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MINIREVIEW

Immunobiology of the Classical Lancefield Group A Streptococcal Carbohydrate Antigen

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ABSTRACT Group A *Streptococcus* (GAS) is a preeminent human bacterial pathogen causing hundreds of millions of infections each year worldwide. In the clinical setting, the bacterium is easily identified by a rapid antigen test against the group A carbohydrate (GAC), a polysaccharide that comprises 30 to 50% of the GAS cell wall by weight. Originally described by Rebecca Lancefield in the 1930s, GAC consists of a polyrhamnose backbone and a *N*-acetylglucosamine (GlcNAc) side chain. This side chain, the species-defining immunodominant antigen, is potentially implicated in autoreactive immune responses against human heart or brain tissue in poststreptococcal rheumatic fever or rheumatic heart disease. The recent discovery of the genetic locus encoding GAC biosynthesis and new insights into its chemical structure have provided novel insights into the assembly of the polysaccharide, its contribution to immune evasion and virulence, and ideas for safely harnessing its natural immunogenicity in vaccine design. This minireview serves to summarize the emerging new literature on GAC, the eponymous cell well antigen that provides structural integrity to GAS and directly interfaces with host innate and adaptive immune responses.

KEYWORDS autoimmunity, cell wall, GlcNAc, group A carbohydrate, group A *Streptococcus*, Lancefield antigen, rheumatic heart disease, *Streptococcus pyogenes*, vaccine, virulence factor

GROUP A *Streptococcus* AND ITS SPECIES-DEFINING ANTIGEN

Streptococcus pyogenes, commonly known as group A *Streptococcus* (GAS), is a preeminent human pathogen, causing hundreds of millions of infections each year worldwide. The tremendous global disease burden of this Gram-positive bacterial pathogen is skewed significantly toward resource-limited parts of the world (1, 2). The most common GAS disease manifestations are superficial mucosal infections, in particular pharyngitis ("strep throat"), and skin infections, notably impetigo, which can be self-limited or effectively managed with oral or topical antibiotics. However, GAS also has significant invasive disease potential and can disseminate through deep tissues or the bloodstream to cause sepsis, necrotizing fasciitis, or streptococcal toxic shock syndrome, potentially life-threatening conditions without urgent medical or surgical intervention. And unique among human bacterial pathogens in terms of scale, GAS can trigger hallmark postinfectious, immunologically mediated pathologies, in particular rheumatic heart disease (RHD), that represent a major source of morbidity and mortality throughout many parts of the developing world. New strategies for effective treatment and prevention of GAS infection and its complications remain a major public health priority, with vaccines targeting the pathogen still in earlier stages of development or evaluation.

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†Deceased.

This article is dedicated to our talented coauthor Ervin Rodas Lima (1999 to 2021).

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The global success of GAS as a human pathogen and its wide array of disease manifestations reflect a complex host-pathogen interaction, with numerous bacterial virulence factors and toxins that act in concert to promote epithelial attachment and barrier disruption, resist innate clearance mechanisms, and provoke host immune and inflammatory responses (2). Among these bacterial factors is a unique and highly abundant cell wall component, group A carbohydrate (GAC), which is universally conserved and indeed defines the species. GAC is the basis for accurate and rapid clinical detection of GAS infection, essential for prescribing prompt and effective treatment to reduce the risk of invasive infection and postinfectious immune-mediated complications with potential lifelong consequences (3, 4). However, despite its utility as an immunodiagnostic and a potential role in triggering autoimmune cross-reactivity post-GAS infection (see below), little else was understood about GAC until recent years. New genetic and chemical structure discoveries have shepherded great progress in understanding the GAC biosynthetic process, its contribution to virulence, and safely harnessing its natural immunogenicity in vaccine design. This minireview serves to summarize the current literature on GAC, the eponymous antigen that both defines and provides structural integrity to GAS.

DISCOVERY OF GAC AND ITS ROLE IN DIAGNOSTICS

In the early days of microbial diagnostics, the different species of beta-hemolytic streptococci were virtually indistinguishable and became grouped together in a single rubric as "*Streptococcus hemolyticus*." However, during the 1920s and 1930s, Rebecca Lancefield discovered a series of bacterial cell wall and surface antigens as a means to identify and classify the beta-hemolytic streptococci. Originally categorized as "C-substance," these "group antigens" were eventually identified to be of a carbohydrate nature through her experiments using enzymatic digestions (5). By the 1930s, a molecular basis for streptococci group classification was established and carried out by antibody-mediated latex bead agglutination and antiserum protection tests (6). Antibody recognition of group antigens quickly found application for identification of clinically relevant pathogenic streptococci (7) and remains to this day the gold standard for rapid antigen detection and diagnostics for GAS.

The original classification of beta-hemolytic streptococci into groups A through M has undergone significant reorganization as the bacterial species were more precisely characterized and genomic comparisons became possible. Though many beta-hemolytic *Streptococcus* group classifications were not specific to a single species, the group A streptococci initially comprised only one species: *Streptococcus pyogenes*, producing GAC as a species-unique identifier. Despite the long history of reliable pathogen identification dependent on GAC, it is only in the last decade that the GAC-encoding operon and biosynthesis process have been characterized largely due to advancements in streptococcal genetics and glycobiology techniques, as well as renewed interest in the potential of GAC as an effective vaccine antigen. Interestingly, anti-GAC antibody cross-reactivity is now recognized from certain rare group C-variant streptococci strains (*Streptococcus. dysgalactiae* subsp. *equisimilis*) (8, 9) and *Streptococcus castoreus*, a recently identified group A streptococcal bacterium isolated from beavers (10); both bacteria have genetic loci homologous to the operon of GAS genes essential for the biosynthetic pathway.

GAC CHEMICAL STRUCTURE AND GENETIC BASIS OF ITS BIOSYNTHESIS

The thick Gram-positive GAS cell wall contains peptidoglycan, lipoteichoic acids, and various structural proteins, but GAC itself makes up a remarkable 30 to 50% of cell wall composition by weight (11). Antibody binding experiments have found that GAC is localized primarily to the outermost surface of the cell wall (11) but also intercalated within a mesh-like structure to the peptidoglycan (12), to which GAC is anchored via phosphodiester bonds (13). Chemical composition analyses indicate that GAC is composed of a linear polyrhamnose chain decorated with *N*-acetylglucosamine (GlcNAc)

side chains (11). Polyrhamnose comprising the GAC backbone are connected by alternating α-L-(1→3) and α-L-(1→2) glycosidic linkages, with the β-D-GlcNAc attached to every other residue on the rhamnose backbone at position 3 (14). A recent study also detected glycerol phosphates (GroP) present on the C6-hydroxyl group of approximately 25% of GlcNAc (15), a significant modification previously undetected due to harsh extraction methods classically used for GAC purification.

GAC biosynthesis is encoded by a 12-gene cluster (*gac* operon) that is highly conserved across GAS genomes. A recent analysis found that 97% of GAS genomes (2,017 of 2,083) had high sequence conservation of >70% sequence homology for the entire 12-gene cluster (16), further supporting conclusions from a smaller data set (17). The first seven genes of the *gac* operon (*gacABCDEFG*) encode for synthesis of the core polyrhamnose structure and are conserved across group A, B, C, and G streptococci (18). In GAS, some genomes possess frameshift mutations within several *gac* genes, suggesting that not all genes are essential for survival and the potential existence of compensatory genes (16, 19). To date, not all the genes or their products have been unambiguously delineated to specific roles, but recent work has expanded our knowledge of individual gene functions and the GAC biosynthesis process (summarized in Fig. 1).

