Published in final edited form as:
Geißner, A., Pereira, C. L., Leddermann, M., Chakkumkal, A., & Seeberger, P. H. (2016). Deciphering Antigenic Determinants of Streptococcus pneumoniae Serotype 4 Capsular Polysaccharide using Synthetic Oligosaccharides. *ACS Chemical Biology, 11*(2), 335-344. doi:10.1021/acschembio.5b00768.

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Streptococcus pneumoniae is a major cause of mortality and morbidity worldwide. More than 90 *S. pneumoniae* serotypes are distinguished based on the structure of their primary targets to the human immune system, the capsular polysaccharides (CPSs). The CPS of the prevalent serotype 4 (ST4) is composed of tetrasaccharide repeating units and is included in existing pneumococcal vaccines. Still, the structural antigenic determinants that are essential for protective immunity, including the role of the rare and labile cyclic *trans*-(2,3) pyruvate ketal modification, remain largely unknown. Molecular insights will support the design of synthetic subunit oligosaccharide vaccines. Here, we identified the key antigenic determinants of ST4 CPS with the help of pyruvated and nonpyruvated synthetic repeating unit glycans. Glycan arrays revealed oligosaccharide antigens recognized by antibodies in the human reference serum. Selected depyruvated ST4 oligosaccharides were used to formulate neoglycoconjugates and immunologically evaluated in mice. These oligosaccharides were highly immunogenic, but the resulting antiglycan antibodies showed only limited binding to the natural CPS present on the bacterial surface. Glycan array and surface plasmon resonance analysis of murine polyclonal serum antibodies as well as monoclonal antibodies revealed that terminal sugars are important in directing the immune responses. The pyruvate modification on the oligosaccharide is needed for cross-reactivity with the native CPS. These findings are an important step toward the design of oligosaccharide-based vaccines against *S. pneumoniae* ST4.

Deciphering Antigenic Determinants of *Streptococcus pneumoniae* Serotype 4 Capsular Polysaccharide using Synthetic Oligosaccharides

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Streptococcus pneumoniae is a Gram-positive bacterium that causes severe and potentially fatal infectious diseases including otitis media, pneumonia, sepsis, and meningitis. Pneumococcal infections killed 1.6 million people including up to one million children under the age of five in 2005.*(1)*. Infectiveness of different strains is governed by their differential expression of virulence factors such as the capsule, a thick layer of repetitive polysaccharide termed capsular polysaccharide (CPS).*(2,3)* CPS plays a crucial role in protecting the bacteria from the human immune system and is a clinically proven vaccine target due to its antigen density and surface exposure.*(4,5)* More than 90 serotypes that differ in their CPS structure have been identified.*(6)* *S. pneumoniae* serotype 4 (ST4) is prevalent worldwide and part of all current pneumococcal vaccines.*(7)* The ST4 repeating unit tetrasaccharide [→3)-β-ManNAc-(1→3)-α-FucNAc-(1→3)-α-GalNAc-(1→4)-α-Gal-(1→] carries a rare *trans*-2,3-(*S*) cyclic pyruvate ketal modification at galactose (Figure 1).*(8,9)* Removal of the ketal by mild acidic treatment alters the antigenic properties of the polysaccharide. Depyruvated CPS cross-reacts with antibodies against pneumococcal cell wall polysaccharide (CWPS) while native CPS does not.*(10)* It is unknown whether the immunodominant and probably highly exposed pyruvate masks other protective epitopes.*(11,12)* *Trans* pyruvate ketals are prone to hydrolysis resulting in CPS micro-heterogeneities. Vaccine production and stability may be negatively impacted by partial pyruvate cleavage that is common and even intentionally performed during production to allow conjugation to immunogenic carrier proteins in glycoconjugate vaccines.*(13)* Tailor-made oligosaccharide subunit vaccines may overcome structural heterogeneities of isolated polysaccharides as defined synthetic oligosaccharides have proven to be promising vaccine candidates.*(14–21)* Epitopes of the respective CPS need to be carefully mapped at monosaccharide resolution to design oligosaccharide vaccines with optimal immunoprotective effects. To date, only limited immunological studies on native ST4 CPS and none on depyruvated preparations or defined glycans have been performed. Therefore, the antigenic determinants that guide protective immunity remain unknown.

We synthesized ST4 CPS-based glycans to reveal the antigenic determinants and protective epitopes of ST4 CPS.*(22)* Numerous oligosaccharide haptens can be envisaged that differ in the terminal monosaccharide of the repeating unit, size and pyruvate modifications. We synthesized the repeating unit containing a linker at the reducing end, β-ManNAc-(1→3)-α-FucNAc-(1→3)-α-GalNAc-(1→4)-α-Gal-(1→1)-5-aminopentanol, with and without the pyruvate ketal, as well as depyruvated deletion sequences subsequently shortened at the non-reducing terminus (Figure 1). We focused on depyruvated structures since the immunodominant pyruvate ketal might suppress other potentially protective glycan epitopes. Comparable modes of epitope suppression are known for proteins and have been circumvented using engineered antigens.*(17)* Depyruvated glycan antigens are easier to

synthesize and would make for more cost-efficient vaccines. Glycan arrays displaying the synthetic antigens were probed with human reference serum for anti-ST4 CPS antibodies. Two non-pyruvated oligosaccharide antigen candidates were identified for the synthesis of neoglycoconjugates and detailed immunological evaluation in mice. Their potential as a vaccine antigen was evaluated by the ability of generated antibodies to bind to native polysaccharide and the surface of bacterial cells. Epitope mapping of monoclonal antibodies using glycan arrays and surface plasmon resonance studies showed the importance of terminal glycan motifs for immunogenicity. Inhibition studies with ST4 CPS and neoglycoconjugates showed the need to divert the immune response from the terminus towards the pyruvate to achieve cross-reactivity with the native polysaccharide. The pyruvate modification emerged as the most important epitope even in ST4 oligosaccharides and has to be included in semi-synthetic or synthetic *S. pneumoniae* vaccines.

