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Significant Impact of Immunogen Design on the Diversity of Antibodies Generated by Carbohydrate-Based Anticancer Vaccine

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Abstract

Development of an effective vaccine targeting tumor associated carbohydrate antigens (TACAs) is an appealing approach toward tumor immunotherapy. While much emphasis has been typically placed on generating high antibody titers against the immunizing antigen, the impact of immunogen design on the diversity of TACA-specific antibodies elicited has been overlooked. Herein, we report that the immunogen structure can significantly impact the breadth and the magnitude of humoral responses. Vaccine constructs that induced diverse TACA-binding antibodies provided much stronger recognition of a variety of Tn positive tumor cells. Optimization of the breadth of the antibody response led to a vaccine construct that demonstrated long lasting efficacy in a mouse tumor model. After challenged with the highly aggressive TA3Ha cells, mice immunized with the new construct exhibited a statistically significant improvement in survival relative to controls (0% vs 50% survival; $p < 0.0001$). Furthermore, the surviving mice developed long-term immunity against TA3Ha. Thus, both the magnitude and the breadth of antibody reactivity should be considered when designing TACA-based antitumor vaccines.

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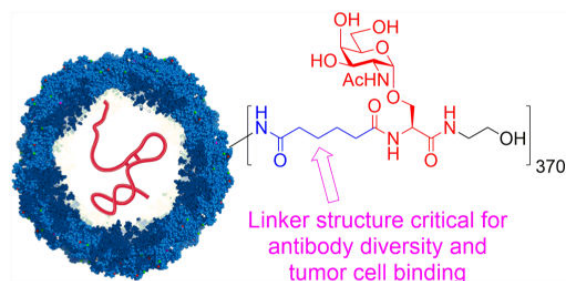
Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acscchem-bio.5b00406. Information on synthesis and characterization of various Q β conjugates. Procedures for CD4 lymphocyte depletion experiment, kinetics study of antibody generation, inhibition of Tn binding experiments and procedures for carbohydrate microarray studies. Supplementary Figures S1–S10. Full microarray data (Table S1)(PDF)

Notes

The authors declare no competing financial interest.

Abstract



Cancer cells often express high levels of characteristic glycan structures, which are referred to as tumor associated carbohydrate antigens (TACAs).¹ An example of TACA is the Tn antigen,² which has been found in 70–90% of breast, lung, prostate, and pancreatic tumors but is rarely expressed in healthy tissues. High levels of Tn antigen expression correlate significantly with shortened disease-free interval and increased metastasis.^{3–5} As a result, Tn antigen has sequence, 17,18 lipid structures of glycolipids, 22 19–21 or neighbor-been ranked among the top 50 tumor-associated antigens,⁶ and ing carbohydrates of the TACAs. For instance, the Tn antigen innovative studies have been performed on Tn-based vaccines either alone or as part of a multiantigen construct.

Development of anti-TACA vaccines is still very challenging due to their low immunogenicity and T-cell independent nature. Despite much effort dedicated to improve anti-TACA antibody responses,^{11–14} no TACA-based vaccines have been approved by FDA yet. Phase III studies of GM2–KLH and STn–KLH have failed to show therapeutic benefits even though significant antibody titers were stimulated in cancer patients.^{15,16} Thus, there is still much yet to be learned about what constitutes effective ant-tumor responses.

In the development of carbohydrate based anticancer vaccines, much emphasis has been placed on the investigation of carrier and modification of antigen structures to enhance humoral responses.^{11–14} One potential complexity in TACA based vaccine design is the heterogeneities of local environments of TACAs on tumor cell surfaces. Antibody recognition of the TACA epitope can be influenced by the glycoprotein sequence,^{17,18} lipid structures of glycolipids,^{19–21} or neighboring carbohydrates of the TACAs.²² For instance, the Tn antigen can be found in a variety of glycoproteins including epiglycanin²³ and mucin-1 (MUC1).²⁴ Even in a protein such as MUC1, because it can contain hundreds of tandem repeats and each repeat region bears five potential glycosylation sites, there are many possible Tn containing structures.²⁵ As a result, a specific antibody generated against the immunizing TACA structure may not recognize the same TACA displayed on tumor cells due to differential conformations. As an example, anti-Tn mAbs MLS128 and 83D4 only interact with clusters of two or three neighboring Tns in glycopeptides but fail to recognize two Tns separated by an unglycosylated amino acid.²⁶ Several anti-Tn IgG mAbs raised by Jurkat cells only recognized Tn antigen in the context of unique peptide motifs.¹⁷ A reinvestigation of the STn–KLH vaccine suggested that induction of anti-STn antibodies targeting a wide range of STn-carrying glycoproteins rather than a single one is critical in controlling tumor growth, suggesting the significance of eliciting diverse TACA-specific

