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Site-Specific Conjugation of Cell Wall Polyrhamnose to Protein SpyAD Envisioning a Safe Universal Group A Streptococcal Vaccine

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Abstract

Development of an effective vaccine against the leading human bacterial pathogen group A *Streptococcus* (GAS) is a public health priority. The species defining group A cell wall carbohydrate (GAC, Lancefield antigen) can be engineered to remove its immunodominant *N*-acetylglucosamine (GlcNAc) side chain, implicated in provoking autoimmune cross-reactivity in rheumatic heart disease, leaving its polyrhamnose core (GAC^{PR}). Here we generate a novel protein conjugate of the GAC^{PR} and test the utility of this conjugate antigen in active immunization. Instead of conjugation to a standard carrier protein, we selected SpyAD, a highly conserved GAS surface protein containing both B-cell and T-cell epitopes relevant to the bacterium that itself shows promise as a vaccine antigen. SpyAD was synthesized using the XpressTM cell-free protein expression system, incorporating a non-natural amino acid to which GAC^{PR} was conjugated by site-specific click chemistry to yield high molecular mass SpyAD-GAC^{PR} conjugates and avoid disruption of important T-cell and B-cell immunological epitopes. The conjugated SpyAD-GAC^{PR} elicited antibodies that bound the surface of multiple GAS strains of diverse M types and promoted opsonophagocytic killing by human neutrophils. Active immunization of mice with a multivalent vaccine consisting of SpyAD-GAC^{PR}, together with candidate vaccine antigens streptolysin O and C5a peptidase, protected against GAS challenge in a systemic infection model and localized skin infection model, without evidence of cross reactivity to human heart or brain tissue epitopes. This general approach may allow GAC to be safely and effectively included in future GAS subunit vaccine formulations with the goal of broad protection without autoreactivity.

Keywords: group A *Streptococcus*; *Streptococcus pyogenes*; group A carbohydrate; Lancefield antigen; conjugate vaccine; XpressCF[TM]; non-natural amino acids; SpyAD

Introduction

Group A *Streptococcus* (GAS, *Streptococcus pyogenes*) is one of the most important human bacterial pathogens, estimated to cause more than 600 million cases of pharyngitis ('strep throat') and 100 million cases of impetigo annually across the globe.[1] In particular, pharyngitis is highly prevalent in school-aged children and a major source of antibiotic prescriptions worldwide, driving selective pressure for antibiotic resistance throughout the human microflora.[2–4] In recent decades, GAS has been increasingly associated with severe invasive forms of infection, sometimes in previously healthy individuals, including sepsis, necrotizing fasciitis, and toxic shock syndrome. Finally, GAS is the trigger for

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important post-infectious immune-mediated diseases, in particular poststreptococcal glomerulonephritis, the most common cause of childhood glomerulonephritis worldwide,[5] and acute rheumatic fever (ARF) and rheumatic heart disease (RHD), a leading cause of cardiovascular mortality in the developing world.[6] At least 33 million people are currently affected by RHD, with approximately 275,000 deaths annually (60% age <70) and 9 million disability-adjusted life years lost.[7] Almost all RHD deaths occur in low-income and middle-income countries.[1]

As a result of all its disease manifestations, GAS ranks among the top ten infectious causes of human mortality.[8] Despite the high disease burden and global demand, there is to date no safe and efficacious commercial vaccine against GAS. A number of phenotypic features of the pathogen pose particular challenges to vaccine development. First, in contrast to the diverse capsular polysaccharides of *Streptococcus pneumoniae* that are the basis of multivalent glycoconjugate vaccines in clinical use worldwide,[9,10] GAS possesses an invariant capsule of high molecular weight hyaluronic acid (HA). The capsule is thus structurally identical to the ubiquitous HA present in human connective tissues, and therefore immunologically inert.[11–14] Instead, GAS serology is classically assigned by its most abundant and highly immunogenic surface-anchored protein, the M protein, which poses a second vaccine challenge—M proteins are highly polymorphic. There are over 220 described sequence variants of the encoding *emm* gene, classified into 48 *emm*-clusters of closely related M proteins sharing functional and structural properties.[15] Heterologous M proteins do not typically elicit cross-protective immunity to M-types from other clusters.[16] Finally, an unique challenge to GAS vaccine development is its link to the serious post-infectious, immune-mediated ARF and RHD, whose precise molecular pathogenesis remains poorly understood.[7,17,18] A prevailing theory holds that GAS molecular mimicry of host cell epitopes mediates B and/or T cell cross-reactions with human tissue antigens in the heart, brain, or other tissues.[19]

Beginning in the late 1970's, the US Food and Drug Administration made the rare decision to suspend GAS vaccine development (21 Code of Federal Regulations 610.19) for nearly three decades. This decision followed an immunization study in which three participants developed suspected or definite ARF.[20] In 2004, the US National Institutes of Allergy and Infectious Diseases convened experts to review epidemiologic, microbiological, and immunologic factors involved in preclinical and clinical development of a safe and effective GAS vaccine that facilitated lifting of the Food and Drug Administration ban.[21] Their summary report concluded that molecular mimicry represented a major obstacle to vaccine development, and that GAS antigens including M proteins and group A carbohydrate (GAC) possess epitopes linked to B and/or T cell reactivity to human tissue antigens. The panel recommended: "*Because the precise role of molecular mimicry in the pathogenesis of ARF has not been established, every effort should be made to exclude tissue cross-reactive epitopes during vaccine development.*"[21] Recently, the World Health Organization, International Vaccine Institute, Wellcome Trust, Bill and Melinda Gates Foundation and other stakeholders convened in advisories on GAS vaccine development to address scientific challenges for this paramount global health need.[4,22]

Ideal candidate antigens for inclusion in a GAS vaccine would be (a) highly immunogenic and elicit antibodies that promote opsonophagocytosis or inhibit virulence, (b) exhibit broad conservation across strains contributing to global disease epidemiology, and (c) be thoughtfully chosen to avoid autoimmune cross-reactivity with human tissue epitopes.[23,24] All GAS strains possess the species-defining Lancefield GAC, composed of a polyrhamnose backbone with an immunodominant *N*-acetylglucosamine (GlcNAc) side chain,[25,26] of which ~25% is decorated with glycerolphosphate.[27] Representing 40%–50% of the GAS cell wall by weight,[25] GAC serves as the basis of current rapid antigen testing for GAS infection.[28] Earlier mouse immunization studies with protein-conjugated native or synthetic GAC vaccines show clear efficacy against multiple GAS M types.[29,30] Serum anti-GAC antibodies are likewise present in healthy individuals and peak around 17 years of age, strongly correlating with decreased GAS infection risk.[31]

For the above reasons of immunogenicity and conservation, GAC has garnered considerable interest as a universal GAS vaccine antigen. However, this has also elicited concern, since experimental evidence for autoreactivity of antibodies that recognize the native GAC GlcNAc side chain against human tissues has been communicated by different research groups. For example, glycoproteins from human heart valves elicit antibodies that bind GAC in a manner blocked by GlcNAc (but not rhamnose or other glycans),[32] and persistence of anti-GlcNAc/GAC antibodies (up to 20 years) are a marker of poor prognosis of RHD valvular disease, whereas antibodies against streptolysin O (SLO) and the polyrhamnose core of GAC fade independently of valvular complications.[33] Also, anti-GAC GlcNAc antibodies that cross-react with heart or brain tissue are present in sera of ARF patients with cardiac or neurological complications.[34,35]

An approach to modify the GAC to eliminate the potential cross-reactive GlcNAc epitope was achieved by Van Sorge et al. upon discovery of the 12-gene GAS *gac* gene cluster encoding the biosynthetic machinery for GAC production.[36] This work generated an isogenic mutant (Δ*gacI*) that expressed only the polyrhamnose backbone of GAC without the GlcNAc side chain.[36,37] This Δ*gacI* mutant was attenuated for virulence in mouse and rabbit infection models, and showed increased sensitivity to killing by human whole blood, neutrophils, and platelet-derived antimicrobials in serum.[36] A biotin conjugate of the modified GAC structure containing only the non-mammalian carbohydrate rhamnose (GAC^{PR}), generated high antibody titers in rabbits that promoted opsonophagocytic killing (OPK) of GAS strains of multiple M types by human neutrophils and protected against systemic GAS challenge in mice upon passive immunization.[36] No cross-reactivity of anti-GAC^{PR} antisera was observed against human heart tissue lysates.[36]

In the present work, we sought to generate a protein conjugate of the GAC^{PR} for use in safe, universal subunit vaccines against this important pathogen, and to test the utility of this conjugate antigen in active immunization. As a novel approach, instead of conjugation to a standard carrier protein to engender T-cell-mediated immunity, such as tetanus toxoid or CRM197, we selected SpyAD, a highly conserved GAS surface protein contains both B-cell and T-cell epitopes relevant to the bacterium and itself shows promise as a vaccine antigen.[38–41] The conjugates SpyAD-GAC^{PR} elicited antibodies that bound the surface of multiple GAS strains of diverse M types and promoted OPK by human neutrophils. Active immunization of mice with a multivalent vaccine consisting of SpyAD-GAC^{PR} in combination with candidate vaccine antigens SLO and C5a peptidase provided significant protection against GAS challenge in a systemic infection model and localized skin infection model, without evidence of cross reactivity to human heart epitopes.

