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A C-Linked Disaccharide Analogue of Thomsen–Friedenreich Epitope Induces a Strong Immune Response in Mice

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Cancer cells can express aberrant cell-surface-glycosylation patterns that makes them to be recognizable by the immune system.[1] This phenotype gives an opportunity to develop carbohydrate-based vaccines for cancer immunotherapy.[2] Thomsen–Friedenreich (TF) antigen is a disaccharide (β-D-Gal*p*-1-3-α-D-GalNA*cp*) oncofetal blood group-related antigen normally O-linked to serines or threonines of mucines (MUC) expressed on cells at the secretory borders of epithelial tissues. The disaccharide is linked to malignancy and plays an important role in docking breast- and prostate-cancer cells onto endothelium.[3] Patients immunized with synthetic TF-Keyhole limpet hemocyanin KLH conjugate vaccines, plus various adjuvants can generate high-titer IgM and IgG antibodies.[4] Antigen **1** constructed from a disaccharide tripeptide cluster conjugated to KLH (+adjuvant QS-21) has been shown by Danishefsky and co-workers to be a relevant antigen target in a multivalent phase II-vaccine trial in patients with high-risk minimal prostate cancer.[5] Because the disaccharide moiety of TF-epitope β-D-Gal*p*-1-3-α-D-GalNA*cp*-O-L-serine is hydrolyzed by β-galactosidases in the body,[6] there is a need for more stable antigens than **1**. Under similar conditions as reported for **1**, we have now found that the C-disaccharide analogue **2** with QS21 as adjuvant[7] induces a strong immune response in mice after two boosts. This suggests that C-disaccharides can be used to construct therapeutic vaccines against cancer and other diseases. Much weaker immune response was observed when using antigen **3** constructed from α-D-Gal*p*-1(CH_2)-3-α-D-GalNA*cp*-O-serine (a α-C-galactoside rather than β-C-galactoside as in **2**; Figure 1).

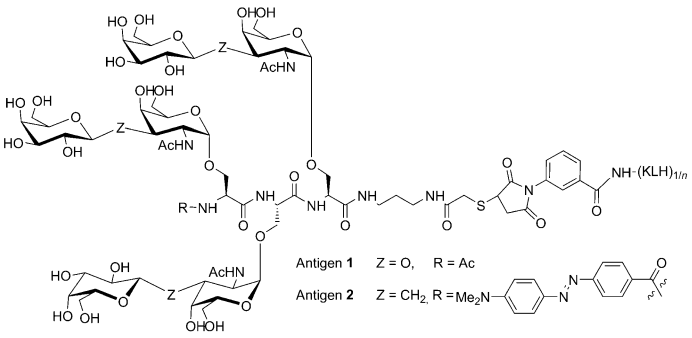

Figure 1. Synthetic TF-antigen **1** clustered as a triglycopeptide conjugated with KLH protein. Antigen **2** is analogue of **1**, in which the TF-disaccharide is a C-disaccharide (this work).

Various strategies towards the incorporation of non-natural hydrolysis-resistant carbohydrate analogues into vaccine constructs have been explored, including the use of C-glycosides[8] and S-glycosides[8b,9] analogues of O-glycosides, as well as O-deoxyfluoroglycosides.[10] It has been proposed also to use β^3-homothreonine conjugates instead of threonine or serine to construct mucin-like glycopeptides antigen analogues.[11] In this report, we have explored whether the replacement of the O-linked disaccharide moiety in a C-linked disaccharide analogue would still induce an immune response, and whether the latter would depend on the β- or α-configuration of the D-galalactopyranoside moiety of the artificial antigen. The exchange of one acetal oxygen atom for a CH_2 group (change from acetal to ether function) modifies the polarity and water solubility of the disaccharide, as well as the population of its conformers about the two interglycosidic bonds. Several studies have suggested that the energy maps of C-linked disaccharides are similar to maps of the corresponding O-disaccharides, but there are differences in the locations and the relative free energies of the minima.[12] For example, with β-D-Gal*p*-(1(CH_2)-4)-β-D-GlcNA*cp*-OMe, the C-disaccharide populates three distinctive conformational families in water solution, the major one being the *anti*-Ψ-conformation, which is only marginally populated for the O-linked disaccharide.[13]