antibodies.²⁷ Unfortunately, current vaccination approaches mostly focused on the magnitude of antibody responses against the immunizing antigen, with little attention paid to the breadth of antibody repertoire. Therefore, strategies that can elicit a diverse range of antibodies capable of binding the target antigen within a variety of contexts are highly desirable to enhance immune recognition and reduce immune escape of cancer cells.

The breadth of antibody response depends on the activation of naive germline B cell pool and subsequent somatic hypermutation in germinal centers, although the exact regulatory mechanism is not well understood.²⁸ Recent studies have revealed the impact of adjuvants or hapten density on the spectrum of antibody responses,^{29–31} while the role of other factors remain to be fully elucidated. Herein we report that the immunogen structure can have a profound effect on the diversity of antibodies. A well-designed Tn immunogen on the virus-like particle (VLP) bacteriophage Q β scaffold improved the antibody titers but perhaps more importantly greatly expanded the diversity of the antibodies induced. As a result, the recognition of Tn-positive tumor cell lines was much enhanced leading to effective protection of mice from tumor development. The results presented provide important design considerations for the development of carbohydrate-based anticancer vaccine.

RESULTS AND DISCUSSION

First Generation Q β -Tn Conjugates Failed To Elicit TA3Ha Reactive Antibodies

To overcome the low immunogenicity of Tn and elicit a powerful humoral response, it is critical to conjugate Tn with a carrier moiety that can potentially induce the activation of CD4+ helper T cells and antibody isotype switching to IgG. We became interested in investigating the utility of VLPs such as bacteriophage Q β as a carrier for Tn. Our first generation construct Q β -Tn **1** was synthesized using the copper-catalyzed alkyne-azide cyclo-addition (CuAAC) click reaction,³² which attached an average of 340 copies of Tn per Q β capsid through a triazole linker (termed internal triazole), as well as 200 copies of triazole without Tn (external triazole) (Figure 1A). Mice were immunized with Q β -Tn **1** with two booster injections on days 14 and 28. On day 35, sera were collected and titrated against BSA-Tn conjugate **2** and BSA-triazole **3** (Figure 1B) to measure the overall antibody responses against the carbohydrate and triazole linker, respectively, in enzyme-linked immunosorbent assays (ELISAs, Figure 1C). The results showed an average Tn titer of 263 600 with a moderate average anti-triazole titer of 35 300. The postimmune sera reacted with Tn-expressing human lymphoma Jurkat cells through FACS analysis. In order to demonstrate the efficacy of antitumor activities, a mouse tumor model using the Tn-expressing murine TA3Ha cells has been established.³³ However, despite binding with Jurkat cells and the high IgG titers against BSA-Tn **2**, none of the sera from mice immunized with Q β -Tn **1** showed significant reactivity with TA3Ha cells even at a relatively high concentration (1:10 dilution). These results suggest immunization of Q β -Tn **1** would be ineffective toward protecting the host from TA3Ha tumor growth.

In order to improve tumor cell recognition, Q β -Tn construct **4** (Figure 1D) was prepared, which contained 360 copies of Tn without any external triazoles to test whether the removal of external triazoles could boost the anti-Tn titers. However, upon immunizing mice following the same protocol as Q β -Tn **1**, lower levels of Tn-specific IgG antibodies

(average titer of 111 000) were found and the titers of antitriazole antibodies increased (average titer of 63 000) (Figure 1E). Next, the density of Tn on Q β was increased from 340 to 540 (Q β -Tn 5, Figure 1D), since higher local antigen density was previously shown to enhance anti-Tn antibody titers.³² of 49 100), while the average triazole titer increased to 175 000 (Figure 1F). The postimmune sera from mice immunized with Q β -Tn 4 or Q β -Tn 5 again failed to bind with TA3Ha cells.

