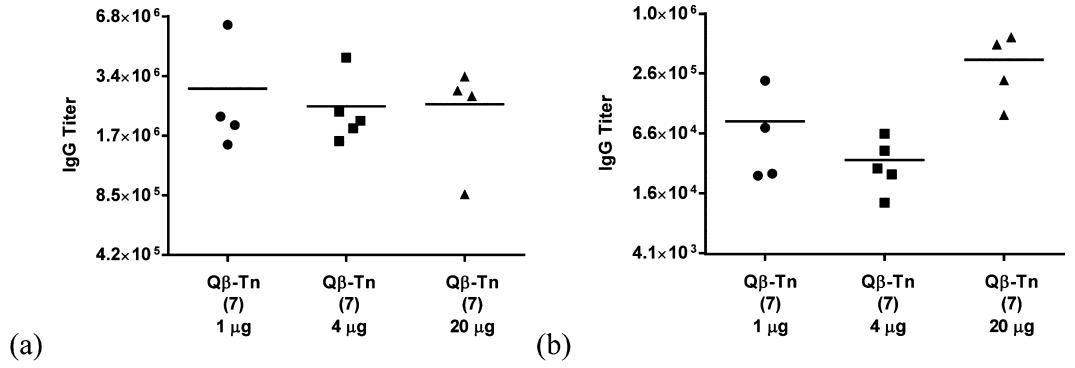

Figure 5. IgG titer against (a) Qβ virion and (b) BSA−triazole conjugate **14**. Sera are from mice immunized with 1, 4, and 20 μg of Qβ−Tn **7**, respectively. The titer numbers were calculated as the highest folds of dilution that gave three times the absorbance of normal mouse sera at 1:1600 dilution.

(a)

Group #	1	2	3	4	5	6
Tn density (particle)	L	M	H	L	M+N	H+N
Vaccine Construct	**11**	**10**	**7**	**11**	**10** + Qβ	**7** + Qβ
Tn Occupancy[a] (%)	23	44	100	23	44 + 0	100 + 0
Total Tn[b] (μg)	0.23	0.44	1	1	1	1
Total Qβ Virion[b] (μg)	18	18	18	75	75	75

[a]Tn occupancy in Qβ-Tn **7**, which displays 340 Tn antigens, was set as 100%.

[b]Amount for each injection in each mouse.

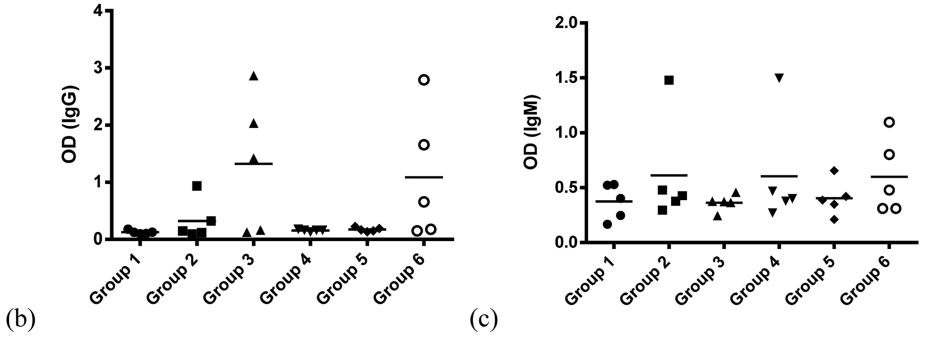

Figure 6. (a) The amounts of Tn and Qβ in six groups of mice for deciphering the importance of antigen display patterns. (b) IgG and (c) IgM responses induced by Qβ conjugated with variable Tn antigen densities (serum dilution 1:6400).

