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Non-Native Amino Acid Click Chemistry-Based Technology for Site-Specific Polysaccharide Conjugation to a Bacterial Protein Serving as Both Carrier and Vaccine Antigen

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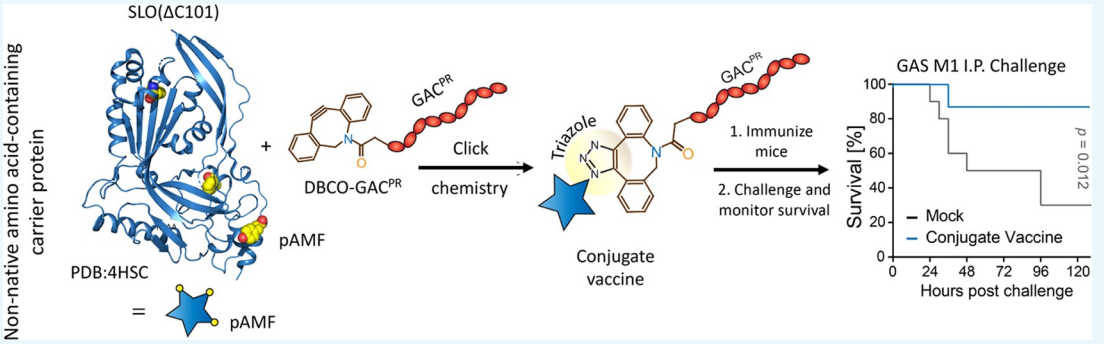

ABSTRACT: Surface-expressed bacterial polysaccharides are important vaccine antigens but must be conjugated to a carrier protein for efficient antigen presentation and development of strong memory B cell and antibody responses, especially in young children. The commonly used protein carriers include tetanus toxoid (TT), diphtheria toxoid (DT), and its derivative CRM197, but carrier-induced epitopic suppression and bystander interference may limit the expanded use of the same carriers in the pediatric immunization schedule. Recent efforts to develop a vaccine against the major human pathogen group A *Streptococcus* (GAS) have sought to combine two promising vaccine antigens—the universally conserved group A cell wall carbohydrate (GAC) with the secreted toxin antigen streptolysin O (SLO) as a protein carrier; however, standard reductive amination procedures appeared to destroy function epitopes of the protein, markedly diminishing functional antibody responses. Here, we couple a cell-free protein synthesis (CFPS) platform, allowing the incorporation of non-natural amino acids into a C-terminally truncated SLO toxoid for the precise conjugation to the polyrhamnose backbone of GAC. The combined immunogen generated functional antibodies against both conserved GAS virulence factors and provided protection against systemic GAS challenges. CFPS may represent a scalable method for generating pathogen-specific carrier proteins for multivalent subunit vaccine development.

1. INTRODUCTION

Bacterial surface-associated polysaccharides are important vaccine targets given their exposure, abundance, and role in immune evasion.[1] However, as immunogens, such purified polysaccharides are generally unable to bind class II major histocompatibility complex (MHC II);[2] consequently, they alone are poor inducers of T-cell responses, especially in infants and young children.[3] To overcome this limitation, covalent conjugation of the polysaccharide to a carrier protein allows follicular dendritic cells to internalize and process the protein−polysaccharide conjugate. The helper peptide epitopes can then be loaded into the MHC II cavity for T-cell presentation.[4] This engagement initiates a reaction in the lymph node germinal center that promotes affinity maturation, isotype switching from IgM to IgG, and development of memory B cells and plasma cells that efficiently produce antipolysaccharide antibodies.[5] Additional advantages of protein conjugation may include mitigating the high solubility and rapid clearance of most pure polysaccharides[6] and the ability to engineer multivalent presentation that could maximize cross-linking of antigen-specific B cell receptors.[7]

For approved *Haemophilus influenzae* type B (HiB), *Streptococcus pneumoniae* (pneumococcus), *Neisseria meningitidis* (meningococcus), and *Salmonella typhi* (typhoid)

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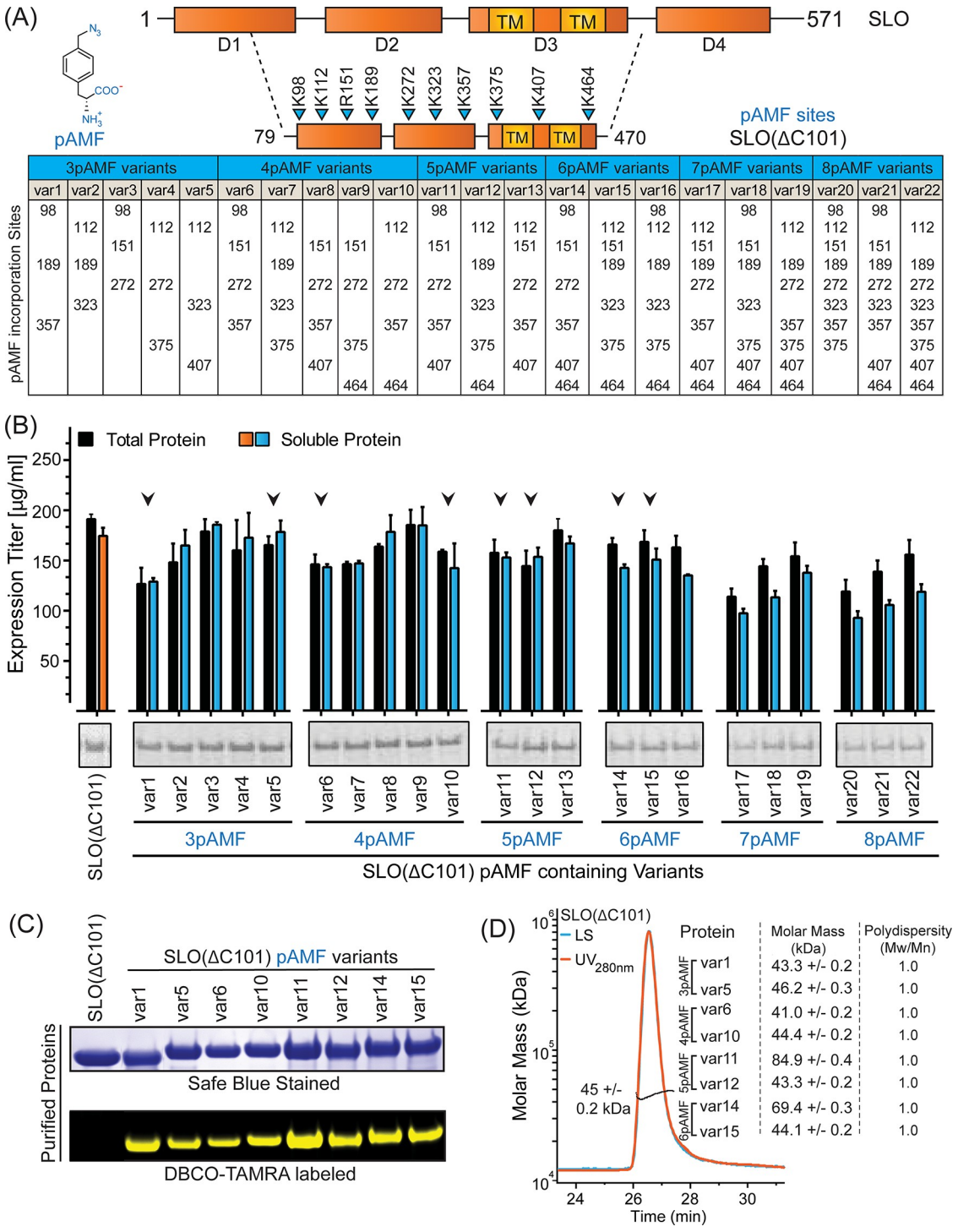

