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Site-Specific Incorporation of Three Toll-Like Receptor 2 Targeting Adjuvants into Semisynthetic, Molecularly Defined Nanoparticles: Application to Group A Streptococcal Vaccines

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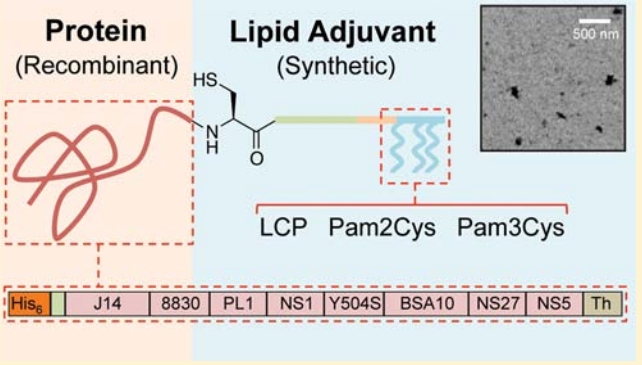
Supporting Information

ABSTRACT: Subunit vaccines offer a means to produce safer, more defined vaccines compared to traditional whole microorganism approaches. Subunit antigens, however, exhibit weak immunity, which is normally overcome through coadministration with adjuvants. Enhanced vaccine properties (e.g., improved potency) can be obtained by linking antigen and adjuvant, as observed for synthetic peptide antigens and Toll-like receptor 2 (TLR2) ligands. As few protective peptide antigens have been reported, compared to protein antigens, we sought to extend the utility of this approach to recombinant proteins, while ensuring that conjugation reactions yielded a single, molecularly defined product. Herein we describe the development and optimization of techniques that enable the efficient, site-specific attachment of three synthetic TLR2 ligands (lipid core peptide (LCP), Pam2Cys, and Pam3Cys) onto engineered protein antigens, permitting the selection of optimal TLR2 agonists during the vaccine development process. Using this approach, broadly protective (J14) and population targeted (seven M protein N-terminal antigens) multiantigenic vaccines against group A streptococcus (GAS; *Streptococcus pyogenes*) were produced and observed to self-assemble in PBS to yield nanoparticules (69, 101, and 123 nm, respectively). All nanoparticle formulations exhibited self-adjuvanting properties, with rapid, persistent, antigen-specific IgG antibody responses elicited toward each antigen in subcutaneously immunized C57BL/6J mice. These antibodies were demonstrated to strongly bind to the cell surface of five GAS serotypes that are not represented by vaccine M protein N-terminal antigens, are among the top 20 circulating strains in developed countries, and are associated with clinical disease, suggesting that these vaccines may elicit broadly protective immune responses.



INTRODUCTION

Traditional whole microorganism vaccines have proven to be a safe and effective means to prevent and control infectious diseases.[1] Safety concerns, however, prevent or limit their use for producing vaccines against many clinically important microorganisms (e.g., human immunodeficiency virus (HIV)).[1,2] The identification of microbial components against which protective immune responses can be mounted represents an exciting means to produce safer, more defined vaccines in these cases. Simplifying vaccine compositions to individual "subunits", however, results in a significant reduction in vaccine potency, which must be overcome in the majority of cases through addition of powerful immunostimulatory adjuvants, of which few have been screened for safety in humans, or are components of vaccines marketed for human use.[1]

A variety of experimental adjuvants have been identified, which vary in their mechanism of action, safety, potency, and capacity to elicit different types of immune responses.[3,4] Toll-like receptor 2 (TLR2) ligands represent a promising class of adjuvants for human use, with clinical trials suggesting such adjuvants are well tolerated, with few or no side effects observed.[5−7] Their structures generally include two or three lipid chains (e.g., lipid core peptides (LCPs), bacterial lipopeptides (MALP-2 and Braun's lipoprotein), or their synthetic analogues dipalmitoyl-*S*-glyceryl cysteine (Pam2Cys) and tripalmitoyl-*S*-glycerol cysteine (Pam3Cys)),[3] with engineered single-chain TLR2 ligands also identified.[8−10]

Administration of TLR2 ligands as a mixture or conjugate with antigens has been demonstrated to elicit antigen-specific cellular- and antibody-mediated immune responses, with the

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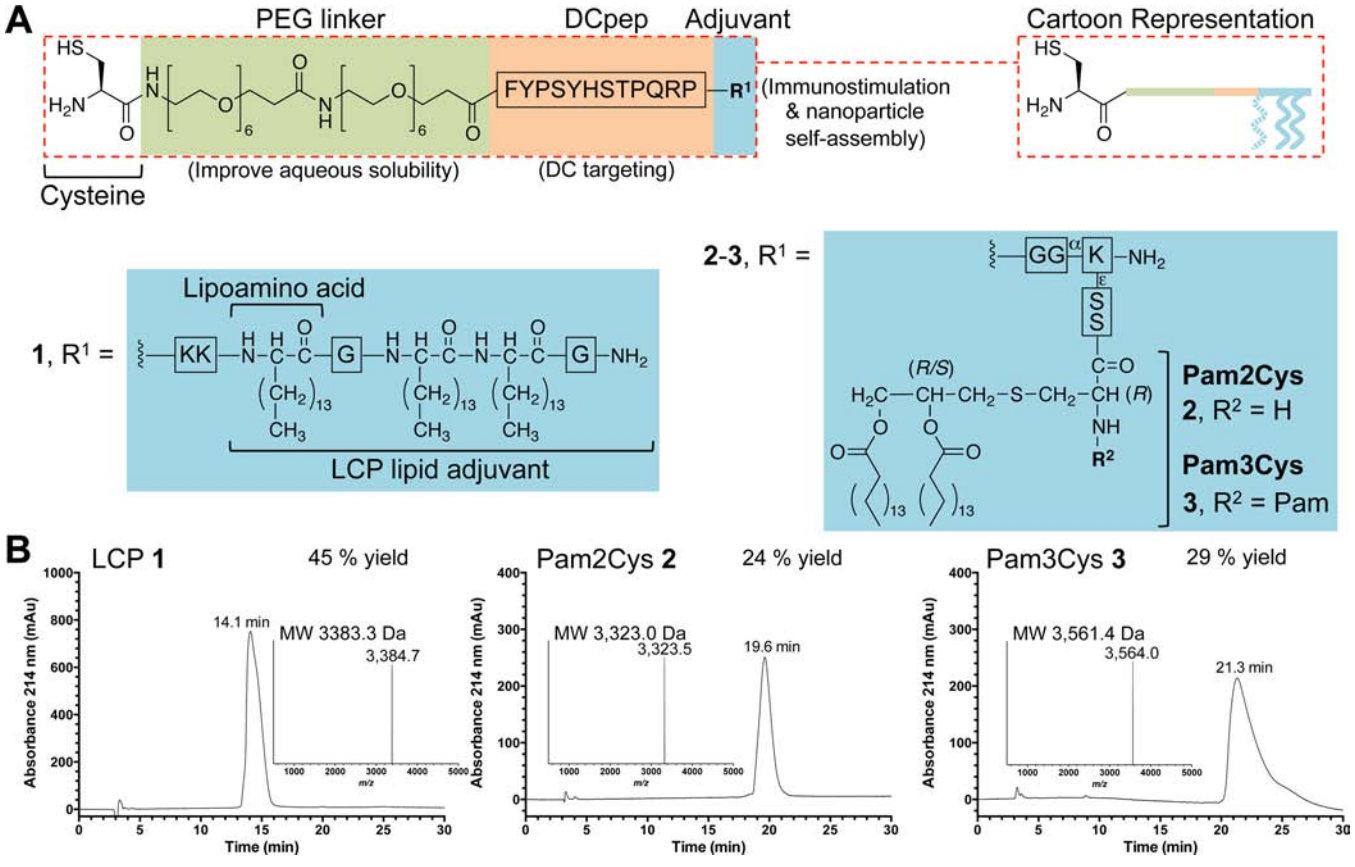

Figure 1. Structure of lipid adjuvant peptides. (A) Lipid adjuvant peptide structures indicating each component and their function. The structures of the LCP, Pam2Cys, and Pam3Cys lipid adjuvants are indicated in R^1. (B) RP-HPLC and deconvoluted ESI-MS data for lipid adjuvant peptides **1**–**3**. Amino acids are written in single letter notation and boxed. Pam, palmitoyl group.

potential for oral or nasal dosing and mucosal immunity.[3,11] TLR2 ligands can therefore be applied to many vaccine applications, including the exciting prospect of immunotherapy to treat cancers, where strong tumor-specific cytotoxic lymphocyte (CTL) responses are necessary.[3,11] TLR2 ligand–antigen conjugates also afford significant increases in vaccine potency compared to mixtures, providing a dose sparing effect that can reduce vaccination costs as well as improve vaccine safety, and in some cases may be necessary to generate effective immunity.[11] Conjugates can also self-assemble through lipid–lipid interactions into particulate antigens,[12,13] improving antigen stability and circulation time *in vivo*, as well as increasing vaccine uptake by antigen presenting cells (APCs), directly activating B cells through the repetitive display of antigen on the particle surface,[14] and potentially targeting antigen to the lymph in a size dependent manner.[14,15]