Results and Discussion

Glycan Array Analysis of ST4 CPS Antibodies

[Place Figure 1 approximately here]

Custom glycan arrays comprising synthetic oligosaccharide subunits of polysaccharides are excellent tools to identify glycan epitopes recognized by antibodies raised against polysaccharides in vaccinated or infected individuals.*(23–25)* We designed an array containing synthetic ST4 oligosaccharides, various pneumococcal polysaccharides isolated from bacterial cultures such as CWPS, native ST4 CPS and depyruvated ST4 CPS, as well as two control proteins for the immunogenicity studies that are described below. (Figure 1 and Supplementary Figures 2 and 3).*(10)* We used *N*-hydroxysuccinimide (NHS)-activated glass slides that allow for covalent orientation-specific immobilization of the synthetic glycans via their primary amino group linkers and proteins via surface amino groups. Pneumococcal CPSs are known to attach to such slides.*(26)* Using similar glycan arrays, we have recently shown that anti-ST4 CPS rabbit serum antibodies specifically bound to pyruvated tetrasaccharide **1** of the synthetic glycans.*(22)* To gain more detailed knowledge regarding the epitopes, we investigated binding patterns of immunoglobulin G (IgG) antibodies from two additional sera: a pooled serum of C57BL/6 laboratory mice immunized with the pneumococcal conjugate vaccine Prevenar 13® and the human reference serum 007sp that is a well-characterized serum pool from 278 adult individuals vaccinated with the *S. pneumoniae* polysaccharide vaccine Pneumovax 23.*(27)*

The antibody binding pattern from the pooled mouse serum was comparable to the aforementioned rabbit typing serum (Supplementary Figure 4). Of the synthetic oligosaccharides, antibodies exclusively recognized pyruvated tetrasaccharide **1** but none of the depyruvated oligosaccharides. Dose-dependent inhibition of antibody binding with native ST4 CPS confirmed that **1** comprises an epitope shared with the natural polysaccharide.

As expected, human 007sp antibodies yielded strong binding signals for isolated CPS of all investigated serotypes (Figure 2). Interestingly, the depyruvated ST4 CPS that was prepared by acidic treatment (Supplementary Figure 3) was also recognized indicating the presence of non-pyruvated epitopes.*(10)* However, low levels of residual pyruvates may have been responsible for antibody binding. Antibodies bound to synthetic oligosaccharide antigens in the following order of fluorescence intensities: pyruvated tetrasaccharide **1** > depyruvated tetrasaccharide **2** > trisaccharide **3** > smaller oligosaccharides (Figure 2 and Supplementary Figure 5). The binding signals for all glycans were dependent on serum dilution. Collectively,

the data indicates that both pyruvated and non-pyruvated glycan epitopes are recognized by human serum antibodies after vaccination with ST4 CPS.

[Place Figure 2 approximately here]

Reference serum 007sp as an adult human serum pool may contain antibodies that cross-react with the synthetic antigens but were generated independently of the vaccination with ST4 CPS. To investigate this possibility, we performed inhibition assays with native and depyruvated ST4 CPS. Serum at different dilutions was pre-incubated with either ST4 CPS, depyruvated ST4 CPS or both to sequester polysaccharide-specific antibodies. Pneumococcal CWPS that commonly co-elutes with vaccine grade CPS preparations was additionally added to every sample to exclude interference from antibodies against this polysaccharide. Antibody binding to the larger synthetic oligosaccharide antigens **1**–**3** was inhibited by native ST4 CPS. Pyruvated tetrasaccharide **1** exhibited the most pronounced signal reduction (60%), followed by depyruvated glycans **2** and **3** (30% and 15%, respectively). This showed that oligosaccharides **1**–**3** share common epitopes with native ST4 CPS. For pyruvated tetrasaccharide **1**, the cross-reactive antibodies constitute the majority of antibodies, while being only the minority for **2** and **3**. No significant inhibition was observed for glycans **4**–**8** (Supplementary Figure 5). Depyruvated CPS also inhibited antibody binding to glycan epitopes, even to pyruvated tetrasaccharide **1.** Residual pyruvate ketals may be responsible for this observation as inhibition was significantly less pronounced at low inhibitor concentrations compared to native CPS (Supplementary Figure 6). Simultaneous inhibition with native and depyruvated CPS did not result in additional signal reductions, indicating that the epitopes located on both polysaccharides are identical.

In summary, glycan arrays revealed the importance of the pyruvate ketal as antigenic determinant of the natural ST4 CPS. Oligosaccharide **1** showed highest antibody binding signals and extensive cross-reactivity to ST4 CPS in mice and humans immunized with the natural polysaccharide. Human serum 007sp additionally contains antibodies to non-pyruvated oligosaccharides, mainly **2** and **3**. As binding of these antibodies could be inhibited by ST4 CPS, non-pyruvated epitopes must be present on the natural polysaccharide and might contribute to protective immunity. These non-pyruvated epitopes are likely less immunogenic than pyruvated epitopes in the conventional vaccine as indicated by lower antibody binding signals to **2** and **3** compared to **1**. Yet, protective epitopes of capsular polysaccharides can be targeted by synthetic oligosaccharides even if they are only occasionally addressed by the native polysaccharide as shown previously for *S. pneumoniae* ST23F CPS.*(18,28)* To investigate this possibility for ST4 CPS, we selected depyruvated glycans **2** and **3** for detailed immunological evaluations in mice. The depyruvated glycans were chosen in light of the synthetic difficulty and low yields of pyruvate-modified glycans

that so far have only been obtained in a scale suitable for glycan array analysis and that would be significantly harder to be produced on large scale than depyruvated oligosaccharides.

Preparation of Neoglycoconjugates

Oligosaccharides **2** and **3** were conjugated to carrier protein CRM_{197} to induce an immune response against the otherwise non-immunogenic oligosaccharides.*(29)* CRM_{197} is a non-toxic mutant of diphtheria toxin and a common carrier in polysaccharide conjugate vaccines such as the pneumococcal vaccine Prevenar 13®.*(30,31)* Covalent coupling between the linker primary amine group of the oligosaccharides and lysine residues on CRM_{197} was performed with an adipate-based crosslinker to afford immunogenic neoglycoconjugates (Figure 3a). An average of seven tetrasaccharides **2** and eight or 13 trisaccharides **3** were conjugated per molecule of CRM_{197} (Supplementary Figures 7-9).

[Place Figure 3 approximately here]

Immunological Analysis of Glycoconjugates

The neoglycoconjugates, formulated with Freund's adjuvant, were used to immunize C57BL/6 mice in a prime/boost regime (Figure 3b). Glycan arrays of polyclonal sera were used to follow the antibody responses. Immunization with both neoglycoconjugates elicited antibodies to CRM_{197} and the generic spacer moiety composed of adipoyl and aminopentyl groups, represented by a BSA-dimannose control neoglycoconjugate.*(32)* Both oligosaccharides **2** and **3** were immunogenic and elicited glycan-specific serum antibodies (Figures 3c and 3d). IgM antibodies were detected two weeks after primary immunization at the latest (not shown). Isotype switching to IgG occurred in all immunized mice, indicating T cell-dependent antibody responses. The boosting immunization after two weeks resulted in increased antibody levels in sera collected at weeks 3 and 8. Based on glycan array-inferred fluorescence signal intensities and antibody titers, trisaccharide **3** was more immunogenic than tetrasaccharide **2**.

