Structure–Activity Relationship of a Series of Synthetic Lipopeptide Self-Adjuvanting Group A Streptococcal Vaccine Candidates

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The development of 16 self-adjuvanting group A streptococcal vaccine candidates, composed of (i) a universal helper T-cell epitope (P25), (ii) a target GAS B-cell epitope (J14), and (iii) a lipid moiety, is described. Systemic J14-specific IgG antibodies were detected following subcutaneous immunization of BALB/c (H-2^d) mice with each construct without the need for an additional adjuvant. The effect of changing the order of P25, J14, and lipid moiety attachment or incorporation of P25 and J14 into a lipid-core peptide system on antibody titers was assessed. The point of lipid moiety attachment had the greatest influence on systemic J14-specific IgG antibody titers. Overall, the best vaccines featured a C-terminal lipid moiety, conjugated through a lysine residue to P25 at the N-terminus, and J14 on the lysine side chain.

Introduction

Group A streptococcus (GAS,[a] *Streptococcus pyogenes*)is responsible for numerous clinical manifestations, of which the most common is streptococcal pharyngitis ("strep throat"). Other clinical manifestations include skin infections, invasive diseases, and nonsuppurative sequelae [rheumatic fever (RF), rheumatic heart disease (RHD), acute glomerulonephritis].[1] A recent independent review commissioned by the World Health Organization emphasized that GAS-associated diseases represent a global health problem, with approximately 18.1 million existing cases of severe GAS disease and approximately 1.78 million new cases each year.[2] GAS-associated diseases are responsible for approximately 517 000 deaths per annum.[2] The development of a vaccine to prevent GAS infection would offer an ideal means to prevent RHD (responsible for the greatest health burden) and other GAS-associated diseases. Numerous research groups[3–23] are therefore working toward the development of prophylactic GAS vaccines, with the most advanced research focusing on the GAS cell-surface M-protein.

The M-protein is an α-helical coiled-coil cell surface protein having both antiphagocytic and adhesive functions.[24] During early clinical trials of GAS vaccine candidates based on the M-protein, several patients developed autoimmune sequelae.[25,26] However, these trials were conducted in high-risk populations with a history of GAS infections. It is not known whether the vaccine or a natural GAS infection was responsible for the manifestation. However, this led to the investigation of M-protein-derived peptides free of autoreactive B- and T-cell epitopes for vaccine development, including amino N-terminal peptides and defined carboxyl C-terminal peptides. Antibodies elicited against the M-protein N-terminus while highly opsonic, are serotype-specific, and thus only protect against homologous GAS strains for which M-protein N-terminal peptides have been included in the vaccine.[27] Because more than 120 GAS strains exist,[28] this approach is not suitable for the development of a vaccine against the majority of GAS strains encountered worldwide. Peptides derived from the M-protein C-terminus are more conserved and thus offer a means to develop broadly protective vaccines. One such peptide is the 29-amino acids chimeric peptide J14 (KQAEDKVK**ASREAKKQVEKALE**QLEDKVK),[29] which contains a B-cell epitope (in bold) derived from C-terminal M-protein enclosed within a helix-promoting sequence. It has been demonstrated that J14, when administered subcutaneously with complete Freund's adjuvant (CFA), elicits immune responses capable of protecting mice against heterologous GAS strains.[4] The conjugation of immunostimulatory lipids to peptides containing B- and T-cell epitopes facilitates the development of lipidated self-adjuvanting vaccines. A variety of lipid moieties, generally derived from bacterial cell walls, have been studied for use in peptide vaccine formulations;[30] two examples are di- and tripalmitoyl-*S*-glyceryl cysteine (Pam_2Cys[31] and Pam_3Cys[32]).

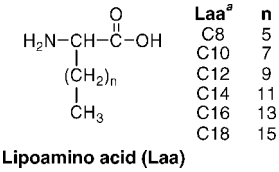

Figure 1. Structure and naming conventions of the lipoamino acids used in this study. The supserscript "a" indicates lipoamino acids that have been described according to the total number of carbons in their skeleton.