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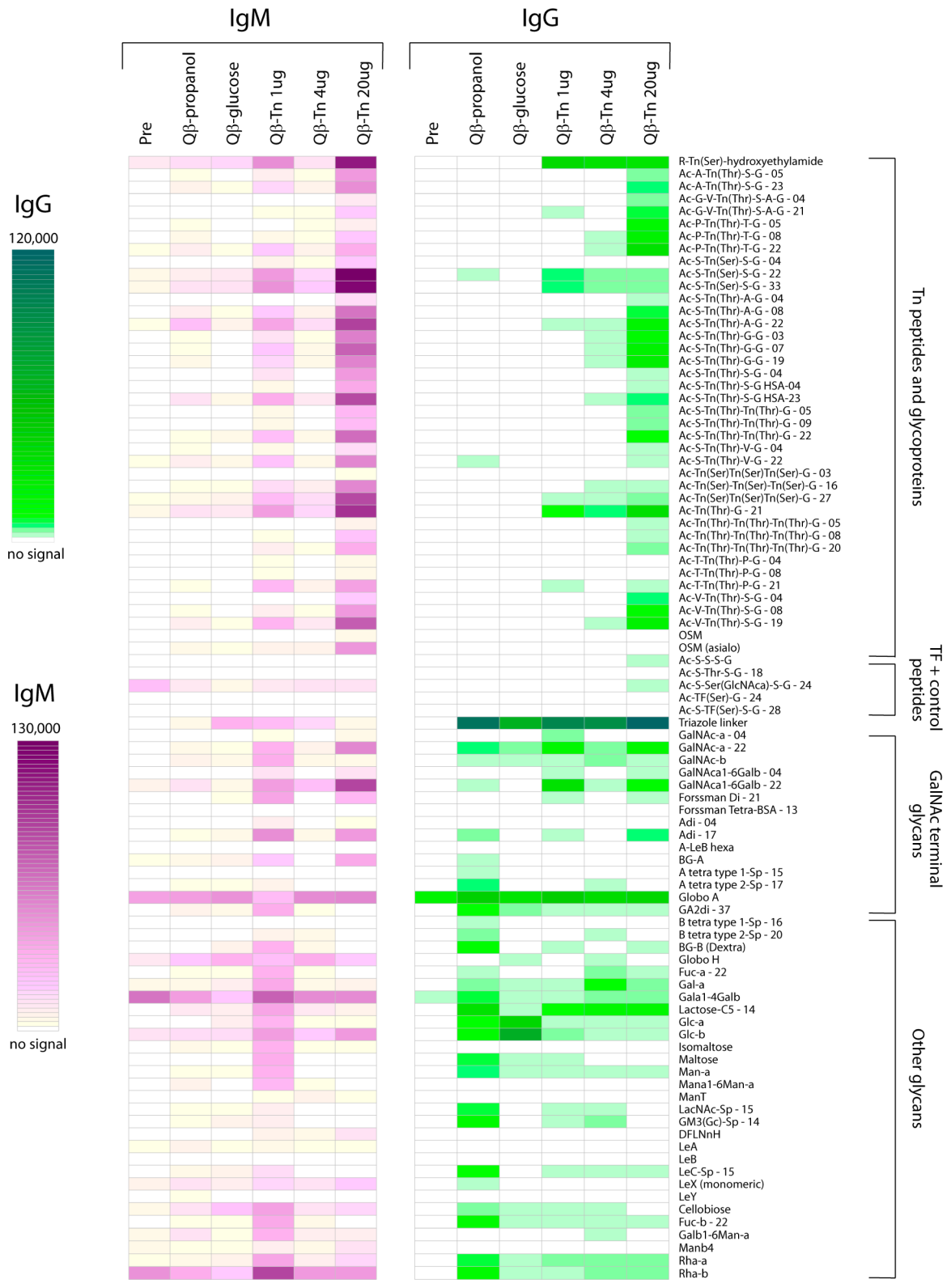

Figure 7. Glyco-microarray profiles comparing pre- and postimmune sera from various groups of mice. The average fluorescence signals from at least 4 mice per group are presented (IgM data shown in magenta and IgG data shown in green). Glycans are attached to BSA prior to printing on the array surface. The numbers after the glycan abbreviation correspond to the average number of glycans per molecule of albumin [for example, Ac-A-Tn(Thr)-S-G-05 is a neoglycoprotein with an average of 5 Tn peptides per molecule of albumin]. Selected array results are shown, and full results can be found in the Supporting Information.

activated T helper cells. The subclasses of IgG were also analyzed (Figure S4, Supporting Information). Substantial amounts of all IgG subclasses were generated with Qβ−Tn 7, indicating the induction of a balanced IgG immune response involving a broad range of cytokines,[47] as well as T helper cell independent IgG3 response.[48]

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Anti-Tn Responses Do Not Correlate with Antibody Levels toward Competing Epitopes. For each group of Qβ−Tn immunized mice, variations were observed in anti-Tn IgG titers. Immune responses to the capsid or the triazole linker may be competitive, suppressing antiglycan immunity in some cases. To test this possibility, we analyzed the levels of antibodies against unmodified Qβ particle and the triazole-containing linker, the latter displayed on BSA as conjugate **14**.