Alkyl Amide-Linked Q β -Tn 6 Generates TA3Ha Reactive Antibodies

The ineffectiveness of the triazole-linked Q β -Tn constructs prompted us to synthesize a new vaccine (Q β -Tn 6) where an alkyl amide linker was utilized to couple Tn antigen with Q β (Figure 2A and Scheme S1). The average number of Tn per capsid was 370, similar to that of Q β -Tn 1. Mice were immunized with Q β -Tn 6 following the same immunization protocol as Q β -Tn 1. ELISA analysis showed that these mice produced primarily an IgG humoral response with exceptionally high average titers (~1 461 000) of anti-Tn IgG (Figure 2B). Analysis of the IgG subtypes demonstrated balanced Th1/Th2 responses with significant titers of all major IgG subclasses (Figure 2B). When the CD4+ helper T cells from the mice were depleted using an anti-CD4 mAb prior to vaccination with Q β -Tn 6, the anti-Tn IgG responses decreased significantly suggesting that CD4+ helper T cells play important roles in regulating class switching of antigenic responses (Figure 2C).

With the superior anti-Tn titers, the binding of the antibodies with multiple tumor cell lines was analyzed by flow cytometry. Although the average anti-Tn IgG titers from Q β -Tn 6 and Q β -Tn 1 differed only by 4-fold, the average mean fluorescence intensities (MFI) of sera binding to Jurkat cells increased more than 50 times with Q β -Tn 6 (Figure 2D and Figure S5). Importantly, the postimmune sera from Q β -Tn 6 immunized mice bound strongly with TA3Ha cells (Figure 2D,E). The breadth of tumor cell recognition by Q β -Tn 6 induced antibodies was further demonstrated through their binding with Ag104-MUC1 cells (Figure 2D and Figure S5).

The generation of a strong anti-Tn humoral response has been very challenging. To induce anti-Tn antibodies, a variety of delivery platforms have been investigated including synthetic multiple antigenic glycopeptide and protein carriers such as keyhole limpet hemocyanin (KLH) and desialylated ovine submaxillary mucin.^{7-10,34-36} It has been found that conjugates bearing the synthetically more accessible monomeric Tn elicited little anti-Tn antibodies presumably due to the small size of the antigen.^{9,36} To boost the antibody levels, Tn clusters have been investigated. However, even with these types of immunogens, most of the anti-Tn titers were still modest^{9,36} with only one study reporting IgG titers over 100 000.¹⁰ Therefore, with its ability to induce super high anti-Tn IgG titers (~1 461 000) using monomeric Tn antigen and the strong binding by the antibodies induced to a wide range of tumor cells, the Q β -Tn 6 construct represents a significant advance in anti-Tn vaccine design.

Q β -Tn 6 Vaccine Greatly Enhanced the Breadth of Antibody Repertoire

The drastic enhancement of tumor cell recognition by Q β -Tn 6 induced antibodies could be due to higher antibody avidity to Tn antigen. To evaluate this, a chaotropic ELISA

procedure was developed. The postimmune sera from Q β -Tn **1** and Q β -Tn **6** immunized mice were incubated with BSA-Tn **2** immobilized in ELISA plates. After removal of unbound antibodies, increasing concentrations of aqueous ammonium thiocyanate solution were added. The concentration of ammonium thiocyanate needed to remove 50% of the antibodies bound to BSA-Tn (IC⁵⁰) was determined as an avidity measure. Both groups were eluted from the plate with similar concentrations of ammonium thiocyanate indicating that there were no substantial differences in avidities of antibodies generated (Figure S6).