A major aim of the current study was to develop a new vaccine delivery system incorporating the J14 peptide, and a universal helper T-cell epitope (P25: KLIPNASLIENCTKAEL[33]), with synthetic lipidic amino acids[34] (see Figure 1). The three components (lipid moiety, J14, and P25) were conjugated to a lysine core *via* its carboxylic acid or the α- or ϵ-amines to give a library of 16 lipopeptides (**1**−**16**, series A−C, Figure 2). The order of the three components was varied to investigate the effects of epitope (J14 and P25) and lipid positions on the systemic J14-specific IgG antibody titers elicited following immunization of BALB/c (H-2^d) mice using a homologous

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[a]Abbreviations: Boc, *tert*-butoxycarbonyl; CFA, complete Freund's adjuvant; DT, diphtheria toxoid; GAS, group A streptococcal; Laa, lipoamino acid; MAP, multiple antigen peptide; PBS, phosphate buffered saline; *p*MBHA, *p*-methylbenzhydrylamine; RHD, rheumatic heart disease.

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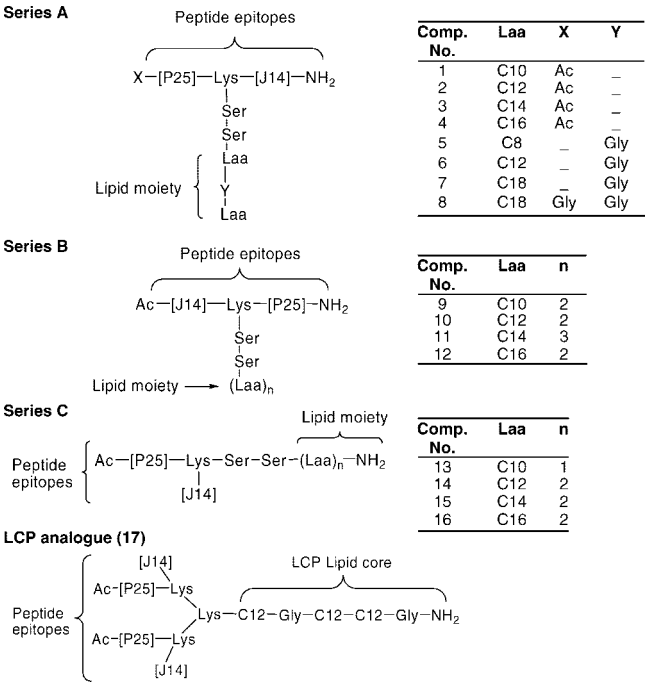

Figure 2. Structures of lipopeptides **1**–**17**. The lipopeptide vaccines incorporate a universal helper T-cell epitope (P25,[33] KLIPNASLIENCTKAEL), as well as the GAS B-cell epitope (J14,[29] KQAEDKVK**ASREAKKQVEKALE**QLEDKVK), and a built-in adjuvant synthesized using the synthetic lipoamino acids[34] (C8−C18; see Figure 1).

prime-boost regimen. In addition, the J14 and P25 peptides were incorporated into a lipid core peptide (LCP) system[35] (**17**, Figure 2), a self-adjuvanting lipopeptide vaccine delivery system incorporating immunostimulatory lipids, a multiple antigen peptide (MAP) system,[36] and peptide epitopes, in order to compare the level of J14-specific IgG antibodies elicited when these peptides are incorporated into the LCP system (**17**) compared to their use in our library (**1**–**16**). The systemic J14-specific IgG antibody titers were also compared between groups prior to each boost and following the immunization regimen in order to determine which structural changes gave rise to a rapid J14-specific IgG antibody response without the requirement of additional boosts. In order to optimize our new lipid moiety, the effect of alterations in the number and length of lipoamino acids (Laa) and the spacing between lipid chains (using glycine residues as spacers) was investigated.