Results

Expression and purification of GAS protein antigens

GAS expresses several secreted and membrane anchored virulence factors that are important for disease pathogenesis in vivo.[42,43] For the present vaccine study, we selected three protein antigens that are strongly conserved with high genomic carriage rate across a globally representative and clinically diverse collection of 2083 GAS genomes, coupled to low amino acid sequence variation[24] and evidence of natural immunogenicity—SLO, C5a peptidase, and SpyAD. SLO is a secreted, pore-forming, cholesterol-dependent cytolysin unique to GAS, and immunization of mice with inactivated SLO toxoid induces SLO-neutralizing antibodies and protects against experimental infection with the pathogen.[44,45] C5a peptidase is a surface-expressed GAS peptidase that cleaves C5a complement to inactivate the chemoattractant, delaying recruitment of phagocytes to the site of GAS infection.C5a peptidase alters clearance and trafficking of group A streptococci by infected mice.[46] C5a peptidase-specific antibody responses strongly correlate with anti-SLO titers in children with pharyngitis,[47] and the protein also elicited protective vaccine responses in murine models.[48,49] Finally, "S. *pyogenes* adhesion and division protein" (SpyAD), which plays dual roles in GAS adherence to host cells and bacterial cell division,[38] was identified as a potential GAS vaccine candidate in several high throughput screens of the GAS surface proteome,[39,50,51] and has been part of a 7-valent GAS vaccine that proved successful in murine immunization studies.[40]

To express the above proteins for use as vaccine antigens, we applied Vaxcyte's proprietary XpressCF^{+TM} cell-free protein synthesis (CFPS) expression platform, which is based on extraction of the *E. coli* cellular machinery required for transcription, translation, and energy production into a cell-free mixture capable of continuous oxidative phosphorylation. With this CFPS platform, we successfully expressed the immunogenic cores of SLO, C5a peptidase, and SpyAD. For native FL-SLO, we expressed both a N-terminal truncation fragment spanning aa 79–571 (designated SLO) as well as a N- and C-terminal truncation fragment comprising aa 79–470 [designated SLO (ΔC101)] (Figure 1A). Truncated SLO were designed to optimize product yield in our CFPS (further improved with truncation of 101 amino acids off the C-terminus) while preserving key

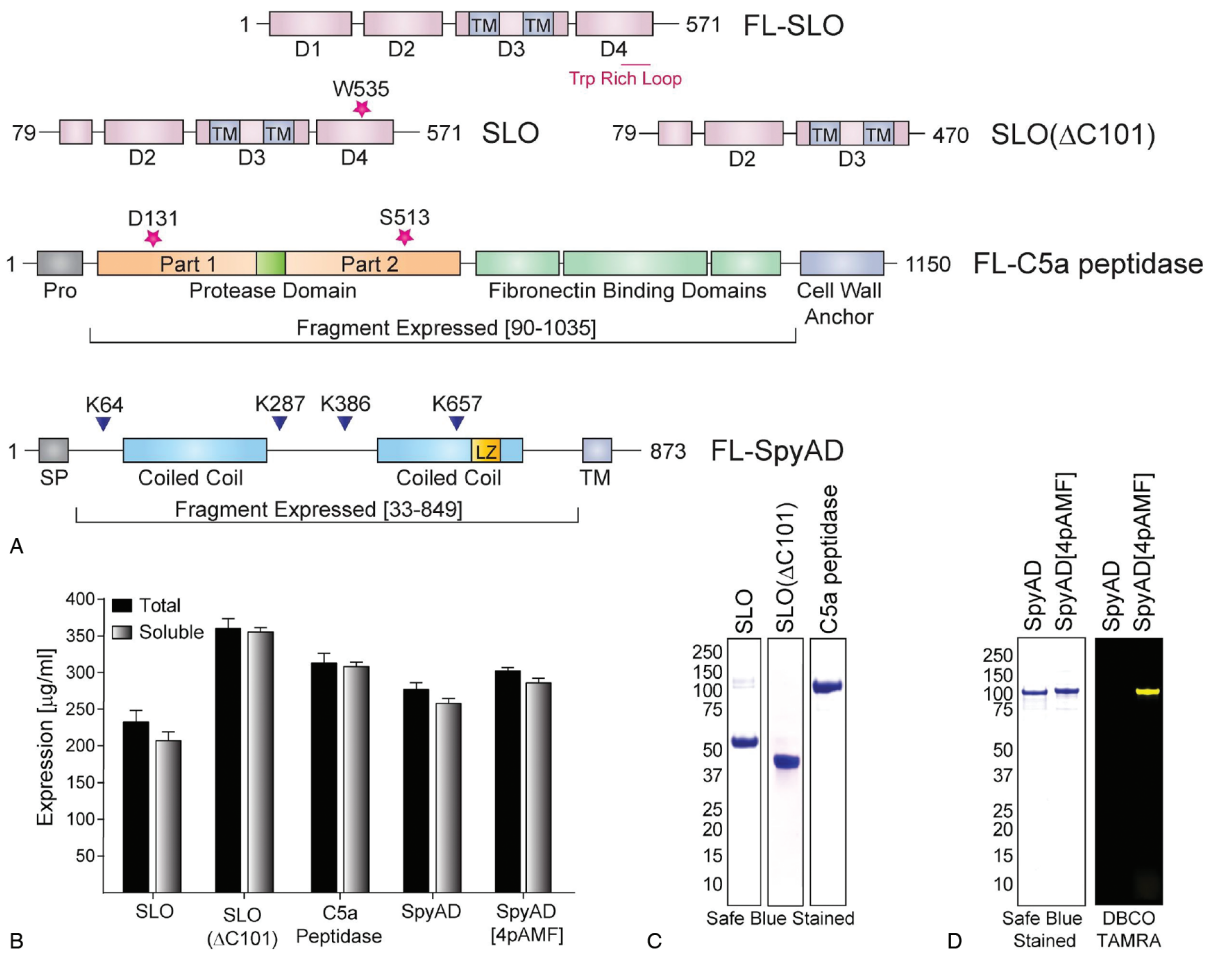

Figure 1. Expression, purification, and analysis of group A *Streptococcus* (GAS) protein antigens. A: Modular architecture of GAS protein antigens is depicted schematically while highlighting amino acid changes (red stars)/truncations in streptolysin O (SLO) or C5a peptidase and pAMF incorporation sites on SpyAD (black triangles) along with the immunogenic core of the proteins expressed in cell free protein synthesis (CFPS). Domains: D#: protein domain; Pro: pro-sequence; SP: signal peptide; TM: transmembrane. B: Protein expression yield in CFPS estimated using incorporation of ^{14}C-leucine into the translating polypeptide. C: Safe blue stained SDS-PAGE of SLO and C5a peptidase (C) and SpyAD (D, left) confirm purity of expressed proteins. pAMF incorporation into SpyAD variant SpyAD[4pAMF] was confirmed through selective labeling with dibenzocyclooctyne-PEG4-tetramethylrhodamine (DBCO-TAMRA) fluorescent dye (D, right).

immunogenic epitopes and detoxifying the cytolysin by removing the tryptophan-rich loop, a domain that mediates insertion into cellular membranes. Additionally, we expressed C5a peptidase and SpyAD with precision mutations for enzymatic inactivation (Figure 1A). Next, using amber stop codon suppression through addition of orthogonal tRNA and tRNA synthetase pair during CFPS, we successfully expressed a variant of SpyAD that contains 4 non-native amino acids (nnAA) in the form of p-azidomethyl phenylalanine (pAMF) in replacement of 4 native amino acids (Figure 1A, marked by black triangles). The quantitative expression yield of each antigen was >200 mg/L as estimated by incorporation of ^{14}C-leucine into the translating polypeptide (Figure 1B). SDS-PAGE analysis of synthesized proteins confirmed high purity fractions of each of the expected peptide antigens (>95% as shown in Figure 1C and 1D). Incorporation of pAMF into SpyAD was confirmed by conjugation to dibenzocyclooctyne-PEG4-tetramethylrhodamine (DBCO-TAMRA) dye and fluorescence readout. Unlike native SpyAD, the variant with 4 pAMF sites gets labeled with the dye to confirm pAMF incorporation (Figure 1D).

Generation of SpyAD-GAC^{PR} conjugate

Using the in-house isolation and purification protocol, highly pure preparations of mutant GAS cell wall carbohydrate containing only polyrhamnose (GAC^{PR}) were generated from a GAS mutant strain that lacked the GlcNAc sidechain of GAC and the surface hyaluronan capsule (Δ*gacI*Δ*hasA*). Using 1-Cyano-4-dimethyl aminopyridinium tetrafluoroborate (CDAP) chemistry, GAC^{PR} was dibenzocyclooctyne (DBCO)-derivatized for use in a conjugation reaction with SpyAD[4pAMF], shown schematically in Figure 2A. SEC-MALS analysis estimated an average molar mass of 7.2 kDa for GAC^{PR} and 87.3 kDa for SpyAD[4pAMF], the latter in close agreement with the theoretical molar mass of a