All sera, including those from mice immunized with control particles **8** and **9**, showed responses to both capsid and the triazole containing linker with no correlations to the levels of anti-Tn antibodies (Figure 5 and Figure S5, Supporting Information). This suggests that the low magnitudes of the anti-Tn IgG responses observed for some Qβ−Tn **7** immunized mice were not due to immune impairment. The anti-Qβ virion and antitriazole antibody titers did not significantly vary with the dose or structures of Qβ constructs administered (Figure S5, Supporting Information). The compatibility of the triazole linker with TACA-based vaccine constructs is consistent with several prior reports.[42,49−51] It is known that rigid structures such as triazole can induce antibodies against themselves, which may consume the Th cells and reduce antiglycan responses.[52] Thus, it is possible that a flexible linker between Tn and Qβ can reduce the amount of antilinker antibodies thus further improving the anti-Tn immunity, which will be explored in the future.

High Local Antigen Density Is Critical for High Antibody Responses. To better understand the origin of immune potentiating effect of Qβ, two additional Qβ−Tn constructs (Qβ−Tn **10** and **11**, Figure 6a) were prepared with lower Tn densities by decreasing the amount of Tn-azide **3** used in the CuAAC conjugation reaction. Relative to particle **7** (high loading, designated "H", 340 Tn per particle), particle **10** bears a medium Tn density (150 copies per particle, "M"), and **11** is lightly loaded (78 Tn molecules per particle, "L"). As each Qβ capsid contains 180 subunits, in the high density Qβ−Tn **7**, there were on average close to 2 Tn per capsid with each pair of the carbohydrates expected within 5 nm of each other. Lower Tn loading on Qβ−Tn **10** and **11** provides more dispersed presentations of Tn.

Two sets of immunization experiments were performed. First, three groups of mice (groups 1−3) were immunized with Qβ−Tn **11**, **10** and **7**, respectively, with the amounts of Qβ capsid kept constant within all three groups (Figure 6a). Because of the differential Tn loading on the capsid, these groups received increasing amounts Tn. ELISA analysis showed that group 3 mice generated the highest IgG antibody responses (Figure 6b). This suggested that although equal amounts of T helper epitopes were presented by the capsid, higher levels of Tn (∼1 μg) were needed to generate strong IgG titers. Interestingly, the Tn specific IgM responses, indicative of early B cell activation, were comparable from all groups (Figure 6c). This is consistent with results by Bachmann and co-workers,[53] who reported that the density of peptide antigens on Qβ did not affect IgM titers but had great influence on the T helper cell dependent IgG responses. In another study, Schiller and co-workers demonstrated that to elicit strong antibodies against tumor necrosis factor-α using papillomavirus VLP as the carrier, a high density of antigen was crucial to break B cell tolerance against this self-antigen.[54]

In the second experiment, the amount of Tn was kept constant, and unmodified Qβ particles (designed "N" in Figure 6a) were added when necessary to equalize the total amount of particle administered. Thus, group 4 received 75 μg of particle **11** in each injection, which delivered 1 μg of attached Tn. Groups 5 and 6 received 33 and 17 μg of particles **10** and **7**, respectively, to carry the same total amount of Tn, along with the unmodified particle to bring the total virion to 75 μg. In group 4, Tn antigen was randomly distributed on all Qβ capsids. In contrast, Tn was present only on 23% of the capsid in group 6 but with much higher local organization. If antigen display patterns were not important, groups 4, 5, and 6 should give similar anti-Tn responses. However, ELISA analysis demonstrated that only group 6 generated significant IgG responses (Figure 6b). As before, IgM response was independent of these variations (Figure 6c).

These results suggest that the organized epitope display is crucial for induction of antibody isotype switching from B cells but not for early B cell activation. This is consistent with the fact that the spacing between many neighboring Tn molecules on Qβ−Tn **7** is approximately around 5 nm, which is suitable for strong BCR cross-linking, leading to potent B cell activation and isotype switching producing IgG antibodies.[3] BCR cross-linking rather than simple binding has been proposed as a requisite signal to induce IgG production.[31] Highly organized antigen geometry cannot be achieved with the traditional amorphous carriers such as KLH and tetanus toxoid (TT) highlighting a key advantage of Qβ as antigen carriers.