Results and Discussion

Lipopeptides **1**–**17** (Figure 2) were synthesized using *in situ* neutralization, *tert*-butoxycarbonyl (Boc) chemistry[37] on *p*-methylbenzhydrylamine (*p*MBHA) resin (see Supporting Information). Lipopeptides **1**–**16** were categorized into three series (series A, B, and C; Figure 2), which differed in their molecular geometries (the orientations of peptide antigens J14 and P25 and the lipid moiety). Series A (**1**−**8**) featured the J14 antigen at the lipopeptide C-terminus, while in series B (**9**−**12**), J14 was attached to the lipopeptide N-terminus and in series C (**13**–**16**) to the side chain N^{ϵ}-amine of the central lysine core. The immunostimulatory lipid moiety incorporated two lipoamino acids (in most cases) to provide a structure similar to, but not identical to, the adjuvant Pam_2Cys. Two serine residues were inserted between the central lysine core and the lipid moiety because this has been reported to improve vaccine immunogenicity for other lipopeptide vaccines.[38,39] The lipid moiety was coupled to the N^{ϵ}-amine of the lysine central core in series A and B, whereas it was incorporated at the C-terminus of series C to mimic the LCP system.[22]

Immunological evaluation was performed in BALB/c ($H\text{-}2^d$) mice primed by lipopeptides **1**–**17**, subcutaneously, without an additional adjuvant. The positive control group was administered J14−diphtheria toxoid conjugate (J14−DT) emulsified with CFA,[3] while the negative control groups were administered CFA or PBS. Mice received three boosts of each lipopeptide at days 21, 31, and 41 after primary immunization. Sera were collected prior to each boost, and 9 days after the final boost, to assess the levels of J14-specific serum IgG elicited using an enzyme-linked immunosorbent assay (ELISA).[29] In order to investigate the effect of varying epitope and lipid orientation (molecular geometry) on J14-specific systemic IgG antibody titers, lipopeptides from different series containing the same number of Laa and the same Laa alkyl chain length were selected for comparison. Finally, lipopeptides **2**,**10**, and **14** (Laa = C12) and **4**, **12**, and**16** (Laa = C16) were compared (Figure 3).

Following priming with lipopeptides **2**, **10**, and **14** (Laa = C12) or lipopeptides **4**, **12**, and **16**(Laa = C16), low-level J14-specific systemic IgG titers were observed (see Supporting Information). However, J14-specific systemic IgG antibody titers rapidly increased after each boost, with the highest antibody titers observed in the case of lipopeptides **14** and **16** after the third boost (Figure 3; **14** vs **16**, $p > 0.05$). Systemic J14-specific IgG titers were significantly lower after the third boost in the groups administered lipopeptides **2** and **10**, or **4** and **12** compared to those administered lipopeptides **14** or **16**, respectively (Figure 3). Lipopeptide **2** was incapable of inducing significant titers of J14-specific IgG following the first and second boosts (see Supporting Information) and only induced low levels of antigen-specific IgG following the third boost. Taken together, these data suggest that the point of lipid attachment significantly affects the level of J14-specific systemic IgG antibodies elicited in response to immunization, with the attachment of Laa to the vaccine's C-terminus (series C) resulting in significantly higher antibody titers compared to vaccines where lipids are attached to the side chain N^{ϵ}-amine of the central lysine core (series A and B). The attachment of the P25 and J14 antigens to the C- or N-terminus in series A and B also had an effect on systemic J14-specific serum IgG titers with the attachment of J14 to the N-terminus (series B) resulting in higher antibody titers than J14 attachment to the C-terminus (series A).

Overall, the aforementioned results suggest that attachment of P25 at the N-terminus, of Laa to the C-terminus, and of J14 to the side chain N^{ϵ}-amine of the central lysine core, as in series C, provides vaccines capable of eliciting higher titers of J14-specific IgG antibodies requiring fewer boosts than other vaccine configurations (series A and B).

The best analogue in the C12 Laa series described above, lipopeptide **14**, was compared to LCP system **17**, the positive control J14-DT emulsified in CFA, and the negative controls PBS and DT emulsified in CFA (Figure 3). Both lipopeptide **14** and LCP system **17** were comparable, with nonsignificant differences in J14-specific systemic IgG antibody titers observed at each time point (see Supporting Information). The level of J14-specific systemic IgG antibodies elicited in response to immunization with **14** and **17** was significantly greater than the antibody titers in the negative control groups ($p < 0.001$ following each boost) and was not statistically different compared to mice immunized with J14-DT emulsified in CFA.