Another factor that can impact tumor cell binding is the diversity of antibodies generated. The epitope profiles were analyzed at a molecular level using a glycan microarray,³⁷ which contains a panel of 328 glycoconjugates derived from glycoproteins or glycolipids with 39 Tn peptides and 44 other GalNAc terminal glycans (for a full list of array components, see Table S1). Sera from mice immunized with Q β -Tn **1** or Q β -Tn **6** were incubated on the microarray and then detected by a fluorescently labeled secondary antibody (Figure 3A). In agreement with the ELISA results, IgG bearing components. In contrast, sera from mice immunized with Q β -Tn **6** exhibited much broader binding. For example, although the immunizing Tn antigen contains serine only, both serine and threonine linked Tn monomers and dimers were recognized well. Tn trimer binding revealed interesting dependence on backbone sequences. The IgG antibodies showed strong reactivity toward the Tn cluster of Tn(Ser)-Tn(Ser)-Tn(Ser) but not its threonine analog. This is consistent with several observations that the recognition of cluster Tn is strongly modulated by the peptide backbone^{17,26} The abilities of Q β -Tn **6** to elicit antibodies capable of binding cluster Tn despite the monomeric Tn utilized for synthesis of Q β -Tn **6** were presumably due to the high density organized display of Tn on Q β mimicking Tn clusters.

Tn antigens are present on tumor cells in a variety of configurations including both monomeric and cluster forms. With a murine Lewis lung cancer model, Matsumoto et al. recently reported that an increased level of Tn clusters in syndecan-1 was closely correlated with enhanced invasion and metastasis.³⁸ On the other hand, overexpression of hypoglycosylated MUC1 is a hallmark in a large variety of epithelial cancers, and the immunodominant epitopes of MUC1 do not contain three consecutive glycosylation sites. In a histochemical study with 322 cases of invasive breast ductal carcinomas, nonconsecutive Tn in MUC1 was implicated in aggressive growth and lymphatic metastasis of breast cancer cells.³⁹ The antibodies elicited by Q β -Tn **6** were capable of recognizing both Tn monomer and clusters (Figure 3), rendering it attractive as a vaccine candidate.

Among all the Tn-containing glycopeptides on the microarray, multiple peptide sequences can be found in glycoprotein epiglycanin, CD43, and MUC1, which are the major Tnbearing proteins in TA3Ha, Jurkat, and Ag104-MUC1, respectively. Antibodies generated by Q β -Tn **6** but not Q β -Tn **1** showed strong signals to these epitopes (Figures 3B and S7), which may account for their dramatic difference in reactivity with these tumor cells. In addition, antibodies generated from Q β -Tn **6** exhibited little bindings to non-Tn carbohydrate epitopes, which highlights their Tn specificity (Figure 3B).

Relative antibody avidity can also be established from the microarray by comparing the intensities of array components due to antibody binding. Consistent with the chaotic

ELISA result, sera generated by Q β -Tn **1** and Q β -Tn **6** showed similar ratios of binding to Tn conjugates arrayed at low and high densities (Figure S8) supporting the aforementioned conclusion that antibodies generated by these two constructs have comparable avidities.

Q β -Tn **1** Rapidly Elicited Antitriazole Antibodies, Which Could Hinder Tn Binding

In order to shine light on the role of immunogen structure on antibody responses, the binding of Q β -Tn **1** and Q β -Tn **6** by soybean agglutinin (SBA, a Tn selective lectin), an anti-Tn mAb Bric 111, or polyclonal antibodies⁴⁰ was compared. The recognition of Tn on both constructs by all these Tn receptors were similar (Figure S9). This suggests that the cyclic triazole moiety in Q β -Tn **1** most likely did not restrict the conformations of Tn for recognition by B cell receptors *in vivo*.

Another possibility examined is that the anti-triazole antibodies induced by Q β -Tn **1** can bind with the triazoles on the vaccine construct, potentially shielding Tn from interacting with B cell receptors. To test this, the binding of Q β -Tn **1** and Q β -Tn **6** by SBA was measured in the presence of serum enriched with polyclonal anti-triazole antibodies. While this serum reduced SBA binding to Q β -Tn **6** by 15%, its effect on Q β -Tn **1** was much more prominent, resulting in 50% inhibition of SBA binding (Figure 4A). Furthermore, we found that in Q β -Tn **1** immunized mice, anti-triazole antibodies were generated much faster than those against Tn (Figure 4B). These results support the possibility that anti-triazole antibodies can partially block the binding of Q β -Tn **1** to Tn-specific B cells *in vivo*, thus potentially reducing the number and types of Tn-specific B cells that can get in contact with the Tn antigen and decreasing the diversity of anti-Tn antibodies.

