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Letters

Epitope Recognition of a Monoclonal Antibody Raised against a Synthetic Glycerol Phosphate Based Teichoic Acid

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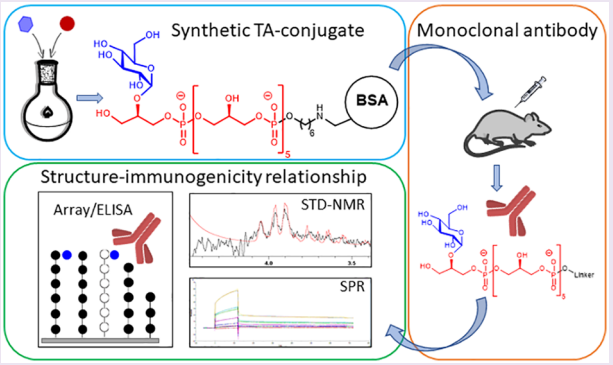
ABSTRACT: Glycerol phosphate (GroP)-based teichoic acids (TAs) are antigenic cell-wall components found in both enterococcus and staphylococcus species. Their immunogenicity has been explored using both native and synthetic structures, but no details have yet been reported on the structural basis of their interaction with antibodies. This work represents the first case study in which a monoclonal antibody, generated against a synthetic TA, was developed and employed for molecular-level binding analysis using TA microarrays, ELISA, SPR-analyses, and STD-NMR spectroscopy. Our findings show that the number and the chirality of the GroP residues are crucial for interaction and that the sugar appendage contributes to the presentation of the backbone to the binding site of the antibody.



Teichoic acids are poly-alditolphosphate cell-wall components found in the majority of Gram-positive bacteria. They are divided into two main classes, differing in the way they are connected to the cell-wall: wall teichoic acids (WTAs) are covalently connected to the peptidoglycan, while lipoteichoic acids (LTAs) are inserted in the lipid bilayer through a lipid anchor via noncovalent, hydrophobic interactions.[1,2] They are involved in various biological processes, and as their structure extends toward the extracellular milieu, they have been appointed as possible antigen candidates for vaccine development.[3−5]

Staphylococci and enterococci are Gram-positive bacteria, associated with a variety of severe infections.[6,7] The prevalence of multidrug resistance strains in the clinical setting, such as methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant enterococci (VRE), has urged the development of alternative strategies, such as active or passive immunization.[8,9] LTAs from staphylococci and enterococci share a common structural motif composed of a glycerol phosphate (GroP) repeating unit.[10] The C-2-OH of the glycerol moiety can be decorated with D-alanine or carbohydrate appendages. Previously, we generated a synthetic TA, featuring an α-glucosyl substituent at the terminal unit of a GroP hexamer (**WH7**), conjugated to the carrier protein Bovine Serum Albumin (BSA) in order to evaluate its immunogenicity and potential as a vaccine candidate.[11] It was observed that WH7-BSA (Figure 1A) was able to induce opsonic and protective cross-reactive antibodies against clinical isolates of *Enterococcus faecalis* 12030, *Enterococcus faecium* E1162, and community acquired *S. aureus* MW2 (USA400), confirming the antigenic property of **WH7**. Microarray analysis showed that the polyclonal serum raised against the conjugate contained antibodies that preferentially bound α-glucosyl functionalized GroP-oligomers.[12] Simultaneously, Snapper and co-workers used a nonglycosylated synthetic GroP-decamer to generate a TA conjugate and showed that this could be used to raise protective antibodies against *S. aureus*.[13]

These studies have revealed synthetic TA fragments as attractive antigens in the generation of well-defined synthetic vaccines. The molecular basis of their immunogenicity however has not been elucidated, and understanding how these fragments are recognized by the generated antibodies will provide valuable insights for the design of improved conjugate vaccines.[14,15] Therefore, in the current study, the aforementioned **WH7** conjugate was employed for the generation of a monoclonal antibody (WH7.01 mAb) to characterize the