The construction of antigens **2** and **3** are outlined in Schemes 1 and 2, respectively, and are described in details in the Supporting Information. The synthesis of the C-disaccharide mimetic of the TF-disaccharide started with aldehyde **4**.[14] Itoh–Nozaki condensation[15] of **4** with isolevoglu-

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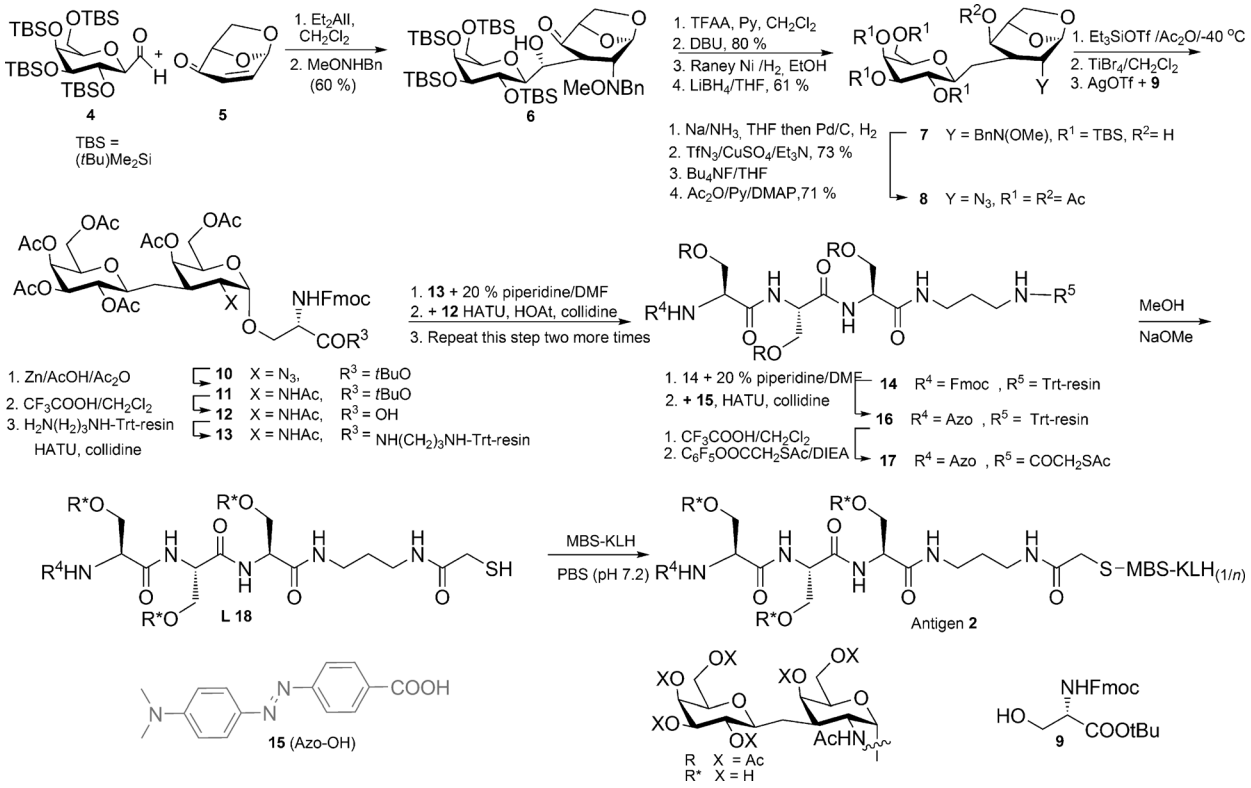