The antibodies generated with both neoglycoconjugates were highly specific for the respective immunogens **2** and **3**. Only minimal cross-reactivity between anti-trisaccharide **3** antibodies and tetrasaccharide **2** or vice versa was observed (Figures 3c and 3d). Shorter deletion sequences and glycans with unnatural stereochemistry at the terminal glycan showed little or no antibody binding indicating highly specific binding. However, cross-

reactivity between the depyruvated tetrasaccharide **2** and pyruvated tetrasaccharide **1** was observed (Figure 3c). Tetrasaccharide **1** not only shares epitopes with the native polysaccharide as observed in glycan array analysis of anti-ST4 polysaccharide antibodies, but also with **2**. Additional experiments to identify the residues that cause this cross reactivity are described below.

Cross-reactivity to Native Polysaccharide

The generation of high-affinity anti-CPS antibodies that opsonize the capsule of extracellular bacteria such as *S. pneumoniae* are the basis of carbohydrate vaccines.*(33)* When using oligosaccharide immunogens, it is necessary to confirm that the induced antibodies bind to a protective epitope of the native polysaccharide. Consequently, we studied if polyclonal serum antibodies of mice vaccinated with the neoglycoconjugates were able to bind to ST4 CPS by glycan array and enzyme-linked immunosorbent assay (ELISA) experiments. Their ability to opsonize *S. pneumoniae* ST4 bacteria of the TIGR4 strain was studied by immunofluorescence microscopy.

Weak antibody binding signals, barely above background in the glycan array experiments, indicated limited reactivity to both native and depyruvated ST4 CPS in mice immunized with trisaccharide **3**-CRM_{197} (Figure 3d). Only one third of the mice immunized with the tetrasaccharide **2** neoglycoconjugate produced antibodies that bind the depyruvated polysaccharide, but not the native ST4 CPS (Figure 3c). ELISA with pooled sera of immunized mice on plates coated with the native polysaccharide confirmed these results (Supplementary Figure 11). The CPS-specific IgG antibody titers increased less than fourfold from pre-immune (week 0) to post-immune (weeks 3 and 8) sera. Previous immunization studies with CPS of other pneumococcal serotypes revealed exponentially increased antibody levels in comparable experimental setups.*(19,20)* ELISA titers remained constant between weeks 3 and 8, whereas glycan arrays showed strongly increasing antibody levels to the oligosaccharides (Figure 3f). Immunofluorescence microscopy confirmed the observations of glycan arrays and ELISA, as the polyclonal sera raised against glycans **2** and **3** were unable to stain UV-inactivated TIGR4 pneumococci (Supplementary Figures 13-15).

Role of Terminal Residues of Glycans

Most anti-glycan antibody crystal structures reveal contacts between the protein and terminal glycan motifs by forming craters and grooves.*(34)* Conjugation of short oligosaccharides to globular carrier proteins such as CRM_{197} mainly exposes the terminal residues for interaction

with B cells. The anti-glycan antibodies elicited with CRM_{197} neoglycoconjugates of oligosaccharides **2** and **3** apparently had a strong preference for the respective terminal residues, indicated by minimal cross-reactivity to other deletion sequences with different terminal sugars.

[Place Figure 4 approximately here]

To gain a better molecular understanding of the interaction of anti-glycan antibodies with oligosaccharides **2** and **3**, we sought to generate monoclonal antibodies (MAbs) from mice immunized with the two different neoglycoconjugates. Antibodies secreted by hybridoma fusion cells confirmed the high specificity similar to polyclonal serum for tetrasaccharide **2**, but clonal isolation failed for this compound (Supplementary Figure 12). Two clones (B3 and H16) reactive to trisaccharide **3** were isolated in multiple rounds of clonal selection to ensure monoclonality. Glycan array analysis of both MAbs mirrored the binding properties of the polyclonal serum raised against **3** (Figure 4). The terminal α-FucNAc residue appeared to be the key structural determinant for antibody recognition of trisaccharide **3.** The trisaccharide used for immunization was bound strongly by both MAbs while any modification at the terminus significantly reduced antibody-glycan interactions. Binding to tetrasaccharides **1** and **2** was detectable, but low fluorescence intensities indicated that the additional $\beta(1\rightarrow3)$ linked ManNAc lowers affinity, probably due to steric hindrance between the larger ligand and the antibody binding site. Lack of the terminal FucNAc in disaccharide **5** also drastically reduced binding. Two additional observations underscore the importance of the contacts between terminal FucNAc and the antibody binding site: Mono-β-FucNAc **8** was bound stronger than tetrasaccharide **2** even though it is much smaller. Trisaccharide **4** that terminates with β-FucNAc instead of α-FucNAc was bound significantly weaker than **3,** which can be attributed to the different stereochemistry of the glycosidic linkage. Weak binding was observed to native and depyruvated ST4 CPS that could not be attributed to the inevitable CPS contamination CWPS as purified CWPS was not bound. These observations again confirm the existence of pyruvate-independent epitopes on native ST4 CPS. Low signal intensities suggest low antibody affinity towards the polysaccharide or limited epitope availability within the repetitive structure. Similar to the polyclonal sera raised against **3**, MAbs B3 and H16 failed to stain inactivated TIGR4 pneumococci (Supplementary Figure 16).

[Place Figure 5 approximately here]

Surface plasmon resonance (SPR) was used to confirm the glycan array studies. SPR allowed for the determination of binding affinities through use of a mouse antibody capture system (Figure 5).*(35)* For a long time, anti-glycan antibodies have been considered weak binders with micromolar affinities.*(5)* In contrast, recent studies showed tight nanomolar antibody-glycan interactions even against small oligosaccharides.*(35–38)* In accordance with these findings, MAbs B3 and H16 showed affinities of 130 ± 20 nM (mean ± SD) and 8 ± 2 nM to trisaccharide **3** as determined by kinetic SPR measurements. Binding affinities to tetrasaccharide **2** could not be uniquely determined as the association rates were below the measurable range of the SPR instrument. For the same reason, a thermodynamic analysis was not possible as the binding did not reach equilibrium state. The results indicate that the affinities of both MAbs for tetrasaccharide **2** are likely two orders of magnitude lower than for immunogen **3**. MAbs B3 and H16 show little binding to isolated ST4 CPS in SPR experiments, in agreement with the results obtained by glycan array (Supplementary Figure 18).