Similar to many polysaccharides exposed on the bacterial outer surface, including capsular polysaccharides and wall teichoic acids, GAC synthesis commences with GacO affixing GlcNAc to undecaprenyl phosphate (UndP) on the intracellular side of the cell membrane (12). The *gacB* gene encodes a rhamnosyltransferase that synthesizes the committed step in the GAC biosynthesis: translocation of the first rhamnose residue onto the membrane-bound UndP-GlcNAc acceptor in the inner leaflet of the bacterial cell membrane (18). Free dTDP-L-rhamnose is produced from α-glucose-1-phosphate by the enzymes encoded by *gacA* and an operon located distally from GAC operon but well studied for rhamnose synthesis, the *rmlABC* operon (20). GacA is a metal-independent dTDP-4-dehydrorhamnose reductase enzyme, and unlike homologues in the RmlD family, GacA uniquely functions as a monomer instead of a homodimer (20). Sequential elongation of the polyrhamnose chain is mediated by glycosyltransferases GacC, GacF, and GacG (18). *gacD* and *gacE* encode heterodimers of an ATP-dependent ABC transporter, actively translocating the completed polyrhamnose chain to the extracellular side of the cell membrane.

The remaining five genes of the *gac* operon are predicted to encode functions extending off the rhamnan chain. GacJ, a small protein associated with the bacterial membrane, complexes with GacI for improved catalytic efficiency to produce free UndP-GlcNAc (18). Though UndP-GlcNAc can freely diffuse across the cytoplasmic membrane on its own, it is also transported by the Wzx family flippase enzyme GacK to the extracellular side of the membrane (21). GacL, a putative glycosyltransferase, uses the UndP-GlcNAc substrate to link GlcNAc to the polyrhamnose (21). GacH cleaves phosphatidylglycerol to release and attach glycerol phosphate to C-6 on approximately 25% of GlcNAc, potentially to reinforce attachment and stability of GAC on the cell wall, though the precise function is unclear (13, 15). Finally, GAC is transferred and covalently linked to the peptidoglycan layer via phosphodiester bond by an enzyme of the Lytr-CpsA-Psr (LCP) family. The final glycan has a reported mass that correlates to an estimated 18 trisaccharide repeating units (22), though different purification methods result in various average polysaccharide sizes (22, 23).

NEW INSIGHTS INTO GAC FUNCTION

For decades, GAC was assumed to function solely in the structural integrity of the streptococcal cell wall, but emerging data has expanded knowledge on the role of the polysaccharide in GAS disease pathogenesis and immunity (Fig. 2). GAC is undeniably a crucial cell wall component, comprising up to 50% of its mass (11). Depletion of L-rhamnose (20) or deletion of genes required for synthesis of the GAC polyrhamnose backbone (17) are lethal to the bacterium, demonstrating the essential role of GAC for cell wall viability. Monte Carlo simulations on nuclear magnetic resonance (NMR) spectroscopy data

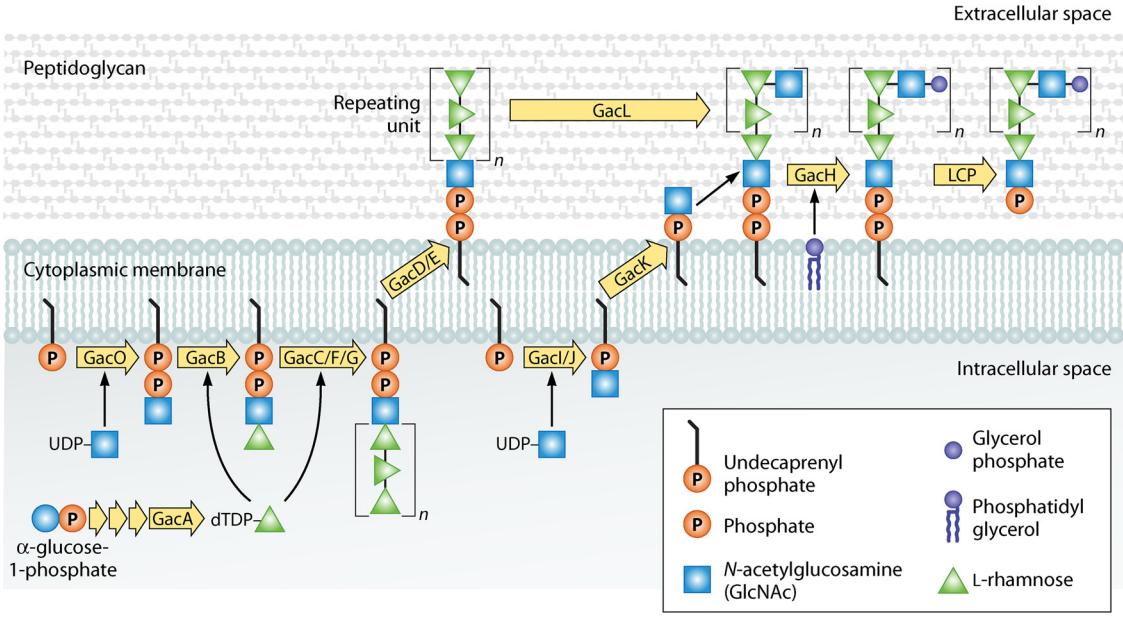

FIG 1 Biosynthesis of the group A carbohydrate (GAC), the most abundant component of the group A streptococcal cell wall. The schematic summarizes current literature on GAC biosynthesis. Enzymatic processes are noted by gene products in yellow arrows. The polyrhaman backbone is assembled on the inner leaflet of the cytoplasmic membrane by GacBCFG and then flipped to the outer leaflet by GacDE complex. The GlcNAc side chain and glycerol phosphates are added onto the GAC by GacL and GacH, respectively, before a Lytr-CpsA-Psr (LCP) family enzyme transfers and attaches the completed GAC to the peptidoglycan via a phosphodiester bond. This figure was created on Biorender.com.

predict a conformationally restricted polysaccharide (24), consistent with GAC acting as a rigid structural support framework for the bacterial cell wall. A functional role(s) of GAC beyond cell wall structure is less well delineated, in part due to the interconnected network that GAC biosynthesis shares with synthesis of other key cell wall glycopolymers, including peptidoglycan (12). However, high conservation of polyrhamnose cell wall polysaccharides (including GAC) in the order *Lactobacillales* provides supporting evidence that GAC may functionally replace wall teichoic or teichuronic acid structures found in other Gram-positive bacteria but absent in beta-hemolytic streptococci (12, 25). This notion implies that GAC performs key roles in pathogenesis, cell shape, regulation of cell division, and other aspects of bacterial cell wall physiology (26).