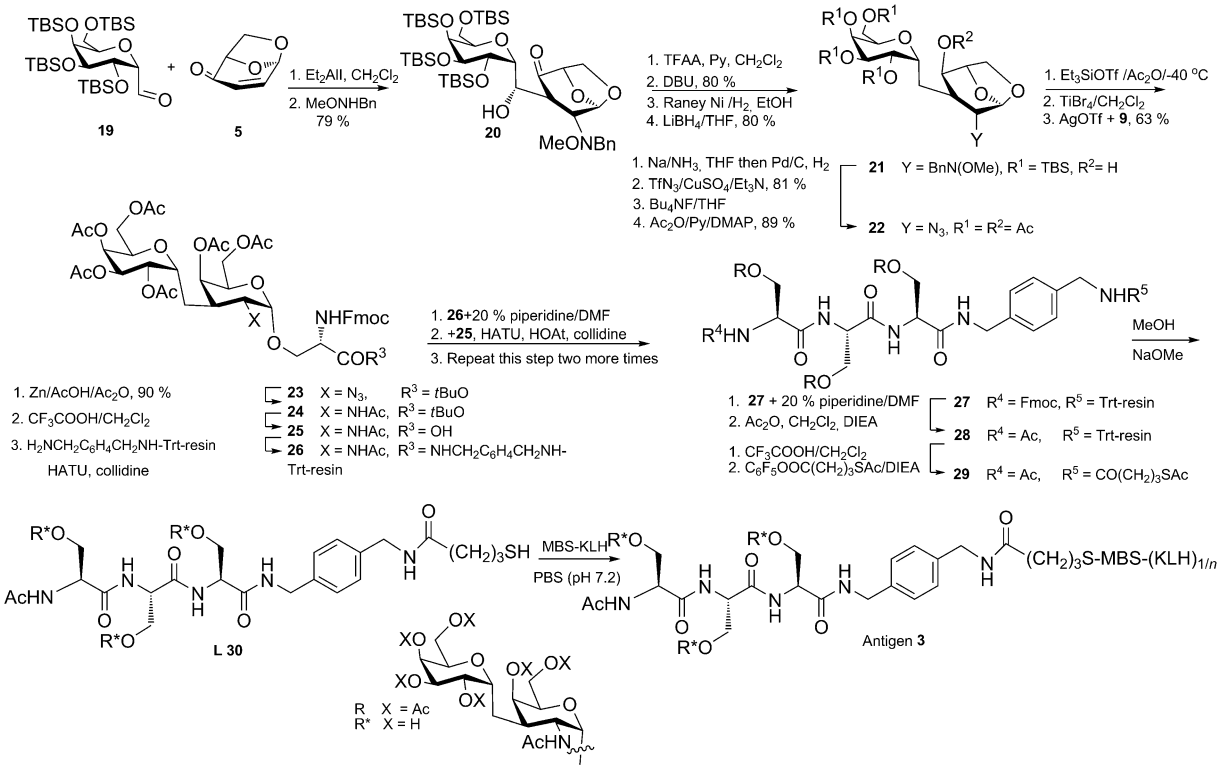
Scheme 1. Synthesis of triglycopeptide **L18** and the preparation of the corresponding antigen **2**: Triphenylmethyl (Trt) ; 2-(1*H*-7-azabenzotriazol-1-yl)–1,1,3,3-tetramethyl uronium hexafluorophosphate methanaminium (HAUT); trifluoroacetic anhydride (TFAA); 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU); 4-dimethylaminopyridine (DMAP); *N,N*-diisopropylethylamine, or Hünig's base (DIEA); phosphate buffered saline (PBS).

cosenone (**5**)[16] induced by Et_2AlI gave a single enone, which reacted with BnONHOMe to give **6**. Water elimination from **6** produced a major enone that was reduced (1,4-reduction) followed by ketone reduction with $LiBH_4$ producing **7**. Further reduction of BnNOMe provided the corresponding amine that was converted into an azide by diazo transfer.[17] After exchange of the silyl group for acetate esters, which produced **8**, the 1,6-anhydrogalactose was used in the α-O-galactosidation of L-serine derivative **9**. This gave the major galactoside **10**, the azido moiety of which was reduced into acetamide **11**. Liberation of the carboxylic acid gave **12** that was coupled to a trityl polymer by using NH_2–$(CH_2)_3$NH–trityl resin giving **13**.

Removal of the Fmoc group of **13** gave an amine that was coupled with carboxylic acid **12** to produce a dipeptide. Repeating the same sequence of reactions, **14** was obtained. Removal of Fmoc group of **14** and capping the primary amine with 4-(dimethylamino)-azobenzene-4′-carboxylic acid **15** gave **16**. Subsequent liberation of the protected triglycopeptides from the polymer and coupling with pentafluorophenyl acetylthioacetate[5a,18] gave **17** (25% over ten steps). Zemplen deacetylation of **17** provided the corresponding deacetylated triglycopeptide **L18**, which was reacted with MBS-KLH by Michael addition to give antigen **2**.