Figure 1. Design, expression, and biophysical characterization of SLO variants. (A) Using SLO c-term truncation variant SLO(ΔC101) as the template sequence, several 3−8 pAMF incorporation combination variants (var1−var22) were generated. (B) Protein expression titers for each variant were estimated using 14C-leucine incorporation assay and (C) pAMF incorporation in a subset of purified variants was confirmed by the successful DBCO-TAMRA labeling. (D) SEC-MALS analysis shows molecular mass estimates for each purified variant.

polysaccharide conjugate vaccines, the most widely used carrier proteins, namely, tetanus toxoid (TT), diphtheria toxoid (DT), and its derivative CRM197, were chosen because of their long established safety records in tetanus and diphtheria vaccination.[8] However, the continued exploitation of the same small set of carrier proteins could eventually result in diminishing returns for the new glycoantigens. Pre-existing immunity to a given carrier protein can blunt the immune response to a new carbohydrate conjugated to the same carrier. Such "carrier-induced epitopic suppression" was revealed when children who received Hib polysaccharide conjugated to diphtheria toxoid had superior immune responses to those who received Hib polysaccharide conjugated to tetanus toxoid when coadministered with a tetanus toxoid-conjugated pneumococcal vaccine.[9] Combining multiple CRM197-conjugate vaccines at once runs a theoretical risk of saturating the

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lymph node's molecular and cellular apparatus for antigen presentation—the so-called "bystander interference" that can extend even to unrelated antigens present within a multivalent subunit vaccine.[10,11]

An attractive alternative to the classical carrier protein repertoire for a novel glycoconjugate vaccine is to conjugate the bacterial polysaccharide antigen to a highly conserved and immunogenic protein antigen from the same bacterial species, whether that be a surface-anchored protein targeted to promote opsonophagocytosis or a bacterial toxin whose neutralization could block virulence. With this approach, the glycoconjugate could represent a "two-for-one" antigen, used in a stand-alone manner or as part of a multivalent subunit vaccine, eliciting functional antibodies that target both the polysaccharide and the autologous carrier protein to protect against diseases. The most frequently used method for carbohydrate conjugation to classical carrier proteins is reductive amination, favored because it achieves hydrolytic stability, covalent linkage, and facile coupling amenable to the production at the industrial scale.[12] However, this approach targets the side-chain ϵ-amine site of lysines in the carrier protein to form the intermediate imine derivatives[13] in an indiscriminate manner that could alter or shield functionally important immunological epitopes. To that end, selective chemistries to employ a protein antigen with the dual role of carrier for polysaccharides have been explored.[14−16]

Here, we explore such a bioconjugation challenge relevant to vaccine development against the leading human bacterial pathogen group A *Streptococcus* (GAS, *S. pyogenes*). Globally, GAS infections and their complications are responsible for more than half a million annual deaths, with low- and middle-income countries suffering the greatest disease burden.[17,18] A desirable surface-exposed GAS antigen for vaccine inclusion is a high-molecular-weight polysaccharide—the universally conserved and species-defining group A carbohydrate (GAC)[19]—or Lancefield antigen, which is essential for bacterial survival[20] comprising 30−50% of the bacterium's cell wall by weight.[21] In preclinical vaccine research, protein conjugates of native GAC, or its polyrhamnose core (GAC^{PR}), used to allay autoimmunity concerns attributed to the GAC *N*-acetylglucosamine (GlcNAc) side chain, elicit antibodies that bind GAS, promote opsonophagocytosis, and protect against infection in murine vaccination models.[20,22−24] In such studies, the purified large-molecular-weight streptococcal polysaccharide was conjugated to carrier proteins tetanus toxoid,[23] CRM197,[25] or pneumococcal elongation factor.[20] Additional investigations generated synthetic fragments of the polysaccharide repeating structure for conjugation to gold nanoparticles[26] or a self-adjuvanting Ac-$PADRE^{\alpha}$-lipid core.[27]