Groups 3 and 6 mice received the same amount of Tn antigen displayed in the same organized manner, while group 6 had four times the amount of Qβ capsids. The IgG levels generated from these two groups were comparable (Figure 6b). In addition, comparison of groups 1 and 2 with 4 and 5 demonstrated that increasing the dose of Qβ and thereby the amount of T helper epitopes available did not compensate for the inefficient IgG response resulting from poorly organized antigen display. These results together suggest excess Qβ did not significantly suppress[55] or improve humoral responses under the conditions evaluated.

Glyco-microarray Results Confirmed the Tn Selectivity in Antibody Recognition. Our ELISA results demonstrated that the postimmune mouse sera contained antibodies capable of recognizing the Tn antigen. To establish the binding specificities of the antibodies, the sera from mice immunized with Qβ−Tn **7** and control particles **8** and **9** were screened against a carbohydrate microarray.[56] This glycan array contained 329 members, which were prepared by attaching a variety of *O*-linked glycopeptides, *N*-linked glycopeptides, glycolipids, and glycoproteins to BSA, followed by printing the BSA conjugates on the slides (a full list of the library components is given in Table S1 in the Supporting Information). After incubation with each mouse serum sample and washing, a fluorescently labeled secondary antibody was used to quantify the relative amounts of serum antibody bound to individual array components. For clarity, the average intensities from each group of mice were presented (Figure 7). Consistent with the ELISA results, sera from all mice immunized with Qβ−Tn **7** reacted well with the immunizing antigen (R-Tn(Ser)-hydroxyethylamide in Figure 7) compared to the control sera and the prevaccination sera. A variety of Tn-containing glycopeptides and GalNAc terminated glycans were also recognized, suggesting the GalNAc moiety was the major element in recognition. Both monomeric Tn and Tn clusters reacted with the Qβ−Tn postimmune sera. The microarray also contained spots with BSA bearing different densities of terminal GalNAc: GalNAc-α-04 (4 GalNAc per BSA) vs GalNAc-α-22

(22 GalNAc per BSA) and GalNAcα1−6Galβ-04 (4 GalNAcα1−6Gal per BSA) vs GalNAcα1−6Galβ-22 (22 GalNAcα1−6Gal per BSA) (see GalNAc terminal glycans group in Figure 7). In both cases, significantly greater binding was observed to the higher-density display, much like the aforementioned ELISA observations using BSA−Tn **12** and Tn-biotin **13**.

Although the dose of Qβ−Tn did not significantly impact the titers against the immunizing Tn antigen **3**, interesting dose-dependent effects were observed on antibody selectivity and specificity. Both IgG and IgM antibodies from the 20 μg group bound strongest with Tn containing array components compared to antibodies from the groups receiving lower doses (Figure 7). Furthermore, antibodies from the 20 μg group showed more specificity toward GalNAc and less cross-reactivities with other glycans. In other words, the higher glycopeptide antigen dose induced a more focused immune response toward the glycan antigen, suggesting the potential advantage of using high antigen dose for immunization.

Induced Anti-Tn Antibodies Reacted with "Native" Tn Epitopes on Tumor Cells. For an effective immunotherapy, it is important that anti-TACA antibodies generated by the synthetic vaccine can recognize the antigens present in the native environments such as the surfaces of cancer cells. To test this, we analyzed the binding of sera showing high IgG titers by ELISA to Jurkat cells, a human leukemia cell line expressing Tn antigens on the surface (Figure 8).[57] Significant cell recognition by these serum antibodies was observed using flow cytometry, whereas serum from mice given control particle **8** or **9** showed no binding (Figure 8d). Consistent with ELISA data, the percentages of cells exhibited positive reactivities with Qβ−Tn in FACS analysis were higher than those from TMV−Tn (Figure S6, Supporting Information).