Site-specific conjugation of TLR2 ligands onto peptide[16,17] and carbohydrate[18] antigens can be readily achieved through synthetic means. The development of peptide vaccines however is limited to cases where broadly protective peptide antigens have been identified, and where the majority of an immunized population can generate protective immunity against the antigen.[3] This is not always the case, with size limits imposed by the synthetic process[19] limiting the amount of immunological information that can be included. Many of these problems can be overcome by techniques that enable site-specific conjugation of TLR2 ligands onto protein antigens. Proteins represent the majority of reported protective antigens, include more protective epitopes than peptides, can be engineered by genetic or chemical means, may be overexpressed on an industrial scale, and permit the correct presentation of folded epitopes.[3] The capacity to engineer genes featuring multiple peptide antigens linked end-to-end (a "polytope" approach)[13,20] also provides a means to ensure the number and order of antigen incorporation, affording advantages over synthetic polymer approaches.[21]

Few techniques that enable the covalent site-specific conjugation of TLR2 ligands onto recombinant antigens have been described.[13,22,23] Many of these approaches rely on nonspecific conjugation chemistries, which generate a mixture of products containing differing linkage sites and numbers of attached TLR2 ligands,[22] or the direct expression of lipoproteins containing a mixture of lipid species.[23] The resulting products are difficult to define, may demonstrate problems with batch-to-batch reproducibility, and therefore could be subject to regulatory issues. Based on the potential advantages afforded by conjugation of TLR2 ligands to protein antigens, we sought herein to optimize techniques to enable the efficient, stable, and site-specific conjugation of three different TLR2 ligands (LCP, Pam2Cys, and Pam3Cys) onto recombinant protein antigens in order to produce a self-adjuvanting, multicomponent, semi-synthetic nanoparticulate vaccine technology platform. Such an approach would generate products with a single defined point of adjuvant attachment, and allow for the assessment and subsequent selection of the most effective TLR2 ligand during the vaccine development process.

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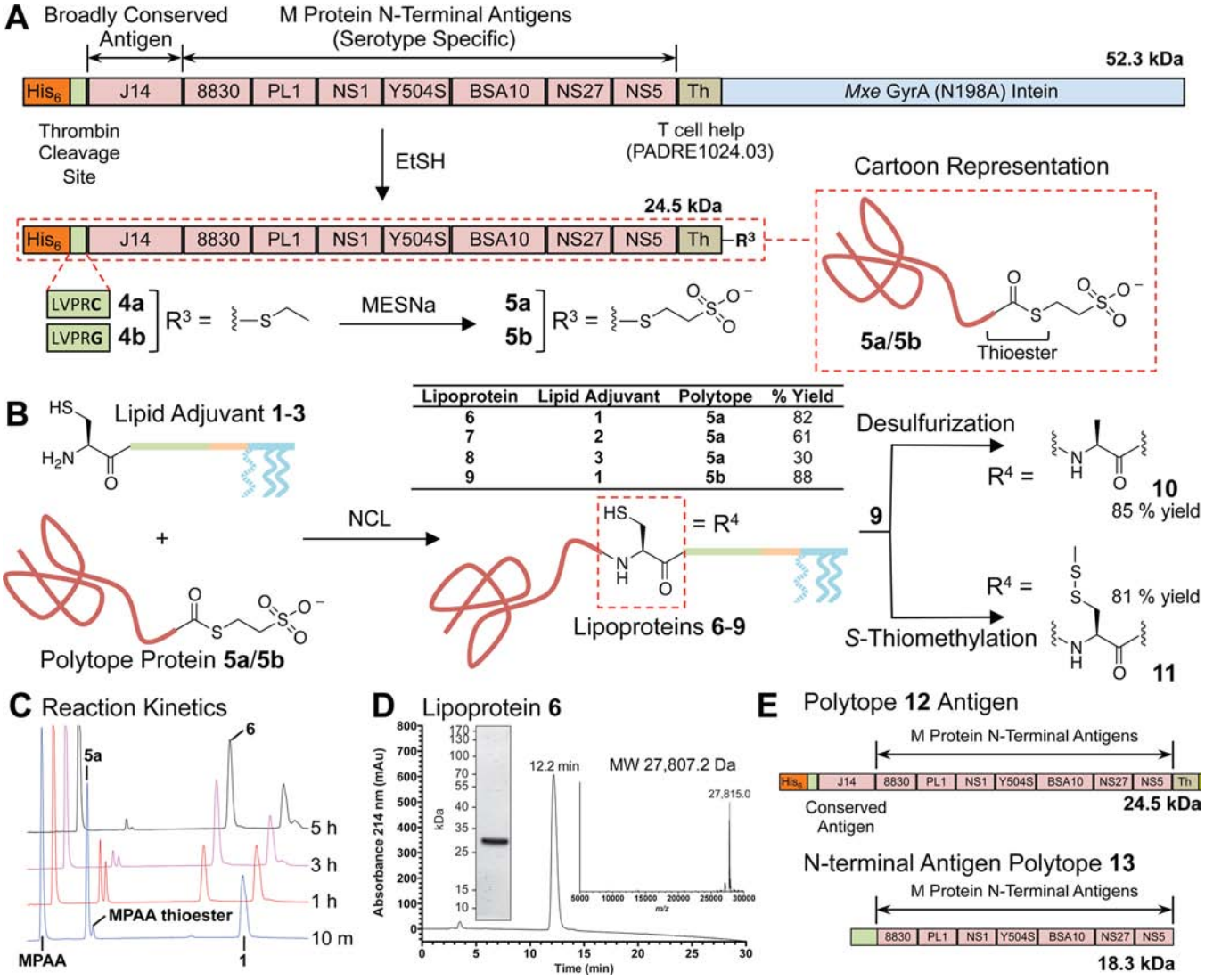

Lipoprotein	Lipid Adjuvant	Polytope	% Yield
6	1	5a	82
7	2	5a	61
8	3	5a	30
9	1	5b	88

Figure 2. Thioester proteins **4**–**5a**/**b** and their ligation to lipid adjuvant peptides **1**–**3**. (A) Scheme depicting the conversion of polytope *Mxe* GyrA (N198A) intein fusion proteins to C-terminal thioester proteins **5a**/**5b**. (B) Conjugation of polytope thioester proteins **5a**/**5b** and lipid adjuvant peptides **1**–**3**, with subsequent desulfuration or *S*-thiomethylation of the ligation site cysteine in **9** depicted. (C) Reaction kinetics for the conjugation of lipid adjuvant **1** and polytope thioester protein **5a** monitored by RP-HPLC. (D) RP-HPLC chromatogram, reconstructed mass spectra, and SDS-PAGE data for purified lipoprotein **6**. (E) Structure of the polytope **12** vaccine antigen and the N-terminal antigen polytope **13** used for ELISA.

To demonstrate the utility of this approach, each TLR2 agonist was conjugated to an engineered recombinant polytope antigen[13] to generate a library of vaccines targeting group A streptococcus (GAS; *Streptococcus pyogenes*). The antigen was designed to incorporate a broadly protective antigen (J14), as well as seven population-targeted antigens.[13] This approach offers protection against common clinically relevant GAS strains that circulate within a specific geographical region, in addition to other circulating strains. Such a vaccine is of clinical importance as GAS is reported to be among the top 10 global causes of infectious mortality in humans, and is responsible for diseases ranging from sore throat to severe, invasive infections (e.g., the "flesh-eating" disease), and kidney and heart damage, with no safe vaccine currently marketed.[24]

Herein, we compare the library of GAS lipoprotein vaccines for their ease of synthesis, capacity to elicit high titer antigen-specific IgG antibodies against each antigen in the polytope sequence, rate of antibody induction, antibody persistence, formation of nanoparticules, and antibody binding to a range of clinically relevant GAS strains, in order to determine the most favorable TLR2 ligand for this GAS vaccine approach.

■ RESULTS AND DISCUSSION

The aim of this work was to produce an optimized multicomponent platform to enable the efficient production of all-in-one nanoparticulate self-adjuvanting semisynthetic vaccines. The platform was optimized for the development of vaccines targeting GAS infection, but could be readily modified to develop vaccines against other pathogens. To achieve this goal, the platform was designed to incorporate (see Figures 1A and 2A): (1) lipid-based TLR2 agonists (LCP **1**, Pam2Cys **2**, or Pam3Cys **3**) to provide immunostimulatory activity, the capacity to form nanoparticles through lipid–lipid interactions,[13] and the possibility for "needle-free" nasal administration[25−27] in the future; (2) known dendritic cell (DC) targeting moieties (though DC targeting peptides (DCpep)[28]

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or TLR2 binding of lipid adjuvants[11]) to potentially improve vaccine potency; (3) a source of promiscuous T-cell help (PADRE1024.03[29]), capable of binding many human major histocompatibility complex (MHC) class II molecules, which may improve the proportion of the population that respond to vaccination, and aid the production of immunological memory; and (4) a reactive handle to permit the site-specific and covalent conjugation of all vaccine components so as to generate a single chemically defined product. Vaccination with this system colocalizes adjuvant and antigen, thereby targeting all vaccine components to the same cell (e.g., DCs through TLR2 targeting and the DC targeting peptide), increasing antigen uptake and T cell stimulation, which in turn may increase the potency of antigen-specific antibody- and cellular-mediated immune responses.[11]

Three different lipid adjuvants (**1**−**3**; Figure 1A), that are known to target TLR2,[30−32] were assessed for their capacity to stimulate a potent systemic antibody mediated immune response toward an engineered GAS polytope antigen.[13] These adjuvants included the synthetic LCP system, and the synthetic bacterial lipopeptide analogues Pam2Cys and Pam3Cys.[3] Each of these adjuvants provides immunostimulatory activity toward attached antigens through TLR2,[30−32] with Pam2Cys and Pam3Cys signaling through heterodimers with TLR6[31] or TLR1,[32] respectively. In comparison, LCP signaling through heterodimers has not been investigated. In addition to providing adjuvant activity, the attachment of lipid adjuvants also promotes the formation of nanoparticles through lipid−lipid interactions,[12,13] which offers the potential for improved antigen uptake by antigen presenting cells (APCs), direct stimulation of B cells through repetitive antigen display, and targeting of antigen to the draining lymph nodes in a size-dependent manner.[14,15]

We have assessed maleimide conjugation as a means for the site-specific conjugation of synthetic peptides, incorporating an LCP adjuvant, onto recombinant protein antigens.[13] Maleimides were readily incorporated into synthetic peptides, and provided rapid, convenient, and high-yielding site-specific reactions with protein antigens that were engineered to incorporate a single C-terminal cysteine residue. Despite these favorable characteristics, maleimide conjugation generates at least two products, as the reaction generates a new chiral center, with the succinimide ring that is formed also sensitive to hydrolysis.[33−35] In addition, site-specific conjugation reactions cannot be guaranteed where proteins contain more than one cysteine, with maleimides also displaying reactivity toward functional groups other than thiols (e.g., amines), which is significantly accelerated as the pH is raised above 7.5.[35] Appropriate quality control measures are therefore necessary to ensure that maleimide conjugation yields the desired product.