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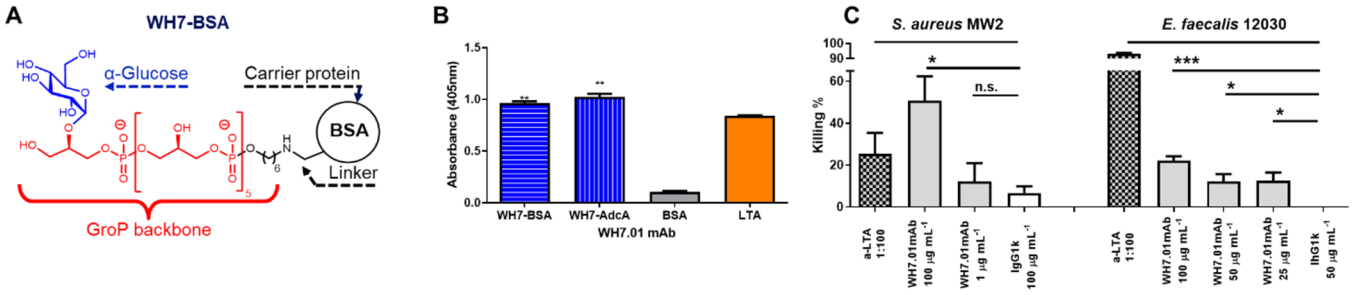

Figure 1. Generation of the monoclonal antibody against WH7-BSA. (A) Schematic representation of the structure of the synthetic teichoic acid glycoconjugate WH7-BSA. (B) Antigen specificity of the supernatant from the hybridoma cell line producing the WH7.01 mAb. The binding was evaluated by ELISA against the synthetic antigen **WH7** (blue) conjugated to BSA (horizontal stripes) or to AdcA (vertical stripes), the unconjugated carrier protein BSA (gray), and the commercially available LTAs from *S. aureus* (orange), all coated in duplicate with 1 μg well^{-1}. The concentration of the WH7.01 mAb in the supernatant was 35 μg mL^{-1}. Bars represent mean data, and the error bars represent the standard errors of the means. Significance was inferred by two tailed unpaired *t* tests between **WH7** conjugates and the carrier protein (**$P < 0.01$). (C) Opsonophagocytic killing activity of the newly generated WH7.01 mAb against *S. aureus* MW2 on the left and *E. faecalis* 12030 on the right. The opsonophagocytic killing activity of the purified monoclonal from the hybridoma cells producing WH7.01 mAb (gray) at different dilutions was evaluated against *S. aureus* MW2 and *E. faecalis* 12030. Polyclonal sera raised in a rabbit against the purified LTA from *E. faecalis* 12030 (black and gray squares) was used as a positive control, and a mAb of the same isotype, IgG1κ, was used as a negative control. Bars represent mean data, and the error bars represent the standard errors of the means. n.s. (not significant), *$P < 0.05$; ***$P < 0.001$, all by one-way ANOVA with Dunnett's multiple comparison to the negative control (monoclonal antibody of the same isotype).