Considering potential autologous GAS protein antigens for the conjugation of GAC/GAC^{PR}, an immediate candidate arises in the pathogen's universally conserved cholesterol-dependent cytolysin, streptolysin O (SLO), a critical virulence factor, and a vaccine target in several ongoing studies of multivalent vaccine formulations.[22,28−30] SLO assembles into and disrupts diverse host cell membranes, triggering rapid cell death by osmotic lysis, apoptosis or pyroptosis, and promoting tissue injury and systemic spread of the pathogen.[31−37] Even at sublethal doses, SLO impairs the antibacterial functions of macrophages and neutrophils, increasing GAS immune evasion.[38−40] Point mutations can be introduced into SLO to render a safe toxoid immunogen that elicits antibodies capable of neutralizing the toxin's membrane-damaging effects,[41] though commercial scale-up has been hampered by a limited yield (1−2 μg/mL) of the recombinant toxoid in cell-based expression systems.[42] Recently, attempts were made by standard reductive amination to allow SLO toxoid to serve as a carrier protein for GAC conjugation, but the total anti-SLO-specific IgG was lower than observed upon immunization with the same dose of unconjugated SLO.[43] Furthermore, conjugation to GAC completely abolished the ability of SLO toxoid to elicit antibodies that neutralized native SLO cytotoxicity.[43]

Here, we describe a successful click chemistry-based approach for the precise conjugation of GAC^{PR} to a C-terminally truncated SLO toxoid, yielding an immunogen that generates functional antibodies against both conserved GAS virulence factors—the cell wall polysaccharide and the pore-forming cytotoxin. Immunization studies in mice were used to verify the performance of this novel dual GAS antigen conjugate head-to-head versus identical concentrations of a classical (CRM197) GAC^{PR} conjugate plus SLO toxoid in terms of antibody responses and protection against systemic GAS challenges.

2. RESULTS AND DISCUSSION

2.1. Generation, Purification, and Biophysical Characterization of SLO Variants with Incorporated pAMF Residues. The Xpress^{+} cell-free protein synthesis (CFPS) platform extracts the *E. coli* cellular machinery required for transcription, translation, and energy production into a cell-free mixture capable of continuous oxidative phosphorylation.[44−46] Typical yields of protein expression and purification with this system have been reported.[47] We previously used Xpress^{+} CFPS to generate an SLO C-terminal truncation variant SLO(ΔC101) (Figure 1A) that elicited SLO neutralizing antibodies and contributed to protection against GAS challenge *in vivo*.[22] To prepare SLO(ΔC101) for use as a carrier protein, we analyzed its sequence and selected several solvent-exposed lysine and arginine residues as sites to incorporate the non-native amino acid (nnAA) *p*-azidomethyl phenylalanine (pAMF). We next combined these sites to generate 3−8 pAMF-containing SLO(ΔC101) variants (Figure 1A) and estimated their expression levels by ^{14}C-leucine incorporation during CFPS and autoradiogram analysis. Most 3−6 pAMF variants expressed >150 μg/mL, while the incorporation of 7 or 8 pAMF sites resulted in lower expression (Figure 1B); thus, we focused on a subset of 3−6 pAMF-containing variants as candidate carrier proteins. For scale-up, each SLO(ΔC101) variant (var1, 5, 6, 10, 11, 12, 14, and 15; Figure 1C) was expressed with a cleavable N-terminal His6-tag using a DASbox bioreactor under controlled conditions, maintaining pH at 7.2 and dissolved oxygen at 30%. Proteins were captured from the clarified supernatant using a 5 mL HisTrap capture column, and their N-terminal purification tags were removed by TEV protease cleavage. Finally, the untagged proteins were subjected to size exclusion chromatography (SEC) on a pre-equilibrated Superdex75 16/60 column, and the highest purity fractions were combined, concentrated, and stored at −80 °C. Click chemistry rhodamine-derived cyclo-octyne (DBCO-TAMRA) labeling gave strong fluorescence signals for each variant, confirming successful pAMF incorporation (Figure 1C). Finally, we used SEC multiangle light scattering (SEC-MALS) to analyze the in-solution oligomeric state of each protein. Similar to native SLO(ΔC101), which is a monomer in solution with a

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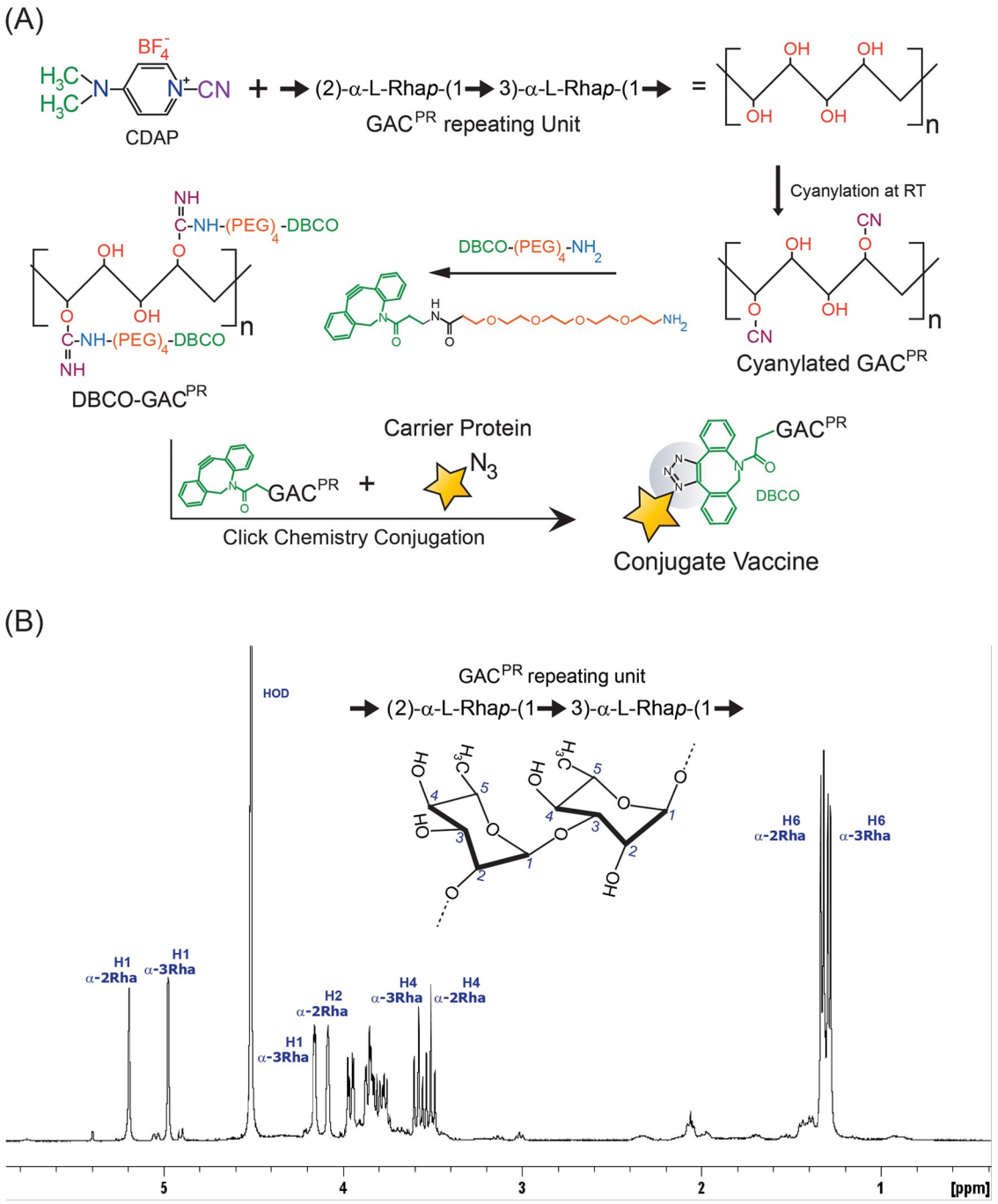