Vaccination with Q β -Tn **6** in Combination with Chemotherapy Confers effective and Long Lasting Protection against Cancer

With the strength and diversity of anti-Tn responses elicited by Q β -Tn **6** established, the ability of the vaccine to protect mice from tumor development was evaluated in a therapeutic model. TA3Ha cells (5000 cells) were injected into the mice intraperitoneally on day 0. The mice were then treated with a low dose of a chemotherapeutic drug, cyclophosphamide (CP), administered 1 day prior to vaccination with Q β -Tn **6**. Control groups received PBS, CP, Q β -Tn **6**, and CP/Q β . As shown in Figure 5A, all mice in the PBS group died within 12 days. Compared with all other treatments, the combination of Q β -Tn **6** and CP provided significantly higher protection against tumor development with 50% of the mice surviving tumor challenge after 50 days. Furthermore, the surviving mice from the CP/Q β -Tn **6** group were challenged with another dose of 5000 TA3Ha cells on day 50. Without any additional treatment, all of these mice rejected the tumor. These results suggest that these CP/Q β -Tn **6** treated mice gained long-lasting immunity against TA3Ha cells.

The observations that the combination of CP and Q β -Tn **6** vaccination provided much higher protection than vaccination or CP treatment alone suggest strong anti-Tn humoral responses were necessary but not sufficient to protect most animals. This is consistent with the observation that direct intraperitoneal injection of an anti-Tn mAb into mice bearing TA3Ha in the peritoneal cavity did not provide much protective benefit.⁴¹ At the dose administered, CP does not impact much the proliferation of TA3Ha cells.⁴¹ It is known that

in C57BL6 mice, the antitumor activities of effector cells are generally weak.⁴² Although the anticancer mechanisms of CP are complex,^{43,44} it can potentially abrogate the immunosuppression imposed by myeloid-derived suppressor cells and activate the effector cells such as natural killer cells to induce cytotoxicity to tumor cells that are bound by Tn-specific antibodies.⁴⁵

Mice Protected by Q β -Tn/CP from Tumor Challenges Have Elevated Levels of Anti-Tn IgG and IgM Antibodies That Can Recognize TA3Ha Cells

To better understand the immunoprotection, sera from mice surviving the tumor challenge were collected and compared with those from dead mice. Although sera from unprotected mice showed binding to TA3Ha cells, tumor cell binding by sera from protected mice was much higher (Figure 6A,B). Glyco-microarray analysis also demonstrated that sera from live mice bound to many more Tn-serine-containing peptides (Figure S10).

To test the specificity of antibodies elicited, we performed competitive flow cytometry analysis. The Tn bearing glycoprotein epiglycanin on TA3Ha cells⁴⁶ is continuously shed and released into ascites.⁴⁷ TA3Ha cells were co-incubated with sera from protected mice and the ascites. The presence of ascites led to an average of 50% reduction in IgG binding to TA3Ha (Figure 6C). Similar phenomena were observed with IgM, with IgM binding to TA3Ha completely inhibited by ascites (Figure 6D). These results indicate that epiglycanin contains the major epitopes recognized by the antibodies.

In conclusion, we present for the first time that immunogen design in a carbohydrate based conjugate vaccine can profoundly alter the breadth of antibody repertoire. Despite the prevalence of the triazole linker in conjugate vaccines,^{32,48-54} our data showed that the inclusion of such a linker could dramatically reduce the diversity of anti-TACA humoral responses. The ability to increase the repertoire of anti-Tn antibodies by removing the triazole linker correlated with substantial enhancement of reactivity with Tn-bearing tumor cells, which ultimately enabled the long lasting protection against tumor challenge in a mouse model. Our findings highlight the importance of immunogen design in eliciting broad anti-TACA responses for the development of effective anticancer vaccines.