Beyond the polyrhamnose backbone that is considered essential, the entire GAS *gac* operon is highly conserved and all human clinical isolates express native GAC with its intact GlcNAc side chain. However, examples in which the polysaccharide will lose GlcNAc after serial passage in mice and rabbits have been reported (27), suggesting a role for the side chain in human-specific pathogenicity or immune evasion. Specific epitopes of various rhamnose cell wall polysaccharides, including other streptococcal group polysaccharides, promote bacterial resistance to immunological clearance, though molecular mechanisms are not yet defined (12). The discovery of the *gac* operon (17) for the first time allowed the generation of an isogenic GAS mutant lacking the GlcNAc side chain but retaining the polyrhamnose backbone, akin to the "A-variant" strains isolated following animal passage (27). The GlcNAc-deficient (ΔGAC) mutant had similar ultrastructural appearance to the parental strain on transmission electron microscopy and did not exhibit a general defect in cell wall integrity, as susceptibility to autolysis, reactive oxygen species, lysozyme, nafcillin, or vancomycin were equivalent to that of the wild type (17). GAS growth in various media was unaffected by loss of the GlcNAc side chain, but the average chain length of the mutant strains was increased (17).

Despite many phenotypic similarities as described above, a ΔGAC mutant in the hypervirulent, globally disseminated M1T1 GAS background was markedly attenuated

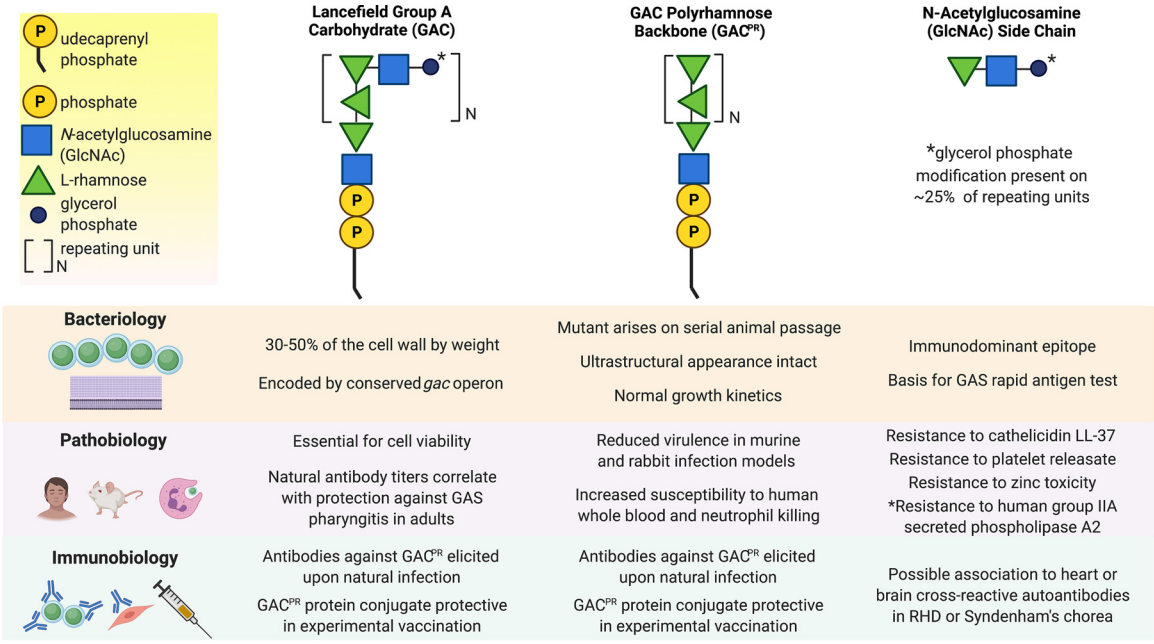

FIG 2 Roles of the group A carbohydrate (GAC) versus its component polyrhamnose backbone (GAC^{PR}) or GlcNAc side chain in the bacteriology, pathobiology, and immunobiology of group A *Streptococcus*. This figure was created on Biorender.com.

for virulence compared to the parent strain in both murine systemic infection and rabbit pneumonia models (17). Moreover, the isogenic ΔGAC mutant was more susceptible than the wild-type GAS strain to killing in human whole blood and by purified human neutrophils, corroborating a function of the GlcNAc side chain in resistance to immune clearance. Mechanistic studies showed increased killing of GAS to human platelet releasate, rich in cationic antimicrobial peptides, and to the cationic human defense peptide cathelicidin (LL-37), including surface plasmon resonance confirmation of greater binding of LL-37 to the ΔGlcNAc mutant GAC versus wild-type GAC polysaccharide (17). Later, it was found that the GroP modification present on some GAC GlcNac sidechains is required for cationic bactericidal enzyme human group IIA secreted phospholipase A2 (hGIIA) to efficiently act against GAS; hGIIA killed GAS strains lacking GlcNAc at 1/10 the concentration lethal for wild-type GAS (15, 28). The GacH required for GroP decoration also conveys resistance to zinc toxicity (15), though the mechanism of protection is not yet understood.

GlcNAc is a common sugar present in glycan structures on mammalian cell surfaces and extracellular matrix, and GAC side chain is hypothesized to play a role in mimicking human epitopes, perhaps helping GAS avoid immune detection by masking polyrhamnose, a nonhuman glycan motif found in bacteria (12), fungi (29), and protozoan parasites (30). Though human lectins with a precise specificity for rhamnose or polyrhamnose have yet to been identified (12), another hypothesis suggests that the GlcNAc may directly bind host receptors to skew the immune response in favor of bacterial survival within the host, as documented with the GlcNAc present in the lipooligosaccharide of *Neisseria gonorrhoeae* (31). It is important to note that the GlcNAc side chain on GAC is not a universal prerequisite for virulence, as the degree to which loss of GlcNAc affects GAS susceptibility to innate immune clearance varied among six isogenic mutants (M1, M2, M3, M4, M28, and M89 serotypes) constructed using a consistent methodology (19). The authors concluded that the relative contribution of the GAC GlcNAc side chain to virulence likely depends on the quorum of other virulence factors that each strain possesses and that an abundance of immune resistance factors expressed or secreted from the surface of GAS can compensate for the loss of the GlcNAc side chain in some strain backgrounds (19).

NATURAL IMMUNOGENICITY OF GAC

Following GAS infection, humans naturally develop antibodies against various GAS antigens, including GAC. Among these antibodies, antibody titers to streptolysin O (SLO), a cholesterol-dependent cytolysin produced by GAS commonly serves as a clinical standard to determine recent GAS infections. When humans develop antibodies against GAC, serum binding assays indicate the anti-GAC antibody peak is slower and more modest compared to that of SLO. For example, one study found the anti-SLO titer increased significantly post-GAS infection (228%) compared to a modest increase in anti-GAC titer (22%) (32). Despite a strong antibody response to GAS infections, the anti-SLO titer level peaks in children and declines with age (32), suggesting that while anti-SLO antibodies protect in acute or active infections, they may not provide lasting immunity or protection. In contrast, mean antibody titers to the GAC in humans increase slowly with age, peaking between 14 and 17 years of age before decreasing slightly in the 20s (32); these serum titers strongly correlate with a reduced rate of GAS infection after 17 years of age (33). Therefore, antibodies recognizing GAC may be important in development of lasting immunity against GAS infections, despite weak natural immunogenicity compared to other GAS antigens.