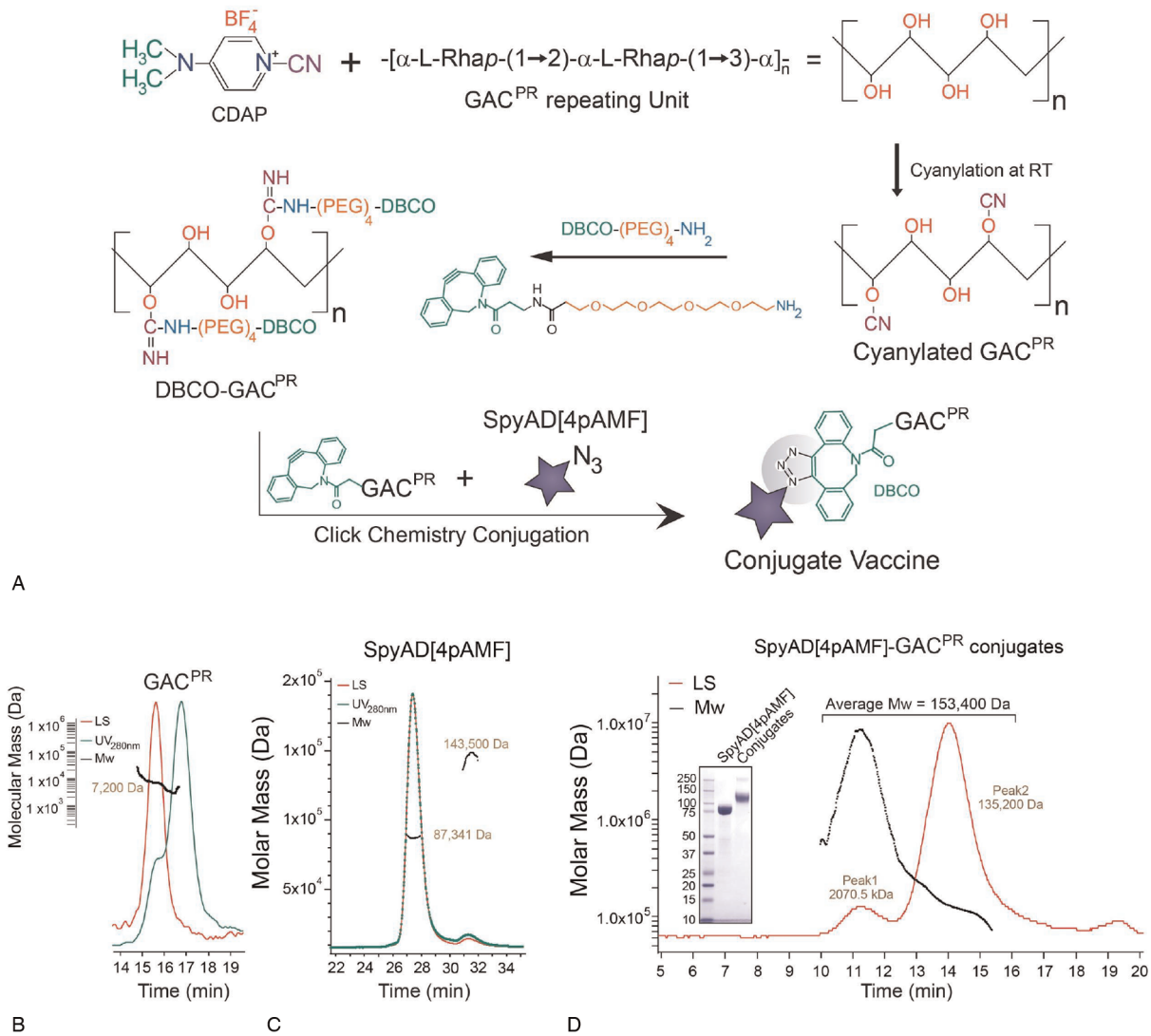

Figure 2. GAC^{PR} derivatization schematic and generation of conjugate vaccine antigen. A: Schematic outlines chemical reactions utilizing 1-Cyano-4-dimethyl aminopyridinium tetrafluoroborate (CDAP) chemistry for dibenzocyclooctyne (DBCO) derivatization of GAC^{PR} and theoretical depiction of Cu^{2+}-free click chemistry reaction for generating conjugate vaccine. B: SEC-MALS analysis of purified native GAC^{PR} estimates an average molecular mass of 7.2 kDa. C: SEC-MALS analysis of purified native SpyAD[4pAMF] estimates an average molecular mass of 87.3 kDa. D: Post-click chemistry SEC-MALS analysis of purified conjugates estimates an average combined molecular mass of 153.4 kDa. Inset shows the safe blue stained SDS-PAGE analysis of SpyAD[4pAMF] pre- (left) and post-conjugation (right).

SpyAD monomer (Figures 2B and 2C). The molar mass of the purified DBCO-derivatized GAC^{PR} was similar to the molar mass of the native polysaccharide (data not shown). For conjugation, DBCO-derivatized GAC^{PR} was incubated with SpyAD[4pAMF] at a 1:1 ratio to facilitate strain-promoted Cu^{2+}-free click chemistry reaction to generate conjugates. After the conjugation reaction and dialysis to remove excess GAC^{PR}, the resulting conjugates are analyzed using SEC-MALS. As shown in Figure 2D, the conjugates elute as 2 separate polydisperse distributions under Peak1 and Peak2 with average molar mass ranging from 2 MDa to 135 kDa respectively, yielding a combined average molar mass of 153.4 kDa for the conjugates, which is significantly higher than the average molar mass of either GAC^{PR} (~7.2 kDa) or SpyAD[4pAMF] (87.3 kDa) alone, thereby confirming successful conjugation into the final product, referred to as SpyAD-GAC^{PR} from here onwards.

Evaluation of GAS vaccine antigens for immunogenicity

Immunization of New Zealand White rabbits was performed to generate antisera for functional evaluation of the candidate GAS vaccine antigens. Rabbits were immunized with 5 μg of individual protein antigen (SLO, C5a peptidase) or SpyAD-GAC^{PR} conjugate adjuvanted with Adju-phos® (Invivogen, San Diego, CA, USA). To determine immunoglobulin G (IgG) titers elicited by vaccination, terminal bleed (day 35) rabbit antisera were evaluated by enzyme-linked immunosorbent assay (ELISA). For all three protein antigens (SpyAD, C5a peptidase, and SLO), the group of rabbits immunized with the recombinant protein showed significantly increased (3- to 4-$\log_{10}$fold) antibody titers against the target antigen compared to either pooled serum from the rabbits before immunization ("pre-immune (pooled)") or the other immunized rabbit groups (Figure 3A), confirming specificity of the IgG response to each respective antigen. The anti-SpyAD protein titer generated by the SpyAD-GAC^{PR} conjugate was not inferior to that of the SpyAD recombinant protein alone, suggesting that key B cell epitopes were not impacted by pAMF sites. Flow cytometry was used to evaluate the binding affinity of rabbit-derived IgG to the surface of eight live wild-type GAS strains of different M protein serotypes (M1-6, M12, M28). For the great preponderance of strains, the respective immunized serum yielded an increase in binding of 100%–400% over the baseline IgG binding of the pre-immune serum (Figure 3B). For six of the eight strains, the GAS surface binding of the antisera raised against the SpyAD-GAC^{PR} conjugate roughly doubled the level of IgG binding seen with antisera raised against SpyAD alone. To examine the post-immunization antibody response to native GAC antigen, we generated a genetic knockout of SpyAD in M1 5448 strain (ΔSpyAD) and tested rabbit serum IgG binding to the cell surface of the live bacterium (Figure 3C). Fluorescent signals detected similar IgG binding of SpyAD or SpyAD-GAC^{PR} antisera to the surface of wild-type M1 GAS. As expected, the IgG fluorescent signal was lost for SpyAD antiserum against the ΔSpyAD, but a clear signal was still present for the SpyAD-GAC^{PR} antiserum, demonstrating that it contains native GAC-binding IgG.

Evaluation of rabbit vaccine antisera in GAS OPK, blocking SLO activity, and passive protection in murine challenge model

The ability of the rabbit antisera raised against the GAS vaccine antigens (SLO, C5a peptidase, SpyAD, and SpyAD-GAC^{PR}) to promote human neutrophil OPK was evaluated using GAS strains of five different M protein serotypes (M1-M5). This assay was performed with 30 min pre-opsonization with the respective heat-inactivated antisera or pre-immune sera control, then 30 min exposure to freshly isolated human neutrophils at multiplicity of infection (MOI) = 0.1 bacteria per neutrophil in the presence of 2% baby rabbit complement (BRC). To test potential protection from a combination of antigens, we tested an antiserum mixture composed of one-third of each antiserum raised against SLO, C5a peptidase, and SpyAD-GAC^{PR} to maintain a consistent total serum concentration (designated as "Combo" in Figure 4A). As a positive control for OPK, we used anti-M1 rabbit serum against M1 GAS and recovered a low percentage of colony forming units (CFU) due to effective killing (Figure 4A, striped bar of first graph). All antisera induced statically significant increases by one-way ANOVA versus pre-immune sera in short-term OPK of GAS (Figure 4A). Of note, our assay uses a 100 to 1000-fold greater GAS inoculum than published OPK assays employing the human promyelocytic leukemia HL-60 cell line at MOI = 0.001[52] or MOI = 0.0001,[53] with our goal being to more accurately recapitulate the immunization target of supporting primary human innate immune cell function. In our ex vivo human neutrophil OPK assay, antisera raised against the SpyAD protein and SpyAD-GAC^{PR} performed similarly. Moreover, the antisera combination "Combo" performed similarly or better than the individual components, indicating no cross-interference in their OPK functions. As SLO is a secreted cytotoxin not anchored to the GAS surface, its contribution to enhanced neutrophil killing is accrued not from increased phagocytic uptake, but rather reducing SLO-mediated membrane damage and impairment of neutrophil antimicrobial functions.[45] We found that the anti-SLO rabbit immune serum, whether elicited by the SLO or SLO (ΔC101) antigens, significantly inhibited hemolytic activity of purified SLO against human red blood cells up to a dilution of 1:2048 (Figure 4B). Furthermore, anti-SLO or anti-SLO (ΔC101) immune serum equally preserved neutrophil oxidative burst function (superoxide generation) against GAS supernatant (SLO)-mediated suppression (Figure 4C).