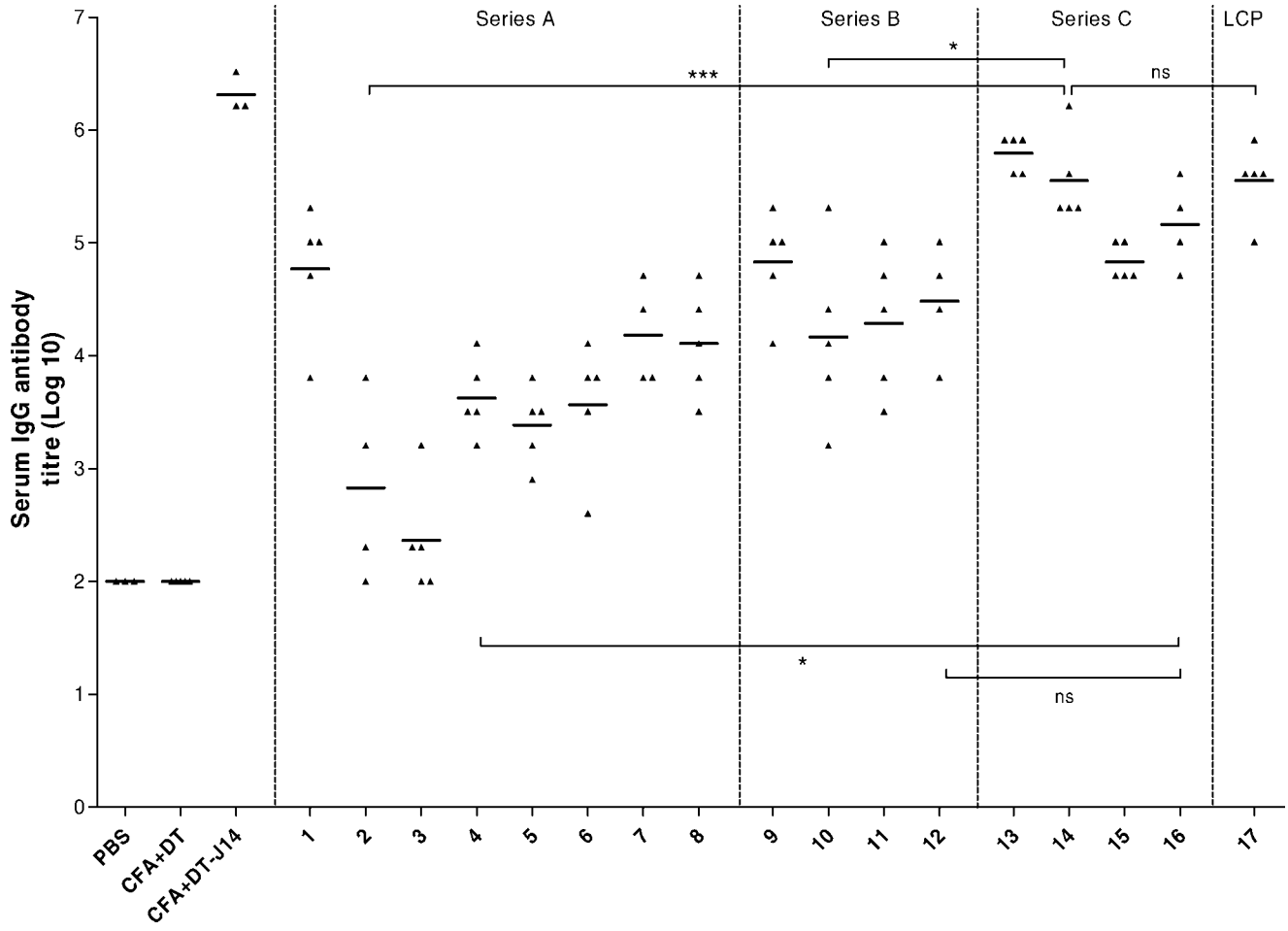

Figure 3. J14-specific serum IgG antibody titers (log 10) at the final bleed (day 50) elicited in response to immunization of BALB/c (H-2^d) mice at the tail base with lipopeptides **1**–**17**, as determined by ELISA. Antibody titers are shown for individual mice to the J14 GAS peptide epitope. Mean antigen-specific serum IgG antibody titers are represented as a bar. Statistical analysis was performed using a two-way ANOVA followed by the Bonferroni post hoc test (ns, $p > 0.05$; ∗, $p < 0.05$; ∗∗, $p < 0.01$; ∗∗∗, $p < 0.001$).