MATERIALS AND METHODS

Synthesis and Characterization of Q β Conjugates

Wild-type Q β particles and Q β -Tn **1**, **4**, and **5** were prepared as described previously,³² except that no extra triazole moiety was introduced in Q β -Tn **4** and **5** by controlling the amount of reagent. Q β -Tn **6** was synthesized by mixing wild-type Q β particles and Tn-NHS³² in a mixture of DMSO and PBS buffer (0.1 M, pH = 7) overnight at RT. All the conjugates were combined and purified by repeated filtration using Millipore 100 000 MWCO filter units against PBS buffer. Total protein concentration was measured using the Coomassie Plus protein reagent (Pierce) with bovine serum albumin as standard. Antigen loading per Q β was determined using electrophoretic analysis and MALDI-TOF. Particle stability after conjugation was shown by size exclusion FPLC on a Superose-6 column.

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 C57BL/6 mice were injected subcutaneously under the scruff on day 0 with 0.1 mL of various Q β 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 of various Q β constructs as emulsions in incomplete Freund's adjuvant (Sigma-Aldrich, F5506). All Tn vaccine constructs administered have the same amounts of Tn antigen (4 μ g). Serum samples were collected on day 0 (before immunization), 7, and 35. The final bleeding was done by cardiac bleed.

Antibody Detection by ELISA and Flow Cytometry

Sera were tested as described previously for anti-Tn and anti-triazole antibodies by ELISA using BSA–Tn 2 or BSA–triazole 3.³² The titer was determined by regression analysis with log 10 dilution plotted with optical density.

Sera were tested by flow cytometry on Tn-bearing tumor cell lines Jurkat (kindly provided by Profs. Barbara Kaplan and Norbert Kaminski, Michigan State University) and fresh TA3Ha cells (kindly provided by Prof. John Hilkens, The Netherlands Cancer Institute) isolated from ascites of A/J mice. 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.).

Antitumor Immunotherapy

Five thousand TA3Ha cells were intraperitoneally injected into eight-week old C57BL6 mice on day 0. Mice were injected with PBS buffer or cyclophosphamide (50 mg/kg) intraperitoneally on day 1. Q β –Tn 6 or Q β particle was intravenously injected with 20 μ g of MPLA on days 1, 4, and 8, while only PBS buffer was injected for PBS group. Survival of mice was monitored for 50 days. Mice free of tumor in Q β –Tn 6 plus cyclophosphamide group were injected with 5000 TA3Ha cells intraperitoneally, and survival of mice was monitored for another 50 days. As a control, a group of four eight-week old C57BL6 mice received same dose of TA3Ha cells at the same time. Statistical analysis of survival was performed with GraphPad Prism using log-rank test.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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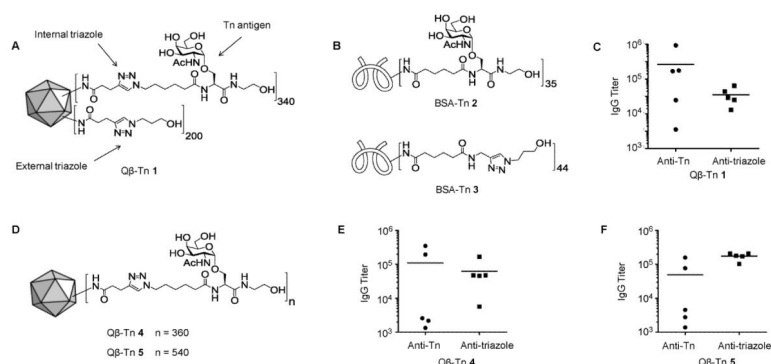


Figure 1. Schematic representations of (A) vaccine construct Q β -Tn 1 and (B) BSA-Tn 2 and BSA-triazole 3. (C) Day 35 IgG titers from mice vaccinated with Q β -Tn 1. (D) Schematic representation of vaccine constructs Q β -Tn 3 and 4. (E, F) IgG titers elicited by Q β -Tn 4 and 5 against Tn and triazole, respectively.