The potential of vaccination using SpyAD-GAC^{PR} alone or in combination with other GAS protein antigens, namely SLO or SLO (ΔC101) plus C5a peptidase, to provide protection against systemic GAS infection was evaluated in murine models of passive immunization. As a first proof-of-principle, rabbit antisera were transferred into adult CD-1 mice intravenously by retro-orbital injection before challenging each animal with 1×10^7 CFU of the virulent serotype M1 GAS strain 89155 injected into the peritoneal cavity (Figure 4D). Control mice receiving pre-immune rabbit serum showed <10% survival within 24 h of infection whereas mice immunized with either the SpyAD-GAC^{PR} antiserum or a combination of SLO + C5a peptidase antisera (volume divided in a 1:1 ratio) showed modest protection (20% and 26.7% survival through day 5, respectively), but most importantly the mice immunized with the multivalent SpyAD-GAC^{PR} + SLO + C5a peptidase (volume divided in a 1:1:1 ratio) had significant protection against mortality with 53.3% survival through day 5 ($P = 0.0048$ vs pre-immune serum group, Figure 4D)

In summary, due to the variation in IgG binding to GAS strains of different M types (Figure 3B), likely due to the variation in antigen surface expression levels, and the superiority of the combination antisera in OPK assays (Figure 4A) and passive immunization (Figure 4D), we concluded that a multivalent vaccine formulation was required to broadly cover GAS strains

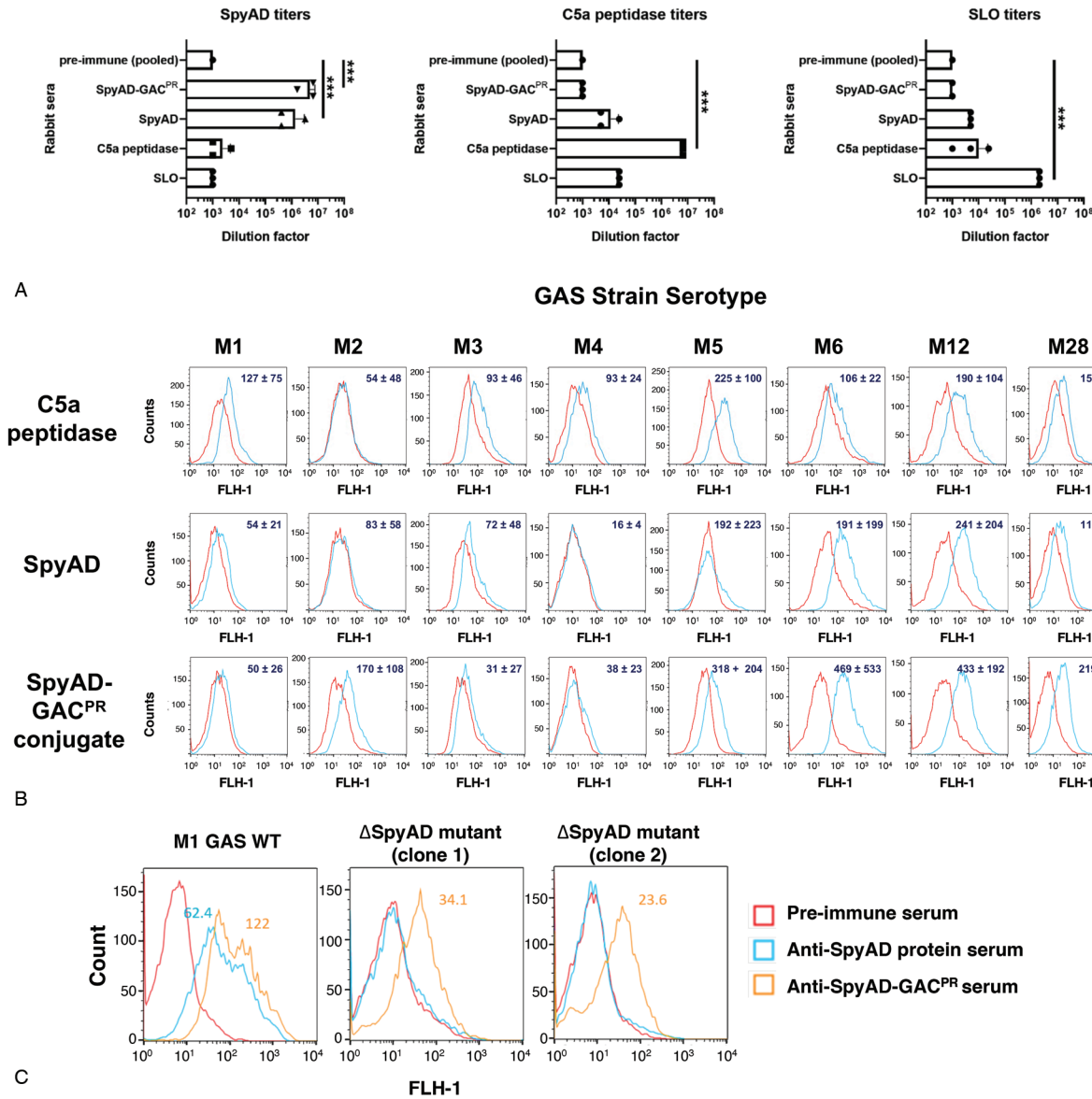

Figure 3. Immunogenicity group A *Streptococcus* (GAS) vaccine antigens in rabbits. A: ELISA was performed on rabbit serum to quantify IgG titers raised against each protein antigen. Each point represents serum derived from one animal, with 2–3 rabbits per group. B: Eight GAS strains of different M-types were used to determine rabbit serum IgG binding to native antigens by flow cytometry. Histograms show representative fluorescent signals from IgG binding; red represents pre-immune sera and blue immune sera. Numbers in dark blue the top right corner of each histogram shows the mean percentage of increased signal of immunized rabbit serum over pre-immunized serum signal. C: Rabbit IgG binding to ΔSpyAD mutant GAS confirms the presence of IgG recognizing native GAC in the SpyAD-GAC^{PR} antiserum.

and proceeded to active immunization experiments with antigen combinations.

Multivalent immunization generates antibody response with improved capacity to bind to native surface antigens and direct OPK

Mouse sera were collected from nine immunized (and ten mock immunized) animals on day 42 following a three dose immunization (intramuscular injections of antigens on days 0, 14, and 28) with SpyAD-GAC^{PR} + SLO (ΔC101) + C5a peptidase or mock immunization control, and the efficacy of these antisera tested for surface IgG binding to 20 GAS strains via flow cytometry. For all 20 GAS strains of differing *emm* types tested, the multivalent combination antisera from all mice bound clearly more IgG to the bacterial surface than the mock immunization animal sera (Figure 5A). Murine IgG binding to methicillin-resistant *Staphylococcus aureus* strain USA300 was comparable between antisera groups, confirming the specificity of bound murine IgG from the SpyAD-GAC^{PR} + SLO (ΔC101) + C5a peptidase for GAS.

The mock and combination immunized mouse serum was subsequently tested for promoting ex vivo OPK killing of M1 89155 GAS by freshly isolated human neutrophils (Figure 5B). When GAS were opsonized with post-immune SpyAD-GAC^{PR} + SLO (ΔC101) + C5a peptidase mouse antisera, only ~20% of the original inoculum of M1 GAS was recovered following neutrophil exposure, compared to ~60% CFU recovery in