Epitopes of Pyruvated Tetrasaccharide **1**

Immunization studies revealed cross-reactivity between tetrasaccharides **1** and **2** (Figure 3c). Pyruvated tetrasaccharide **1** contains an epitope that is cross-reactive with the native ST4 CPS as seen from serum screenings (Figure 2, Supplementary Figure 6 and Pereira et al. *(22)*). Human serum 007sp additionally contains antibodies to **1** that are not cross-reactive to native ST4 CPS as indicated by incomplete inhibition (Figure 2c). We employed an inhibition assay using the neoglycoconjugates to examine whether these human serum antibodies are cross-reactive to depyruvated tetrasaccharide **2** and likely bind its non-reducing end. If the epitopes are cross-reactive, the **2**-CRM_{197} conjugate should be able to efficiently inhibit antibody binding due to multivalent presentation of the terminal epitope. Indeed, pre-incubation of serum dilutions with **2**-CRM_{197} resulted in a loss of signal for tetrasaccharide **1** and double inhibition with both glycoconjugate and ST4 CPS almost completely abrogated the binding signal (Figure 6). Pre-incubation with the glycoconjugate of trisaccharide **3** had a much smaller effect on binding in support of the notion that antibodies that are not sequestered by ST4 CPS bind to the non-reducing end. Therefore, at least two competitive epitopes are present on a comparably small molecule. A semi-synthetic or synthetic vaccine against ST4 based on an oligosaccharide requires an immune response directed towards the cross-reactive pyruvate-containing epitope. Therefore, the pyruvate has to be placed in a yet-to-be-determined optimal position and capping the non-reducing end with non-immunogenic sugars might be advantageous to prevent an immune response against the unwanted, non-cross-reactive epitope.

[Place Figure 6 approximately here]

Conclusions

The antigenic determinants of the repeating unit of *S. pneumoniae* serotype 4 CPS were identified. Glycan array analysis of ST4 CPS reactive mouse sera revealed exclusive binding to tetrasaccharide **1** demonstrating the importance of the *trans*-2,3(*S*)-pyruvate ketal as ST4 epitope. Human reference serum 007sp contained ST4-directed antibodies that recognized both pyruvate-dependent and independent epitopes. Immunization of mice with neoglycoconjugates of two selected non-pyruvated ST4 oligosaccharides elicited highly specific immune responses. Antibodies from post-immune sera failed to recognize the natural polysaccharide on the surface of *S. pneumoniae* ST4 bacteria. These murine studies indicate that another oligosaccharide immunogen might be required for a synthetic vaccine. Investigations of both the polyclonal serum as well as monoclonal antibodies revealed that the anti-oligosaccharide antibodies were mainly directed to epitopes at the non-reducing end. Antibodies to both terminal and internal epitopes confer protection to bacteria that are coated with repetitive polysaccharides.*(39–41)* Targeting internal epitopes of large polysaccharides such as pneumococcal CPS may be advantageous since such epitopes are more abundant than terminal epitopes.*(42)* While frameshifts and larger oligosaccharides might expose such epitopes in depyruvated glycans, the obvious candidate for an internal epitope for ST4 is the pyruvate ketal as we verified its important role for ST4 directed immunity. A larger set of pyruvated compounds has to be obtained in sufficient amounts for conjugation for a detailed analysis of the immunological properties of this rare and interesting glycan modification, including identification of a minimal hapten that can be obtained with minimal synthetic effort.

Materials and Methods

Glycan Array Analysis

Glycan array analysis was performed as described previously.*(24,43,44)* Briefly, oligosaccharides, proteins and polysaccharides were immobilized on NHS activated glass slides (CodeLink, Surmodics) using a piezoelectric printer (S3, Scienion) according to the printing pattern shown in Supplementary Figure 2. Remaining reactive groups on the surface were quenched and slides stored at 4 °C until use. Slides were blocked with 1 % (w/v) BSA in phosphate-buffered saline (PBS) before applying antibody dilutions. Sera or monoclonal antibodies were diluted in 1 % (w/v) BSA in PBS and incubated in a 64 well format on the slide. For the inhibition studies, the respective inhibitors were added to the solutions used for dilution and dilutions were incubated for 20 min at room temperature (RT) before incubation on the slide. After primary antibody incubation, slides were washed with PBS containing 0.1 % (v/v) Tween-20 (v/v). The secondary antibody was applied to the slide and incubated for 30 min at RT protected from light. Slides were washed to remove unbound secondary antibody, dried by centrifugation and fluorescence was read out with a GenePix 4300A microarray scanner (Molecular Devices). See Supporting Information for details.

Neoglycoconjugate Preparation

Glycoconjugates were prepared as described earlier.*(43,24,44,32,45)* Briefly, glycan and activated spacer (di-*N*-succinimidyl adipate) were separately dissolved in dry DMSO. Triethylamine was added to the spacer solution and the glycan was added slowly under stirring over 30 min. After additional 90 min reaction time under stirring at RT, conjugation buffer (100 mM sodium phosphate, pH 7.5) was added and the non-reacted spacer and DMSO were extracted twice with chloroform. The aqueous phase was added to a 1 mg/mL solution of CRM_{197} (Pfénex, Inc.) in conjugation buffer and the solution was stirred at RT overnight. The conjugate was desalted using Amicon centrifugation filter devices (MWCO 10,000, Merck Millipore) by repeatedly adding ultrapure water to the upper compartment and centrifugation at 3300 x *g*. The resulting neoglycoconjugates were characterized by SDS-PAGE and MALDI-TOF mass spectrometry and stored at 4 °C until use. See Supporting Information for details.

Immunizations and Development of Monoclonal Antibodies

All animal experiments were carried out according to the ethical guidelines of the Landesamt für Gesundheit und Soziales, Berlin, Germany. In the first round of immunizations, six female C57BL/6J mice (Charles River), three each for neoglycoconjugates of **2** and **3**, but one

mouse of the latter group died shortly after priming, were immunized subcutaneously with neoglycoconjugates formulated with Complete Freund's Adjuvant. Glycoconjugate containing ~3 µg of antigen in a total volume of 100 µL was administered per mouse. Boosting after two weeks was performed with the same amount of neoglycoconjugate formulated in Incomplete Freund's adjuvant. Blood was collected before the first injection, and after 1, 2, 3 and 8 weeks. For the development of monoclonal antibodies against trisaccharide **3**, three female C57BL/6J mice were primed and boosted as described above. A second boost was performed five weeks post-priming. Another 10 days later, the mouse with the highest titer against **3** received a final boost of the same amount of conjugate intraperitoneally without adjuvant and was sacrificed four days later. The spleen was removed and splenocytes were fused to X63.Ag8 cells using standard hybridoma techniques.*(46)* Clonal selection based on repeated single cell plating and subsequent glycan array analysis of supernatants was carried out to isolate glycan-specific hybridomas. Stable clones were cultivated in serum-free medium (ISF-1, Biochrom) and monoclonal antibodies were purified with Protein A/G sepharose affinity chromatography (BioVision, for details see Supporting Experimental Procedures). For all immunizations, anti-glycan antibody titer evaluation was performed using glycan arrays. Antibody levels of each mouse were analyzed and represented as mean fluorescence intensity at a particular serum dilution. See Supporting Information for details.