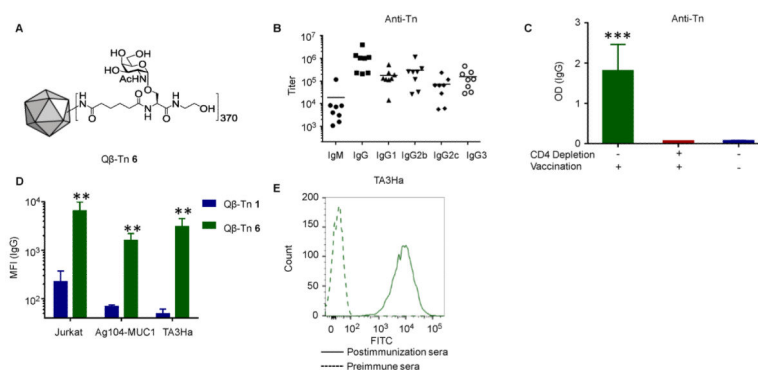


Figure 2.

(A) Schematic representation of vaccine $Q\beta$ -Tn **6**. (B) Anti-Tn titers from mice vaccinated with $Q\beta$ -Tn **6**. (C) Mice were depleted of CD4⁺ T cells prior to vaccination, and anti-Tn IgG responses were compared with control mice without depletion or vaccination. The results show that CD4⁺ T cells are critical for high antibody responses. (D) Sera from mice immunized with $Q\beta$ -Tn **6** showed significantly higher binding with multiple Tn positive tumor cells than those with $Q\beta$ -Tn **1**. Results are expressed as the mean of individual \pm SEM; ** $p < 0.01$ and *** $p < 0.001$ using Student's t test. (E) Recognition of TA3Ha cells by serum from mice immunized with $Q\beta$ -Tn **6** (For clarity, only one representative example of binding by postimmune serum is shown).

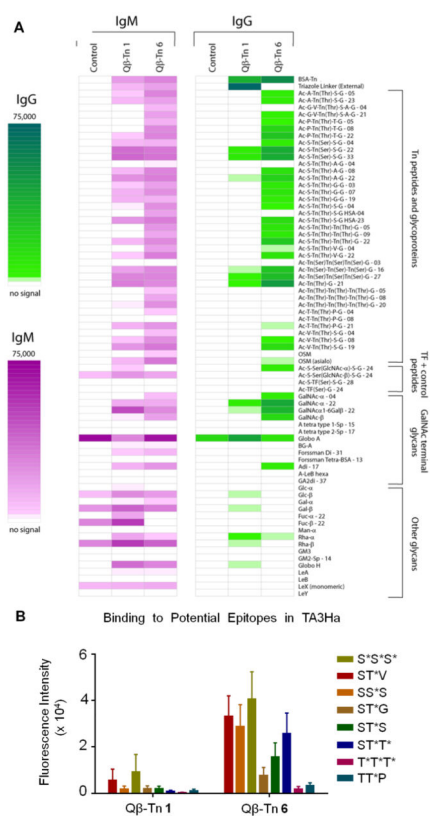


Figure 3.

(A) Glyco-microarray profiles comparing pre- and postimmune sera from control mice and mice immunized with Q β -Tn 1 and Q β -Tn 6. The average fluorescence signals from at least four 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. (B) Sera from mice immunized with vaccine Q β -Tn 1 and Q β -Tn 6 was analyzed against potential epitopes present in epiglycanin (TA3Ha cell) by microarray.

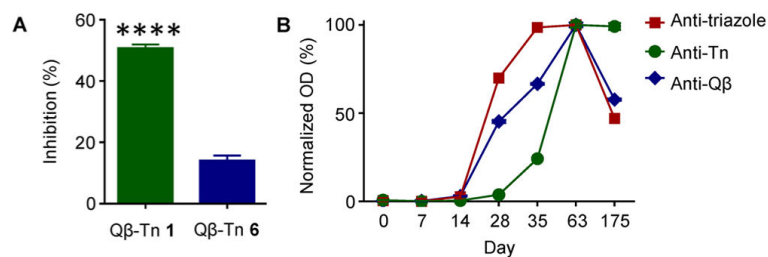


Figure 4.