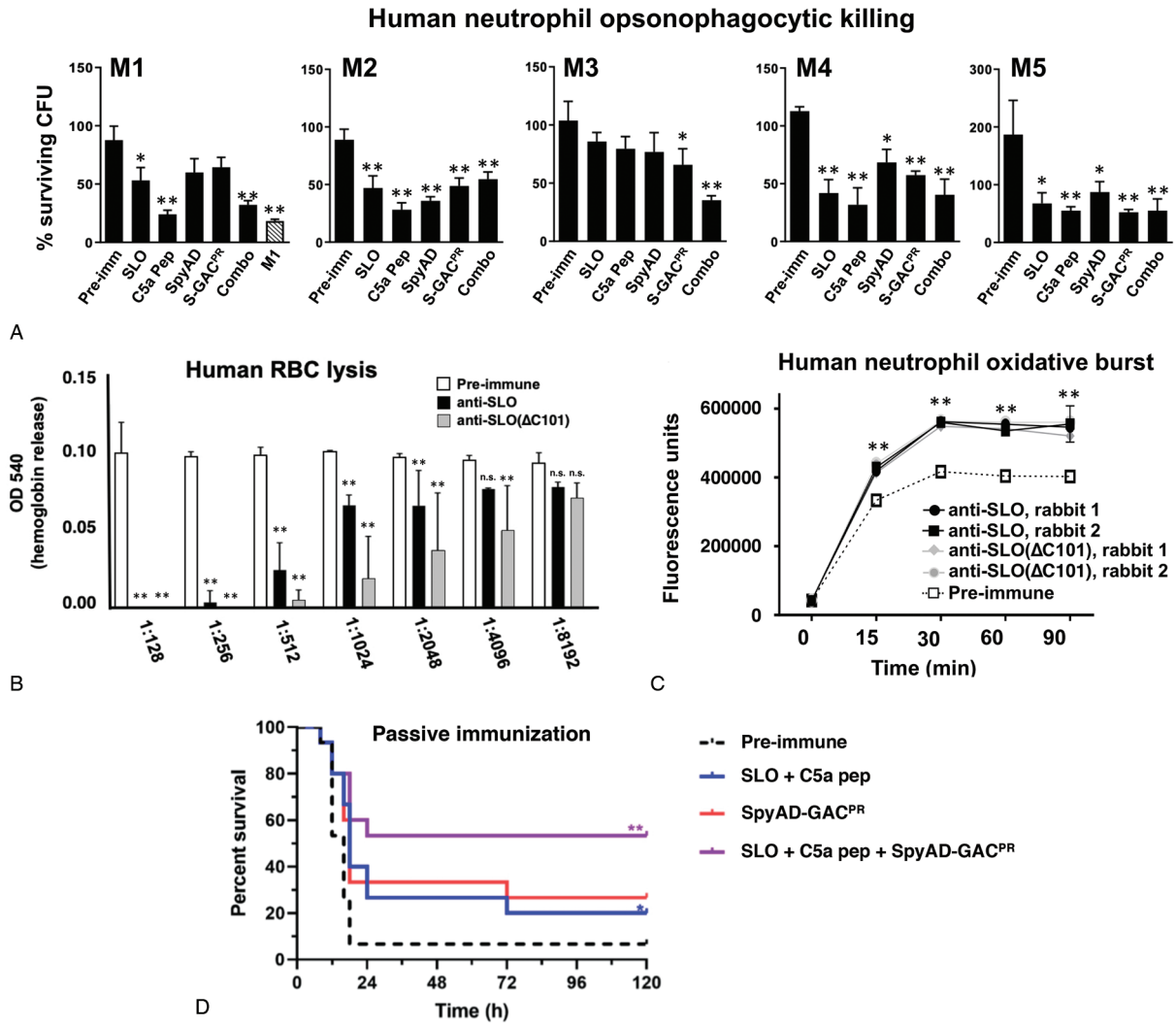

Figure 4. **Evaluation of rabbit vaccine antisera in group A *Streptococcus* (GAS) opsonophagocytic killing, blocking streptolysin O (SLO) activity, and passive protection in murine challenge model**. A: Opsonophagocytic killing of GAS of different M serotypes by human neutrophils in the presence of immune serum from immunized rabbits compared to serum from pre-immunized rabbit serum. B: Anti-SLO or anti-SLO (ΔC101) antisera block lysis of human red blood cells by purified SLO. C: Anti-SLO or anti-SLO (ΔC101) antisera enhance oxidative burst capacity of human neutrophils exposed to GAS supernatants containing SLO as quantified by 2,7-dichlorofluoroscein diacetate fluorescence. Statistical analyses were performed by one-way ANOVA compared to pre-immune serum; $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.001$ (***). D: CD-1 female mice receive an intravenous dose of rabbit antisera followed by intraperitoneal lethal challenge with 1×10^7 M1 89155 strain GAS. Data shown are from two independent experiments with N = 15 (combined) mice per group; statistics are calculated from log rank Mantel Cox test for Kaplan-Meier plots.

control studies in which GAS were opsonized with mock immune mouse sera (Figure 5B, $P < 0.01$).

Immunization with a multivalent glycoconjugate vaccine provides significant protection against systemic and intradermal GAS challenge

Active immunization of mice was performed with intramuscular injections of antigens at days 0, 14, and 28 followed by intraperitoneal (i.p.) M1 GAS challenge on day 42 (Figure 6A). In this model, the SpyAD-GAC^{PR} + SLO + C5a peptidase triple combination vaccine yielded striking 100% protection against the lethal challenge ($P = 0.0004$), whereas SpyAD-GAC^{PR} alone (20% protection) or two protein SLO + C5a peptidase formulation (40% protection) showed modest increases in mouse survival that did not achieve statistical significance (Figure 6A).

Mice immunized with SpyAD-GAC^{PR} + SLO (ΔC101) + C5a peptidase or mock (PBS and adjuvant alone) control were challenged using intradermal M1 GAS infection. The size of necrotic lesions generated by the resulting acute inflammatory response did not differ significantly between the two groups (Figure 6B and 6C), but the recovered bacterial CFU/gram was reduced more than two-fold in the debrided tissue at the site of infection in the SpyAD-GAC^{PR} + SLO (ΔC101) + C5a peptidase immunized group (Figure 6D, $P = 0.0012$).

SpyAD-GAC^{PR} immunization does not induce antibodies cross-reactive to human heart or brain tissue epitopes

GAS vaccine development programs have the unique challenge of ensuring the formulation does elicit cross-reactive immune responses capable of recognizing self-antigens in heart tissue implicated in the pathogenesis of RHD. We performed western

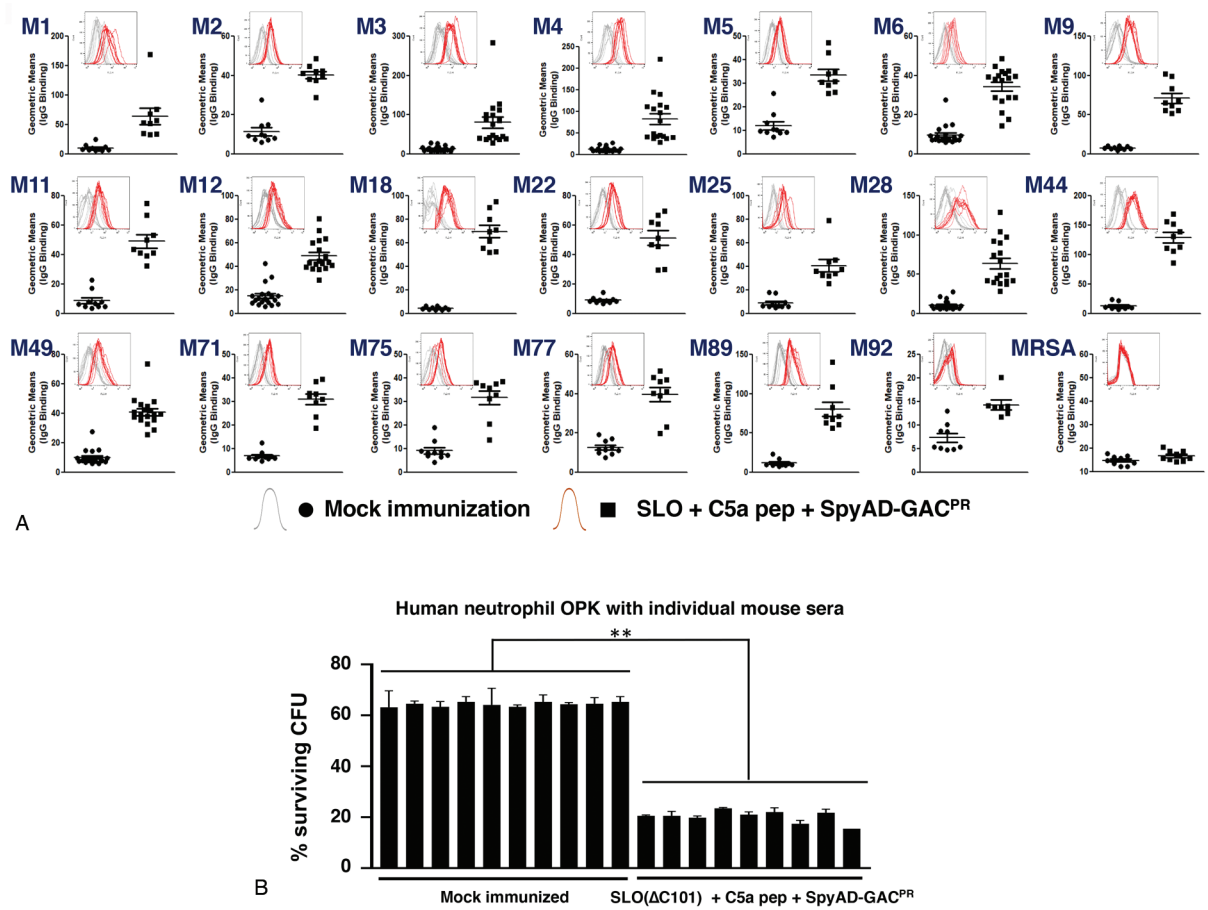

Figure 5. A multivalent vaccine SpyAD-GAC^{PR} + SLO(ΔC101) + C5a peptidase vaccine elicits IgG responses in mice that recognize the surface of diverse group A *Streptococcus* (GAS) strains and promote opsonophagocytic killing of M1 GAS. Serum was collected from female CD-1 mice immunized with three doses of combination SpyAD-GAC^{PR} + SLO (ΔC101) + C5a peptidase vaccine or mock immunization control. A: Fluorescent intensities of murine IgG from mock antisera (gray on inset histogram) or multivalent combination antisera (red on inset histograms) bound to GAS surface antigens of multiple serotypes are quantified via flow cytometry. Mean fluorescence intensities are summarized in column scatter plots, with each point representing an individual mouse serum. Methicillin-resistant *Staphylococcus aureus* USA300 served as a control to show specificity of IgG binding to GAS. B: M1 GAS bacteria was pre-opsonized with murine post-immune serum and tested in a human neutrophil opsonophagocytic killing (OPK) assay. Each bar shown is the result from an individual mouse serum, with error bars representing technical replicates. $P < 0.01$ (**) for each mouse by one-way ANOVA versus mock immunized.

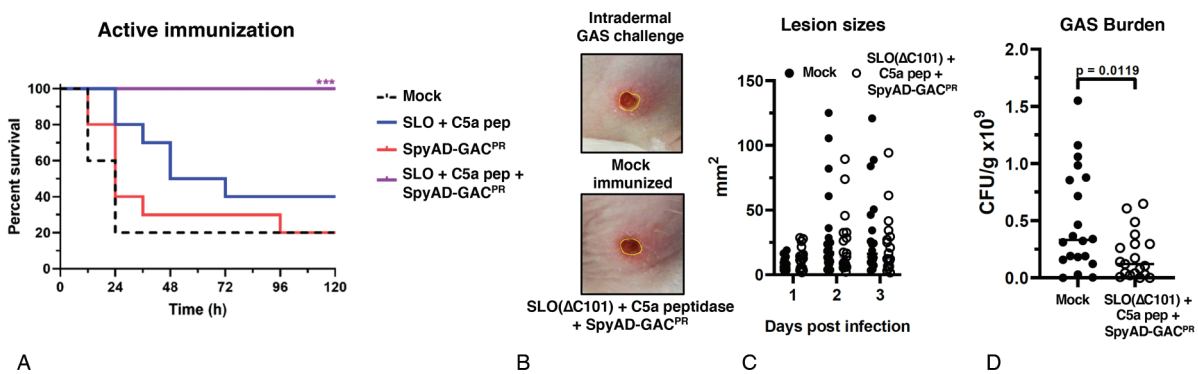

Figure 6. Mice immunized by multivalent conjugate combination are protected when challenged by lethal group A *Streptococcus* (GAS) systemic infection or intradermal skin infection. Female, wild-type CD-1 mice received three intramuscular doses of alum-adjuvanted vaccine formulations before infectious challenge with M1 GAS. A: Immunized mice were subjected to lethal intraperitoneal challenge with 1×10^7 CFU M1 89155 strain GAS and tracked for survival. N = 10 mice per group for lethal challenges, with statistics calculated from log rank Mantel Cox test for Kaplan-Meier plots. Immunized mice were intradermally challenged with 1×10^6 CFU M1 GAS and tracked for skin lesion development. Representative photos of lesions at day 3 are shown in (B), with the total affected area for each mouse quantified in (C). D: Skin lesions were harvested by day 3 post-infection and homogenized to enumerate bacterial burden. For intradermal infections, data shown reflects two independent experiments with groups of 10; statistics are calculated with combined data by unpaired *t*-test with Welch correction (C and D).