Figure 2. (A) Schematic showing the chemical steps for DBCO derivatization and conjugation of GAC^{PR} to carrier proteins[1] and (B) ^{1}H NMR spectrum of purified GAC^{PR} recorded at 400 MHz, 50 °C. Diagnostic peaks for H1, H2, H4, and H6 for a-2Rhap and a-3Rhap of the disaccharide repeating unit are labeled.

molecular mass of 45 ± 0.2 kDa, the estimated molecular mass for each of the 3 and 4 pAMF variants verified a monomeric state in solution (Figure 1D). Interestingly, and unexpectedly, the SEC-MALS analysis of 5 pAMF (var11)- or 6 pAMF (var14)-containing SLO(ΔC101) variants revealed higher-order oligomeric states in solution. For these reasons, we focused our efforts only on 3 and 4 pAMF-containing SLO(ΔC101) variants as carrier proteins for conjugating GAC^{PR}.

2.2. Biophysical Characterization and Immunogenicity of GAC^{PR} Conjugates to SLO(ΔC101) Carrier Proteins. To generate conjugate polysaccharide vaccine antigens, we used a dibenzocyclooctyne (DBCO)-derivatized form (Figure 2A) of the purified GAS cell wall GAC polyrhamnose (Figure 2B) core (DBCO-GAC^{PR}) in a Cu^{2+}-free strain promoted azide−alkyne cycloaddition reaction with 3 pAMF (var1 and var5)- and 4 pAMF (var6 and var10)-containing SLO(ΔC101) variants (Figure 3A, inset). Typical yields of glycoconjugation reaction and purification with this method have been reported in our work with a 24-valent pneumococcal capsule conjugate vaccine.[47] Each carrier protein was mixed with DBCO-GAC^{PR} in a 1:1 mass ratio (0.5 mg/mL each) for 4 h at room temperature with constant stirring. Thereafter, the reactions were harvested, and the

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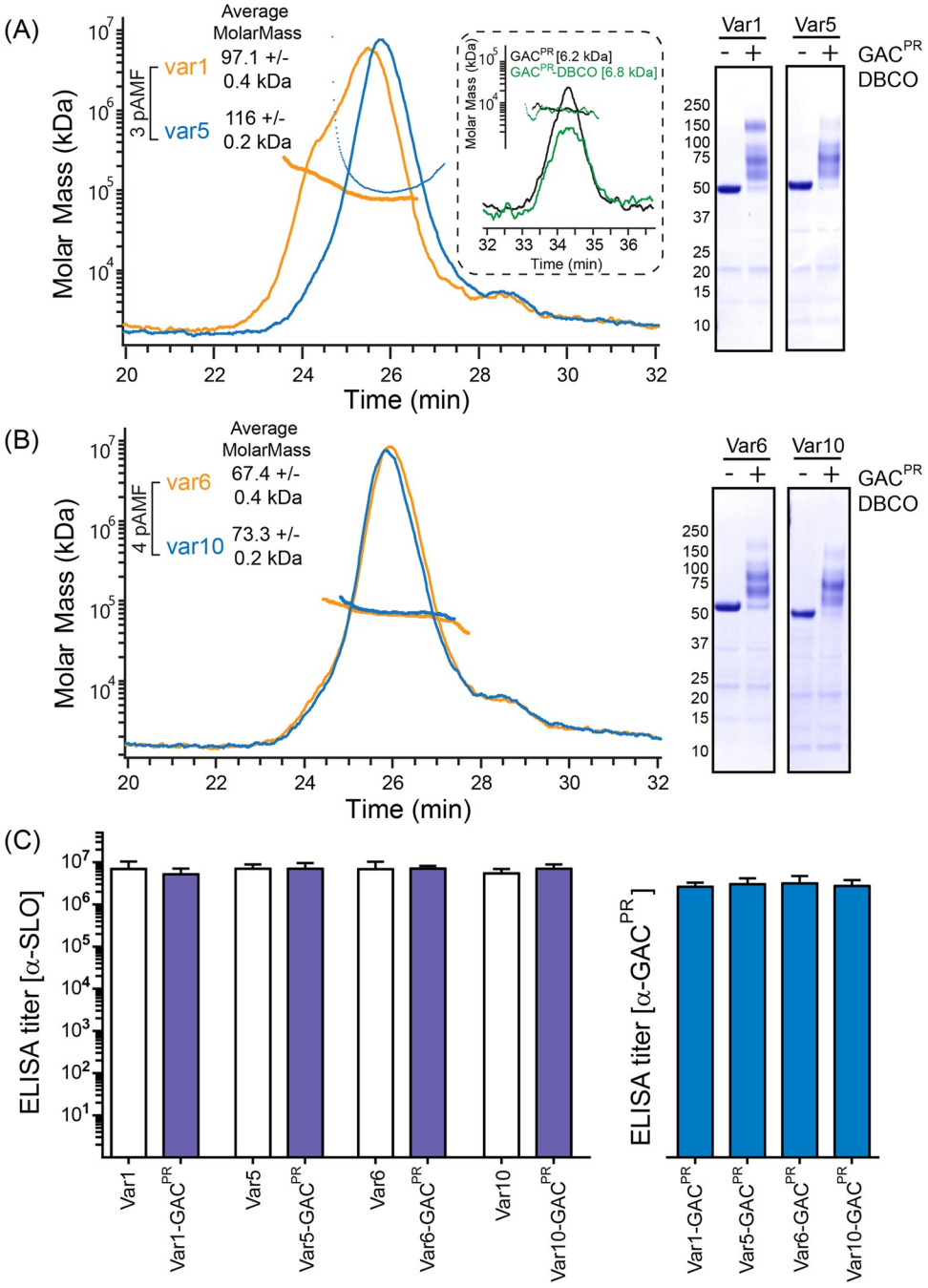