Where bioconjugation reactions yield several products, each product may display different therapeutic activities when administered to humans. It is therefore desirable that conjugation reactions produce a defined linkage chemistry and site of conjugation between the adjuvant and antigen. Further, the selected chemistry should not require extensive protein manipulation to enable incorporation of a suitable reactive handle. To fulfill these requirements, and allow for site-specific adjuvant conjugation where proteins contain more than one cysteine, we selected expressed protein ligation (EPL)[36,37] as a means to overcome these problems. This technique extends the principles of native chemical ligation (NCL)[38] to enable the use of recombinant proteins as one (or both) of the reaction components. Successful NCL requires two unprotected polypeptides, with one containing a C-terminal thioester and the other an N-terminal cysteine. Mixing these two species together under neutral aqueous conditions results in their conjugation through a "native" peptide bond.[38]

Lipid Adjuvant Peptides 1−3 Were Synthesized by Solid Phase Peptide Synthesis (SPPS). To allow for the C-terminal attachment of lipid adjuvants onto recombinant protein antigens using NCL, the lipid adjuvant peptides (**1**−**3**) were synthesized by Fmoc (9-fluorenylmethyloxycarbonyl)-SPPS to include an N-terminal cysteine (Figure 1A). The lipid adjuvants (LCP, Pam2Cys, or Pam3Cys) were incorporated at the C-terminus of the peptides, with a poly(ethylene glycol) (PEG) linker and a peptide that has the potential to target dendritic cells (DCpep[28]) incorporated internally (Figure 1A). The PEG linker was necessary to improve the solubility of the hydrophobic lipid adjuvants under the aqueous conditions required for NCL. Where a PEG linker was not included, no reactivity was observed under NCL conditions (data not shown). The DCpep peptide also improved the aqueous solubility of each lipid adjuvant peptide, and has previously been demonstrated to improve the immune responses toward attached polypeptide antigens.[13]

The synthesis of LCP lipid adjuvant **1** (Figure 1A) could be easily achieved using standard SPPS conditions and three copies of a Dde (1-(4,4-dimethyl-2,6-dioxocyclohexylidene)-ethyl)-protected 2-aminohexadecanoic acid lipoamino acid building block (Dde-C16-OH)[13] in 45% overall yield following purification by reversed-phase high-performance liquid chromatography (RP-HPLC) (Figure 1B; Figure S1A). The Pam2Cys and Pam3Cys adjuvants in peptides **2** and **3**, respectively, were attached to the side-chain of a C-terminal lysine residue through a diserine linker (Figure 1A). This linker was included, as it is found in the natural version of Pam3Cys (Braun's lipoprotein), helps to improve solubility,[39] and for the serine that is attached to Pam2Cys, forms a hydrogen bond with TLR2-F325,[31] which is important for maximal immunopotency. The attachment of Pam2Cys and Pam3Cys was achieved using an *N*-Fmoc-protected *S*-glycerol cysteine building block (Fmoc-Dhc-OH),[40] to which two ester-linked copies of palmitic acid were conjugated to yield **2**. Following Fmoc-deprotection with DBU, to minimize ester hydrolysis, an amide-linked palmitic acid was coupled to give **3**. Lipid adjuvant peptides **2** and **3** were obtained in 24% and 29% overall yield following RP-HPLC purification (Figure 1B; Figure S1B−C).

Expression of Engineered α-Thioester Polytope Antigens 4a/4b for EPL. In order to conjugate the recombinant polytope species[13] onto the N-terminal cysteine modified lipid adjuvant peptides (**1**−**3**; Figure 1A; Figure 2B), recombinant polytope antigens **4a**/**4b** incorporating a C-terminal thioester were required (Figure 2A). This was achieved by expressing the recombinant polytope antigen as a C-terminal fusion with the *Mycobacterium xenopy* (*Mxe*) DNA gyrase (GyrA) (N198A) intein (Figure S2A-C).[36] Two versions of this polytope were produced (**4a**/**4b**), with **4a** designed to yield an N-terminal cysteine residue following its treatment with thrombin (Figure 2A). This cysteine could be used for a second NCL reaction, to enable the incorporation of additional components where desired.

The fusion proteins were efficiently overexpressed as soluble proteins (Figure S2B), which could be readily enriched using nickel-nitrilotriacetic acid (Ni-NTA) immobilized metal affinity

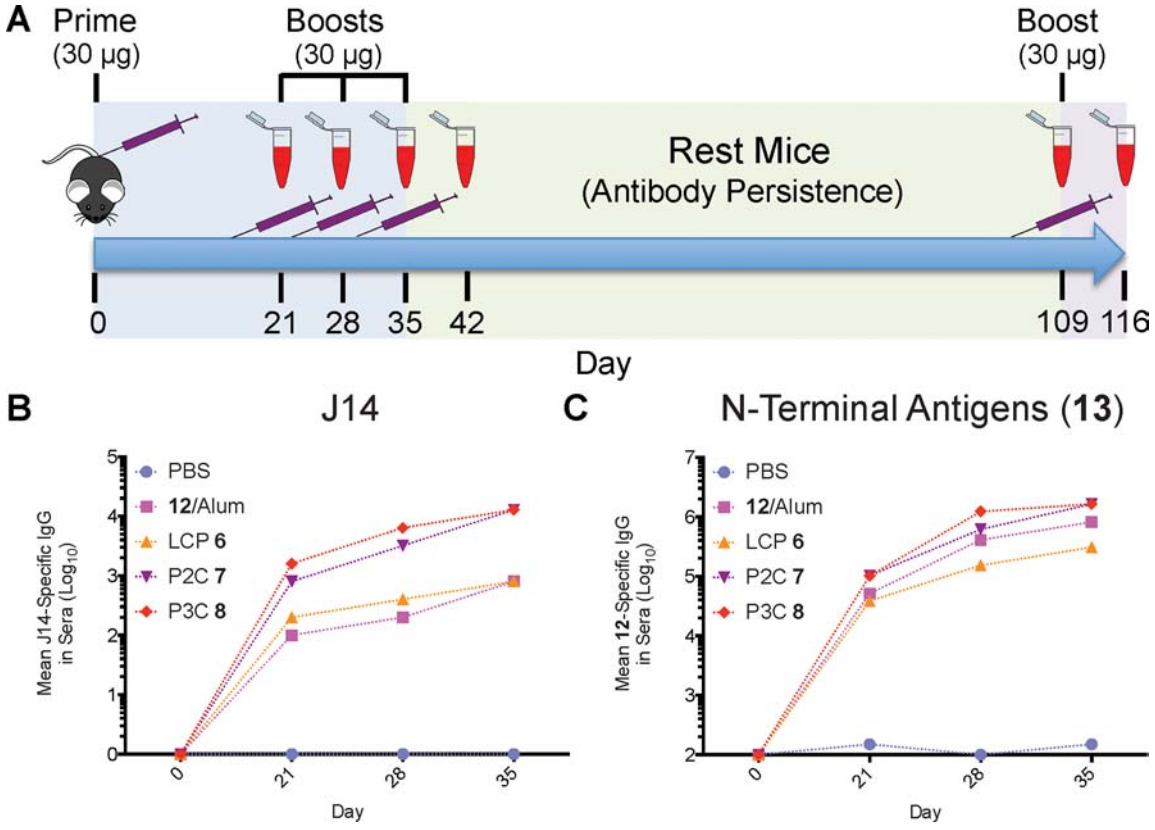

Figure 3. (A) Immunization/bleeding schedule. (B–C) Serum antigen-specific IgG antibody titers against (B) J14 and (C) polytope M protein N-terminal antigens **13** before subcutaneous immunization of C57BL/6J mice, and prior to each boost with lipoprotein vaccines **6**–**8**, polytope **12** (administered with alum), or PBS. Antibody titers were determined by ELISA using pooled sera from each group, and represent the average titer from three replicate experiments.

chromatography (IMAC). The proteins were subsequently eluted using 500 mM imidazole, which was removed by dialysis against three changes of PBS. This step was performed prior to converting the intein fusion proteins into thioesters as imidazole has been demonstrated to catalyze thioester hydrolysis and aminolysis.[41] Conversion to the thioester species was then achieved by adding ethanethiol (EtSH) to 3% (v/v) to the mixture, which was left to react overnight at RT. This led to complete conversion to the ethyl α-thioester species **4a**/**4b** (Figure 2A; Figure S2D-E; Figure S3A-B), which could be readily purified by preparative RP-HPLC to yield >18 mg of protein per liter of culture. Conversion to the poorly reactive ethyl α-thioester species was selected to ensure protein stability during subsequent purification steps, and to ensure long-term storage stability.[42] These species were subsequently converted to highly reactive ethanesulfonate α-thioesters **5a**/**5b** (Figure 2A; Figure S2F; Figure S3C–D) prior to their use for NCL. This process resulted in a shift in retention time by RP-HPLC (Figure S2F), which could aid in further enriching the reactive protein by RP-HPLC purification; otherwise, the conversion reaction could be dialyzed to remove excess reagents prior to NCL.