ELISA

ELISA was performed with dilutions of poooled sera collected from the two immunized groups of mice. High binding Immulon 4 HBX plates were directly coated with ST4 CPS (SSI, Denmark), sera were overlaid and incubated for specific time points. See Supporting Information for details.

SPR

All SPR experiments were performed using a Biacore T100 instrument (GE Healthcare) at 25 °C using the capture system described previously.*(35)* Briefly, goat anti-mouse IgG was immobilized on CM5 sensor chips (both GE Healthcare) at high levels (reference cell: blank immobilization). Kinetic evaluations were performed by first passing purified monoclonal antibodies and subsequently the glycan analyte (both in PBS) through the flow cells. The surface was regenerated after each cycle. Double-referenced binding curves were obtained by subtracting both reference cell signal and signal from buffer (PBS) only cycles from samples containing the glycans. The Biacore T100 Evaluation software supplied with the instrument was used for kinetic binding analysis using the built-in one-to-one binding model. See Supporting Information for details.

Other Experimental Procedures

Experimental procedures for the depyruvation of ST4 CPS by acidic treatment and immunofluorescence staining of inactivated TIGR 4 pneumococci are described in the Supporting Information.

Acknowledgements

The authors gratefully acknowledge generous funding by the Max Planck Society and the European Research Council (ERC Advanced Grant AUTOHEPARIN). C.A. acknowledges the support by the German Federal Ministry of Education and Research (Grant 0315447). We are grateful to Pfénex, Inc. for providing recombinant CRM_{197} at a discounted price, to M. Akkoyunlu (FDA/CBER) for providing pneumococcal reference serum 007sp for free, and to K. Reppe (Charite Berlin) for providing UV-inactivated TIGR4 pneumococci. Pooled serum of Prevenar 13® immunized mice was a kind gift of B. Monnanda and dimannoside **9** a kind gift of H. Tanaka. We acknowledge C. Martin and F. Broecker for MALDI-TOF MS measurements and A. Reinhardt for valuable and fruitful discussions. B. Lepenies is acknowledged for advice on institutional animal ethics.

Supporting Information

Detailed experimental procedures and supplementary figures can be found in the Supporting Information. This material is available free of charge *via* the Internet at http://pubs.acs.org.

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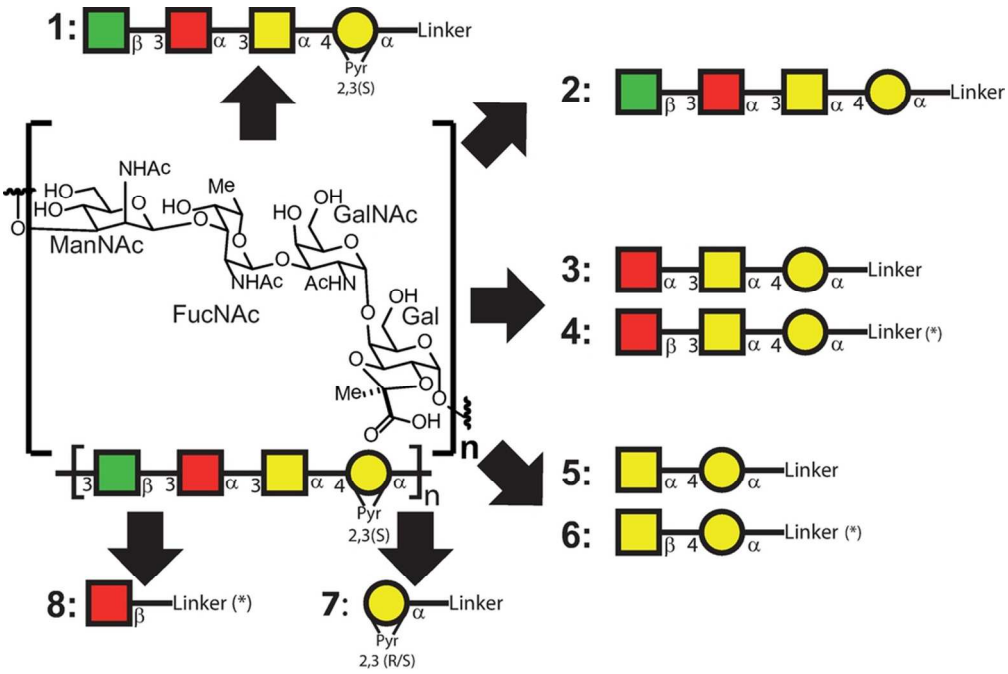