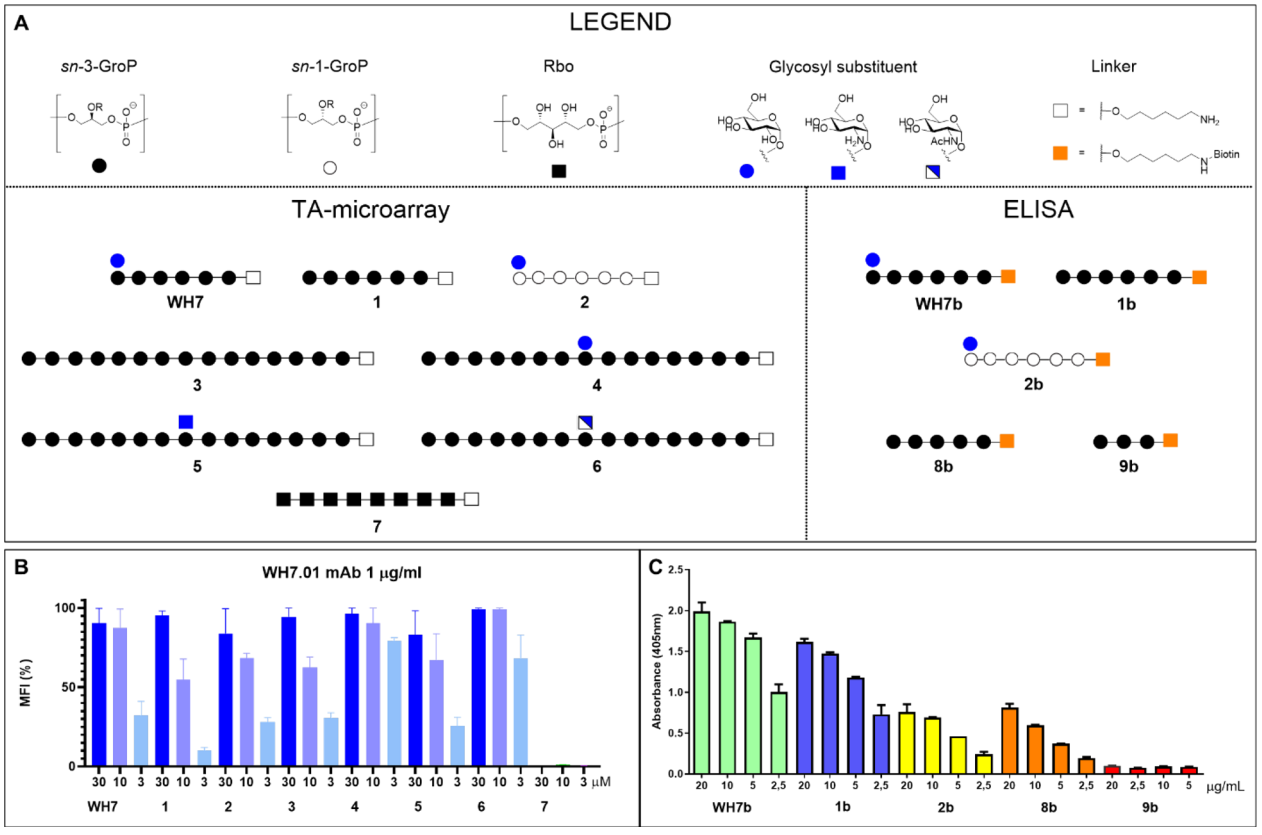

Figure 2. TA-library overview and binding assay results from microarray and ELISA. (A) Overview of the synthetic fragments used for the binding analysis of WH7.01 mAb. (B) TA-microarray results with WH7.01 mAb at 1 μg mL^{-1}. Compounds **WH7**, **1** to **7** were immobilized on an epoxide functionalized glass slide in three different concentrations: 30 μM (dark blue), 10 μM (shade blue), and 3 μM (light blue). The average of the triplicate spots was normalized to the highest intensity on the array. (C) ELISA results of WH7.01 mAb at different concentrations against **WH7b** (light green), **1b** (blue), **2b** (yellow), **8b** (orange), and **9b** (red). All biotin derivatives were coated at the same concentration (1 μM) on a streptavidin ELISA plate. Bars represent mean data, and the error bars, the standard errors of the means.

binding with synthetic GroP-based fragments at the molecular level using TA-microarrays, ELISA, SPR-binding studies, and STD-NMR. This work represents the first case study for a synthetic GroP-based TA immunogen, and our detailed binding studies have revealed that all structural components, the length and chirality of the GroP backbone, as well as the presence of the carbohydrate substituent contribute to binding of the antibody.

First, we implemented the hybridoma technology, as described previously, in order to generate a mAb against