Recombinant Polytope Antigens Are Efficiently Conjugated to Lipid Adjuvants Using NCL. NCL[38] was assessed as a means to conjugate lipid adjuvant peptides (**1**–**3**) onto the recombinant thioester polytope antigen **5a**/**b** (Figure 2B). Because this polytope mainly contains linear peptide antigens, which generate antibodies against their sequence rather than structure, NCL reactions (Figure 2B) could be performed in the presence of denaturant (6 M guanidine) to increase the solubility of the reaction components. The use of denaturants also increases the reaction rate by allowing the use of higher concentrations of each reaction component, and improves thioester accessibility and reactivity by denaturing aggregated protein.[19] The NCL reactions were performed with an excess of the lipid adjuvant peptides **1**–**3** (4–5 equiv), with any unreacted peptide partially recovered during purification. Reactions were catalyzed by 4-mercaptophenylacetic acid (MPAA),[43] which generates a highly reactive thioester population through *trans*-thioesterification of the thioester proteins **5a**/**5b**. This gave rise to rapid ligation reactions that were essentially complete by 5 h at 37 °C for **6** (Figure 2C) and **7** (Figure S4A), and more than 50% complete for **9** (Figure S4B). The lipoprotein products were subsequently obtained in medium to high yield (**6**: 82%; **7**: 61%; **8**: 30%; **9**: 88%) following purification by semipreparative RP-HPLC, and characterized by ESI-MS, analytical RP-HPLC, and SDS-PAGE (Figure 2D for **6**; Figure S5).

Ligation Site Thiol Can be Readily Desulfurized or Blocked to Improve Vaccine Stability. The use of NCL to site-specifically attach lipid adjuvants to recombinant thioester proteins results in products that contain a cysteine at the ligation site (Figure 2B). The cysteine thiol can subsequently be used as a handle for other bioconjugation reactions (e.g., to attach additional adjuvants or antigens), maintained in the reduced state, used to produce larger dimeric systems through disulfide bond formation, desulfurized to generate an alanine residue, or blocked to prevent the formation of disulfide bonds.

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While oxidation of the cysteine thiol was not observed for our lyophilized products, in certain cases it may be desirable to block or desulfurize the cysteine residue to prevent the formation of disulfide-linked species. The compatibility of radical-mediated desulfurization with our lipoprotein products was assessed using LCP **9** according to the conditions described by Danishefsky[44] (VA-044 radical initiator, tris(carboxyethyl)-phosphine (TCEP), EtSH, and 2-methyl-2-propanethiol (*t*-BuSH)). The desulfurized product **10** was obtained in excellent yield (85%) after an overnight reaction at 37 °C and purification by semipreparative RP-HPLC (Figure 2B; Figure S6A; Figure S7). As an alternative method, we assessed the capacity to block the thiol group with small molecules. Methylmethanethiolsulfonate (MMTS)[35] proved suitable for this purpose, did not add any additional chiral center to our products, and was site-specific, simple, and inexpensive. The addition of MMTS to reduced LCP **9** generated the desired *S*-thiomethylated protein **11** within 30 min in similar yield (81%) to desulfurization following semipreparative RP-HPLC purification (Figure 2B; Figure S6B; Figure S7).

Lipid Adjuvant Conjugation Produces Self-Adjuvanting Vaccines Capable of Stimulating Systemic IgG Antibodies Targeting the Eight Polytope Antigens. In order to compare the effects of individual lipid adjuvants on immune responses toward the eight antigens in the polytope sequence, C57BL/6J mice (5/group) were immunized subcutaneously with each lipoprotein vaccine (**6**–**8**) in 1× Dulbecco's PBS (DPBS); 1× DPBS alone (negative control), or with the recombinant polytope antigen **12**[13] (Figure 2E) formulated with the commercial alum adjuvant Alhydrogel (aluminum hydroxide wet gel suspension; positive control). The alum formulation is representative of currently marketed vaccines for human use, where alum is mainly used as an adjuvant.[3]

Conjugation of TLR2-ligands to the polytope structure affords several important advantages over alum for GAS vaccine development. These include (i) the capacity for nasal delivery, reducing the need for administration by trained personnel;[3,45] and (ii) stimulation of mucosal immunity, reducing GAS colonization of the nose and throat, which represent common sites of GAS infection, and subsequent progression to systemic infection.[46] Alum also stimulates a Th2-biased immune response, and minimal cell-mediated immunity.[4] Therefore, the conjugation of lipid adjuvants is preferable where antigen-specific CTL responses are desired (e.g., development of anticancer vaccines or vaccines against intracellular pathogens).

The vaccines were administered using an optimized homologous prime-boost regimen[47] (30 μg antigen/dose; Figure 3A), with mice primed on day 0 and then administered three boosts at one-week intervals starting on day 21. Immediately prior to each boost (days 21, 28, and 35), and 1 week after the final boost (day 42), sera was sampled and used to assess antigen-specific serum IgG antibody titers against each polytope represented antigen using an enzyme-linked immunosorbent assay (ELISA). Increases in antibody titers following each boost (Figure 3B) were assessed using a synthetic J14 peptide as well as a recombinant polytope protein **13** (Figure 2E), corresponding to the seven M protein N-terminal antigens (without J14) as coating antigens and pooled sera from each vaccine group.

Mice immunized with Pam2Cys **7** or Pam3Cys **8** vaccines demonstrated the fastest increase in serum J14-specific IgG antibody levels (Figure 3B), with 8-fold (8.0×10^2) and 16-fold (1.6×10^3) higher average titers observed in these groups prior to boosting (day 21) compared with the alum formulated polytope **12** group (1.0×10^2). This difference was maintained throughout the vaccination protocol (Figure 3B), with 16-fold higher J14-specific IgG antibody average titers observed in the **6** and **7** lipoprotein groups (1.3×10^4 each) prior to the final boost (day 35) in comparison to the alum and LCP **6** groups (8×10^2 each).

A smaller difference was observed between groups when investigating the level of serum M protein N-terminal antigen **13**-specific IgG antibodies at each time point, with 2-fold higher antibody titers observed prior to the final boost (day 35) in the Pam2Cys **7** and Pam3Cys **8** groups (1.6×10^6 each) compared to the alum (8.2×10^6) group (Figure 3C). These experiments suggested that the alum and LCP formulations were similar in their ability to elicit serum IgG antibodies against the J14 and M protein N-terminal antigens, with Pam2Cys **7** and Pam3Cys **8** adjuvants proving superior at eliciting high titer antibodies against the J14 antigen.

Although the recombinant polytope approach permits the expression of antigens of a size that would not be readily synthetically accessible, and in large amounts, the end-to-end linkage of antigens has the potential drawback of generating nonfunctional antibodies against the junctional regions that exist between each antigen (see structure in Figure 2A). These antibodies will also be quantified when polytope **13**, which contains only the M protein N-terminal antigens, is used as a coating antigen (as above). To determine the level of serum IgG antibodies against each of the seven individual M protein N-terminal antigens in the polytope sequence, synthetic peptides corresponding to each antigen were used to coat ELISA plates, with antibody titers assessed using sera from individual mice in order to investigate the variance in antibody titers within each group (Figure 4), and to assess if any mice failed to respond to immunization (Figure S8).

Each adjuvant stimulated production of antigen-specific serum IgG antibodies capable of targeting all seven M protein N-terminal antigens (Figure 4; Figure S8). A trend toward higher average antibody titers in the Pam2Cys **7** and Pam3Cys **8** groups compared to the alum and LCP **6** groups was also observed in this experiment (Figure 4), with no significant difference observed between these groups ($p > 0.05$). The LCP **6** and alum/**12** groups also elicited similar antibody titers against each antigen, with the exception of the NS1 and NS5 antigens, which demonstrated 35- and 54-fold lower average IgG antibody titers in the LCP **6** group (Figure 4). In addition, the Y504S antigen demonstrated low titers of antigen-specific antibodies in all groups. This antigen has previously been shown to be poorly immunogenic,[13] and may be subject to epitope suppression effects in the context of the polytope construct.