Tagging with the azocarboxamide permitted an accurate determination of the degree of conjugation. It was found by UV/Vis spectroscopy that 230 ± 20 molecules of the triglycopeptide were grafted per molecule of KLH protein.

Antigen **3** was constructed in the same manner (Scheme 2) starting with aldehyde **19**, which was reacted with isolevoglucosenone (**5**) and Et_2AlI to produce a single enone that was converted into α-C-disaccharide **22** applying the same sequence of reactions that converted **6** into **12** (Scheme 1). The free carboxylic acid **25** was coupled with trityl resin monoprotected diamine 1,4-di(aminomethyl)benzene to give **26**; this linker was selected to offer chromophore for better detection in HPLC. After Fmoc cleavage of **26**, the free primary amine was coupled with acid **25**. Repeating the same sequence of reactions and capping the last amine moiety as an acetamide gave polyacetylated triglycopeptide **28**. Its liberation from the solid polymer and subsequent coupling with pentafluorophenyl 4-acetylthiobutanoate (prepared in situ from 4-acetylthiobutyric acid and pentafluorophenol[19]) gave **29** (22%, nine steps). Zemplen deacetylation of **29** provided the corresponding unprotected triglycopeptide **L30**, which was conjugated with MBS–KLH[5a] (Scheme 2) by Michael addition to give antigen **3**. The degree of conjugation was assumed to be $n = 200 \pm 50$ molecules[20] of triglycopeptide per molecule of KLH as confirmed by comparison of the ^{1}H NMR spectra in D_2O of **2** and **3** with that of MBS–KLH.



Scheme 2. Synthesis of triglycopeptide **L30** and the preparation of the corresponding antigen **3**.

Vaccine candidates **β-V** and **α-V** were prepared out of 25 μg of antigen **2** and antigen **3**, respectively, and 125 μg of adjuvant QS21 per 1 mL of water. The pH of these solutions was 6.8 (first injections), but was then adjusted to pH 7.0 with 2×PBS (all following injections). One blank solution without the antigen **B-QS-21** was prepared out of 125 μg of **QS-21** per 1 mL of water. These solutions were stored at −20 °C. BALB/c adult mice (nine weeks old, ten females/group) were housed at the EPFL-animal facility, and all experiments were performed according to Swiss legislation (permission obtained). Mice received subcutaneous (s.c.) injections of the different vaccine preparations or the adjuvant **QS21** batch. Injections of 100 μL/dose (except the first one, which used 200 μL) consisted of 5 μg antigen/25 μg **QS21**/dose. Injections were done on five occasions with a two-weeks interval between each immunization (at day 0, 15, 28, 42, and 56). Mice from all groups showed a normal growth with time (the analysis of variance ANOVA repeated measure, $P=0.001$). Even before starting the treatment, the group treated with **β-V** vaccine candidate had a slightly higher body weight (two-ways ANOVA repeated measure, $P=0.071$, interaction group×time, $P=0.5$). No treatment effect on the body weight was observed (see Figure on p. 58 in the Supporting Information).

Total IgG titers from sera (bleeding at day −7, 7, 21, 35, 49, and 63) were analyzed by enzyme-linked immunosorbent assay (ELISA) by using plates coated with **β-V** or **α-V** (Figure 2). The sera of the mice immunized with **β-V** showed a strong immune response against **β-V** already after the second injection at day 21 and 35 (Figure 3A). Total IgG titers were significantly higher than titers in mice immunized with the **B-QS-21** (adjuvant alone) (two-ways ANOVA, Bonferroni posttest $P<0.0001$) at all days after day seven. A boost effect was observed at day 21 (two-ways ANOVA, days effect $P<0.0001$), and then the response increased slightly with each boosting.