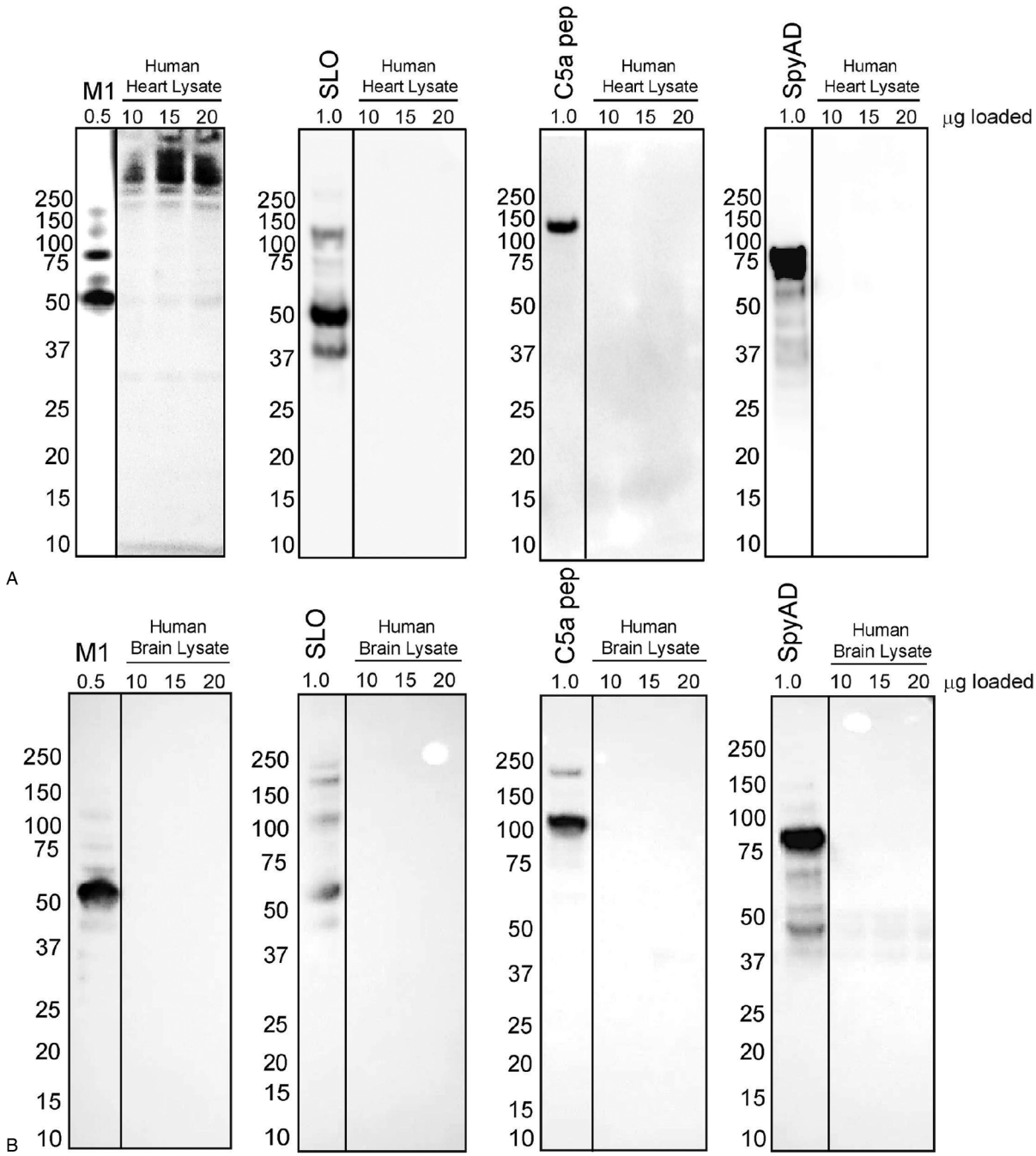

Figure 7. Assessment of antigen-specific antisera for cross-reactivity to human heart lysate. Antisera from rabbits immunized with M1 protein, full length streptolysin O (SLO), C5a peptidase or the SpyAD-GAC^{PR} conjugates were used in western blot analysis against (A) human heart or (B) brain lysate. Unlike antisera raised against purified M1, antisera generated against each of the group A *Streptococcus* (GAS) vaccine antigens only react to the corresponding recombinant protein immunogen but do not possess detectable cross-reactivity to human heart lysate.

blot analysis of the SLO, C5a peptidase, and SpyAD-GAC^{PR} rabbit immune sera on normal human heart lysates separated by polyacrylamide gel electrophoresis; antisera raised against the GAS M1 protein using the same rabbit immunization protocol served as a control. As shown in Figure 7, while anti-M1 antisera reacted to very high molecular weight components of the lysate, no cross-reactivity to the human heart tissue was seen for the SLO, C5a peptidase, and SpyAD-GAC^{PR} antisera, although they recognized the respective cognate GAS protein (or protein carrier in the case of SpyAD-GAC^{PR}). This analysis is consistent with (a) the lack of sequence homology of SLO, C5a peptidase or SpyAD to human proteins, and (b) knowledge that GAC^{PR} is comprised solely of repeating rhamnose, a sugar absent in humans, following the genetic deletion of its GlcNAc side chain, which represented a common mammalian sugar epitope. Though our data reveals a lack of cross-reactivity of rabbit serum antibodies to human tissues, this does not exclude the possibility of cross-reactivity in humans nor does it alone guarantee vaccine safety.

Discussion

In the present work, we deployed a novel conjugation technology to a universally conserved, highly abundant GAS cell wall carbohydrate molecule (GAC^{PR}) that has been engineered to eliminate a suspected cross-reactive epitope (GlcNAc). To functionalize SpyAD as a carrier, the protein was synthesized using the Xpress™ cell-free system, incorporating a non-natural amino acid (nnAA) to which GAC^{PR} could be conjugated in a site-specific manner using click chemistry, generating high molecular mass SpyAD-GAC^{PR} conjugates. The sites of nnAA incorporation were chosen to avoid disruption of important immunological epitopes, including both T-cell and B-cell moieties. The successful outcome expands the proven utility of this conjugation platform, which has previously been applied in a 32-valent preclinical pneumococcal conjugate vaccine program[54] and in studies of an experimental malaria conjugate vaccine.[55] The use of bio-orthogonal attachment chemistry incorporated into the non-natural amino acids theoretically allows for more efficient and potent antigen presentation to the immune system, simplified purification, and more well-defined structure of these semi-synthetic immunogens.

We also applied a cell-free in vitro transcription-translation system for GAS protein antigen production that has high potential for speed and linear scalability. An advantage of this cell-free system is that viability of a microbial expression strain is not required; removal of this constraint creates unique avenues for optimizing protein production, especially for antigens that might confer cytotoxicity in a cell-based system. Indeed, expression yields for the GAS protein antigens studied herein using the cell-free system were much higher than previously reported in the literature, in particular for the cytotoxin SLO (~200-fold improvement). Additionally, the lack of a cellular membrane also allows for the exogenous addition of components to manipulate transcription, translation, and folding, and for site-specific incorporation of non-native amino acids into the translating polypeptide. The protein expression process can be modulated and easily sampled over time with standard industrial microbial fermentation and process equipment.

Another innovative aspect of this work was our aim to devise a GAC^{PR} protein conjugate that avoided using the common pneumococcal vaccine carrier protein antigen CRM197,[56] a non-toxic mutant of diphtheria toxin.[57] As the ultimate goal of a GAS vaccine development program would be to enter the pediatric immunization schedule, simultaneous vaccination with different glyco-antigens on the same carrier protein is potentially associated with dampened immune responses. First, pre-existing immunity to a carrier protein may diminish the ensuing immune response to a new antigen conjugated to the same carrier, that is, carrier-induced epitopic suppression.[58] This was seen in children immunized simultaneously with tetanus toxoid-conjugated pneumococcal vaccine and either (a) *Haemophilus influenzae* type B (Hib) polysaccharide conjugated likewise to tetanus toxoid or, (b) Hib conjugated to diphtheria toxin, and the anti-Hib immune response was demonstrably superior in the latter group.[59] Second, combining different vaccines with CRM197 as carrier can induce "bystander interference" extending even to unrelated antigens present in a multivalent subunit vaccine, speculated to derive from competition for limited molecular and cellular resources for antigen presentation within the lymph node.[58,60,61] In our study, GAC^{PR} was conjugated to the surface-anchored GAS protein (SpyAD), itself a highly conserved vaccine antigen candidate for the pathogen.[39–41] SpyAD retained its immunogenicity following rational targeted introduction of non-natural amino acids for site-specific conjugation that left its critical human B and T cell epitopes exposed.