The J14 antigen was included in the polytope sequence to elicit antibodies that can provide broad protection against the majority of circulating GAS serotypes.[48] An aim of this study was to optimize the type of lipid adjuvant in order to achieve the highest levels of serum IgG antibodies toward J14. This was necessary, as the titer of antibodies against the closely related J8 antigen has been demonstrated to directly correlate with protective efficacy.[49] Because J14 (KQAEDKVKASREAKKQVEKALEQLEDKVK) is a chimeric peptide, with the protective conformational M protein antigen (J14.0; underlined) enclosed within peptide sequences that encourage an α-helical conformation,[50] it is necessary to ensure that antibodies

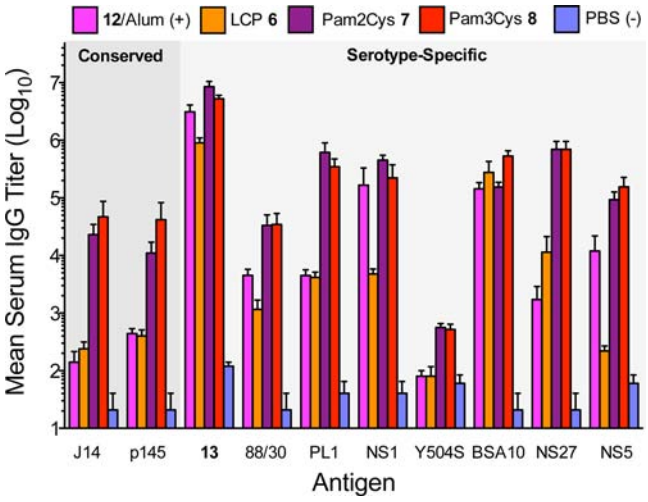

Figure 4. Quantification of serum IgG antibody titers (log_{10}) targeting the eight individual polytope antigens by ELISA, one week after the third boost (day 42), in mice (n = 5/group) immunized subcutaneously with polytope **12** (with alum), lipoproteins **6**–**8**, or PBS. Synthetic peptide antigens and a recombinant polytope consisting of the seven serotype-specific antigens **13** were used to coat plates. Binding of antibodies targeting J14 to the clinically relevant J14.0 conformational epitope was demonstrated using the α-helical p145 peptide. Data is presented as mean serum antigen-specific IgG titer ± standard error of the mean (SEM).

generated against J14 recognize the correctly folded J14.0 epitope, rather than binding to the flanking sequences or junctional epitopes alone. A longer M protein sequence (p145: LRRDLDASREAKKQVEKALE),[50] which forms an α-helix and contains the J14.0 epitope (underlined) flanked by the native M protein sequence, was used as an ELISA coating antigen to assess the level of antibodies targeting the correctly folded J14.0 antigen. Similar titers of p145- and J14-specific serum IgG antibodies were observed within each group (Figure 4), suggesting that these antibodies may afford broad protection. The Pam2Cys **7** and Pam3Cys **8** groups showed higher antibody titers against p145 and J14 (Figure 4), which were within the range previously demonstrated to provide significant protection in GAS challenge experiments.[48]

Lipoprotein Vaccines Aggregate to Form Nanoparticulate Formulations. In addition to providing direct immunostimulatory activity, the attachment of lipidic TLR2 ligands affords the capacity for vaccines to self-assemble into particles through lipid–lipid interactions.[13] Particle formation improves vaccine potency through improved antigen stability, repetitive antigen display (providing optimal B cell stimulation), and improved antigen uptake.[14] Particle size has proven important for optimal vaccine potency, with smaller particles (20–200 nm) claimed to stimulate more potent antibody-mediated immunity due to their potential for direct and rapid transport to the draining lymph nodes.[15] In comparison, larger particles (>200–500 nm) usually require cellular transport to reach the lymphatic system, reducing the amount of antigen interacting with B cells in the lymph nodes.[15]

Because particle size can have significant effects on the potency of immune responses targeting particulate vaccine formulations,[15] the size of each lipoprotein vaccine was assessed using two unrelated techniques: transmission electron microscopy (TEM) (Figure 5A) and dynamic light scattering (DLS) (Figure 5B). Size measurements were performed on the formulations that were used for immunization in order to control for the effects of vaccine and salt concentration, pH, and buffer type on particle size. Particles ranging from 24 to 458 nm were observed (Figure 5) following the addition of 1× DPBS to the freeze-dried lipoprotein vaccines. The type of lipid moiety attached to the recombinant antigen was observed to affect the mean particle size and distribution (Figure 5), with LCP lipoprotein **6** yielding the smallest particles (69.4 ± 3.7 nm by DLS) and the narrowest size distribution (PDI: 0.175; Range: 24–164 nm) (Figure 5B). The largest particles (123.9 ± 11.0 by DLS) and size distribution (PDI: 0.324; Range: 24–458) were observed for Pam3Cys lipoprotein **8**, with Pam2Cys lipoprotein **7** demonstrating intermediate size (101.3 ± 3.7 nm by DLS) and distribution (PDI: 0.225; Range: 24–342) (Figure 5B). The majority of particles in each vaccine formulation were within the desired range (20–200 nm) for potent antibody mediated immunity.[15] The differences in antibody titers against each vaccine antigen were therefore primarily attributable to the type of lipid adjuvant, or due to differences in the spatial positioning of the lipid moiety in each vaccine design (Figure 1A). Overall, the combination of TLR2-targeted immunostimulation with repetitive antigen display and self-assembly to form particles in the 20–200 nm size range suggests that these systems represent ideal platforms for the development of vaccines aimed at eliciting potent B cell mediated immunity against attached recombinant antigens.

Antibodies Elicited Against Each Vaccine Formulation Demonstrate Persistence. In order to develop a clinically relevant vaccine, the vaccine must elicit long lasting immunity capable of protecting against future exposure to the infectious organism. This necessitates that antibodies targeting the vaccine are produced over long periods of time, and/or that immunological memory is generated to enable a rapid immunological response against subsequent encounters with the associated infectious organism.[51] The life-span of antigen-specific plasma cells generated in response to immunization is short, leading to a rapid decline in antibody levels over the first few weeks to months following immunization.[51] Some of these plasma cells migrate to the bone marrow and become long-lived plasma cells that produce antibodies for years (>1 year), with the number of these cells associated with the duration of the antibody response.[51] We therefore chose to investigate the decline in serum IgG antibody titers toward the J14 and N-terminal polytope antigens over time to investigate antibody persistence in response to vaccination with lipoproteins **6**–**9**.

In order to assess antibody persistence, mice were rested for 74 days following the third boost (day 35) to let the short-lived plasma cells and their associated antigen-specific IgG antibody levels subside (Figure 3A). At this point (day 109) the mice were bled (Figure 3A) and the mean serum IgG antibody titers against J14 and the N-terminal polytope antigen compared by ELISA versus their levels on day 42 (Figure 6). The majority of the antibody response at this point would be associated with long-lived plasma cells.[51]

A decline in mean serum antibody titers was observed in all groups at day 109 except for J14 in the Alum/**12** group (Figure 6), with decreases of up to 4.6-fold for the J14 antigen (P2C > P3C > LCP > Alum; Figure 6A) and a 1.8–10-fold decrease for the N-terminal polytope antigens **13** (LCP > P2C > P3C > Alum; Figure 6B). The reduced antibody titers were only significant (α = 0.05) for antibodies targeting the polytope N-terminal antigens in the LCP **6** ($p < 0.01$) and Pam3Cys **8** ($p < 0.01$) groups. It was also observed that while two mice in the

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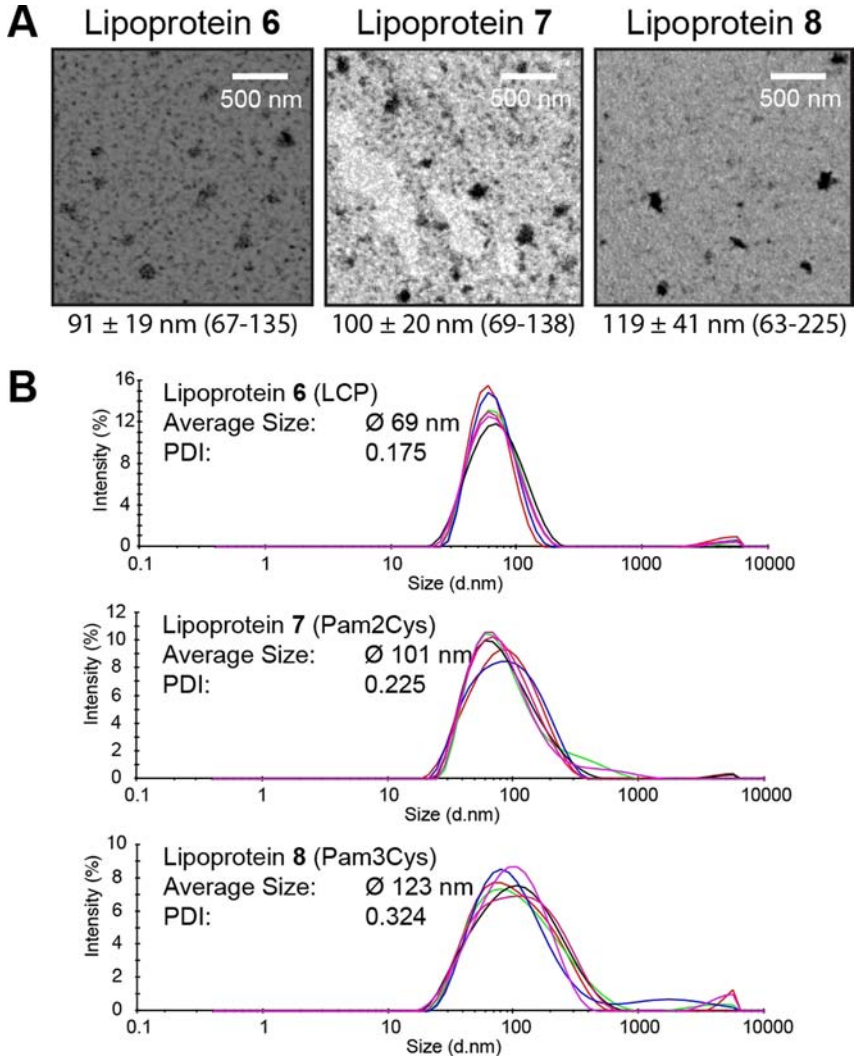

Figure 5. Lipoproteins **6**–**8** aggregate to form nanoparticulates in 1× DPBS. (A) TEM images showing the formation of nanoparticulates. Average particle size (nm) ± standard deviation and the range of particle sizes counted (in brackets) is reported. (B) DLS sizing of lipoproteins (6 replicate experiments). Average particle diameter (nm) and polydispersity index (PDI) is reported.