Figure 1: Design of synthetic glycans used in this study. Center: Chemical structure of the ST4 CPS repeating unit.*(8)* Around it, the synthetic glycans are depicted in their symbolic notation with the asterisk designating compounds with unnatural stereochemistry at the terminal glycan.
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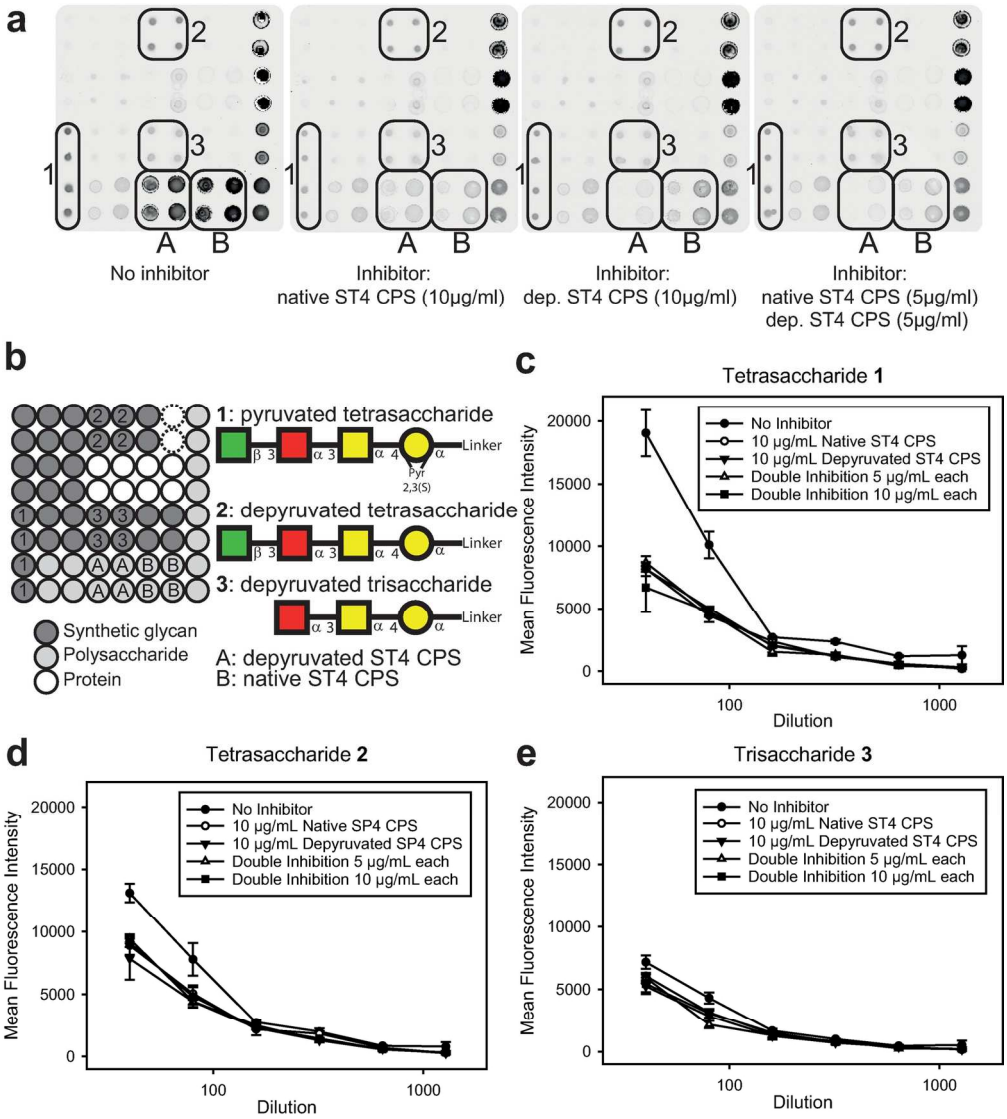

Figure 2: Glycan array analysis of IgG antibodies from human reference serum 007sp. a) Representative images from microarray wells at serum dilution factor 80 and different inhibitors used for pre-incubation as outlined under the images. b) Simplified microarray printing pattern only showing discussed compounds. Detailed printing pattern is depicted in Supplementary Figure 2. c–e) Fluorescence intensities of spots printed with 0.1 mM synthetic glycan depending on serum dilution and inhibitor for oligosaccharides **1**–**3** (Mean of four spots with error bars representing standard deviation (SD)).
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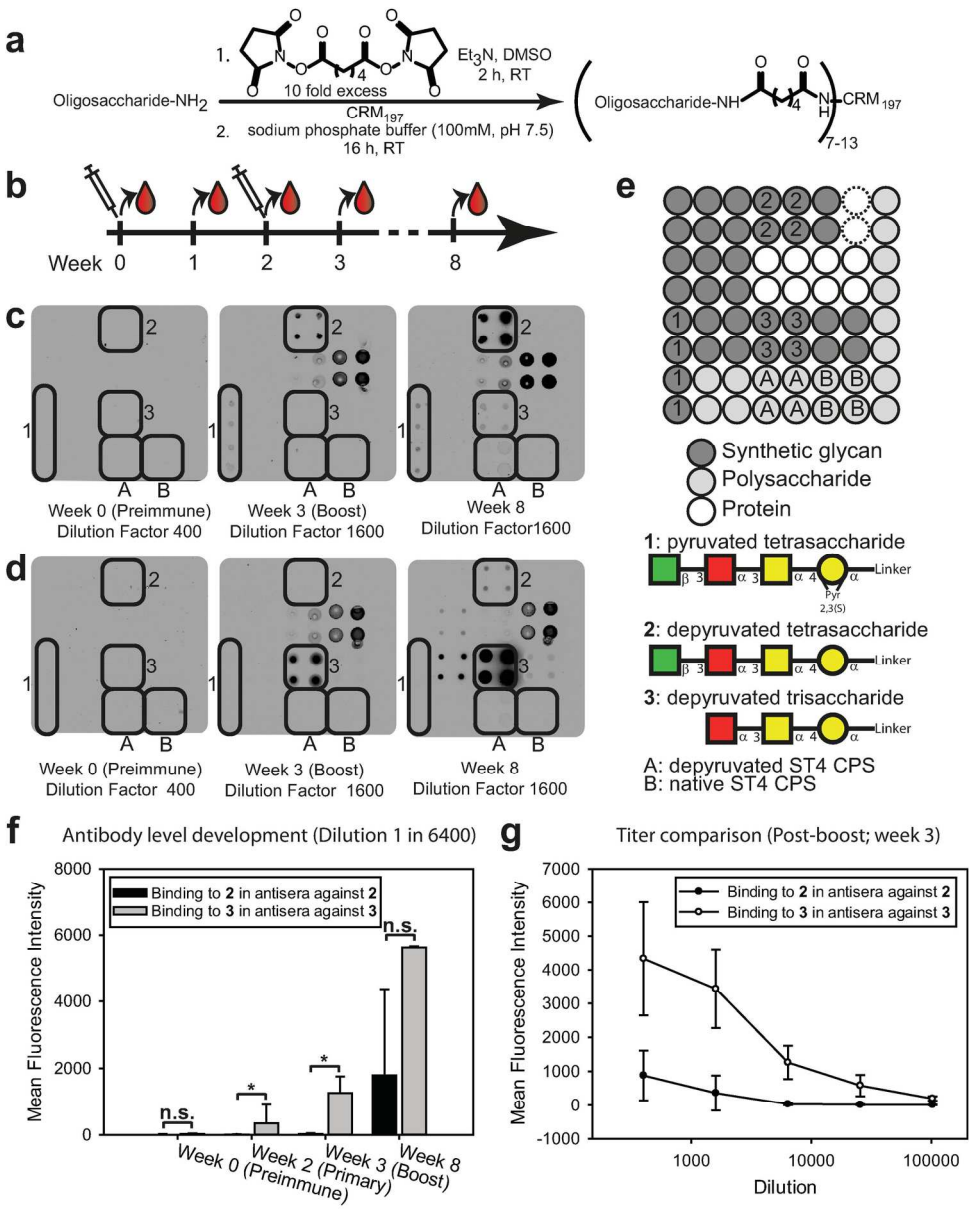