Because both lipopeptide **14** and **17** are self-adjuvanting, they eliminate the use of any adjuvant, which is one of the major obstacles facing the development of many subunit peptide-based vaccines. Furthermore, these analogues do not include a traditional carrier, like DT, and therefore may not suffer from problems including the administration of many unnecessary carrier associated epitopes and epitope suppression due to the existence of antibodies against the carrier.[40] Lipopeptide **14** also offers many advantages over LCP system **17**, including ease of synthesis, as lipopeptide **14** does not contain a MAP system, and because it contains only one copy of each peptide antigen, it would be less expensive to manufacture.

A previous study to optimize the LCP system for systemic IgG antibody elicitation demonstrated that increases in the length of the Laa alkyl side chains and the use of glycine spacers between Laa resulted in increased humoral immunity against the attached antigens. [35,41] In order to investigate the effect of altering the length of the Laa alkyl side chains in the current system on systemic J14-specific IgG antibody titers, a comparison was performed between lipopeptides within each series that differed only in the Laa alkyl side chain length (see Figure 3, **1**−**4** in series A, **9**, **10**, and **12** in series B, and **14**−**16** in series C). In series A, mice immunized with lipopeptide **1** (Laa = C10) demonstrated the highest level of J14-specific serum IgG antibodies, followed by mice immunized with lipopeptide **4** (Laa = C16), whereas mice immunized with lipopeptides **2** (Laa = C12) and **3** (Laa = C14) did not demonstrate significant levels of J14-specific serum IgG antibodies when compared to PBS immunized mice. In contrast, there was not a significant difference in the levels of J14-specific serum IgG antibodies elicited by lipopeptides in series B and C, therefore suggesting that in these series, changes in the length of the Laa alkyl side chain do not have a significant effect on humoral immune responses against attached peptide antigens. Similarly, increasing the distance between lipid chains, through the insertion of glycine residue, was found to have no significant effect on the final J14-specific serum IgG titer (**2** *vs* **6**, $p > 0.05$).

Conclusion

The current study, through the synthesis and immunological evaluation of the library of J14-based GAS vaccines, has resulted in the optimization of a new self-adjuvanting peptide vaccine delivery system incorporating a new lipid moiety. By investigation of the structure–activity relationships of the synthesized vaccines, it was demonstrated that the point of lipid moiety attachment has the greatest effect on vaccine immunogenicity. The orientation of the J14 and P25 antigens was also found to have an effect on antibody titers. In comparison, altering the length of the Laa alkyl side chains had minimal to no effect on antibody titers. Overall, the study revealed that vaccines featuring C-terminal lipids, P25 at the N-terminus, and J14 on the lysine side chain produced the

highest titers of systemic J14-specific IgG antibodies. Mice immunized with vaccines featuring this molecular structure elicited comparable levels of systemic J14-specific IgG antibodies to mice immunized with an LCP-system featuring the J14 and P25 antigens. The current vaccine delivery system has several advantages over other systems (e.g., the LCP system, and Pam_2-/Pam_3-Cys lipopeptides), for example, the use of Laa instead of complex lipids and the need for only one copy of each antigen, which would ultimately simplify the synthesis of these vaccines and reduce the cost of vaccine. The present study strongly suggests that the orientation of the lipid adjuvant or peptide epitopes may have a greater effect on antigen-specific systemic IgG antibody titers compared to increasing the length or number of lipids. The information obtained from this study would thus prove valuable for the design of an optimal J14-based lipopeptide GAS vaccine capable of protecting against the majority of GAS strains.