Figure 3. Biophysical characterization and immunogenicity assessment of conjugate vaccines generated using SLO(ΔC101) variants as carrier proteins. SEC-MALS analysis of (A) native or DBCO-derivatized GACPR (inset) and conjugate vaccines generated using 3 pAMF- or (B) 4 pAMF-containing SLO(ΔC101) variants followed by SafeBlue-stained SDS-PAGE analysis of purified conjugates. (C) ELISA analysis was performed to estimate titers against SLO or GAC^{PR}.

conjugated polysaccharides were dialyzed against the buffer to remove excess free GAC^{PR}. Next, the SEC-MALS analysis was performed on the purified conjugates, which estimated an average molar mass of 97 or 116 kDa for conjugates generated using SLO(ΔC101) variants containing 3 pAMF sites (Figure 3A) and 68 or 74 kDa for conjugates generated using SLO(ΔC101) variants containing 4 pAMF sites (Figure 3B). SDS-PAGE analysis of the purified conjugates followed by SafeBlue staining corroborated the results and confirmed the efficient consumption of the carrier proteins for the generation of each conjugate post the Cu^{2+}-click chemistry reaction (Figure 3A,B).

Post conjugation and purification, the PS concentration in the recovered conjugates was 0.33 mg/mL, as estimated by the anthrone assay. Since the molecular mass of the GACPR-DBCO is 6800 g/mol (Figure 3A, inset), we estimate (0.33/6800) = 48.5 μmol of GACPR-DBCO in the recovered conjugates. In addition, the concentration of the protein in the recovered conjugates was 0.5 mg/mL. Since the molecular mass of SLO(ΔC101) is 43,300 g/mol, we estimate (0.5/43,300) = 11.54 μmol of SLO(ΔC101) in the recovered conjugates. Finally, there are about 48.5/11.54 = 3.8 GACPR molecules per SLO(ΔC101). Since each protein subunit only has 3 pAMF sites, this suggests that we are at 100% occupancy

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of the pAMF sites in the CV. Finally, for the purified conjugates, we recovered 0.325 mg/mL of GACPR and 0.5 mg/mL of SLO carrier protein. Thus, in the final purified conjugate, we get a saccharide/protein ratio of 0.33/0.5 = 0.66.

To test *in vivo* immunogenicity, alum-adjuvanted formulations of the purified conjugates were injected intramuscularly into groups of CD-1 mice. After three doses were administered 2 weeks apart (days 0, 14, and 28), terminal bleeds were analyzed on day 35 by ELISA assay to estimate antibody titers against SLO and GAC^{PR}. Similarly, robust antibody titers (>10^6) against SLO were present in antisera generated by the SLO(ΔC101) variant protein alone or by the GAC^{PR} conjugate in which the protein served as the carrier (Figure 3C); thus, site-selective conjugation was accomplished without disrupting critical immunogenic epitopes on SLO(ΔC101). Robust antibody responses (>10^6) against GAC^{PR} were also generated by all of the conjugates (Figure 3C), showing how CFPS can provide an effective platform for designing pathogen-specific antigens containing nnAA, which can be used to generate conjugates that elicit strong antibody responses against both the carrier protein and the polysaccharide. Note that we present the titer based on the signal in the ELISA being 3 standard deviations above the buffer background, which results in a high titer value; in this analysis, the SLO protein serves as a positive control. In two variants, the immunogenicity of SLO is increased, and in one variant, the immunogenicity of the protein remains the same following conjugation with GAC, in stark contrast to previous reports of a decrease in immunogenicity of SLO following conjugation with polysaccharides and consistent with efficacy data below.

2.3. Conjugate GAC^{PR} Vaccine Antigen Using SLO-(ΔC101) as the Carrier Protein Protects against GAS Infection. Using a GAS-specific protein antigen as a carrier protein for GAC^{PR} could reduce the complexity of a multivalent subunit vaccine against the pathogen.[22,29,48] Recently, the use of conventional chemistries to prepare SLO as a carrier protein for GAC yielded reduced titers of non-neutralizing antibodies,[43] possibly due to the disruption of important immunogenic epitopes on the protein through non-site-specific conjugation. This led the authors to use inactivated diphtheria toxin variant CRM197 as the carrier protein for the GAS polysaccharide. We sought to evaluate the vaccine efficacy of our SLO(ΔC101)−DBCO-GAC^{PR} (variant 1) as a single conjugate antigen versus SLO(ΔC101) protein plus CRM197-conjugated GAC^{PR} as two separate antigens. First, we used the Xpress$^+$ CFPS system to make a modified CRM197 carrier protein with nnAA (eCRM)[8] and conjugated it to GAC^{PR}-DBCO. We then performed vaccinated mice using alum-adjuvanted formulations of mock (saline alone), SLO-(ΔC101)−GAC^{PR} conjugate, or a combination vaccine of SLO(ΔC101) + eCRM-GAC^{PR} conjugate. Mice were immunized on days 0, 14, and 28 followed by intraperitoneal (i.p.) challenge on day 42. For each immunization, 5 μg of PS was administered while dose-matching the amount of SLO-(ΔC101) for each group. Vaccination with the SLO-(ΔC101)−GAC^{PR} conjugate provided significant protection against a lethal challenge with an invasive M1 serotype GAS strain (p = 0.012 versus mock group), indeed appearing to outperform the combination of eCRM-GAC^{PR} conjugate + SLO(ΔC101) (Figure 4). This result indicates that conjugate polysaccharide vaccine antigens using a GAS-specific protein as a carrier may be formulated without compromising *in vivo* efficacy.