The critical factors for development of immunity against GAC are not well understood. As is the case for other polysaccharides, molecular weight of GAC fragments plays a role in immunogenicity and the accompanying inflammatory response. Immunization of rabbits with synthetic GAC polysaccharides of various lengths showed a significant protective response with a hexasaccharide, equivalent to two repeat units (22). The hexasaccharide GAC may represent the minimum sufficient antigen, as it contains all possible structural motives of the repeating subunits (22). In a rat arthritis model, rats injected with larger polysaccharide chains experienced acute edema and arthritis, while an equivalent mass of polysaccharides composed of smaller fragments induced arthritis but no edema (34). The inflammatory response to GAC thus varies depending on the length of the chain, likely due to the differences in molecular recognition and processing of the polysaccharide antigen. The trisaccharide unit branch point and the size of the total polysaccharide determine the epitopes to which anti-GAC antibodies bind (24).

Most antibodies directed against the GAC are specific to the GlcNAc moiety, the immunodominant epitope of the GAC and the basis for the Lancefield group assignment (33). Early immunization experiments with A-variant (animal-passaged) GAS strains lacking GlcNAc confirmed that these strains induced antisera against the polyrhamnose on GAS (35). Antibodies to purified native GAC are protective in various infection models and opsonizing across different M-protein serotypes in phagocytosis assays (33). Likewise, antirhamnan antibodies raised against ΔGAC enhanced neutrophil opsonophagocytic killing of multiple *emm* serotypes GAS *in vitro* and protected against lethal challenge in a murine passive immunization model (17). As the terminal, beta-linked GlcNAc sugars play such a dominant role in the serological specificity of the polysaccharide, concerns for immunological cross-reactivity with mammalian connective tissues containing this sugar motif have been raised (35) and are discussed in the subsequent section.

SUSPECTED ROLE OF GAC IN GAS-INDUCED AUTOIMMUNITY

GAS can trigger significant postinfectious, immune-mediated disease sequelae, in particular acute rheumatic fever (ARF), which if recurrent can lead to rheumatic heart disease (RHD), a major cause of cardiovascular morbidity and mortality in several resource-poor regions of the world. ARF may arise following 3 to 6% of GAS pharyngitis infections that are not promptly treated, with the autoimmune reaction affecting the heart, joints, skin and/or central nervous system (36), the latter including the syndrome of Sydenham's chorea, reflective of basal ganglia dysfunction (37). Uncommon in adult subjects, ARF is most frequently seen in children and adolescents 5 to 15 years of age (4, 38), coinciding with the peak incidence of GAS

pharyngitis cases. ARF and progression to RHD can be prevented with the use of primary and secondary antibiotic prophylaxis to target GAS, respectively (39). Current diagnostic criteria for ARF, originally published in 1944 as the Jones criteria and updated in 1992, feature a list of major and minor clinical manifestations but also require documentation of recent GAS infection via positive throat culture, positive rapid antigen test, or elevated/rising streptococcal antibody titer (typically anti-streptolysin O and sometimes anti-DNase B) (40).

Original studies by Goldstein isolated glycopeptides from human heart valves that share immunological properties with the GlcNac side chain of GAC (41). Anti-streptococcal monoclonal antibodies derived from human ARF patients that reacted with heart cells were then noted to bind the glycoprotein laminin found in the basement membrane underlying human heart valve endothelium (42). Further studies found that responses against GlcNAc were strongly linked to antibody responses against cardiac myosin and other alpha-helical coiled-coil proteins, including streptococcal M proteins (43, 44); these alpha-helical peptide motifs are hypothesized to underpin cross-reactivity between GAC epitope and the myocardium or heart valves (42, 45).

Individual monoclonal antibodies against the GlcNAc epitope have been isolated from patients with rheumatic carditis (45) and Sydenham's chorea, one of the Jones criteria for ARF that affects neuronal cell signaling (46), suggesting that the same epitope may be responsible for multiple ARF manifestations. Of note, one such monoclonal antibody from an ARF patient that recognized native GAC on the wild-type GAS surface did not recognize an isogenic GAS knockout mutant that lacked the GlcNAc side chain (17). Overall, ARF patients had two- to threefold-higher concentrations of anti-GAC antibodies at the initial time point of illness compared to GAS pharyngitis patients (47), and elevated levels of serum antibodies recognizing GAC persisted for longer periods of time in patients with RHD compared to the normal rate of decline in ARF patients without carditis (48).

However, the presence of cross-reactive antibodies in and of itself does not establish a central role in promoting ARF/RHD pathogenesis; a sustained immune response must also be driven to break tolerance. Models have been proposed in which anti-GlcNAc antibodies attach to the valvular endothelium and trigger upregulation of CXC chemokine ligand 9 (CXCL9)/Mig, which attracts $CD8^+$ and $CD4^+$ T cells, and vascular cell adhesion molecule-1 (VCAM-1), which facilitates the extravasation of these T cells into the heart valve tissue (reviewed in reference 49). However, a comparison of sera from healthy rabbits and GAC-immunized rabbits demonstrated no significant difference in antibody binding to fixed human tissues (50) or human cardiac tissue lysates via enzyme-linked immunosorbent assays (ELISAs) (17), suggesting that GAC may not be sufficient or solely responsible for the development of ARF. A clinical trial for a M-protein-based GAS vaccine in the 1960s sounded alarm when 3 of the 21 participants developed suspected or definite ARF (51), leading the U.S. Food and Drug Administration to issue a ban on GAS vaccine development that was not lifted until after an expert panel brought together in 2005 by the U.S. National Institute of Allergy and Infectious Diseases demonstrated the therapeutic potential and need for a GAS vaccine (52). Research can proceed; however, the panel clearly established the need for safety and testing for autoreactivity as a special requirement specific for GAS vaccines. The incompletely understood mechanism(s) of ARF/RHD immunopathology remains a critical knowledge gap(s), and next-generation biomarkers to detect early signs of these diseases are urgently needed to help steer the development of a safe and effective GAS vaccine (53).

GAC AS A CANDIDATE GAS VACCINE ANTIGEN

GAS differs from other prominent human streptococcal pathogens, notably group B *Streptococcus* (GBS) and *Streptococcus pneumoniae*, which express immunodominant exopolysaccharide capsules that confer serotype specificity, promote virulence, and are validated as vaccine antigens (54). Rather, the polysaccharide capsule expressed by the vast majority of GAS strains is composed of high-molecular-weight hyaluronic acid, a ubiquitous component of host tissues, and therefore immunologically inert (55).