Experimental Section

Materials and Methods. Protected L-amino acids and *p*MBHA resin were purchased from Novabiochem (Läufelfingen, Switzerland) or Reanal (Budapest, Hungary). Peptide synthesis grade dichloromethane (DCM), *N*,*N*-dimethylformamide (DMF), 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), and TFA were purchased from Auspep (Melbourne, Australia). Solvents for HPLC [acetonitrile (MeCN) and isopropyl alcohol (IPA)] were purchased from Honeywell-Burdick and Jackson (Morristown, NJ) or Labscan (Dublin, Ireland). All other reagents were purchased at the highest available purity from Sigma-Aldrich (Castle Hill, NSW, Australia). ESI-MS was performed with a Perkin-Elmer-Sciex API3000 instrument using Analyst 1.4 (Applied Biosystems/MDS Sciex, Toronto, Canada) software. Samples were introduced into MeCN−water mobile phases containing 0.1% (v/v) acetic acid. Analytical RP-HPLC was performed using Shimadzu Instrumentation (Class Vp 6.12 software, SCL-10AVp controller, SIL-10A autoinjector, LC-10AT pump, LC-10AD pump, Waters 486 tunable absorbance detector) with a 1 mL/min flow rate and detection at 214 nm. Separation was achieved on either a Vydac analytical C4 column (214TP54; 5 μm, 4.6 mm × 250 mm) or a Vydac analytical C18 column (218TP54; 5 μm, 4.6 mm × 250 mm). Analysis was run in gradient mode using 0.1% TFA/H_2O as solvent A and either 90% MeCN/0.1% TFA/H_2O (solvent B1) or 90% IPA/0.1% TFA/H_2O (solvent B2). Three methods were run over 30 min: method 1, 0–100% solvent B1; method 2, 0–100% solvent B2; method 3, 20–80% solvent B1. Preparative RP-HPLC was performed on a Waters Delta 600 system using a 5 mL/min flow rate and detection at 230 nm. Separations were performed in gradient mode with 0.1% TFA/H_2O as solvent A and 90% MeCN/0.1% TFA/H_2O as solvent B on either a Vydac preparative C4 column (214TP1022; 10 μm, 22 mm × 250 mm) or a Vydac preparative C18 column (218TP1022; 10 μm, 22 mm × 250 mm).