In the present proof-of-principle studies, the novel SpyAD-GAC^{PR} conjugate was combined with two additional universally conserved GAS virulence factors, the surface anchored C5a peptidase and the potent secreted cytotoxin SLO, yielding a 3-component, 4-valent combination vaccine rationally designed to avoid cross-reactivity with human heart muscle epitopes, here corroborated by western blot analysis. This vaccine elicited antibodies that bound the surface of intact GAS of different serotypes, promoted human neutrophil OPK, and showed protection in a murine model of systemic and localized skin M1 GAS infection.

Other multi-subunit formulations are currently under preclinical exploration for GAS vaccination. Among the protein antigens we selected, C5a peptidase and SpyAD were two of the seven proteins selected for inclusion in a multicomponent vaccine (Spy7) that showed efficacy in reducing GAS dissemination in a murine intramuscular infection model,[40] SpyAD and SLO were included in a five protein component vaccine that reduced murine GAS skin lesion development and accelerated lesion recovery,[41] and SLO and C5a peptidase were included in a formulation (Combo5) that produced a reduction in pharyngitis and tonsillitis in a GAS nonhuman primate mucosal infection model.[62] The use of GAS proteins, including SLO, SpyCEP or SpyAD, as both antigen and carrier protein to conjugate GAC, has also recently been reported,[63] but this approach (i) use non-specific conjugation methods that can disrupt the polysaccharide backbone through periodate mediated oxidation, and (ii) were applied to native GAC, containing the potentially cross-reactive GlcNAc side chain epitope. Our approach instead utilizes CDAP to only derivatize polysaccharide backbone hydroxyls, which allows a site-specific conjugation strategy that can preserve critical protective immune epitopes of the carrier protein when known.

In sum, our general approach may allow the signature, species defining GAC antigen to be safely and effectively included in future GAS subunit vaccine formulations with the goal of broad protection without autoreactivity.

Methods

Bacterial strains and generation of SpyAD knockout strain

The main GAS strain used in the paper was M1 (*emm1*) strain 89155, an invasive disease isolate of U.S. origin from the World Health Organization Collaborating Center for Reference and Research on Streptococci at the University of Minnesota showing the common small fragment chromosomal restriction enzyme analysis pattern 1c.[64] Additional U.S. Centers for Disease Control and Prevention reference strains used were 3752-05 (*emm2*), 4041-05 (*emm3*), 3979-05 (*emm4*), 4623-05 (*emm5*), 4045-05 (*emm6*), 3749-05 (*emm9*), 3979-05 (*emm11*), 4523-05 (*emm12*), 4626-05 (*emm22*), 4039-05 (*emm28*), 3756-05 (*emm44*), 3487-05 (*emm49*), 4044-05 (*emm77*), and 4264-05 (*emm89)* were kindly provided by B.W. Beall at the CDC Streptococcal Reference Laboratory. M1 5448 is an *emm1* GAS isolate from a patient with severe invasive GAS disease.[65] All GAS strains were propagated in liquid Todd-Hewitt broth (THB) and either THB agar plates (THA) or tryptic soy agar plates with 5% sheep's blood (Hardy Diagnostics, Santa Maria, CA, USA, #A10) overnight at 37°C in ambient air without shaking. To

generate the genetic knockout of SpyAD (5448Δ*SpyAD*) via plasmid integration into the chromosome, an intragenic fragment (300 bp) of *spyAD* (original locus tag Spy0269) was amplified from M1T1 GAS 5448 chromosome using forward primer Spy0629_For_EcoRI (5'-GAATTCAGCAGATCGTAATCGC-3') and reverse primer Spy0629_Rev_EcoRI (5'-GAATTCC-CACGTTTAATACC-3'). The PCR product was recovered by TA cloning into pCR2.1-TOPO (Invitrogen), and then subsequently cloned into the temperature-sensitive erythromycin (Erm) resistant plasmid pHY304. The resultant plasmid was transformed into wild-type M1 GAS strain 5448 by electroporation and transformants were plated on THA-Erm 2 μg/mL. Single-crossover chromosomal insertions were identified by shifting to the non-permissive temperature (37°C) while maintaining Erm selection. Integrational knockouts were confirmed by PCR. Used as a control, methicillin-resistant *S. aureus* USA300 strain TCH1516 was originally isolated from an adolescent patient with severe sepsis syndrome at the Texas Children's Hospital in Houston.[66]

GAC purification

GAC^{PR} was purified from GAS 5448Δ*hasA*Δ*gacI*, a M1 serotype strain genetically engineered to lack both the HA capsule and the GlcNAc sidechain on GAC. A bacterial cell pellet from 6.25L growth culture was resuspended and sonicated in ice cold 48% aqueous hydrogen fluoride, stirred at 4°C for 48 h, then dialyzed against ice cold deionized H_2O, centrifuged to remove cellular debris, and supernatants lyophilized. Cell wall material was treated with proteinase K overnight, dialyzed in deionized water to remove salts and proteins before supernatants were once again lyophilized. GAC^{PR} was purified from these lyophilized supernatants by size-exclusion chromatography (SEC), with positive fractions pooled and re-lyophilized. GAC^{PR} identification and purity were confirmed by gas chromatography/mass spectrometry of alditol acetate derivatives and linkage analysis performed on partially methylated alditol acetate derivatives as a service of the UC San Diego Glycotechnology Core.

Cloning, expression, and purification of SLO, C5a peptidase, and SpyAD[4pAMF]

Genes were designed using Biomax ProteoExpert (https://ssl.biomax.de/ ProteoExpert/index.jsp) or DNA 2.0 GeneDesigner (https://www.dna20.com/genedesigner2/) algorithms (Welch et al. 2009) and re-synthesized (DNA 2.0, Menlo Park, CA). The codon-optimized gene for expression of native SLO (79-571), SLO (ΔC101) (79-470) and C5a peptidase (90-1035) or SpyAD (33-849) without or with non-native amino acid namely pAMF incorporation sites [K64,287,386,657] to generate SpyAD[4pAMF] variant 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 his6-tag followed by a TEV protease site [ENLYFQG] for purification of untagged protein. In vitro protein expression using Xpress cell free protein synthesis (CFPS) or XpressCF+TM platform was performed as described elsewhere.[55] For titer estimates, expression of SLO (79-571), SLO (ΔC101) (79-470), C5a peptidase (90-1035) or SpyAD[4pAMF] was quantitated through incorporation of ^{14}C-leucine (GE Life Sciences, Piscataway, NJ) into the translating polypeptide during CFPS at 25°C. 4 μL of either the complete CFPS reaction or the reaction supernatant were blotted onto an anion exchange filter membrane, extensively washed to remove unbound material, and heat dried for 30 min. Finally, the filter membrane was evenly coated with scintillation fluid, air dried and the counts recorded to estimate the total and soluble yield of the expressed proteins.

Controlled large-scale antigen expression utilized a DASbox mini bioreactor system for 10 h at 25°C with constant 650 rpm stirring, pH 7.2, dissolved oxygen 30%. After 10 h, reactions were harvested and spun down at 15,000 × *g* at 4°C for 30 min, passed through a 0.45 μm filter, filtrate loaded on a 5 mL HisTrap excel column (Cytiva) equilibrated and extensively washed with Buffer A (50 mM Tris, 150 mM M NaCl, 10 mM imidazole) until absorbance returned to baseline. Proteins were eluted using a 50% Step gradient of Buffer B (50 mM Tris, 150 mM M NaCl, 500 mM Imidazole), and elution fractions pooled, concentrated, and incubated with excess purified his6-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 (Cytiva Life Sciences, Logan, UT, USA) and untagged proteins collected in the flow-through fractions. Thereafter, the flow-through was concentrated and loaded onto a Superdex 200 26/60 SEC column pre-equilibrated with Buffer S (50 mM Tris pH 8.0, 150 mM NaCl) and purity of eluted fractions assessed by SDS-PAGE gel analysis. Purified SpyAD [4pAMF] was incubated with excess DBCO-TAMRA dye for 1 h at room temperature to confirm pAMF sites by SDS-PAGE gel and fluorescence readout was recorded using a Syngene G-box gel imager.

Multi-angle light scattering (MALS) analysis

SEC MALS-UV-RI was performed with an Agilent HPLC 1100 degasser, temperature-controlled auto-sampler (4°C), column compartment (25°C) and UV-VIS diode array detector (Agilent, Santa Clara, CA) in line with a DAWN-HELEOS multi-angle laser light scattering detector and Optilab T-rEX differential refractive interferometer (Wyatt Technology, Santa Barbara, CA) coupled to three TOSOH columns in series: TSKgel Guard PWXL 6.0 mm ID × 4.0 cm long, 12 μm particle; TOSOH TSKgel 6000 PWXL 7.8 mm ID x× 30 cm long, 13 μm particle; and a TSKgel 3000 PWXL 7.8 mm ID × 30 cm long, 7 μm particle. A mobile phase consisting of 0.2 μm filtered 1x PBS with 5% (v/v) acetonitrile was used at a 0.5 mL/min flow rate and 50–100 μg sample was injected for analysis. Agilent Open Lab software was used to control the HPLC, and Wyatt Astra 7 software was used for data collection and molecular weight analysis.

Dibenzocyclooctyne DBCO-derivatization of GAC^{PR} and conjugation to SpyAD[4pAMF]

To a 6 mM solution of GAC^{PR} in 100 mM Borate Buffer pH 8.5, three equivalents [to the polysaccharide repeating unit (PSRU)] of 1-cyano-4-dimethylaminopyridinium tetrafluoroborate (CDAP; from 100 mg/mL solution in acetonitrile) were added with vigorous stirring to facilitate cyanylation at reactive hydroxyl groups. 5 min after addition of CDAP, 2 molar equivalents of dibenzocyclooctyne-amine linker stock in dimethyl sulfoxide (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 zeba spin desalting 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 complete removal of excess DBCO linker and other reaction byproducts. Finally, the polysaccharide concentration was measured using 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, %DBCO derivatization of the GAC^{PR} was kept between 5% and 10%. Thereafter, SpyAD[4pAMF] was mixed with DBCO-derivatized GAC^{PR} 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 polysaccharide. The recovered conjugates were analyzed by SEC MALS and the concentration was estimated using an anthrone assay.