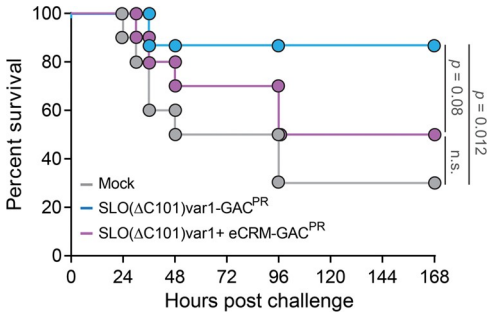

Figure 4. In vivo assessment of conjugate vaccines. Survival kinetics of actively immunized mice (mock or vaccine antigens) challenged with a lethal dose of an M1 strain of group A *Streptococcus*. Data shown are from an experiment with n = 10 animals per group, and statistics are calculated from the log-rank Mantel−Cox test or Kaplan−Meier plots.

3. CONCLUSIONS

In the current study, we used CFPS for the site-specific incorporation of nnAA to generate conjugate vaccine antigens with an autologous pathogen-specific protein carrier. For SLO, an important conserved GAS virulence factor and favored antigen for inclusion subunit vaccine formulations, the method yielded a high-level expression of a variety of SLO(ΔC101) variants containing multiple pAMF sites and corresponding SLO(ΔC101)−GACPR conjugates. Unlike SLO−GAC conjugates generated using conventional reductive amination chemistry, we retained the immunogenicity of the protein carrier in antibody generation and *in vivo* protection. As corroborated by our successful conjugation of GAC^{PR} to another conserved surface-expressed GAS antigen SpyAD that likewise retained dual antigen immunogenicity,[22] CFPS may provide a viable and scalable method for generating pathogen-specific carrier proteins in the development of new multivalent subunit vaccines.

4. MATERIALS AND METHODS

4.1. Cloning, Expression, Titer Analysis, and Purification of Native and pAMF-Containing SLO(ΔC101) Variants. The codon-optimized gene for the expression of native SLO(ΔC101) (79−470) or non-native amino acid, namely, *p*-azidomethyl phenylalanine (pAMF)-containing variants of SLO(ΔC101), was synthesized at ATUM (Menlo Park, CA) and subcloned with an N-terminal methionine into a proprietary vector. Each of the genes contained an N-terminal his_6-tag followed by a TEV protease site [ENLYFQG] to help capture and eventually purify untagged proteins. *In vitro* protein expression using the Xpress cell-free protein synthesis (CFPS) platform was performed at 25 °C and pH 7.2, as described elsewhere.[1,2] For protein expression titers, ^{14}C-leucine (GE Life Sciences, Piscataway, NJ) was cotranslationally added to the CFPS reaction and its incorporation into the translating polypeptides was estimated. Post reaction, 4 μL of either the complete CFPS reaction or the supernatant recovered post spinning the reactions at 4500 rpm for 15 min was blotted onto an anion-exchange filter membrane. The membrane was extensively washed to remove the unbound material and heat-dried for 30 min. Finally, the filter membrane was evenly coated with scintillation fluid, air-dried, and the counts were recorded to estimate the total and soluble yield of the expressed proteins. For the controlled large-scale expression of each of the antigens, a DASbox mini bioreactor

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ACS Omega 2022, 7, 24111−24120

system was utilized. Expression was performed at 25 °C for 10 h with constant stirring at 650 rpm while maintaining the pH at 7.2 and sparging a blend of air and oxygen through the reaction mixture to maintain the dissolved oxygen at 30%. After 10 h, the reactions were harvested and spun down at 15,000*g* at 4 °C for 30 min followed by filtration using a 0.45 μm amicon filter (Cat # Z717185, Millipore Sigma). The filtrate was then loaded onto a 5 mL HisTrap excel column (Cat # 17-5248-02, Cytiva, Sweden) equilibrated with buffer A [50 mM tris, 150 mM M NaCl, 10 mM imidazole]. After washing extensively with buffer A to bring the absorbance back to baseline, proteins were eluted using a 50% step gradient of buffer B [50 mM tris, 150 mM M NaCl, 500 mM imidazole]. The elution fractions were pooled, concentrated, and incubated with excess in-house-purified his_6-tagged TEV protease overnight while dialyzing against buffer A. The dialyzed cleavage reaction was loaded back onto a pre-equilibrated HisTRAP excel 5 mL column, and the untagged protein was collected in the flow-through (FT) fractions. Thereafter, the FT was concentrated and loaded onto a Superdex75 26/60 size exclusion chromatography (SEC) column (Cat # 28-9893-34, Cytiva, Sweden) pre-equilibrated with buffer S [50 mM tris pH 8.0, 150 mM NaCl]. The highest purity fractions (>95% as assessed by SDS-PAGE and SafeBlue Staining) were combined, concentrated, and aliquoted for −80 °C storage. pAMF incorporation was confirmed by incubating purified proteins with excess of the dibenzocyclooctyne-PEG4-tetramethylrhodamine (DBCO-TAMRA) dye (Cat # A131-25, Click Chemistry Tools, Scottsdale, AZ) for 1 h at RT. Thereafter, the reactions were analyzed by SDS-PAGE gel and fluorescence readout was recorded on a Syngene G-box gel imager (Figure 1C).