TABLE 1 Group A streptococcal immunization strategies utilizing the group A carbohydrate (GAC)

Vaccine	GAC	Formulation	Reference(s)
GAS CHO-TT	Native wild-type	Conjugated to tetanus toxoid (TT)	Sabharwal et al. (50)
CRM197 conjugates	Synthetic wild-type of various isomers (6-mer and 12-mer)	Conjugated onto CRM197	Kabanova et al. (22)
GAC-SP0435 conjugate	Native wild-type and mutant	Conjugated to recombinant pneumococcal protein SP_0435 (elongation factor)	van Sorge et al. (17)
ΔGAC-ADI	Native mutant	Conjugated to arginine deiminase (ADI)	Rivera-Hernandez et al. (60)
C5a peptidase-GAC	Synthetic wild-type in various oligomers (3-mer, 6-mer, 9-mer)	Conjugated to C5a peptidase	Zhao et al. (63), Wang et al. (59)
GAC glycoconjugates	Native wild-type	Random conjugation to CRM197, streptolysin O, SpyCEP, or SpyAD	Di Benedetto et al. (64)
Nanoparticle formulation	Synthetic polyrhamnose	Conjugation to gold nanoparticles	Pitirollo et al. (65)
SpyAD-GAC^{PR}	Native mutant	Site-directed conjugation to SpyAD	Gao et al. (61)
Trirhamnosyl-lipopeptide	Synthetic trirhamnose	Conjugation to a self-adjuvanting Ac-PADRE[a]–lipid core	Khatun et al. (62)

[a]Ac-PADRE, acetyl pan-HLA-DR binding epitope.

Instead, GAS serotype specificity is conferred by the hypervariable N-terminal domains of the surface M protein, encoded by more than 200 *emm* gene variants, among which cross-protection is not ensured (56, 57). Against these complexities, the species defining GAC antigen has several hallmarks of an ideal GAS vaccine candidate. GAC is universally conserved, essential for GAS survival, abundant on the bacterial cell surface, and accessible to antibody binding regardless of degree of encapsulation (22). Whereas human sera rarely contain antibodies to multiple M-protein serotypes, IgG antibody against GAC, present in sera from children from diverse geographic areas, promoted opsonophagocytosis of several M-type-specific GAS strains in a titer-dependent manner (33). That said, native GAC has two key limitations as a vaccine antigen: (i) large polysaccharides alone are not sufficiently immunogenic to generate a robust vaccine immune response, and (ii) potential safety concerns due to potential autoreactivity related to ARF/RHD discussed above.

Polysaccharides covalently linked to a carrier, including immunogenic proteins, lipopeptides, or gold nanoparticles, can become effective vaccine antigens, such as the approved polysaccharide capsule protein conjugates found in approved vaccines for *S. pneumoniae*, *Haemophilus influenzae* type B, and *Neisseria meningitidis*. To boost immunogenicity of the native GAC, several studies have employed conjugation methods that generated protective antibody responses in animals (summarized in Table 1). Classic protein carriers employed include modified bacterial toxins such as tetanus toxoid (50, 58) or CRM_{197}, a nontoxic mutant diphtheria toxin (22). Original murine challenge studies using two different M serotype GAS strains showed that immunization with tetanus toxoid-GAC conjugates protected against intranasal colonization and intraperitoneal lethal infection (50). Subsequently, synthetic GAC molecules of various lengths were conjugated to CRM_{197} and used to determine size-dependent immunogenicity—a synthetic dodecasaccharide (equivalent to four trisaccharide repeating units of native GAC) was sufficient to generate antibody titers equivalent to purified native GAC and elicited a protective response in infectious challenge (22). GAC sequences of various sizes were also conjugated to GAS surface protein C5a peptidase and induced IgG1 antibodies that promoted opsonophagocytic killing; immunized mice showed reduced lung injury and mortality following GAS challenge in a pneumonia model (59).

The discovery of the GAS *gac* operon allowed targeted mutagenesis of the bacterium to eliminate the GlcNAc side chain on GAC and subsequent purification of the polyrhamnose backbone (GAC^{PR}) from the resulting mutant (17). GAC^{PR} has been explored as a potential universal GAS vaccine antigen theoretically devoid of the autoimmunity concerns ascribed to the GlcNAc moiety. Using an immunogenic pneumococcal protein as a carrier for wild-type GAC and GAC^{PR}, it was found upon conjugation

that GAC^{PR} was sufficient to generate anti-GAC antibody responses comparable to native GAC (17); the GAC^{PR} conjugated onto arginine deiminase, a streptococcal protein and virulence factor, also induced strong anti-GAC antibody titers in BALB/c mice and afforded partial protection in mouse bacteremia and skin infection models (60). For enhanced immunogenicity and broad coverage of GAS strains, our own recent study (61) examined the use of a multivalent vaccine formulation composed of C5a peptidase, SLO, and GAC^{PR} conjugated to GAS surface protein SpyAD. This use of multiple, highly conserved GAS proteins in addition to GAC^{PR} induced broad immunity to multiple GAS strains of different serotypes as demonstrated by opsonophagocytic killing assays and two different murine models of GAS infection without evidence of cross-reactivity to human heart or brain tissue lysates (61). Finally, a new study successfully elicited anti-GAC antibodies using a synthetic rhamnose-GlcNAc trisaccharide conjugated to a lipopeptide that was engineered to self-assemble into immunogenic, self-adjuvanting lipid-core complexes (62). Opsonic activity resulting from immunized mice showed 75 to 97% protection against four different clinically relevant strains of GAS and proved that GlcNAc was not required for the vaccine antigen to induce protective immunity (62). Collectively, these studies suggest that GAC (wild type or modified to lack GlcNAc) may potentially be sufficient as a universal vaccine antigen for protective immunity against GAS but requires boosted immunogenicity achieved by a variety of different adjuvants or conjugation strategies.

CONCLUSIONS

The Lancefield GAC antigen has played a prominent historical role in medical microbiology and a crucial clinical role in the laboratory diagnosis of GAS. For nearly a century, the abundance, conservation, and universal presence of GAC in human GAS isolates implied an essential function in cell wall structure and bacterial survival. However, it has only been with the recent discovery of the biosynthetic *gac* operon that mechanistic research into GAC assembly and other potential functional roles in host immune evasion and resistance to bactericidal proteins or metals became possible. Development of natural immunity against GAC spurred its potential as a universal GAS vaccine antigen, but this has met with caution due to correlations between its GlcNAc side chain and important autoimmune sequelae of RHD or Sydenham's chorea. Identification and functional understanding of the genetically encoded biosynthetic process and chemical approaches to GAC component synthesis have enabled immunogenicity studies of modified GAC (e.g., GAC^{PR}) that might allay these safety concerns.

A number of unanswered questions remain that can guide future research. What are the roles of conserved genes in the GAC operon to which a biochemical function has yet to be ascribed? What GAS strain-specific differences impact the variable contribution of the GAC GlcNAc side chain to innate immune resistance and virulence? Since GAS strains lacking the GAC GlcNAc are identified on animal passage and retain some measure of experimental virulence, what unique aspect(s) of human infection and immunity explains its universal conservation among human clinical isolates? What are the specific host antigen molecules expressed in heart or brain tissue that could be targets of cross-reactive autoimmunity induced by GAC GlcNAc, and is there an immunogenetic basis to identify the subset of individuals in which such reactions could be of concern? Finally, can antibodies directed against the polyrhamnose core prove sufficient to recognize GAC in all strains, even those expressing thick hyaluronic acid capsules?

Ultimately, should further elucidation of the immunobiology of the eponymous species defining GAC contribute to GAS disease prevention as well as diagnostics, it would indeed be a fitting tribute to the outsize legacy of Rebecca Lancefield.