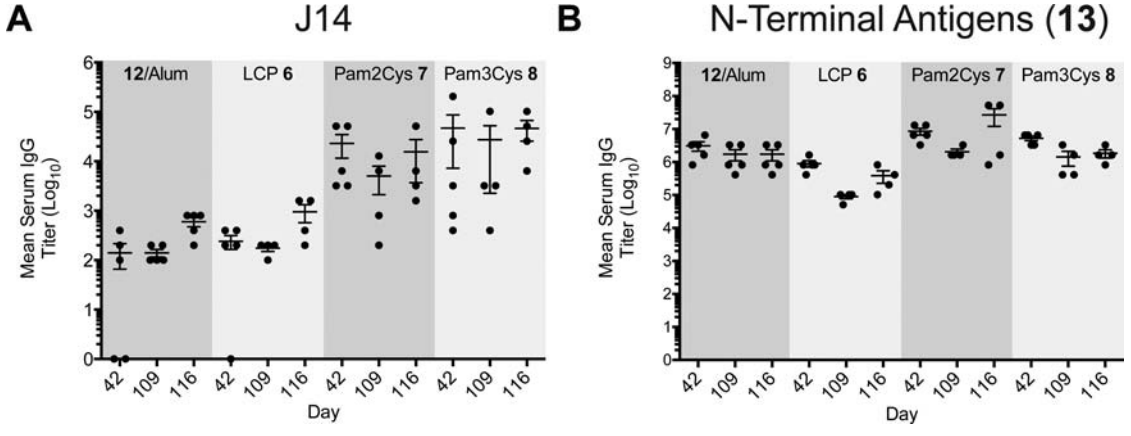

Figure 6. Comparison of serum IgG antibody titers (log_{10}) targeting the (A) J14 and (B) polytope N-terminal antigens **13** by ELISA: 1 week after the third boost (day 42); after resting for 74 days (day 109); and 1 week after boosting on day 109 (day 116). Synthetic peptide antigens and a recombinant polytope consisting of the seven serotype-specific antigens **13** were used to coat plates. Data is presented for each mouse (n = 5/group) along with mean serum antigen-specific IgG titer ± standard error of the mean (SEM).

alum/**12** group and one mouse in the LCP **6** group exhibited anti-J14 serum IgG antibody titers below the detection limit (<200) on day 42, by day 109 all mice showed responses against the J14 antigen. This is of importance, since this antigen is responsible for broad protective coverage against non N-terminal M protein antigen represented GAS serotypes.

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Overall, the antibody titers targeting each epitope in the polytope remained high, despite resting mice for 74 days. This data suggests that persistent antibody responses are elicited toward each of the lipoprotein vaccines **6**−**9**. This was expected for the N-terminal antigens, which have been described to generate long-lasting serotype-specific serum IgG responses that last up to 30 years.[52]

In addition to demonstrating antibody persistence, we wanted to assess if mice could be boosted after resting for 74 days. Mice in each group received a boost at day 109 and were subsequently bled after 7 days (day 116) to assess for increases in serum IgG antibody titers against J14 and the N-terminal polytope antigen. The aim of this experiment was to probe for possible antigen-specific memory B cell activation, which would lead to a rapid increase in serum IgG antibody titers toward J14 and the M protein N-terminal antigens after boosting. By assessing antibody titers toward these antigens after 1 week, we were able to provide evidence for a memory response, which takes 4−5 days to occur compared with the 10−14 days for a new primary immune response.[53,54]

No increase in J14-specific IgG serum antibody titers was observed on day 116 for the alum/**12** group. In comparison, 4.3-, 13.1-, and 1.3-fold increases were observed in the LCP **6**, Pam2Cys **7**, and Pam3Cys **8** groups, respectively, with the large increase in the **7** group suggesting recall of a memory B cell response. Increases of 4.3-, 5.4-, 3.1-, and 1.7-fold were observed with respect to serum IgG antibodies targeting the M protein N-terminal polytope antigen in mice immunized with alum/**12**, **6**, **7**, and **8**, respectively. This data suggests that boosting with the lipoprotein vaccines can elicit a rapid increase in antigen-specific serum IgG antibodies toward the broadly conserved J14 antigen as well as the serotype-specific M protein N-terminal polytope antigens, with the largest increases observed in the LCP **6** and Pam2Cys **7** lipoprotein groups. This is of importance, as recent studies[55] have demonstrated that antibody titers against the J8 peptide, which is related to J14, are not critical for protection at the time of GAS challenge. The presence of memory B cells specific to J8 can respond to antigenic stimulus from the GAS infection to generate a rapid increase in J8-specific IgG antibodies that are capable of clearing GAS infection.

Because Pam2Cys lipoprotein **7** provided a higher yielding synthesis than Pam3Cys analogue **8**, and produced high titer serum IgG antibodies targeting J14 and each of the M protein N-terminal antigens, which were demonstrated to persist, and could be rapidly increased in response to boosting, this analogue was selected as a lead compound for further characterization.

Antibodies Elicited Against the J14 Antigen Offer the Capacity for Broad Protective Efficacy Against Vaccine and Non-Vaccine Represented GAS Serotypes. As there are more than 200 known GAS genotypes (*emm*-type),[56] which vary with respect to their geographical distribution,[57] vaccines based solely on serotype-specific M protein N-terminal antigens are unlikely to protect entire populations against all circulating serotypes. The N-terminal antigens, however, elicit highly opsonic antibodies, which have been demonstrated to kill GAS, and have the most advanced clinical data supporting their safety and capacity to protect against GAS infection in humans.[58]

The protective coverage of vaccines based on N-terminal antigens could be enhanced through the addition of highly conserved, ubiquitously expressed antigens. A large number of highly conserved protein antigens have been identified that provide at least partial protection against GAS challenge in animal models.[58] Clinical data supporting their use to treat human infection, however, is lacking, and most have not been assessed in combination with N-terminal M protein antigens.[58] Incorporation of the J14 peptide into our polytope antigen represents one of the simplest means to add broad protective coverage to our system, as J14 is relatively small, does not require a complex fold, and can be readily expressed as part of a fusion protein.

Antibodies elicited against the polytope antigen have been demonstrated to bind to peptides corresponding to each of the seven polytope M protein N-terminal sequences as well as the representative GAS strains.[13] The binding of such antibodies to GAS serotypes that are not represented by the polytope M protein N-terminal antigens has not been assessed. This data would indicate the potential for these vaccines to elicit broad protective coverage. The Pam2Cys **7** and Pam3Cys **8** vaccines elicited antibodies against J14 that were within a range that has been previously demonstrated to provide protection against GAS challenge.[48] As the Pam2Cys version of this vaccine proved more efficient to synthesize, pooled anti-sera from mice immunized against this construct were tested for their capacity to bind to a library of five clinically relevant GAS serotypes (*emm*1, 3, 12, 28, and 53) that are not represented by N-terminal M protein antigens within the polytope construct. The library included a strain obtained from the 2011 Hong Kong scarlett fever outbreak (HKU1 (*emm*12), strains from invasive infections (5448 (*emm*1), 90254 (*emm*3), and NS13 (*emm*53)), and a serotype associated with puerperal sepsis (6180 (*emm*28)). Representative serotypes from this library are among the top 20 most common circulating *emm* types in high-income countries (*emm*1, 3, 12, 28, and 53), Africa (*emm*1, 3, 12, 28), and the Pacific (*emm*1).[57] Further, strains were selected to ensure that members of each *emm* pattern-type (A−C (*emm*1, 3, and 12); D (*emm*53); and E (*emm*28)), which have different M protein structures,[59] were represented. This is important, as while all M proteins contain C-repeats, from which the conformational B cell epitope (J14.0) in J14 was derived, not all M proteins include the J14.0 sequence[59] (C repeat sequences for *emm*1, 3, 12, 28, and 53 are reported in Table S2 and Figure S9). This is the case for pattern E strains (~37% of circulating strains),[59] which includes *emm* 28 (6180). Further, as most M proteins contain 3 C-repeats, which include similar sequences to J14.0, there is the potential for antibodies targeting J14.0 to also bind to these other sequences, offering improved protective efficacy, or protection against strains where J14.0 is absent (e.g., *emm*28).