4.2. Multiangle Light Scattering (MALS) Analysis. The SEC-MALS−UV−RI setup consists of an Agilent HPLC 1100 degasser, a temperature-controlled autosampler (4 °C), a column compartment (25 °C), and a UV−vis diode array detector (Agilent, Santa Clara, CA) in line with a DAWN-HELEOS multiangle laser light scattering detector and an Optilab T-rEX differential refractive interferometer (Wyatt Technology, Santa Barbara, CA). The system was coupled to a Superdex75 10/30 GL column (Cat # 17-5174-01, Cytiva, Sweden) for analyzing native and pAMF-containing variants of SLO(ΔC101). A mobile phase consisting of 0.2 μm filtered 50 mM tris pH 8 and 150 mM NaCl was used at a 0.5 mL/min flow rate. Approximately 50−100 μg sample was injected for analysis. Agilent Open Lab software was used to control the HPLC, and Wyatt Astra 7 software (Wyatt Technology Corp., Santa Barbara, CA) was used for data collection and molecular weight analysis.

4.3. Dibenzocyclooctyne (DBCO) Derivatization of GAC^{PR} and Conjugation to pAMF-Containing SLO-(ΔC101) Variants. To a 6 mM solution of GAC^{PR} (purified as shown elsewhere[2]) in 100 mM borate buffer pH 8.5, 3 equivalents (to the polysaccharide repeating unit) of 1-cyano-4-dimethylaminopyridinium tetrafluoroborate (CDAP; from 100 mg/mL solution in acetonitrile) was added with vigorous stirring to facilitate cyanylation at reactive hydroxyl groups. Five minutes after the addition of CDAP, 2 molar equivalents of dibenzocyclooctyne-amine linker stock in DMSO was added such that the final DMSO concentration was 5% (v/v). After DBCO derivatization, 200 mM glycine was added to the reaction to quench unreacted cyanate esters. The DBCO-derivatized polysaccharide was purified via a zeba spin column, and the purity of the recovered material was assessed by reverse phase. A single peak in HPLC when absorbance was monitored at 309 nm confirmed the complete removal of excess DBCO linker and other reaction byproducts. Finally, the polysaccharide concentration was measured using an anthrone assay and dibenzocyclooctyne concentration was measured using absorbance at 309 nm. These two values were combined to give an estimate of the percentage of polysaccharide derivatized with a dibenzocyclooctyne functional group. For conjugation, the % DBCO derivatization of the mutant PS was kept between 5 and 10%. Thereafter, 3 and 4 pAMF-containing SLO(ΔC1010) variants were mixed with DBCO-derivatized GAC^{PR} (GAC^{PR}-DBCO) at a 1:1 ratio [0.5 mg/mL each] to facilitate conjugation via click chemistry. Post conjugation, the reaction mixture was dialyzed against a 50 kDa cutoff membrane to remove excess unreacted free PS. The recovered conjugates (or the native and GAC^{PR}-DBCO) were analyzed by SEC-MALS, and the concentration was estimated using an anthrone assay.[49]

4.4. GAC^{PR} Isolation and Purification Procedure. GAS mutant cell line, namely, 5448Δ*gacl*Δ*hasA* (lacking the gene cassette to express the GlcNAc side chain), was grown for 20−24 h and then utilized to inoculate a 10 L Eppendorf BioFlo 320 bioreactor containing growth medium with primary components being lactose (Spectrum, Cat # LA103, 300 g/L), soytone (Teknova, Cat # S9050, 20 g/L), yeast extract (BD biosciences, Cat # 212720, 20 g/L), and vegetable special infusion powder (Sigma-Aldrich, Cat # 95757, 10 g/L). Fermentation was performed in a fed-batch mode at 37 °C with 30% dissolved oxygen at 150 rpm with lactose supplied exponentially to the growing culture. After 14−18 h, the culture was harvested, inactivated with formaldehyde, and subjected to microfiltration, utilizing a 750 kDa WaterSep hollow-fiber membrane. For GAC isolation and separation from the cell wall, base hydrolysis was performed using 4 N NaOH and 25 mM $NaBH_4$ with constant agitation at 65 °C for 2 h. Next, the solution was centrifuged for 30 min at 14,000*g* at 25 °C, the pellet was discarded, and the supernatant was collected. Using concentrated HCl, the supernatant was pH-adjusted to 3 followed by incubation for 1 h at room temperature, after which the precipitate was removed. Thereafter, the pH was readjusted to 6.5 ± 0.3 followed by mutanolysin (Millipore Sigma Cat # M9901-10KU) treatment to further separate GAC from the peptidoglycan. Next, 40 IU/mL proteinase K (Millipore Sigma Cat # 03115828001) was added to the sample followed by incubation at 50 °C for 12−16 h to degrade copurifying proteinaceous impurities. Next, 1% CTAB (v/v) was added, and the recovered solution was incubated at 30 °C for 1 h to remove all nucleic acid-based comigrating impurities. Thereafter, the buffered solution was centrifuged, and the filtered supernatant was utilized to purify GAC^{PR} using hydrophobic interaction chromatography (HIC) using a HiPre Butyl Fast Flow 16/10 pre-equilibrated with 50 mM sodium phosphate pH 6.8 and 3 M sodium chloride. Load sample in the equilibration buffer was applied to the HIC resin, and the flow-through containing purified GAC^{PR} was collected. Finally, 3 kDa tangential flow filtration (TFF) was conducted utilizing a Sartorius TFF cassette to remove salts from the sample by diafiltration against 9 diavolumes of MilliQ water. The purified PS solution was then 0.22 μm filtered and stored for further use.