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REFERENCES

1. Sims Sanyahumbi A, Colquhoun S, Wyber R, Carapetis JR. 2016. Global disease burden of group A *Streptococcus*, p 480–500. *In* Ferretti JJ, Stevens DL, Fischetti VA (ed), *Streptococcus pyogenes*: basic biology to clinical manifestations. University of Oklahoma Health Sciences Center, Oklahoma City, OK. https://www.ncbi.nlm.nih.gov/books/NBK333415/.
2. Walker MJ, Barnett TC, McArthur JD, Cole JN, Gillen CM, Henningham A, Sriprakash KS, Sanderson-Smith ML, Nizet V. 2014. Disease manifestations and pathogenic mechanisms of group A *Streptococcus*. Clin Microbiol Rev 27:264–301. https://doi.org/10.1128/CMR.00101-13.
3. Banerjee S, Ford C. 2018. Rapid tests for the diagnosis of group A streptococcal infection: a review of diagnostic test accuracy, clinical utility, safety, and cost-effectiveness. Canadian Agency for Drugs and Technologies in Health, Ottawa, Canada.
4. Cunningham MW. 2014. Rheumatic fever, autoimmunity, and molecular mimicry: the streptococcal connection. Int Rev Immunol 33:314–329. https://doi.org/10.3109/08830185.2014.917411.
5. Lancefield RC. 1928. The antigenic complex of *Streptococcus haemolyticus*. J Exp Med 47:469–480. https://doi.org/10.1084/jem.47.3.469.
6. Lancefield RC. 1933. A serological differentiation of human and other groups of hemolytic streptococci. J Exp Med 57:571–595. https://doi.org/10.1084/jem.57.4.571.
7. Lue YA, Howit IP, Ellner PD. 1978. Rapid grouping of beta-hemolytic streptococci by latex agglutination. J Clin Microbiol 8:326–328. https://doi.org/10.1128/jcm.8.3.326-328.1978.
8. Shulman ST, Ayoub EM. 1983. Serologic cross-reactions among streptococcal group A, A-variant, and C polysaccharides. Clin Immunol Immunopathol 28:229–242. https://doi.org/10.1016/0090-1229(83)90157-5.
9. McCarty M. 1956. Variation in the group-specific carbohydrate of group A streptococci. II. Studies on the chemical basis for serological specificity of the carbohydrates. J Exp Med 104:629–643. https://doi.org/10.1084/jem.104.5.629.
10. Lawson PA, Foster G, Falsen E, Markopoulos SJ, Collins MD. 2005. *Streptococcus castoreus* sp. nov., isolated from a beaver (*Castor fiber*). Int J Syst Evol Microbiol 55:843–846. https://doi.org/10.1099/ijs.0.63433-0.
11. McCarty M. 1952. The lysis of group A hemolytic streptococci by extracellular enzymes of *Streptomyces albus*. J Exp Med 96:569–580. https://doi.org/10.1084/jem.96.6.569.
12. Mistou M-Y, Sutcliffe IC, van Sorge NM. 2016. Bacterial glycobiology: rhamnose-containing cell wall polysaccharides in Gram-positive bacteria. FEMS Microbiol Rev 40:464–479. https://doi.org/10.1093/femsre/fuw006.
13. Heymann H, Manniello JM, Barkulis SS. 1967. Structure of streptococcal cell walls. V. Phosphate esters in the walls of group A *Streptococcus pyogenes*. Biochem Biophys Res Commun 26:486–491. https://doi.org/10.1016/0006-291X(67)90574-8.
14. Kreis UC, Varma V, Pinto BM. 1995. Application of two-dimensional NMR spectroscopy and molecular dynamics simulations to the conformational analysis of oligosaccharides corresponding to the cell-wall polysaccharide of *Streptococcus* group A. Int J Biol Macromol 17:117–130. https://doi.org/10.1016/0141-8130(95)92678-j.
15. Edgar RJ, van Hensbergen VP, Ruda A, Turner AG, Deng P, Le Breton Y, El-Sayed NM, Belew AT, McIver KS, McEwan AG, Morris AJ, Lambeau G, Walker MJ, Rush JS, Korotkov KV, Widmalm G, van Sorge NM, Korotkova N. 2019. Discovery of glycerol phosphate modification on streptococcal rhamnose polysaccharides. Nat Chem Biol 15:463–471. https://doi.org/10.1038/s41589-019-0251-4.
16. Davies MR, McIntyre L, Mutreja A, Lacey JA, Lees JA, Towers RJ, Duchêne S, Smeesters PR, Frost HR, Price DJ, Holden MTG, David S, Giffard PM, Worthing KA, Seale AC, Berkley JA, Harris SR, Rivera-Hernandez T, Berking O, Cork AJ, Torres RSLA, Lithgow T, Strugnell RA, Bergmann R, Nitsche-Schmitz P, Chhatwal GS, Bentley SD, Fraser JD, Moreland NJ, Carapetis JR, Steer AC, Parkhill J, Saul A, Williamson DA, Currie BJ, Tong SYC, Dougan G, Walker MJ. 2019. Atlas of group A streptococcal vaccine candidates compiled using large-scale comparative genomics. Nat Genet 51:1035–1043. https://doi.org/10.1038/s41588-019-0417-8.
17. van Sorge NM, Cole JN, Kuipers K, Henningham A, Aziz RK, Kasirer-Friede A, Lin L, Berends ETM, Davies MR, Dougan G, Zhang F, Dahesh S, Shaw L, Gin J, Cunningham M, Merriman JA, Hütter J, Lepenies B, Rooijakkers SHM, Malley R, Walker MJ, Shattil SJ, Schlievert PM, Choudhury B, Nizet V. 2014. The classical Lancefield antigen of group A *Streptococcus* is a virulence determinant with implications for vaccine design. Cell Host Microbe 15:729–740. https://doi.org/10.1016/j.chom.2014.05.009.
