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Structure–Activity Relationship for the Development of a Self-Adjuvanting Mucosally Active Lipopeptide Vaccine against *Streptococcus pyogenes*

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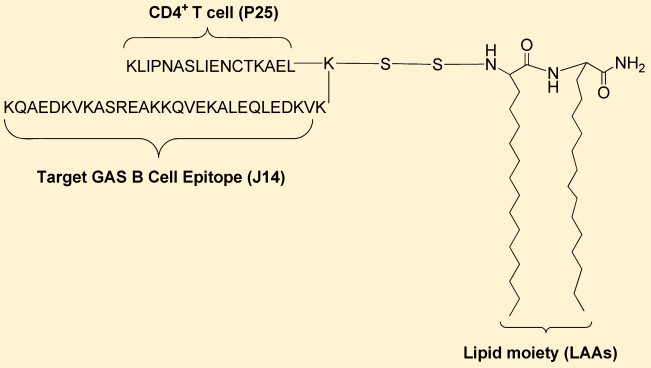
ABSTRACT: Infection with group A streptococcus (GAS) can result in a number of diseases, some of which are potentially life-threatening. The oral–nasal mucosa is a primary site of GAS infection, and a mucosally active vaccine candidate could form the basis of an antidisease and transmission-blocking GAS vaccine. In the present study, a peptide from the GAS M protein (J14) representing a B cell epitope was incorporated alongside a universal T cell helper epitope and a Toll-like receptor 2 targeting lipid moiety to form lipopeptide constructs. Through structure activity studies, we identified a vaccine candidate that induces J14-specific mucosal and systemic antibody responses when administered intranasally without additional adjuvants. The systemic antibodies elicited were capable of inhibiting the growth of GAS. In addition, J14-specific mucosal antibodies corresponded with reduced throat colonization after respiratory GAS challenge. These preclinical experiments show that this lipopeptide could form the basis of an optimal needle-free mucosal GAS vaccine.



INTRODUCTION

Group A Streptococcus (*Streptoccus pyogenes* or GAS) associated disease represents a global health problem (predominantly in the poorest countries of the world) and is responsible for approximately 517000 deaths per annum.[1,2] GAS is among the top 10 pathogens responsible for global morbidity and mortality, with approximately 18.1 million existing cases of severe GAS disease with an additional 1.78 million new cases each year. GAS infection can result in a number of disease states, ranging from relatively benign (e.g., pharyngitis) to serious invasive disease (e.g., necrotizing fasciitis) and serious postinfectious complications (e.g., rheumatic fever (RF) and rheumatic heart disease (RHD)).[3] RF/RHD are autoimmune diseases where autoreactive T cells and antibodies are induced by GAS virulence factors, including the cell surface M protein.[3]

Vaccines have been regarded as the most cost-effective way to improve public health. To date, successful vaccines have relied on attenuated or killed organisms. Unfortunately, an attenuated or killed GAS vaccine may pose a significant risk of inducing autoimmune disease.[4] New technological advances in vaccine design and delivery are essential to alleviate the global health burden associated with GAS. There are a number of different vaccine targets under consideration for GAS,[2] including: the cell surface M protein,[5−7] C5a peptidase,[8] streptococcal extracellular cysteine protease,[9] and fibronectin-binding protein.[10] Currently, more than 180 different serotypes of GAS have been defined by sequence differences at the amino (N) terminus of the M protein.[11] However, the highly conserved carboxyl (C) terminus of the M protein is considered a promising candidate because it will enable protection against multiple GAS strains.

The peptide p145 (a 20-mer with the sequence: LRRDLDASREAKKQVEKALE) has been defined as an epitope within the conserved C terminus of the M protein and the target of antibodies in both mice and humans.[12] However, immunization with p145 could potentially lead to the development of cross-reactive T cells due to sequence similarity with human cardiac myosin. Further investigation resulted in the identification of the minimal peptide epitope J14 (a 29-mer with the sequence KQAEDKVK***ASREAKKQVEKALE***Q-LEDKVK), that does not contain the potentially deleterious T cell epitope from p145. This B cell epitope consists of 14 amino acids from p145 (sequence in bold) enclosed within a nonstreptococcal sequence, designed to maintain the natural helical conformation of the epitope. It has been demonstrated that J14 or its analogues combined with appropriate vaccine