In contrast, no IgG titers toward **α-V** were detected in the group immunized with the **α-V** at all days (Figure 2B). Only at days 49 and 63, very weak total IgG titers were observed (2-way ANOVA, Bonferroni posttest $P<0.0001$), but they were too low to be considered as an immune response. To verify that the detected antibodies rose against **β-V** were specific to antigen **2**, sera were analyzed on plate coated with pure ligand **L18**. Anti-**L18** IgG titers were strong at all days (Figure 3A). A boost effect was observed at day 21 and 35. As a control, sera of three bleeding days (7, 35, and 65) of the group treated with **B-QS-21** were also analyzed in the same ELISA test. In these control sera, no immune response was detected against the adjuvant (Figure 3A). The cross reaction of the antibodies generated by each vaccine candidate **β-V** and **α-V** were analyzed against the different antigens **2** and **3** (Figure 3B). Sera of day 63 of mice immunized with **β-V** or with **α-V** were analyzed on plates coated with triglycopeptide **L18** or with triglycopeptide **L30**. Sera of mice immunized with antigen **2** showed a big difference in binding **L18** versus **L30** ($P<0.005$). Thus, IgG antibodies raised against **β-V** did not cross react with **L30**. There were very low levels of IgG antibodies against either **L30** or **L18**

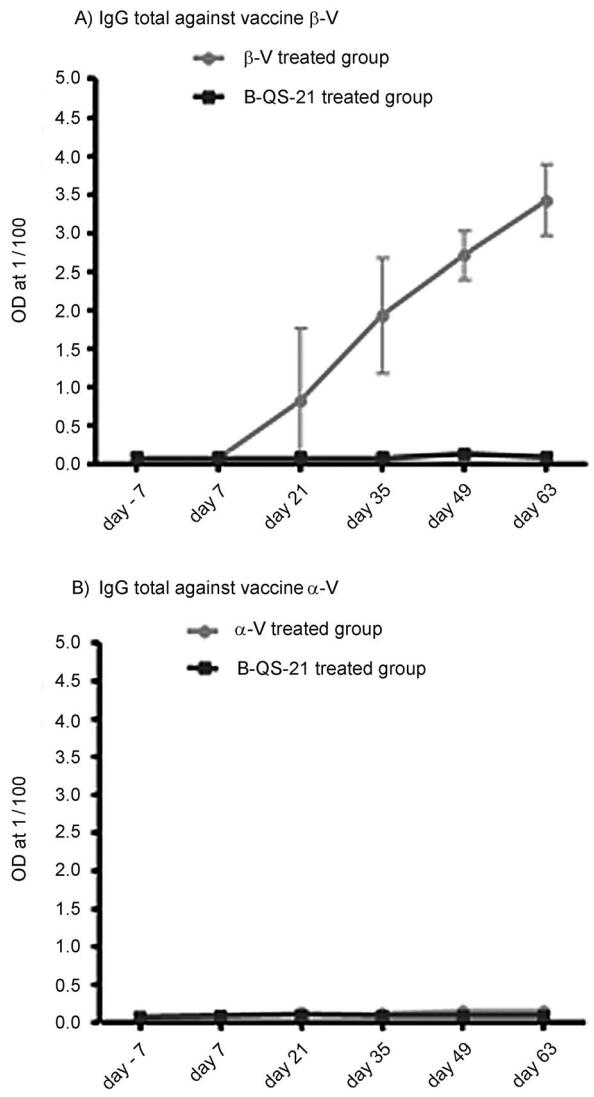


Figure 2. Immune response in mice immunized with **β-V** or with **α-V** vaccines. Analysis of IgG antibodies raised against: A) **β-V**; B) **α-V** in the sera of mice at day −7, 7, 21, 35, 49, and 63. Mean of antibodies + standard deviations are shown.

in the sera of mice immunized with **α-V**. Taken together, these results suggest a specific binding of the **β-V**-derived antibodies to antigen **2**.

To further verify the specificity of the antibodies raised against **β-V** and **α-V**, respective sera were analyzed on plates coated with KLH alone (Figure 4). Anti-KLH IgG titers were barely detected before day 63. At that day, the sera showed a very weak interaction with KLH alone. The sera of mice immunized with **B-QS-21** (adjuvant alone) did not show any interaction with KLH in this ELISA test at any day. As a positive control, **Ab12B4**-specific anti-KLH antibody was used.

Specific IgG responses were determined by ELISA. Plates were coated with the vaccines **β-V** and **α-V** (5 μM). Other plates were coated with triglycopeptides **L18** and **L30** (5 μg mL^{-1}), or with KLH (25 μM mL^{-1}; Figure 4).