4.5. Anthrone Assay for Total Polysaccharide Concentration. A stock of 2 mg/mL of the anthrone reagent

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ACS Omega 2022, 7, 24111−24120

(Sigma-Aldrich, CAS # 90-44-8) was prepared in cold sulfuric acid, while a 1 mM stock of polysaccharide repeating unit (PSRU) comprising 2× rhamnose was prepared in water. Thereafter, 100 μL/well of serially diluted PSRU stock or the 3× diluted unknown is plated (96-well plate) in triplicate followed by the addition of 200 μL/well of the anthrone reagent stock. Thereafter, all of the reactions are mixed to homogeneity and sealed with a plate cover followed by incubation at 95 °C for 10 min. Post incubation, the plate is placed at 4 °C for 10 min to facilitate cooling to an ambient temperature. Finally, absorbance is measured at λ = 620 nm using a UV/vis plate reader. To determine the concentration, the average absorbance values and %RSD (<10%) of the triplicate wells of the working standards are plotted on the *Y*-axis, with the corresponding concentrations (μmol/mL) on the *X*-axis. The data are fit using the least-squares fit ($y = mx + b$) with the targeted $R^2 > 0.95$. Finally, using the slope and *y*-intercept of the least-squares fit, the concentration of the unknown samples is calculated using the average absorbance value measured at λ = 620 nm.

4.6. NMR Analysis of the Purified GAC^{PR}. The purified GAC^{PR} sample (2 mL or 8 mg) was lyophilized and dissolved in 1 mL of 99.96% D_2O (CAT # 450410-100ml), and 0.7 mL was introduced into a 5 mm NMR tube for data acquisition. The NMR spectrum was recorded on a 400 MHz Bruker spectrometer operating at 50 °C using a 90° pulse and a 5 s delay; 40 scans were recorded. The spectrum was processed using Topspin 3.2 and referenced using DSS as an internal reference (0 ppm).[49]

4.7. Mouse Immunization Studies. Adult (6−8 weeks old) female CD-1 mice (n = 10 per group) at Bayside Biosciences Inc. (Santa Clara, CA) were immunized intramuscularly on days 0, 14, and 28 with 5 μg of each antigen adsorbed to Adju-Phos (4.8% v/v) in a 100 μL volume. Blood was collected on day 1 (1 day prior to vaccination) and day 35 (postvaccination) for serum antibody measurement. Control groups received PBS alone. All active immunization mouse experiments followed by a lethal challenge to track survival were approved by the UC San Diego Institutional Animal Care and Use Committee and conducted per accepted veterinary standards. For this, wild-type female CD-1 mice (Charles River) were immunized every 14 days for a total of three doses starting at the age of 5 weeks. Intramuscular immunizations delivered consisted of 100 μL of total volume per mouse per dose, including 50 μL of alhydrogel and 2% of aluminum hydroxide adjuvant (Invivogen) prepared per the manufacturer's instructions. Fourteen days after final immunization, mice were infected with 1×10^7 colony forming units of the M1 serotype GAS strains 89155 by i.p. injection and tracked for survival. Statistics of the Kaplan−Meier survival curves were calculated using the log-rank Mantel−Cox test.

4.8. Antibody Titer Measurements Using ELISA and Statistical Analysis. Antigen-specific serum IgG titers specific for SLO were measured by ELISA, as previously described.[22] Briefly, NUNC ELISA plates (NUNC Maxisorp) were coated with in-house-purified SLO, as reported elsewhere,[22] or with a conjugate vaccine using eCRM[47] as the carrier protein at 1 and 5 μg/mL, respectively, in PBS pH 7.4 and incubated overnight at 4 °C. The next day, the plates were washed 3× with standard buffer (Cat # AQUAMAX2000, Molecular Devices) and blocked with blocking buffer (Cat # 37516, Thermo Fisher) for 1 h at room temperature. Plates were washed three times. Fivefold serial dilutions of the sera were made in PBS pH 7.4, and 50 μL of sera was added to the plate. The plates were incubated for 1 h at 37 °C, washed six times, and 100 μL of 1:5000 diluted peroxidase-conjugated donkey antimouse IgG (H+L) (Cat. # 115-035-062, Jackson ImmunoResearch) was added to the plates, incubated for 1 h at room temperature, washed six times, and TMB substrate solution (Cat. #N301, Thermo Fisher) was added followed by incubation for 30 min at room temperature. Finally, after 30 min, the plates were read at λ = 650 nm. Antibody titer was calculated as the highest serum dilution where the signal exceeded the signal of blank wells plus 3 standard deviations, and all samples were run in triplicate for mice per immunization group. The estimated antibody titers were analyzed by paired *t*-test. Differences were considered statistically significant at $p < 0.05$. All statistical analysis was conducted using GraphPad Prism 9 (GraphPad Software, La Jolla, CA).

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Author Contributions

N.K., J.F., and V.N. designed the research. T.R. and M.W. performed the expression screening, while N.K., L.P., L.B., L.Y., and M.R. purified the proteins and performed the conjugation. E.C. performed ELISA assays. A.S. purified GACPR. S.U. performed the *in vivo* immunizations. N.K. and V.N. wrote the manuscript: all authors provided the critical review and feedback and approved the final manuscript.

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Notes

The authors declare the following competing financial interest(s): NK, LP, LB, AS, LY, MR, EC, TR, MW, PD and JF are employees of Vaxcyte, Inc. Some studies at UC San Diego were supported by a sponsored research agreement with Vaxcyte, Inc. VN previously received fees as a consultant for SutroVax, Inc., a former name of Vaxcyte.
N.K., L.P., L.B., A.S., L.Y., M.R., E.C., T.R., M.W., P.D., and J.F. are employees of Vaxcyte, Inc. Some studies at the UC San Diego were supported by a sponsored research agreement with Vaxcyte, Inc. V.N. previously received fees as a consultant for SutroVax, Inc., a former name of Vaxcyte.

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■ ABBREVIATIONS

GAS, group A *Streptococcus*; GAC, group A carbohydrate; SLO, streptolysin O; CFPS, cell-free protein synthesis; pAMF, *p*-azidomethyl phenylalanine; nnAA, non-native amino acid; DBCO, dibenzocyclooctyne; TAMRA, tetramethyl rhodamine; CV, conjugate vaccine

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