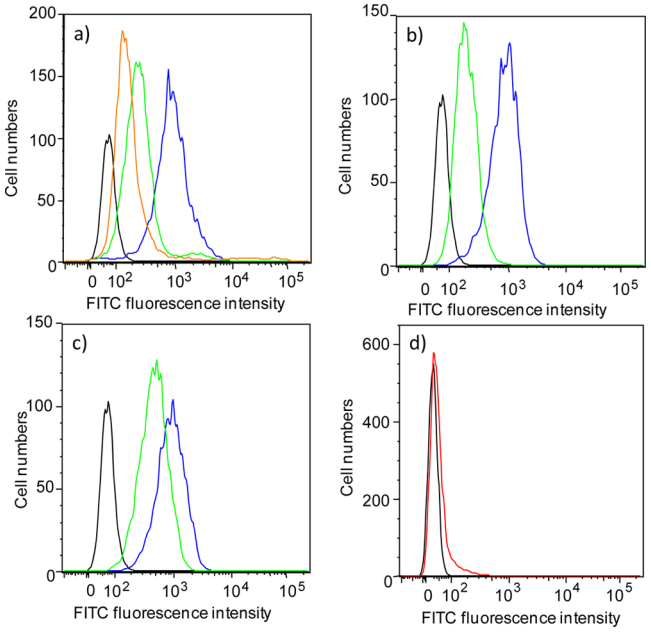

Figure 8. Flow cytometry analysis of Jurkat cell binding by sera (1:10 dilution) from representative mice immunized with increasing amounts of Qβ−Tn **7**, (a) 1 μg Tn, (b) 4 μg Tn, and (c) 20 μg Tn, and (d) control mouse immunized with **8**. Black curve (negative control) represents the fluorescence obtained with FITC conjugated antimouse IgG antibodies in the absence of serum. Colored curves represent results for different mice in the same group.

CONCLUSION

We demonstrate here that the bacteriophage Qβ capsid is a powerful antigen delivery platform capable of boosting the humoral immune responses to a very weak TACA, the Tn antigen. The addition of external Freund's adjuvant to Qβ−Tn conjugates produced higher IgG titers, although strong immune responses were also obtained without exogenous adjuvants. The IgG antibodies produced were highly selective toward Tn antigen binding and reacted strongly with native Tn antigens on human leukemia cells demonstrating the physiological relevance of the IgG antibodies induced by the Qβ−Tn vaccine construct. Once the density of Tn antigen administered passed a threshold level, the pattern of Tn antigen display, rather than the total amount of Tn antigen administered, appeared to be crucial for the induction of high anti-Tn IgG titers. The knowledge gained from the current study will facilitate the rational design and optimization of carbohydrate-based anticancer vaccines using virus-like particles such as Qβ as a powerful antigen delivery system.

METHODS

General Procedures for Mouse Immunization. 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 (Fisher), TiterMax Gold adjuvant (Sigma Aldrich), Imject Alum (Thermo Scientific), or Incomplete Freund's adjuvant (Fisher) according to the manufacturer's instructions. Boosters were given subcutaneously under the scruff on days 14 and 28 (for complete Freund's adjuvant group, incomplete Freund's adjuvant was used). Serum samples were collected on days 0 (before immunization), 7 and 35. The final bleeding was done by cardiac bleed.

Procedures for ELISA. A 96-well microtiter plate was first coated with a solution of BSA−Tn, BSA−triazole, or wide type Qβ viron in PBS buffer (10 μg mL^{-1}) and then incubated at 4 °C overnight. The plate was then washed four times with PBS/0.5% Tween-20 (PBST), followed by the addition of 1% (w/v) BSA in PBS to each well and incubation at RT for one hour. The plate was washed again with PBST and mice sera were added in 0.1% (w/v) BSA/PBS. The plate was incubated for two hours at 37 °C and washed. A 1:2000 dilution of horseradish peroxidase (HRP)-conjugated goat antimouse IgG, IgM, IgG1, IgG2b, IgG2c, or IgG3 respectively (Jackson ImmunoResearch Laboratory) in 0.1% BSA/PBS was added to each well. The plate was incubated for one hour at 37 °C, washed and a solution of 3,3,5′,5′-tetramethylbenzidine (TMB) was added. Color was allowed to develop for 15 min, and then a solution of 0.5 M H_2SO_4 was added to quench the reaction. The optical density was then measured at 450 nm. The titer was determined by regression analysis with $\log_{10}$ dilution plotted with optical density. The titer was calculated as the highest dilution that gave three times the absorbance of normal mouse sera diluted at 1:1600 (about 0.1 for all sera).

ASSOCIATED CONTENT

Supporting Information

Information on synthesis, Qβ, CPMV conjugation and characterization. Procedures for glycan microarray and FACS analysis and full array data. Figures S1−S16, NMR spectra, and Table S1. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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