18. Zorzoli A, Meyer BH, Adair E, Torgov VI, Veselovsky VV, Danilov LL, Uhrin D, Dorfmueller HC. 2019. Group A, B, C, and G Streptococcus Lancefield antigen biosynthesis is initiated by a conserved α-d-GlcNAc-β-1,4-l-rhamnosyltransferase. J Biol Chem 294:15237–15256. https://doi.org/10.1074/jbc.RA119.009894.
19. Henningham A, Davies MR, Uchiyama S, van Sorge NM, Lund S, Chen KT, Walker MJ, Cole JN, Nizet V. 2018. Virulence role of the GlcNAc side chain of the Lancefield cell wall carbohydrate antigen in non-M1-serotype group A Streptococcus. mBio 9:e02294-17. https://doi.org/10.1128/mBio.02294-17.
20. van der Beek SL, Le Breton Y, Ferenbach AT, Chapman RN, van Aalten DMF, Navratilova I, Boons G-J, McIver KS, van Sorge NM, Dorfmueller HC. 2015. GacA is essential for Group A *Streptococcus* and defines a new class of monomeric dTDP-4-dehydrorhamnose reductases (RmlD). Mol Microbiol 98:946–962. https://doi.org/10.1111/mmi.13169.
21. Rush JS, Edgar RJ, Deng P, Chen J, Zhu H, van Sorge NM, Morris AJ, Korotkov KV, Korotkova N. 2017. The molecular mechanism of N-acetylglucosamine side-chain attachment to the Lancefield group A carbohydrate in *Streptococcus pyogenes*. J Biol Chem 292:19441–19457. https://doi.org/10.1074/jbc.M117.815910.
22. Kabanova A, Margarit I, Berti F, Romano MR, Grandi G, Bensi G, Chiarot E, Proietti D, Swennen E, Cappelletti E, Fontani P, Casini D, Adamo R, Pinto V, Skibinski D, Capo S, Buffi G, Gallotta M, Christ WJ, Campbell AS, Pena J, Seeberger PH, Rappuoli R, Costantino P. 2010. Evaluation of a group A *Streptococcus* synthetic oligosaccharide as vaccine candidate. Vaccine 29:104–114. https://doi.org/10.1016/j.vaccine.2010.09.018.
23. Hamada S, Okahashi N, Yamamoto T, Morisaki I, Michalek SM, McGhee JR. 1983. Isolation and immunochemical characterization of carbohydrate antigens prepared from group A *Streptococcus pyogenes*. Zentralbl Bakteriol Mikrobiol Hyg A 256:37–48.
24. Stuike-Prill R, Pinto BM. 1995. Conformational analysis of oligosaccharides corresponding to the cell-wall polysaccharide of the *Streptococcus* group A by Metropolis Monte Carlo simulations. Carbohydr Res 279:59–73. https://doi.org/10.1016/0008-6215(95)00279-0.
25. Caliot É, Dramsi S, Chapot-Chartier M-P, Courtin P, Kulakauskas S, Péchoux C, Trieu-Cuot P, Mistou M-Y. 2012. Role of the Group B antigen of *Streptococcus agalactiae*: a peptidoglycan-anchored polysaccharide involved in cell wall biogenesis. PLoS Pathog 8:e1002756. https://doi.org/10.1371/journal.ppat.1002756.
26. Brown S, Santa Maria JP, Jr, Walker S. 2013. Wall teichoic acids of gram-positive bacteria. Annu Rev Microbiol 67:313–336. https://doi.org/10.1146/annurev-micro-092412-155620.
27. McCarty M, Lancefield RC. 1955. Variation in the group-specific carbohydrate of group A streptococci. I. Immunochemical studies on the carbohydrates of variant strains. J Exp Med 102:11–28. https://doi.org/10.1084/jem.102.1.11.
28. van Hensbergen VP, Movert E, de Maat V, Lüchtenborg C, Le Breton Y, Lambeau G, Payré C, Henningham A, Nizet V, van Strijp JAG, Brügger B, Carlsson F, McIver KS, van Sorge NM. 2018. Streptococcal Lancefield polysaccharides are critical cell wall determinants for human Group IIA secreted phospholipase A2 to exert its bactericidal effects. PLoS Pathog 14:e1007348. https://doi.org/10.1371/journal.ppat.1007348.
29. Martinez V, Ingwers M, Smith J, Glushka J, Yang T, Bar-Peled M. 2012. Biosynthesis of UDP-4-keto-6-deoxyglucose and UDP-rhamnose in pathogenic fungi *Magnaporthe grisea* and *Botryotinia fuckeliana*. J Biol Chem 287:879–892. https://doi.org/10.1074/jbc.M111.287367.
30. Singh BN, Hayes GR, Lucas JJ, Sommer U, Viseux N, Mirgorodskaya E, Trifonova RT, Sassi RRS, Costello CE, Fichorova RN. 2009. Structural details and composition of *Trichomonas vaginalis* lipophosphoglycan in relevance to the epithelial immune function. Glycoconj J 26:3–17. https://doi.org/10.1007/s10719-008-9157-1.
31. van Vliet SJ, Steeghs L, Bruijns SCM, Vaezirad MM, Snijders Blok C, Arenas Busto JA, Deken M, van Putten JPM, van Kooyk Y. 2009. Variation of *Neisseria gonorrhoeae* lipooligosaccharide directs dendritic cell-induced T helper responses. PLoS Pathog 5:e1000625. https://doi.org/10.1371/journal.ppat.1000625.
32. Zimmerman RA, Auernheimer AH, Taranta A. 1971. Precipitating antibody to group A streptococcal polysaccharide in humans. J Immunol 107:832–841.
33. Salvadori LG, Blake MS, McCarty M, Tai JY, Zabriskie JB. 1995. Group A *Streptococcus*-liposome ELISA antibody titers to group A polysaccharide