Figure 3: Immunizations to obtain antisera against depyruvated ST4 oligosaccharides. a) Conjugation reaction. Glycans are linked to lysines of CRM_{197} by a two-step reaction via the amine linker. b) Immunization regime: Mice received a prime immunization with the glycoconjugates formulated in Complete Freund's Adjuvant and a boost immunization (Incomplete Freund's Adjuvant) 14 days later. Blood for analysis was taken at the indicated time points. In later trisaccharide immunizations, a second boosting injection was given after four weeks. c, d) Representative images of glycan array wells to analyze the development of the immune response of mice immunized with glycoconjugates of depyruvated tetrasaccharide **2** (c) and depyruvated trisaccharide **3** (d). e) Simplified microarray printing pattern only showing discussed compounds. f) Development of IgG levels towards glycans used for immunization presented as bars of fluorescence intensities towards the respective immunogen at different time points. Significance levels were calculated using Mann-Whitney rank sum test (*: $p<0.05$; n.s.: $p>0.05$). g) Comparison of IgG titers at week 3 (post-boost) between **3** and **2** determined by glycan array fluorescence at different serum dilutions. Higher titers for **3** can also be observed at week 2 and 8 (Supplementary Figure

10). Values in panels f and g represent means of n=3 mice for tetrasaccharide **2** and n=5 mice for trisaccharide **3** with the exception of week 8 for trisaccharide **3** (n=2). Error bars represent SD.
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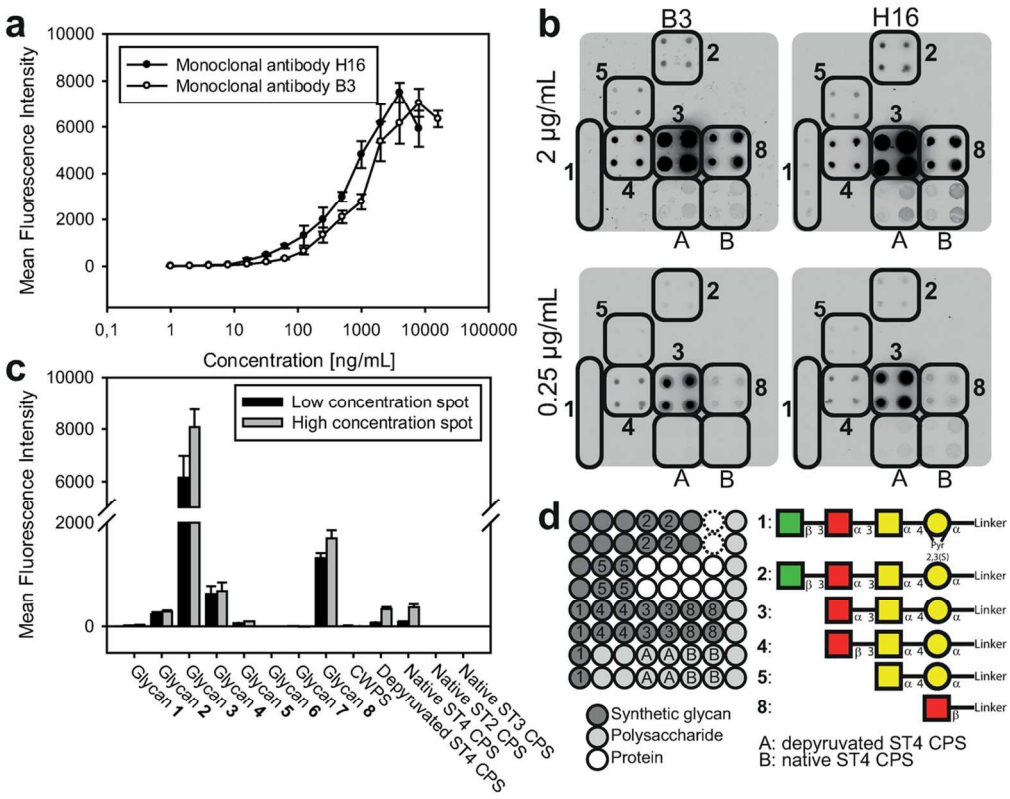

Figure 4: Glycan array-based epitope mapping of monoclonal antibodies B3 and H16 against trisaccharide **3**. a) Both antibodies bind **3** in a concentration dependent matter whereby H16 shows the higher apparent avidity. Crowding effects on the array lead to a signal decline at very high concentrations. Mean of four spots per value with error bars representing SD. b) Binding patterns of both antibodies on glycan array mirror the serum of mice immunized with glycoconjugates of **3**. Signals are strongest for **3** – at 2 µg/mL already almost saturated – while weaker binding is seen for a number of other synthetic structures including **2**. Binding to native and depyruvated ST4 CPS is weak. c) Binding profile of 2 µg/mL monoclonal antibody H16 to visualize differences between weakly bound structures (Mean of four spots per value; error bars: SD). Binding profile of B3 is very similar (Supplementary Figure 17). d) Simplified microarray printing pattern only showing discussed compounds.
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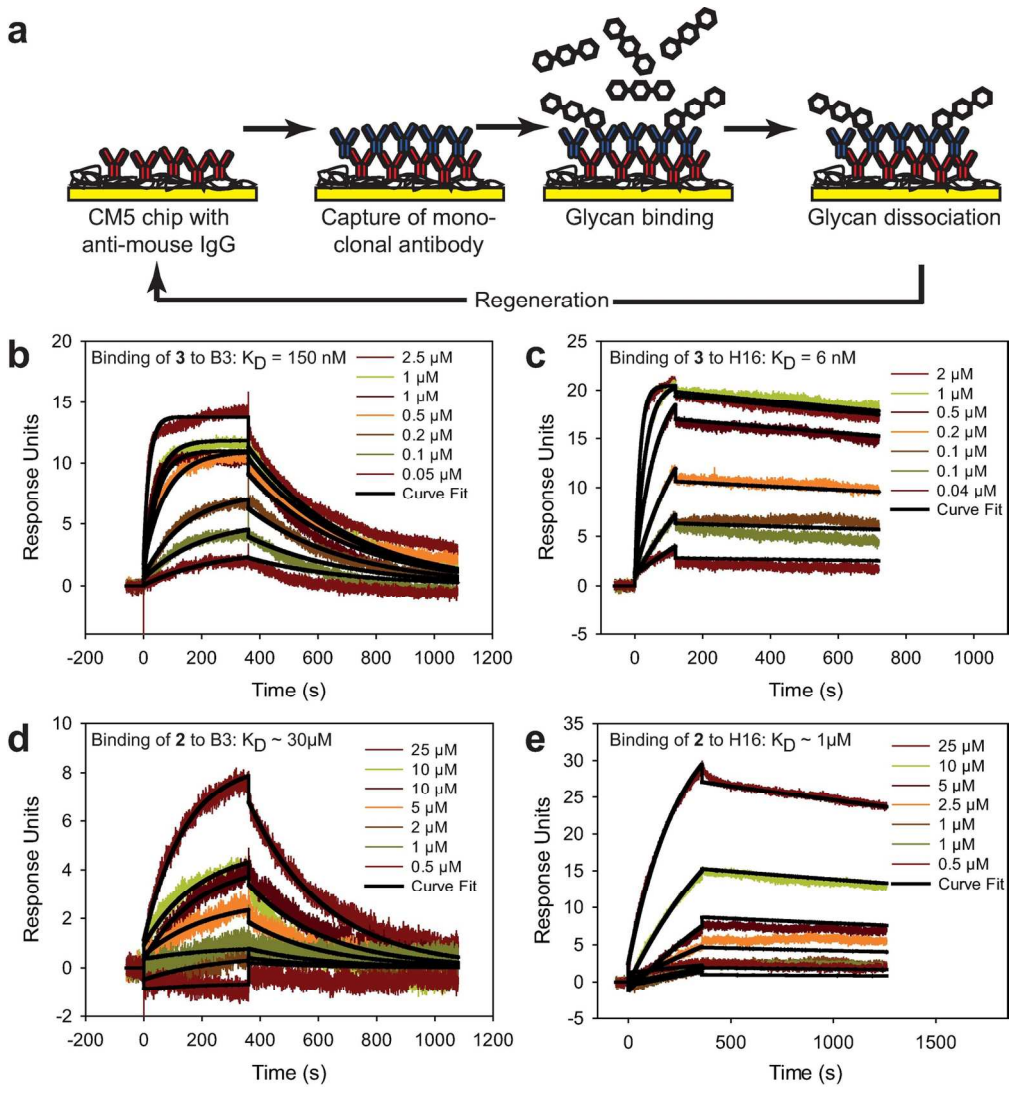