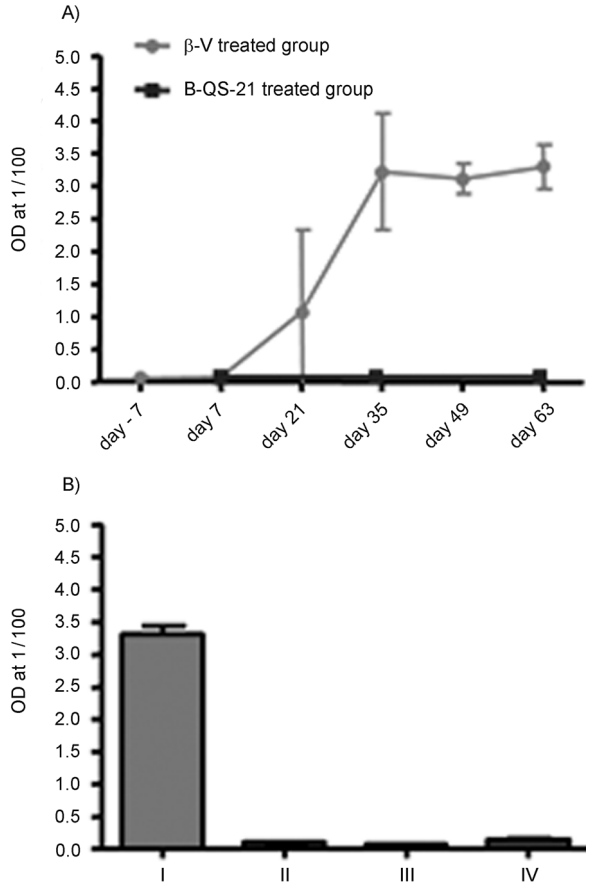


Figure 3. A) Anti-**L18** titers in mice immunized with **β-V** vaccine or with adjuvant (**QS-21**) alone. B) Cross reaction of antibodies induced by **β-V** and **α-V** against **L18** and **L30**: I) Sera/**β-V** on **L18**. II) Sera/**β-V** on **L30**. III) Sera/**α-V** on **L18**. IV) Sera/**α-V** on **L30**. Sera at day 63 were used in these analyses. Mean of antibodies + standard deviations are shown.

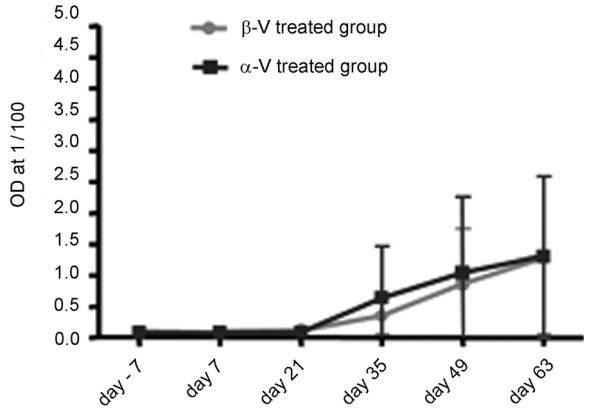


Figure 4. Anti-KLH titers in mice immunized with **β-V** or **α-V** vaccine candidates or with **QS-21**. Analysis of anti-KLH IgG antibodies in the sera of mice at day −7, 7, 21, 35, 49, and 63 of **β-V** and **α-V** treated mice. Mean of antibodies + standard deviations are shown.

To conclude, the vaccine candidate **β-V** is immunogenic in mice and induces specific IgG titers against triglycopeptide **L18**.

This work demonstrates for the first time that a C-disaccharide analogue of an O-disaccharide sugar epitope can be used to construct vaccine candidates. This opens the possibility to obtain antigens, which are not metabolized too quickly in the body and thus should lead to vaccines capable to induce a desired immune response with a single or limited number of injections. Very interesting is the finding that the β-C-disaccharide analogue of the TF disaccharide sugar epitope (β-D-Gal*p*-(1-3)-α-D-GalNAc*p*-O-L-serine) has a much stronger immune response than its α-C-disaccharide stereoisomer. We believe that the use of different linkers, different N-terminus capping of the conjugates are not causing difference in immunogenicity.[21]

It is thus possible that the efficiency and high stereoselectivity of the immune response observed with our vaccine candidate **β-V** corresponds to a stimulation of the natural immune response that mice normally have toward the genuine TF epitope (auto-antigen) of naturally formed cancer cells. This leaves the possibility to construct therapeutic vaccines based on C-disaccharide analogues of natural O-disaccharide epitopes.

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