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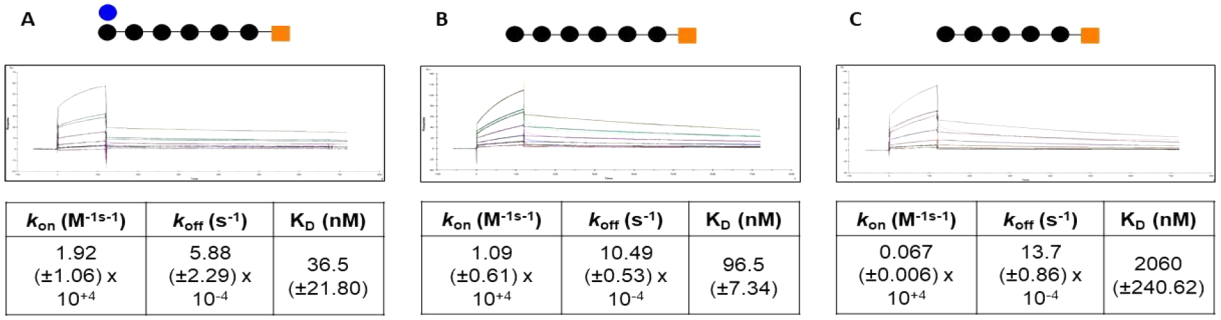

k_{on} ($M^{-1}s^{-1}$)	k_{off} (s^{-1})	K_D (nM)
1.92 (±1.06) x 10^{+4}	5.88 (±2.29) x 10^{-4}	36.5 (±21.80)

k_{on} ($M^{-1}s^{-1}$)	k_{off} (s^{-1})	K_D (nM)
1.09 (±0.61) x 10^{+4}	10.49 (±0.53) x 10^{-4}	96.5 (±7.34)

k_{on} ($M^{-1}s^{-1}$)	k_{off} (s^{-1})	K_D (nM)
0.067 (±0.006) x 10^{+4}	13.7 (±0.86) x 10^{-4}	2060 (±240.62)

Figure 3. Binding kinetics and kinetic and affinity constants of WH7.01 mAb against **WH7b** (A) and compounds **1b** (B) and **8b** (C). Serial dilutions of the analyte, WH7.01 mAb (2000-62.5 nM), were run. The numbers in parentheses represent the standard deviations of k_{on} and k_{off}. Results are representative of three independent experiments.

WH7.[16−20] Briefly, mice were immunized with the conjugated form of **WH7** to the commonly used carrier protein BSA. The generated hybridomas were selected by ELISA against the conjugated form of **WH7** and by negative selection with BSA (Supplementary Figure S3). The secreted mAb from the resulting hybridoma cell line, named, WH7.01 mAb, was confirmed by ELISA to be of the IgG1 class for the heavy chain and kappa for the light chain. WH7.01 mAb exhibited strong binding in ELISA (Figure 1B) against the WH7-BSA conjugate as well as toward a second conjugate that was constructed using the zinc ABC transporter substrate-binding lipoprotein AdcA[21] from *E. faecium*. It also bound native LTAs from *S. aureus* (Sigma), but it did not recognize BSA either in ELISA or in Western blot (Supplementary Figure S4). These results confirmed the development of a new mAb that specifically recognized the synthetic teichoic acid **WH7** antigen. This monoclonal was evaluated in an opsonophagocytic assay, and it was observed that the WH7.01 mAb exhibited opsonic killing activity against *E. faecalis* 12030 and *S. aureus* MW2, with most killing of the latter species (Figure 1C).

Next, the binding specificity of the newly generated monoclonal was qualitatively assessed using a TA-microarray. This tool allows the simultaneous screening of the binding interaction of a designated protein with a number of substrates using only minute amounts of materials.[22] In line with our previous TA-microarray studies,[12,23] different synthetic GroP fragments, each equipped with an aminohexanol linker, were covalently immobilized on an epoxide functionalized microarray glass slide. The compounds were printed on the slides in three different concentrations (30 μM, 10 μM, and 3 μM) in triplicate. As shown in Figure 2A, the tested library comprises GroP-based fragments varying in length (hexamers or pentadecamers), nature of the glucosyl substituent (α-glucose, α-glucosamine, or α-*N*-acetylglucosamine), the position of the carbohydrate on the chain (terminal or in the middle), and the stereochemistry of the glycerol unit (*sn*-1 vs *sn*-3). Compound **7**, a ribitol phosphate chain resembling the structure of *S. aureus* WTAs,[24] was included as a negative control. As can be observed from the results shown in Figure 2B, the WH7.01 mAb recognized all printed GroP-based TA fragments well, indicating that the GroP backbone is the main recognition element for the mAb. The sugar appendage also seems to contribute to binding with **WH7** being recognized slightly better than its nonglucosylated counterpart **1** and α-glucosyl pentadecamer **4** showing the strongest binding among the longer GroP fragments.