(A) Inhibitory effect of anti-triazole antibodies on Tn recognition by SBA. Vaccines were coated on ELISA plates, then treated with anti-triazole antibodies, which was followed by detection by SBA. The inhibitory effect was calculated as the percentage of signal intensity decrease for each vaccine. (B) Kinetics of the generation of anti-triazole, anti-Q β , and anti-Tn antibodies in Q β -Tn 1 immunized mice. The OD values of each group were normalized.

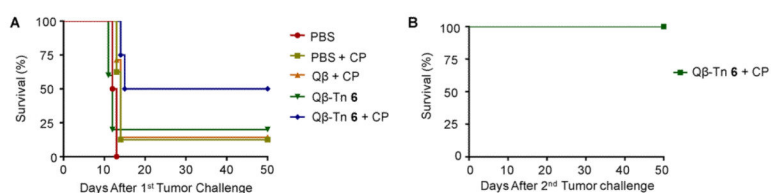


Figure 5.

Active vaccination with Q β -Tn 6 in combination of chemotherapy improved the survival of mice. (A) Groups of mice were intraperitoneally injected with 5000 TA3Ha cells on day 0, with or without intraperitoneal treatment of cyclophosphamide (50 mg kg^{-1}). PBS buffer, Q β particle plus MPLA, or Q β -Tn 6 plus MPLA was administrated intravenously. The survival of mice was followed for 50 days. PBS group ($n = 8$), PBS+CP group ($n = 8$), Q β +CP group ($n = 7$), Q β -Tn 6 group ($n = 5$), Q β -Tn 6 + CP group ($n = 8$). There is significant difference between Q β -Tn 6 + CP group and PBS group ($p < 0.0001$) or all other treatment group ($p < 0.05$). Statistical analysis of survival was performed with GraphPad Prism using log-rank test. (B) Mice surviving tumor challenge from CP/Q β -Tn 6 group were rechallenged with 5000 TA3Ha cells. All mice survived without any further treatment.

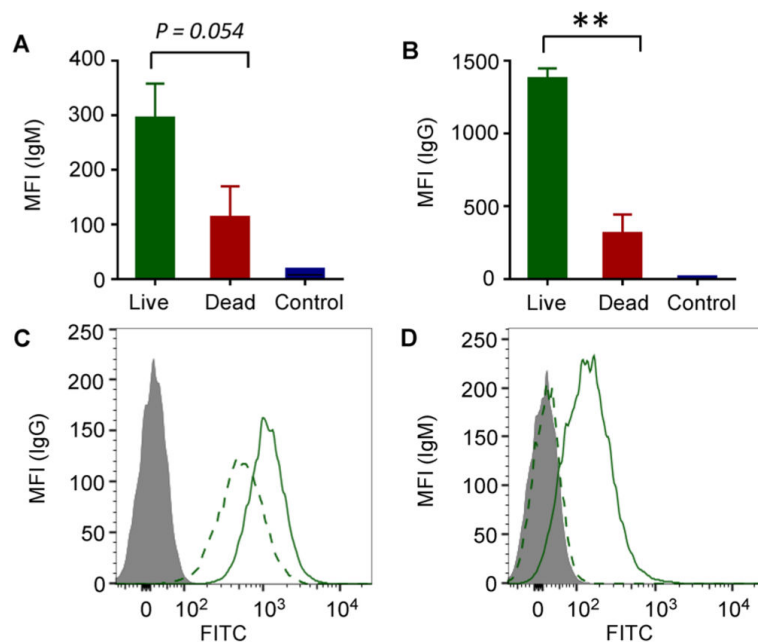


Figure 6.

Analysis of anti-Tn responses in live or dead mice immunized with $Q\beta$ -Tn 6 plus cyclophosphamide. For live mice, sera were bled on day 23. Sera were collected from dead mice right after mice were sacrificed due to the heavy tumor burden. IgG and IgM binding with TA3Ha tumor cells were analyzed at 1:20 dilution, and mean fluorescence intensities for (A) IgG and (B) IgM binding were shown. Preimmune sera were used as control. Competitions of IgG and IgM antibodies binding with TA3Ha by ascites fluid were shown in panels C and D, respectively. Solid curve, without ascites incubation; dashed curve, with ascites co-incubation; gray filled, preimmune serum binding; $**p < 0.01$ using Student's *t* test.