Anthrone assay for total polysaccharide concentration

A stock of 2 mg/mL of the anthrone reagent (Sigma-Aldrich, St. Louis, MO, USA, CAS#90-44-8) was prepared in cold sulfuric acid while a 1 mM stock of PSRU comprising 2x rhamnose was prepared in water as a standard. In triplicate wells, 100 μL of PSRU stock (serially diluted into reference standards) or the unknown samples (diluted 1:3) were plated (96-well plate) followed by addition of 200 μL/well of the anthrone reagent stock. All reactions were thoroughly mixed and sealed with a plate cover for incubation at 95°C for 10 min. The plate was briefly placed on ice to cool to ambient temperature before absorbance is measured at 620 nm using a UV/VIS plate reader. To determine concentration of unknown samples, PSRU standard concentrations and absorbances were used to generate a least-square fit regression.

Generation of immunized rabbit serum

Age-matched New Zealand white rabbits were intramuscularly injected with 5 μg of protein antigens (SLO, C5a peptidase, M1) or SpyAD-GAC^{PR} conjugate (equivalent of 5 μg of polysaccharide) in succinate buffer adjuvanted with Adju-phos® (Invivogen). Immunizations (250 μL per injection per dose) were performed on day 0, 14, and 28 with terminal bleed for serum performed on day 35.

Antibody titer of rabbit serum by ELISA

Anti-protein antibody titers of rabbit antisera were determined by ELISA. 3 μg recombinant protein in PBS per well was incubated overnight in a high-binding flat bottom 96-well plate (Corning #3361) at room temperature, antigen-coated plates washed three times in PBS with 0.05% Tween-20 (PBST), blotted dry, and blocked for 2 h in PBS + 1% bovine serum albumin (R&D Systems cat#DY995). Plates were washed ×3 with PBST and blotted dry before addition of rabbit antisera, serially diluted in PBS with 1% BSA starting from 1:1000 dilution. Antisera were incubated for 2 h at room temperature, plates washed three times with PBST, blotted dry, then incubated with horseradish peroxidase (HRP)-conjugated goat-anti rabbit IgG secondary antibody (Southern Biotech cat#4050-05), diluted 1:4000 in PBS with 1% BSA, for 2 h at room temperature. Plates were washed three times with PBST and blotted dry before addition of TMB substrate (BD cat#555214) per manufacturer's instruction and incubated in the dark for 5 min. HRP reaction was stopped by the addition of 2N sulfuric acid (Sigma) before optical density was read at 450 nm. Antibody titer calculated as highest serum dilution where the signal exceeded the signal of blank wells plus three standard deviations, and all samples were run in at least duplicate for 2–3 rabbits per immunization group. For subsequent experiments, serum from the rabbits with the highest anti-protein antigen titers were used: 1:2,050,000 for SLO, 1:6,550,000 for C5a peptidase, 1:3,280,000 for SpyAD, and 1:6,550,000 for SpyAD-GAC^{PR}. Statistics shown use one-way ANOVA with Dunnett multiple comparisons test to compare each immunized group with the dilution factor of the pooled pre-immune system.

IgG binding to GAS strains

All GAS strains were grown to mid-logarithmic growth phase (OD600 nm = 0.4) and washed in PBS before blocking incubation in 10% donkey serum for 1 h at room temperature. Murine or rabbit antisera was added to bacteria to 2% final volume and incubated for 1 h at room temperature to allow antibodies to bind to bacteria surfaces. Samples were washed with PBS and incubated with 1:200 donkey anti-rabbit IgG conjugated with AlexaFluor 488 fluorophore (Thermo Fisher, Waltham, MA, USA #21206), protected from light for 30 min at room temperature. Samples were washed in PBS once and run on a BD FACSCalibur. Signal intensity was analyzed using FlowJo software (Tree Star) and reported as a percentage of increased mean fluorescence intensity signal in individual rabbit antiserum compared to pooled pre-immunized rabbit sera.

Primary human neutrophil OPK assay

Neutrophils were isolated from blood drawn from healthy human donors with consent, as approved by UC San Diego institutional review board (Protocol #131002X). Neutrophils were pre-incubated with BRC (PelFreez cat#31061) and heat-inactivated fetal bovine serum for 10 min. All GAS strains were grown to mid-logarithmic growth phase (OD600 nm = 0.4), washed in PBS, and incubated with heat-inactivated murine or rabbit antisera for 30 min at 37°C. For combination of rabbit serum, total anti-serum volume was kept the same but consisted of an even pool of serum from multiple rabbits. Neutrophils were added to bacteria at a MOI = 0.1 bacteria per neutrophil, briefly centrifuged to ensure contact, and allowed to incubate for 30 min at 37°C with 5% CO_2. Final concentrations of components were 20% murine or rabbit serum, 2% fetal bovine serum, and 2% BRC, and the remaining volume comprised bacteria and neutrophils in PBS. At experiment termination, samples were serially diluted in PBS and plated onto THB agar plates for CFU enumeration. Sera from pre-immunized rabbits were pooled and used as control to measure non-specific, baseline bacterial killing by neutrophils for the rabbit antisera samples. At minimum, each serum or serum combination was tested in triplicate assays to ensure statistical confidence.

Primary human neutrophil oxidative burst assay

Primary human neutrophils were isolated as described for the OPK assay. 2×10^6/mL human neutrophils were loaded with 20 μM 2,7-dichlorofluorescein diacetate (Thermo Fisher, Waltham, MA, USA) in Hank balanced salt solution (Corning Cellgro, Glendale, AZ, USA) without Ca^{2+} and Mg^{2+} and incubated with rotation at 37°C for 20 min. Neutrophils were resuspended in Hank balanced salt solutionwith Ca^{2+} and Mg^{2+} to a density of 1×10^6 cells/well before treatment with rabbit

serum (1:64 final concentration) followed by supernatant from mid-logarithmic growth phase M1 89155 bacterial cultures (diluted 1:10). Lastly, 50 ng/mL phorbol myristate acetate was added to wells, and incubated at 37°C/5% CO_2, and fluorescence intensity at 485 nm excitation/520 nm emission quantified on an Enspire plate reader (Perkin Elmer, Waltham, MA, USA) at the indicated time points.

In vivo mouse immunization studies

All mouse experiments were approved by the UC San Diego Institutional Animal Care and Use Committee (Protocol #S00227M) and conducted per accepted veterinary standards. For passive immunization experiments, 200 μL of rabbit antisera was intravenously delivered via retro-orbital injection in anesthetized wild-type female CD-1 mice (Charles River), 8–10 weeks of age. After 5 min, mice were challenged with 1×10^7 CFU M1 strain 89155 GAS by i.p. injection and tracked for survival. For active immunization, wild-type female CD-1 mice (Charles River, Wilmington, MA, USA) were immunized every 14 days for a total of three doses starting at 5–7 weeks of age. Intramuscular immunizations delivered consisted of 100 μL total volume per mouse per dose, including 50 μL of Alhydrogel 2% aluminum hydroxide adjuvant (Invivogen), prepared per manufacturer's instructions. 14 days after final immunization, mice were infected with 1×10^7 CFU M1 89155 GAS by i.p. injection and tracked for survival. Statistics of Kaplan-Meier survival curves calculated using log-rank Mantel-Cox test.[67]

For intradermal infection, female CD-1 mice were immunized as previously mentioned. Mice were shaved and chemically depilated under isoflurane anesthesia and allowed to recover for a day prior to infection. Isoflurane-anesthetized mice were injected with 20 μL prepared M1 89155 culture (1×10^6 CFU) intradermally using 26½ gauge needles on Hamilton 1000 μL glass syringe (cat# 81320) with PD600 repeating dispenser (cat# 83700) for reproducible precision. Developing lesions was tracked and photographed daily. On day 3 post-infection, mice were euthanized, lesions excised and homogenized using MagNaLyser (Roche, Indianapolis, Indiana, USA) and serially diluted in technical triplicate onto tryptic soy agar plates with 5% sheep's blood (Hardy Diagnostics A10) for CFU enumeration. Lesion sizes were quantified using FIJI.[68] Statistics of both lesion sizes and recovered CFU were calculated using unpaired *t*-test with Welch correction.

Western blot analysis for heart/brain lysate cross reactivity

Varying amounts of normal adult human heart tissue lysate (Novus Biologicals, Littleton, CO, USA, cat# NB820-59217) or brain lysate (Novus Biologicals cat # NB820-59177) incubated with SDS-containing denaturing loading dye were separated by SDS-PAGE using 4-12% Bis-Tris gels before transfer onto a PVDF membrane using the manufacturer's protocol on iBlot (Thermo Fisher). The blot was blocked at ambient temperature for 1 h, followed by probing with rabbit antisera generated against each of the GAS antigens (diluted 1:1000). After three 30 min washes, HRP conjugated anti-rabbit (Jackson ImmunoResearch Laboratories, Inc., Cat # 211-035-109) secondary antibody (diluted 1:10,000) was added and chemiluminescence recorded on an Syngene G-Box F3 image scanner after incubation of the blot with the pico substrate (Thermo Fisher Scientific Cat # 34580). All blot blocking, washes, and antibody/serum dilutions were performed in TBS + 5% BSA.

Statistical analysis

All statistical analyses were performed using GraphPad Prism version 8.0.0 for Windows, GraphPad Software, San Diego, California USA, www.graphpad.com. *P* values were summarized for respective analyses as: $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.001$ (***).

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