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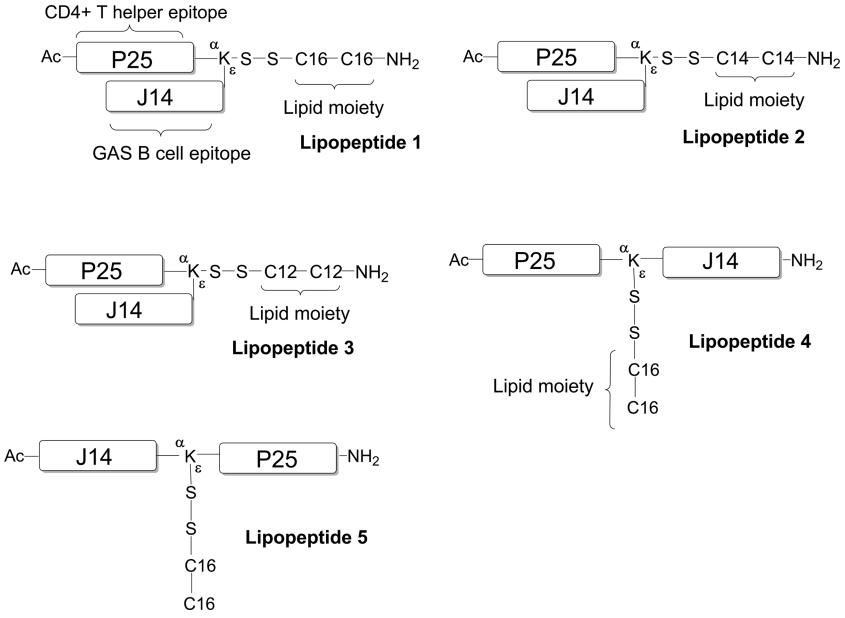

Figure 1. Schematic representation of the lipopeptide GAS vaccine candidates evaluated in the present study. The vaccines incorporate a universal helper T cell epitope (P25: KLIPNASLIENCTKAEL), GAS B cell epitope (J14: KQAEDKVKASREAKKQVEKALEQLEDKVK), and two copies of LAAs with differing alkyl chain length (C16, C14, or C12 carbons) as a built-in lipidic adjuvant. Ac = acetyl group.

delivery systems conferred a protective immune response in mice against heterologous GAS strains.[12,13]

GAS primarily colonizes the epithelial surfaces of the upper respiratory tract and the skin,[14] resulting in the aforementioned disease states. On mucosal surfaces (immunoglobulin A or IgA) is a primary host defense for the prevention of bacterial attachment and colonization.[15] Although several candidate GAS vaccines are currently approaching or undergoing human clinical trials,[6,13] the preclinical evaluation of these vaccine candidates was primarily based on their ability to induce systemic protection (IgG) following parenteral immunization. Immunity conferred by these vaccine candidates was proven effective in preventing GAS dissemination and associated disease at systemic sites through opsonic IgG antibodies. However, it may not be the optimal approach for inducing protection against GAS due to lack of IgA preventing adherence of GAS to mucosal epithelium.[16] An optimal GAS vaccine therefore needs to elicit IgA, preventing pharyngeal colonization, and an opsonic IgG systemic response to facilitate GAS clearance. Several studies have tested the mucosal activity of GAS vaccine candidates when delivered to the mucosal surface.[17−20] While some of these studies identified promising, mucosally active GAS vaccine candidates, most required use of animal-restricted mucosal adjuvants to stimulate an IgA response. These studies also identified the potential of the intranasal route for the mucosal delivery of a GAS vaccine.[21] Vaccination at this mucosal surface has the additional advantage of stimulating antibody responses in both mucosal secretions and at systemic sites (IgA and IgG), whereas parenteral vaccine delivery results only in systemic immune responses (IgG).[22]