By using confocal immunofluorescence microscopy (Figure 7), it was observed that antibodies elicited toward lipoprotein **7** bound the surface of all five GAS serotypes assessed (Figure 7B). In comparison, no fluorescence was observed with sera from PBS sham immunized mice (Figure 7). This data, when considered in combination with the significant increase in anti-J14 titers that were elicited against the Pam2Cys adjuvant containing polytope **7** (Figure 4), suggests that the strong antibody binding observed in this experiment was due to the antibodies binding to J14.0 and related antigens. In the case of the *emm*28 strain, which does not contain J14.0, strong antibody binding was still observed (Figure 7B). This was most likely because the J14.0 conserved epitope has 71%, 57%, and 64% sequence similarity with the J14.1, J14.29, and J14.41 sequences (Figure S9) in the *emm*28 C-repeats (Table S2). The data therefore supports the capacity of J14 to provide broadly

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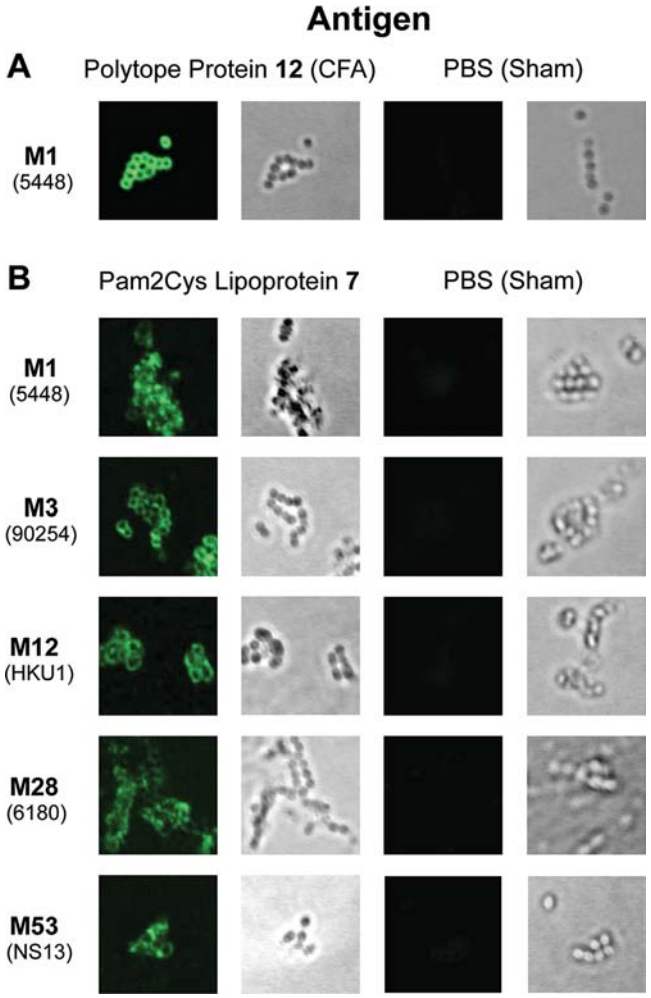

Figure 7. Serum IgG antibodies elicited toward Pam2Cys lipoprotein **7** bind a broad range of GAS serotypes that are not represented by M protein N-terminal antigens in the polytope sequence. Confocal immunofluorescence (left panel) data for antibody binding and DIC (right panel) imaging of bacteria is shown. Stationary-phase bacteria were incubated with pooled anti-sera (1:200 dilution) from mice immunized with polytope **12** (primed with complete Freund's adjuvant (CFA)), PBS, or lipoprotein **7**, labeled with goat anti-mouse IgG-FITC conjugate, and viewed with a 100× oil-immersion objective. (A) Positive control (**12**/CFA) and negative control (PBS) anti-sera binding to M1 GAS. (B) Anti-sera from lipoprotein **7** or PBS immunized mice (background control) binding to a library of clinically relevant GAS serotypes (strains in brackets; *emm* pattern below).

protective immunity, including toward strains that do not express the J14.0 antigen.

CONCLUSIONS

The covalent attachment of ligands targeting pattern-recognition receptors (e.g., TLRs) to antigens provides a means to produce potent vaccines through the targeting of all vaccine components to the same APC, and is of significant utility for developing various anticancer vaccines.[3,11] Despite the advantages afforded by conjugation, the potency and safety of TLR2 targeting adjuvants,[5−7] and the large number of protein antigens reported in the literature, few methods have been described to enable the efficient conjugation of hydrophobic TLR2 ligands onto protein antigens. Of the described methods, most generate conjugates incorporating various numbers of nonspecifically attached TLR2 ligands,[22] or yield TLR2 ligands that include mixtures of different fatty acids.[23] The heterogeneous nature of such constructs is undesirable. To overcome this problem, we sought to develop an efficient method to enable the site-specific incorporation of a defined number of TLR2 ligands onto engineered recombinant protein antigens, with the nature of the TLR2 ligand fatty acids guaranteed through their chemical synthesis.

In this work we extended the robust and efficient EPL approach[36,37] to enable the site-specific conjugation of three different TLR2 ligands onto recombinant protein antigens. This method provided moderate to high yields of molecularly defined, homogeneous lipoprotein products, which self-assembled upon their addition to PBS, yielding nanoparticulate formulations of an ideal size to stimulate potent antibody-mediated immunity.[14,15] With each TLR2 ligand using slightly different signaling pathways,[3,30−32] the potential for differences in the type and potency of stimulated immune responses exists. This approach thereby provides a simple method to compare different TLR2 ligands during the vaccine development process to determine which ligand possesses the most desirable characteristics for use with a particular antigen (e.g., ease of synthesis, potency, and the correct type of immune response). In this case, the Pam2Cys and Pam3Cys adjuvants yielded the highest titer antigen-specific IgG antibodies toward each polytope-represented antigen. The yield of Pam3Cys vaccine **8** however was significantly less than that of Pam2Cys analogue **7**, leading to the selection of **7** as a lead compound for further development. Of significance, serum IgG antibodies elicited in response to immunization with **7** were capable of binding various common, clinically relevant GAS serotypes that were not represented by serotype-specific M protein N-terminal antigens in the polytope. This data thus lends support to the approach of combining TLR2 ligands, M protein N-terminal antigens from commonly circulating GAS serotypes, and the broadly conserved J14 antigen for producing broadly protective vaccines targeting GAS infection and associated disease.

EXPERIMENTAL PROCEDURES

Materials. N^{α}-Fmoc amino acids and 1-[bis-(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-*b*]-pyridinium 3-oxid hexafluorophosphate (HATU) were from Mimotopes (Clayton, VIC, Australia). Fmoc-NH-PEG_6-CH_2CH_2COOH and Fmoc-Lys(ivDde)-OH were from ChemPep Inc. (Wellington, FL, USA). Rink amide 4-methylbenzhydrylamine (MBHA) resin (100−200 mesh; 0.59 mmol/g), *N*,*N*-dimethylformamide (DMF), and trifluoroacetic acid (TFA) were from Merck Millipore (Kilsynth, VIC, Australia). 2-[[1-(4,4-Dimethyl-2,6-dioxocyclohexylidene)ethyl]amino]-hexadecanoic acid (Dde-C16-OH) was synthesized as previously described.[13] *S*-(2,3-Dihydroxypropyl)-*N*-[(9*H*-fluoren-9-ylmethoxy)carbonyl]-L-cysteine (Fmoc-Dhc-OH) was synthesized as previously described[40] with modifications (see Supporting Information). Polytope gene synthesis and expression of **12** were previously described.[13] pTXB1 and pET-28b(+) vectors were from NEB (Ipswich, MA, USA) and Merck Millipore, respectively. 2,2′-Azobis[2-(2-imidazolin-2-yl)propane]dihydrochloride (VA-044) was from Wako Pure Chemical Industries (Osaka, Japan). Alhydrogel 2% (aluminum hydroxide wet gel suspension) was from InvivoGen (San Diego, CA, USA). All other reagents were purchased from Sigma-Aldrich (Castle Hill, NSW, Australia) and Thermo

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Fisher Scientific (Scoresby, VIC, Australia) at the highest available purity.

Synthesis of Lipid Adjuvant Peptides 1−3. Lipid adjuvant peptides (Figure 1A) were synthesized by microwave-assisted Fmoc SPPS with HATU *in situ* neutralization chemistry[60] on Rink amide-MBHA resin. Synthesis of the LCP lipid adjuvant in **1**, incorporating an N-terminal dilysine linker, was achieved using Dde-C16-OH as previously described.[13] Peptides **2** and **3** were synthesized with Fmoc-Lys(ivDde) at the C-terminus followed by a diglycine linker. After the synthesis of DCpep[28] (FYPSYHSTPQRP), two copies of Fmoc-NH-PEG_6-CH_2CH_2COOH and a Boc-Cys(Trt)-OH residue were coupled. For **2** and **3**, ivDde deprotection was subsequently performed (2% (v/v) hydrazine-DMF; 8 × 20 min), followed by attachment of a diserine linker. Incorporation of Pam2Cys into **2** was achieved by coupling Fmoc-Dhc-OH[40] (4 equiv; 2 h) using DIC (*N*,*N*′-diisopropylcarbodiimide)-HOBt (1-hydroxybenzotriazole) activation in DMF, followed by palmitoylation of the two free *S*-glycerolcysteine hydroxyl groups (20 equiv palmitic acid, 25 equiv DIC, 2 equiv 4-(dimethylamino)pyridine (DMAP) in dichloromethane (DCM); 12 h), and Fmoc-deprotection (2.5% (w/v) DBU (1,8-diazabicyclo[5.4.0]undec-7-ene)-DMF; 3 × 5 min) as described.[16] To generate Pam3Cys peptide **3**, palmitic acid was coupled to the *S*-glycerolcysteine amine residue in **2** using HATU *in situ* neutralization chemistry.[60] Peptides were cleaved from the solid-support using 95% (v/v) TFA, 2.5% (v/v) triisopropylsilane (TIPS), 2.5% (v/v) water for 2 h at RT, precipitated with diethyl ether, and purified by RP-HPLC on C4 stationary phases. Detailed synthetic procedures and characterization data (Figure S1) are provided as Supporting Information.