The ribitol phosphate-based *S. aureus* WTA fragment **7** was not recognized by the monoclonal. To further validate the binding, ELISA assays were performed using the biotinylated derivatives **WH7b**, **1b**, and **2b** as well as the shorter pentamer **8b** and trimer **9b** (Figure 2A). The synthetic fragments, tagged with biotin, were immobilized at a concentration of 1 μM on streptavidin coated plates, and different dilutions of the monoclonal were employed (20 μg mL^{-1}, 10 μg mL^{-1}, 5 μg mL^{-1}, 2.5 μg mL^{-1}) in duplicate. These ELISA studies (Figure 2C) revealed that the binding of WH7.01 mAb is influenced by three structural elements: the length of the GroP chain (**1b** > **8b** ≫ **9b**), the presence of the glucosyl substituent (**WH7** > **1b**) and the stereochemistry[23] of the glycerol backbone (**WH7** ≫ **2b**). These results overall confirm the observations from the microarray analysis, that the GroP backbone is the major structural feature that is recognized by this monoclonal antibody. At the same time, it becomes more prominent that the number of repeating units, the relative position of the C-2-OH (*sn*-1 vs *sn*-3 GroP), and the presence of the glucosyl substituent also have an impact. Previously, we have observed that the GroP backbone stereochemistry did play an important role in IgG binding of the polyclonal sera, raised against **WH7** antigen or native *E. faecalis* LTA.[23] In these cases, the major epitope recognized was the glycosyl substituent, while binding of the monoclonal antibody generated here is dominated by the GroP backbone (**1b** and **8b**), likely driven by ionic interaction with the phosphate moiety. The preferential binding of the monoclonal to **WH7** compared to its diastereoisomer **2** is determined by the different GroP stereochemistry, but it also suggests a role of the glucosyl appendage to the antibody interaction. Without the glucosyl substituent, the influence of the chirality of the internal GroP residues of the *sn*-1-GroP and *sn*-3-GroP oligomers is lost. In order to investigate binding in more detail, a quantitative analysis of binding parameters was performed by measuring the antibody−ligand interaction by SPR.[25] To this end, **WH7b**, nonglucosylated hexamer **1b** and pentamer **8b** were immobilized on a streptavidin functionalized sensor chip. Figure 3 reveals a higher affinity toward the hexameric fragments (**WH7b** and **1b**) than to the pentamer (**8b**). The length of the TA backbone significantly affects the k_{on} with the value for compound **8b** being 16 and 28 times lower than **1b** and **WH7b**, respectively. While **WH7b** and **1b** have the same number of GroP repeating units, the affinity slightly increases when the glucosyl substituent is present. The carbohydrate appendage contributes positively to the binding by increasing the k_{on} and decreasing the k_{off} value.

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STD-NMR spectroscopy has been widely used in past decades for structural epitope mapping.[26] Therefore, we continued our binding study between the monoclonal and its ligand **WH7** using this technique. The experiment was carried out using a 2.5 μM concentration of WH7.01 mAb with a protein/ligand ratio of 1:100, at 303 K to avoid overlapping of the glucosyl anomeric proton with the HDO signal. Figure 4