Peptide Synthesis. Each lipopeptide vaccine (**1**−**17**, Figure 2) was synthesized on *p*MBHA resin (0.4 mmol of NH_2/g, 0.5 mmol scale) using manual stepwise solid-phase peptide synthesis, HBTU/*N*,*N*-diisopropylethylamine (DIPEA) in situ neutralization, and Boc chemistry.[37] Coupling cycles consisted of 2 × 1 min treatments with neat TFA for Boc deprotection, a 1 min DMF flow wash, and then 20–60 min couplings with 4 equiv of 1 min preactivated amino acid. Amino acid activation was achieved by dissolving each amino acid (2.2 mmol, 4.4 equiv) in 0.5 M HBTU/DMF solution (4 mL, 2.0 mmol, 4 equiv) followed by the addition of DIPEA (500 μL, 2.87 mmol). Coupling yields were assessed using the quantitative ninhydrin test,[42] with couplings repeated where coupling yields were less than 99.6%. Boc-amino acids with the following side chain protection were utilized: Arg(Tos), Asn(Xan), Asp(OcHx), Cys(p-MeBzl), Gln(Xan), Glu(OcHx), Lys(2-Cl-Z), Lys(Fmoc), Ser(Bzl), Thr(Bzl), and Tyr(2-Br-Z). The following Boc-protected lipoamino acids (Boc-Laa-OH) were synthesized as described by Gibbons et al.:[34] 2-(*tert*-butoxycarbonylamino)-D,L-octanoic acid (Boc-C8-OH), 2-(*tert*-butoxycarbonylamino)-D,L-decanoic acid (Boc-C10-OH), 2-(*tert*-butoxycarbonylamino)-D,L-dodecanoic acid (Boc-C12-OH), 2-(*tert*-butoxycarbonylamino)-D,L-tetradecanoic acid (Boc-C14-OH), 2-(*tert*-butoxycarbonylamino)-D,L-hexadecanoic acid (Boc-C16-OH), and 2-(*tert*-butoxycarbonylamino)-D,L-octadecanoic acid (Boc-C18-OH). After the coupling of glutamine and asparagine residues, the resin was washed with DCM before and after Boc deprotection to prevent high-temperature catalyzed pyrrolidone carboxylic acid (Pca) formation.[37] Acetylation of peptide epitopes was achieved by treating the resin with a mixture of acetic anhydride (0.5 mL, 5.29 mmol), DIPEA (0.47 mL, 2.70 mmol), and DMF (14 mL) for 5 min and repeating for 30 min. Lysine N^{ϵ}-Fmoc deprotection was achieved using 20% piperidine in DMF for 5 min and repeating twice for 30 min. Benzyloxycarbonyl protection was achieved by agitating the resin for 1.5 h in a mixture containing 2 equiv of *N*-(benzyloxycarbonyl)succinimide and 1.5 equiv of DIPEA in DMF (10 mL). After the synthesis of each lipopeptide vaccine was complete, the peptidyl resins were washed with DMF, DCM, and MeOH and then dried under vacuum prior to cleavage with anhydrous HF. HF cleavage (10 mL HF/g resin) was performed at 0 °C in the presence of 5% (v/v) *p*-cresol and 5% (v/v) *p*-thiocresol. After 2 h, the HF was removed under reduced pressure and the peptides were precipitated in ice-cold diethyl ether, filtered, dissolved in 40% aqueous MeCN containing 0.1% TFA, and lyophilized. The lyophilized product (150 mg) was then purified by preparative RP-HPLC on either a C18 or C4 column using a gradient of 10% solvent B to 100% solvent B over 60 min. The fractions were analyzed by ESI-MS and analytical RP-HPLC and where appropriate combined to give pure product (details provided as Supporting Information).

Mice and Subcutaneous Immunization. All protocols were approved by the Queensland Institute of Medical Research Animal Ethics Committee and were carried out according to Australian National Health and Medical Research guidelines. Immunization was performed as reported elsewhere.[10,20,21,43] Female BALB/c (H-2^d) mice (4–6 week-old, Animal Resource Centre, Perth, Western Australia, Australia) were used for immunization. Mice (n = 5/group) were injected subcutaneously at the tail base on day 0 with 30 μg of immunogens in a total volume of 50 μL of sterile-filtered phosphate buffered saline (PBS). Mice received three further boosts at 10-day intervals (days 21, 31, and 41) with 30 μg of immunogens in a total volume of 50 μL of PBS. A positive control received 30 μg of J14-DT emulsified in a total volume of 50 μL of CFA/PBS (1:1). Two negative controls were administered a 50 μL of either CFA/PBS (1:1) or PBS alone. All controls were boosted with 50 μL of PBS.

Collection of Sera. Blood was collected from the tail artery of each mouse 1 day prior to each injection and 9 days after the last immunization. The blood was left to clot at 37 °C for 1 h and then centrifuged for 10 min at 3000 rpm to remove clots. Sera were then stored at −20 °C.

Detection of Systemic IgG Antibodies by ELISA. Determination of serum IgG antibodies against the J14 epitope included in the vaccine was performed using a previously described ELISA.[44] Briefly, serial dilutions of sera were produced in 0.5% skim milk/PBS-Tween-20 buffer, starting at 1:100 concentration with 2-fold dilutions. Optical density was read at 450 nm in a microplate reader following the addition of peroxidase-conjugated goat antimouse IgG, and *O*-phenylenediamine. The antibody titer was defined as the lowest dilution with an optical density of more than 3 standard deviations greater than the mean absorbance of control wells containing normal mouse serum.

Statistics. Statistical analysis of antibody titers between groups was performed using a two-way ANOVA followed by the Bonferroni post hoc test. GraphPad Prism 4 software was used for statistical analysis, with $p < 0.05$ taken as statistically significant.

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Supporting Information Available: Synthesis of lipopeptides **1–17** and their detailed immunological data. This material is available free of charge via the Internet at http://pubs.acs.org.

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