and opsonophagocytic capabilities of the antibodies. J Infect Dis 171:593–600. https://doi.org/10.1093/infdis/171.3.593.
34. Chetty C, Klapper DG, Schwab JH. 1982. Soluble peptidoglycan-polysaccharide fragments of the bacterial cell wall induce acute inflammation. Infect Immun 38:1010–1019. https://doi.org/10.1128/iai.38.3.1010-1019.1982.
35. McCarty M. 1969. The streptococcal cell wall. Harvey Society Lectures. Rockefeller University Digital Commons https://digitalcommons.rockefeller.edu/harvey-lectures/42/.
36. Esposito S, Bianchini S, Fastiggi M, Fumagalli M, Andreozzi L, Rigante D. 2015. Geoepidemiological hints about *Streptococcus pyogenes* strains in relationship with acute rheumatic fever. Autoimmun Rev 14:616–621. https://doi.org/10.1016/j.autrev.2015.03.001.
37. Punukollu M, Mushet N, Linney M, Hennessy C, Morton M. 2016. Neuropsychiatric manifestations of Sydenham's chorea: a systematic review. Dev Med Child Neurol 58:16–28. https://doi.org/10.1111/dmcn.12786.
38. Cunningham MW. 2016. Post-streptococcal autoimmune sequelae: rheumatic fever and beyond. *In* Ferretti JJ, Stevens DL, Fischetti VA (ed), *Streptococcus pyogenes*: basic biology to clinical manifestations. University of Oklahoma Health Sciences Center, Oklahoma City, OK. https://www.ncbi.nlm.nih.gov/books/NBK333434/.
39. Bright PD, Mayosi BM, Martin WJ. 2016. An immunological perspective on rheumatic heart disease pathogenesis: more questions than answers. Heart 102:1527–1532. https://doi.org/10.1136/heartjnl-2015-309188.
40. Burke RJ, Chang C. 2014. Diagnostic criteria of acute rheumatic fever. Autoimmun Rev 13:503–507. https://doi.org/10.1016/j.autrev.2014.01.036.
41. Goldstein I, Rebeyrotte P, Parlebas J, Halpern B. 1968. Isolation from heart valves of glycopeptides which share immunological properties with *Streptococcus haemolyticus* group A polysaccharides. Nature 219:866–868. https://doi.org/10.1038/219866a0.
42. Galvin JE, Hemric ME, Ward K, Cunningham MW. 2000. Cytotoxic mAb from rheumatic carditis recognizes heart valves and laminin. J Clin Invest 106:217–224. https://doi.org/10.1172/JCI7132.
43. Cunningham MW. 2000. Pathogenesis of group A streptococcal infections. Clin Microbiol Rev 13:470–511. https://doi.org/10.1128/CMR.13.3.470.
44. Shikhman AR, Greenspan NS, Cunningham MW. 1994. Cytokeratin peptide SFGSGFGGGY mimics N-acetyl-beta-D-glucosamine in reaction with antibodies and lectins and induces in vivo anti-carbohydrate antibody response. J Immunol 153:5593–5606.
45. Adderson EE, Shikhman AR, Ward KE, Cunningham MW. 1998. Molecular analysis of polyreactive monoclonal antibodies from rheumatic carditis: human anti-N-acetylglucosamine/anti-myosin antibody V region genes. J Immunol 161:2020–2031.
46. Kirvan CA, Swedo SE, Heuser JS, Cunningham MW. 2003. Mimicry and autoantibody-mediated neuronal cell signaling in Sydenham chorea. Nat Med 9:914–920. https://doi.org/10.1038/nm892.
47. Martins TB, Hoffman JL, Augustine NH, Phansalkar AR, Fischetti VA, Zabriskie JB, Cleary PP, Musser JM, Veasy LG, Hill HR. 2008. Comprehensive analysis of antibody responses to streptococcal and tissue antigens in patients with acute rheumatic fever. Int Immunol 20:445–452. https://doi.org/10.1093/intimm/dxn004.
48. Dudding BA, Ayoub EM. 1968. Persistence of streptococcal group A antibody in patients with rheumatic valvular disease. J Exp Med 128:1081–1098. https://doi.org/10.1084/jem.128.5.1081.
49. Dooley LM, Ahmad TB, Pandey M, Good MF, Kotiw M. 2021. Rheumatic heart disease: a review of the current status of global research activity. Autoimmun Rev 20:102740. https://doi.org/10.1016/j.autrev.2020.102740.
50. Sabharwal H, Michon F, Nelson D, Dong W, Fuchs K, Manjarrez RC, Sarkar A, Uitz C, Viteri-Jackson A, Suarez RSR, Blake M, Zabriskie JB. 2006. Group A *Streptococcus* (GAS) carbohydrate as an immunogen for protection against GAS infection. J Infect Dis 193:129–135. https://doi.org/10.1086/498618.
51. Massell BF, Michael JG, Amezcua J, Siner M. 1968. Secondary and apparent primary antibody responses after group A streptococcal vaccination of 21 children. Appl Microbiol 16:509–518. https://doi.org/10.1128/am.16.3.509-518.1968.
52. Bisno AL, Rubin FA, Cleary PP, Dale JB, National Institute of Allergy and Infectious Diseases. 2005. Prospects for a group A streptococcal vaccine: rationale, feasibility, and obstacles—report of a National Institute of Allergy and Infectious Diseases workshop. Clin Infect Dis 41:1150–1156. https://doi.org/10.1086/444505.
53. Martin WJ, Steer AC, Smeesters PR, Keeble J, Inouye M, Carapetis J, Wicks IP. 2015. Post-infectious group A streptococcal autoimmune syndromes and the heart. Autoimmun Rev 14:710–725. https://doi.org/10.1016/j.autrev.2015.04.005.
54. Sadarangani M. 2018. Protection against invasive infections in children caused by encapsulated bacteria. Front Immunol 9:2674. https://doi.org/10.3389/fimmu.2018.02674.
55. Wessels MR. 2016. Cell wall and surface molecules: capsule. *In* Ferretti JJ, Stevens DL, Fischetti VA (ed), *Streptococcus pyogenes*: basic biology to clinical manifestations. University of Oklahoma Health Sciences Center, Oklahoma City, OK. https://www.ncbi.nlm.nih.gov/books/NBK333410/.
56. Steer AC, Law I, Matatolu L, Beall BW, Carapetis JR. 2009. Global emm type distribution of group A streptococci: systematic review and implications for vaccine development. Lancet Infect Dis 9:611–616. https://doi.org/10.1016/S1473-3099(09)70178-1.
57. Sanderson-Smith M, De Oliveira DMP, Guglielmini J, McMillan DJ, Vu T, Holien JK, Henningham A, Steer AC, Bessen DE, Dale JB, Curtis N, Beall BW, Walker MJ, Parker MW, Carapetis JR, Van Melderen L, Sriprakash KS, Smeesters PR, M Protein Study Group. 2014. A systematic and functional classification of *Streptococcus pyogenes* that serves as a new tool for molecular typing and vaccine development. J Infect Dis 210:1325–1338. https://doi.org/10.1093/infdis/jiu260.
58. Sabharwal H, Nelson D, Michon F, Zabriskie J. 2006. The streptococcal group A carbohydrate protects against nasal colonization with group A streptococci in mice. Int Congr Ser 1289:329–331. https://doi.org/10.1016/j.ics.2005.11.002.
59. Wang S, Zhao Y, Wang G, Feng S, Guo Z, Gu G. 2020. Group A Streptococcus cell wall oligosaccharide-streptococcal C5a peptidase conjugates as effective antibacterial vaccines. ACS Infect Dis 6:281–290. https://doi.org/10.1021/acsinfecdis.9b00347.
60. Rivera-Hernandez T, Pandey M, Henningham A, Cole J, Choudhury B, Cork AJ, Gillen CM, Ghaffar KA, West NP, Silvestri G, Good MF, Moyle PM, Toth I, Nizet V, Batzloff MR, Walker MJ. 2016. Differing efficacies of lead group A streptococcal vaccine candidates and full-length M protein in cutaneous and invasive disease models. mBio 7:e00618-16. https://doi.org/10.1128/mBio.00618-16.
61. Gao NJ, Uchiyama S, Pill L, Dahesh S, Olson J, Bautista L, Maroju S, Berges A, Liu JZ, Zurich RH, van Sorge N, Fairman J, Kapoor N, Nizet V. 2021. Site-specific conjugation of cell wall polyrhamnose to protein SpyAD envisioning a safe universal group A streptococcal vaccine. Infect Microb Dis 3:87–100. https://doi.org/10.1097/IM9.0000000000000044.
62. Khatun F, Dai CC, Rivera-Hernandez T, Hussein WM, Khalil ZG, Capon RJ, Toth I, Stephenson RJ. 2021. Immunogenicity assessment of cell wall carbohydrates of group A *Streptococcus* via self-adjuvanted glyco-lipopeptides. ACS Infect Dis 7:390–405. https://doi.org/10.1021/acsinfecdis.0c00722.
63. Zhao Y, Wang S, Wang G, Li H, Guo Z, Gu G. 2019. Synthesis and immunological studies of group A *Streptococcus* cell-wall oligosaccharide–streptococcal C5a peptidase conjugates as bivalent vaccines. Org Chem Front 6:3589–3596. https://doi.org/10.1039/C9QO00651F.
64. Di Benedetto R, Mancini F, Carducci M, Gasperini G, Moriel DG, Saul A, Necchi F, Rappuoli R, Micoli F. 2020. Rational design of a glycoconjugate vaccine against group A *Streptococcus*. Int J Mol Sci 21:8558. https://doi.org/10.3390/ijms21228558.
65. Pitirollo O, Micoli F, Necchi F, Mancini F, Carducci M, Adamo R, Evangelisti C, Morelli L, Polito L, Lay L. 2020. Gold nanoparticles morphology does not affect the multivalent presentation and antibody recognition of group A Streptococcus synthetic oligorhamnans. Bioorg Chem 99:103815. https://doi.org/10.1016/j.bioorg.2020.103815.