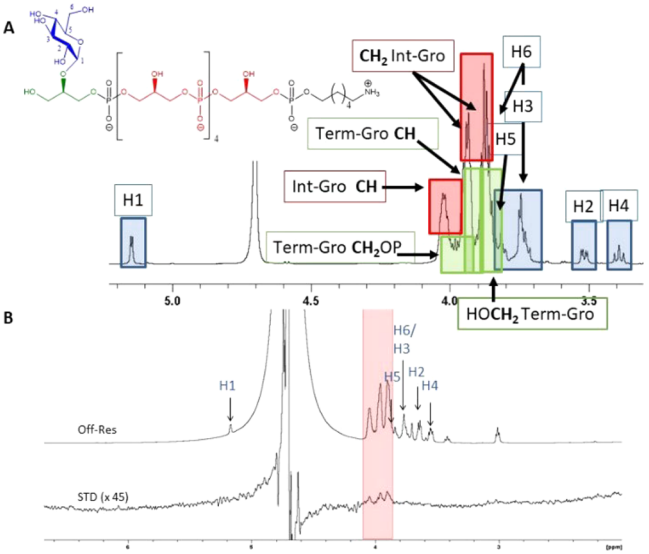

Figure 4. STD-NMR analysis. (A) Assignment of the ^{1}H NMR spectrum of **WH7** in D_2O at RT. H peaks for the intermediate GroP (Int-Gro CH/CH_2), terminal GroP (Term-Gro CH/CH_2OP/$HOCH_2$), and α-glucose (H1 to 5) are assigned with red, green, and blue, respectively. (B) Off- (top) and on-resonance (bottom) of ^{1}H-STD-NMR spectra between WH7.01 mAb (2.5 μM) and **WH7** (0.5 mM) at 303 K.

shows the ^{1}H NMR spectrum of the ligand alone (A) and the STD-NMR experiment (B). Because of the repetitive nature of the ligand, there is significant signal overlap of the GroP repeating units, but the signals of the glucosyl H1, H2, and H4 can be clearly distinguished. Major STD effects can be observed for signals related to CH/CH_2 of the glycerol units, while no significant involvement of the glucosyl substituent is detected. This indicates that the sugar substituent is not involved in the binding site of WH7.01 mAb. Taking into account the SPR results, showing that the presence of the glucosyl moiety provides a better GroP binder for the case-study antibody, it may be speculated that the glucosyl moiety reduces the conformational freedom of the terminal glycerol unit and/or provides a conformation that enables stabilizing additional contacts with the mAb, which has previously been observed in the immunorecognition of other polysaccharides.[27−29]

In conclusion, the first mAb raised against a synthetic glycosylated GroP-based TA has been generated by hybridoma technology. This mAb showed high binding specificity toward both the synthetic antigen and the commercially available LTAs from *S. aureus*. Different techniques were used to reveal the key structural elements for ligand recognition. A first screening using a TA-microarray showed the GroP backbone as the main recognition element. Further analysis using ELISA and SPR indicated the importance of the number of GroP residues as well as a role for the glucosyl substituent. STD-NMR spectroscopy revealed interactions with the GroP backbone, but no interaction of the glucose residue with the mAb was detected. This suggests a possible indirect contribution of the substituent to the binding. The carbohydrate may impose a particular conformational geometry to the GroP residue or backbone, leading to increased binding of the glucosylated TA. Due to the high structural heterogenicity in TAs from native sources, the availability of more well-defined TA fragments[30] can provide insights on the structural elements (the backbone, the stereochemistry, carbohydrates, as well as D-Ala substituents) required for TA-antibody binding. In particular, the workflow here presented can be used in the future for the generation of a library of mAbs[15] with high specificity to a variety of different poly-alditolphosphate antigen candidates and to analyze the structure−immunogenicity relationship for future TA-based vaccine development.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acschembio.1c00422.

Full experimental details, characterization and spectra of compounds and glycoconjugates, materials and methods (PDF)

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[+]F.B. and E.K. contributed equally to the manuscript. F.B., D.v.E, S.A.: synthesis of the TA compounds. E.K., D.L., K.M., S.M., T.L.R., S.J.: antibody manufacturing and characterization. D.v.E., F.R.-S.: generation of WH7 conjugates. F.B., A.v.D.: microarray assays. E.K., F.B.: ELISA on synthetic fragments. E.K., F.C.: SPR measurement. F.B., A.G.: STD-NMR analysis. J.D.C.C., J.H., F.B., E.K.: conceived the study and planned the experiments. J.D.C.C., G.A.v.M., H.S.O., C.H.H., R.A., J.H., J.J.B.: supervised the study. F.B., E.K.: drafted the manuscript. All authors were involved in analysis of the results, reviewing the manuscript, and giving approval to the final version.

Notes

The authors declare the following competing financial interest(s): F.C. and R.A. are employees of the GSK group of companies.

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