Expression of Polytope Ethyl α-Thioester (4a/4b) and M Protein N-Terminal Antigen Polytope (13). Plasmids encoding for C-terminal *Mxe* GyrA (N198A) fusions with the polytope gene (pTXB1_GAS polytope_GyrA; Amp^R; Figure 2A) or the polytope M protein N-terminal antigens **13** (pET-28 N-terminal antigens; Kan^R; Figure 2E) were expressed in BL21-codonplus (DE3)-RIL *E. coli* (Cm^R) using LB (Miller) broth with dual antibiotic selection. Protein expression was performed at 37 °C, 250 rpm for 3−4 h following induction at OD_{600} 0.6 with 0.5 M isopropyl β-D-1-thiogalactopyranoside (IPTG). Proteins were enriched by Ni-NTA IMAC after cell lysis (Constant Systems cell disruptor or sonication) in lysis buffer (50 mM Tris pH 7.5, 500 mM NaCl, 10 mM imidazole, 1 mM phenylmethanesulfonyl fluoride (PMSF)) containing 1 mM TCEP (for intein fusion protein) or 2 mM 2-mercaptoethanol (M protein N-terminal antigen polytope **13**). Proteins were eluted with lysis buffer containing 500 mM imidazole (without PMSF), and dialyzed (3 kDa MWCO) against PBS (for thioester proteins) or 20 mM Tris pH 8, 100 mM NaCl (M protein N-terminal antigen polytope) at 4 °C. The intein fusion proteins were converted to ethyl α-thioester proteins (**4a**/**4b**) by gentle mixing with EtSH (to 3% (v/v)) overnight at RT (Figure 2A; FigureS2D-E). The M protein N-terminal antigen polytope **13** was treated with human serum thrombin (~2 U/mg protein) for 1 h at RT to cleave the N-terminal hexahistidine tag. The proteins were purified by RP-HPLC on C18 stationary phases to yield 18 mg per L culture of polytope ethyl α-thioester proteins **4a**/**4b** and 42 mg per L culture of M protein N-terminal antigen polytope **13**. Characterization data (Figure S3A-B; Figure S6D) is provided as Supporting Information.

Site-Specific Conjugation of Lipid Adjuvant Peptides 1−3 onto Recombinant Polytope Antigens 5a/5b. Bioconjugation reactions (Figure 2B) were performed at 37 °C in denaturing ligation buffer (6 M guanidine-HCl, 50 mM NaPi pH 7.5, 20% (v/v) acetonitrile (MeCN), 40 mM TCEP, 20 mM MPAA) containing ~12 mg/mL of the recombinant thioester protein (**5a** or **5b**) and 4−5 equiv of the lipid adjuvant peptide (**1**−**3**). Recombinant ethyl α-thioester proteins **4a** and **4b** were converted to the more reactive ethanesulfonate α-thioester proteins **5a** and **5b** (Figure 2A) by incubation in 6 M guanidine-HCl, 0.1 M NaPi pH 7.5, 0.1 M NaCl, 0.1 M 2-mercaptoethanesulfonate sodium (MESNa) at ~3 mg/mL concentration for 2 h at RT, followed by purification by RP-HPLC on a C18 stationary phase (Figure S3C−D) or dialysis against 0.1% (v/v) TFA-water, followed by freeze-drying. Lipoprotein products (**6**−**9**; Figure 2B; Figure S5) were purified by RP-HPLC on a C4 stationary phase.

Radical Mediated Desulfurization of LCP 9 to Yield 10. Desulfurization[44] (Figure 2B) of LCP **9** was performed at ~8 mg/mL concentration in desulfurization buffer (6 M guanidine-HCl, 0.2 M NaPi pH 6.7, 0.28 M TCEP, 0.1 M EtSH, 0.33 M *t*-BuSH, 2 mM VA-044) for 16 h at 37 °C. The desulfurization product **10** (Figure S6A; Figure S7) was subsequently purified by RP-HPLC on a C4 stationary phase.

***S*-Thiomethylation of LCP 9 to Generate 11.** LCP **9** was dissolved in 0.1 M NaPi pH 7.5 to 1 mg/mL, to which TCEP (10 μmol/mg **9**) was added. Reduction was performed at 37 °C for 30 min, followed by *S*-thiomethylation (Figure 2B) by addition of MMTS (20 μmol/mg **9**) and incubation at 37 °C for 30 min. The product **11** (Figure S6B; Figure S7) was purified by RP-HPLC on a C4 stationary phase.

Immunization. Immunization was performed according to protocols approved by the Griffith University (BDD/06/10/AEC) and University of Queensland (SCMB/GRIFFITH/005/12/MERCK/NIH/NHF/NHMRC) animal ethics committees in accordance with the Australian code for the care and use of animals for scientific purposes. Vaccines (**6**−**8** and **12**[13]) were administered (30 μg/dose in 50 μL total volume) subcutaneously to C57BL/6J ($H\text{-}2^b$) mice (5/group) at the tail-base. Antigens were formulated at 0.6 mg/mL in DPBS (**6**−**8**) or in 1:1 DPBS-alhydrogel (**12**). Sham immunization with DPBS was performed as a negative control. Mice were boosted on days 21, 28, 35, and 109 after priming. Mice were bled (tail snip) prior to each boost (days 21, 28, and 35) to investigate the effect of boosting on serum IgG antibody titers against J14 and the M protein N-terminal polytope antigens (**13**) (Figure 3B−C); on day 42 to investigate serum IgG antibody titers targeting each polytope antigen (Figure 4); on day 109 to investigate antibody persistence (Figure 6A−B); and on day 116 to investigate the capacity to boost mice after resting (Figure 6A−B).

Enzyme-Linked Immunosorbent Assay. ELISA was performed as previously described[13] using 96-well flat-bottomed PVC microplates. Synthetic peptides representing each of the eight antigens found in the recombinant polytope (sequences in Table S1) and the conformational p145 antigen, as well as a recombinant polytope antigen **13** incorporating the seven M protein N-terminal antigens were used as coating antigens. Antibody titers were defined as the lowest sera dilution with an OD_{450} value greater than 3 standard deviations above the mean absorbance of blank control wells. ELISA data is presented as mean antigen-specific IgG antibody titer ± standard error of the mean (SEM). Raw data is provided as Supporting Information (Figure S8).

Confocal Immunofluorescence Microscopy. Confocal immunofluorescence microscopy (Figure 7) was performed as described[13] using pooled antibodies (day 42; 1:200 dilution) from mice immunized with Pam2Cys lipoprotein **7** to investigate the binding of antibodies to GAS strains (5448 (*emm1*), 90254 (*emm3*), HKU1 (*emm12*), 6180 (*emm*28), and NS13 (*emm53*)) that are not represented by M protein N-terminal antigens in the polytope sequence. Calibration for positive and negative binding was achieved using 5448 (M1) GAS and murine anti-sera from polytope **12** (primed with CFA)[13] or PBS sham immunized mice, respectively (Figure 7A).

Statistical Analysis. Statistical analysis of antibody titers between groups was performed using a one-way analysis of variance (ANOVA) with Tukey's multiple comparison posthoc test. GraphPad Prism 6 was used for statistical analysis, with $P < 0.05$ considered to be significant.

■ ASSOCIATED CONTENT

Supporting Information

Detailed synthesis, cloning and expression procedures, as well as characterization data for Fmoc-Dhc-OH, lipid adjuvants (**1**–**3**), polytope antigens (**4a**/**b**, **5a**/**b**, **12**, **13**), and lipoprotein vaccines (**6**–**11**). Immunization, ELISA, and confocal microscopy methods. Raw ELISA data for day 42. Polytope antigen sequences. C repeat sequences for the GAS library (*emm1*, *emm3*, *emm12*, *emm28*, and *emm53*). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

APCs, antigen presenting cells; *t*-BuSH, 2-methyl-2-propanethiol; CFA, complete Freund's adjuvant; DBU, 1,8-diazabicyclo[5.4.0]undec-7-ene; DC, dendritic cell; DCM, dichloromethane; Dde, 1-(4,4-dimethyl-2,6-dioxocyclohexylidene)ethyl; Dhc, *S*-(2,3-dihydroxypropyl)-cysteine; DIC, differential interference contrast; DLS, dynamic light scattering; DMAP, 4-(dimethylamino)pyridine; DPBS, Dulbecco's PBS; EPL, expressed protein ligation; FITC, fluorescein isothiocyanate; Fmoc, 9-fluorenylmethyloxycarbonyl; GAS, group A streptococcus; GyrA, DNA gyrase; HATU, 1-[bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-*b*]-pyridinium 3-oxide; IMAC, immobilized metal affinity chromatography; LCP, lipid core peptide; MBHA, 4-methylbenzhydrylamine; MeCN, acetonitrile; MESNa, 2-mercaptoethanesulfonate sodium; MHC, major histocompatibility complex; MMTS, methylmethanethiolsulfonate; MPAA, 4-mercaptophenylacetic acid; *Mxe*, *Mycobacterium xenopy*; NCL, native chemical ligation; Ni-NTA, nickel-nitrilotriacetic acid; OD, optical density; PDI, polydispersity index; Pam, palmitoyl; Pam2Cys, dipalmitoyl-*S*-glyceryl cysteine; Pam3Cys, tripalmitoyl-*S*-glyceryl cysteine; PMSF, phenylmethylsulfonyl fluoride; SPPS, solid phase peptide synthesis; TCEP, tris(carboxylethyl)-phosphine; TFA, trifluoroacetic acid; TIPS, triisopropylsilane; TLR, Toll-like receptor

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