Figure 5: SPR analysis of monoclonal antibodies a) Antibody capture setup used for SPR studies allowing for one-to-one binding evaluation. b, c) Representative kinetic SPR measurements to determine affinities of antibodies B3 (b) and H16 (c) for trisaccharide **3**. d, e) Similar evaluation for tetrasaccharide **2**. Association rates were outside the instrument's specifications, therefore the KD is only an approximate value, but around two orders of magnitude higher than for **3**. Thermodynamic (steady state) evaluation was impossible as the kinetics were too slow to reach equilibrium.
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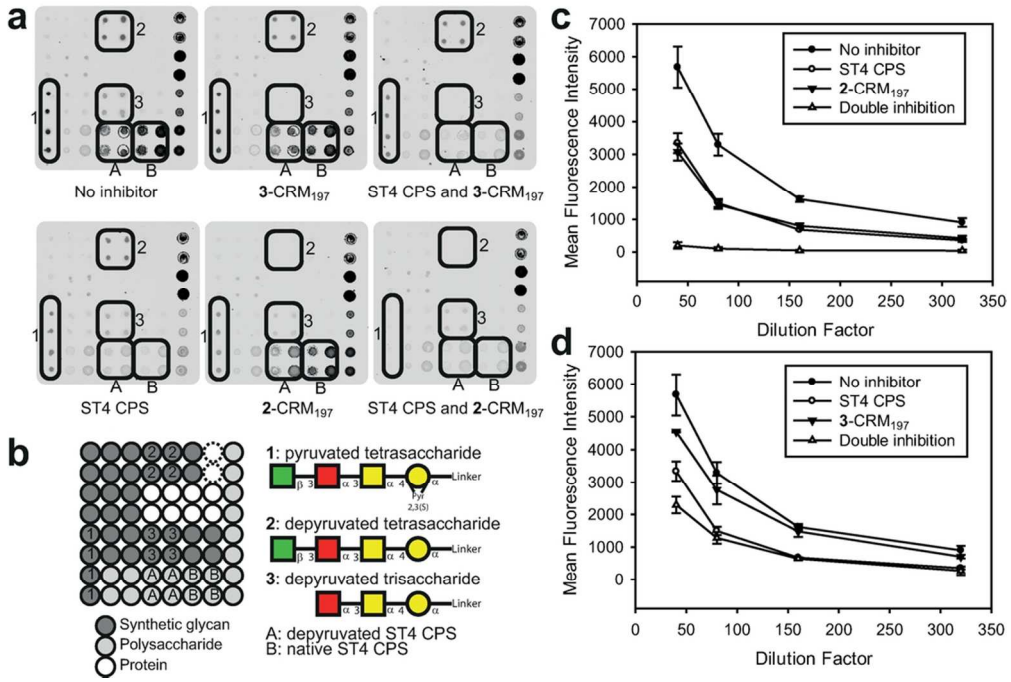

Figure 6: Inhibition assay on glycan arrays with neoglycoconjugates of **2** and **3**. a) Representative images of wells incubated with serum 007sp diluted 1 : 80 and the inhibitors as outlined below the image. b) Simplified microarray printing pattern only showing discussed compounds. c) Antibody binding level from serum 007sp towards tetrasaccharide **1** at different dilutions. For inhibition studies, serum dilutions were pre-incubated in buffer containing native ST4 CPS, neoglycoconjugate **2**-CRM_{197}, or both. d) Same experiment as described in (c) using neoglycoconjugate **3**-CRM_{197}.
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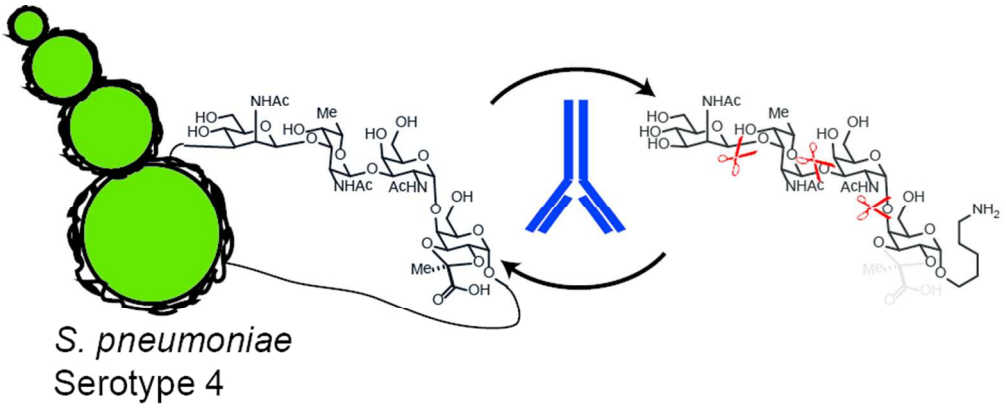

Graphical abstract
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