The development of a J14-based, mucosally active GAS vaccine has been hindered by the lack of a suitable mucosal adjuvant for the administration of peptides. A variety of lipid moieties have been studied to achieve mucosal adjuvant activity when chemically coupled to target peptides. These include: Pam_2Cys (a synthetic version of the lipid moiety from macrophage-activating lipopeptide 2 derived from *Mycoplasma fermentans*),[23] Pam_3Cys (a synthetic analogue of Brauns' lipoprotein found in Gram-negative cell walls), and simpler palmitoyl groups.[24] These lipid moieties have demonstrated very potent adjuvanting activity by activating toll-like receptor 2 (TLR2), which recognize pathogen-associated molecular patterns, consequently leading to the induction of an immune response.[25] However, lipopeptides incorporating the aforementioned lipid moieties cannot be synthesized using standard peptide synthesis protocols and are prone to damage by oxidation and esterase enzymes.[25]

The current vaccine candidates (Figure 1) are designed as branched lipopeptide constructs incorporating the GAS conserved peptide (J14), as well as a universal CD4+ helper T cell epitope (P25), and a lipid moiety based on racemic lipoamino acids (LAAs). The T helper epitope (P25) was used generate the proliferation of T cells, negating the need for carrier proteins.

LAAs are synthetic α-amino acids bearing an alkyl side chain (Figure 2).[26] The use of LAAs as lipid moieties is particularly

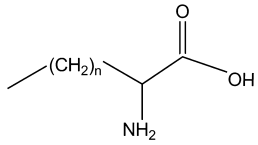

Figure 2. The structure of lipoamino acid (LAA). LAAs are described according to the total number of carbon atoms in their skeleton: C12 (n = 9), 2-amino-D,L-dodecanoic acid; C14 (n = 11), 2-amino-D,L-tetradecanoic acid; C16 (n = 13), 2-amino-D,L-hexadecanoic acid.

beneficial because they can be readily synthesized in large quantities and the length, branching, and saturation of the side chain can be easily modified.[26] LAA can also be coupled together and to other amino acids using standard peptide synthesis protocols, including solid-phase peptide synthesis (SPPS). LAA are also stable against oxidation and esterases enzymes.[25] More recently, it has been identified that LAA-based lipid moieties exert self-adjuvanting activity similar to that

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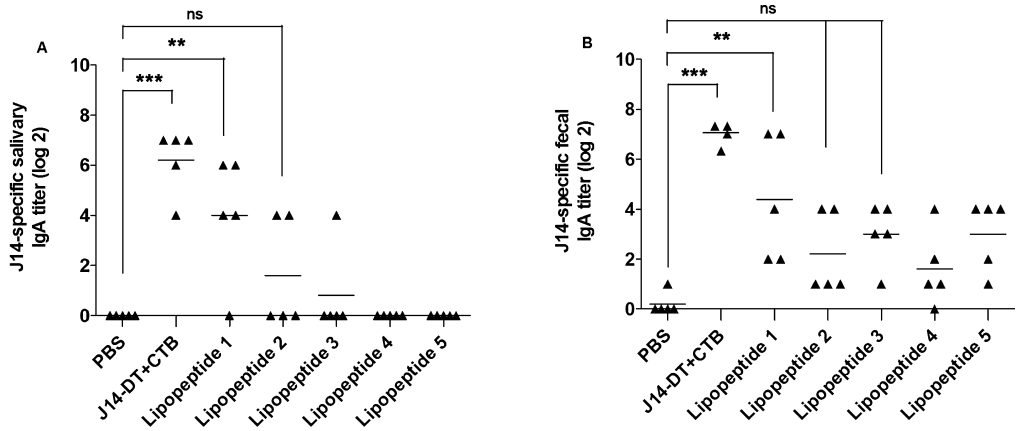

Figure 3. J14-specific IgA titer at day 47 following intranasal administration of lipopeptides to inbred B10.BR mice. (A) Salivary titer. (B) Fecal titer. Mean J14-specific IgA titer is represented as a bar. Cohorts of B10.BR mice (n = 5/group) received a primary immunization on day 0 followed by two further boosts on days 21 and 41. Samples were collected on days 27 and 47 after primary immunization. Statistical analysis was performed using a one-way ANOVA followed by the Tukey post hoc test (ns, $p > 0.05$; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

of other lipopeptides via TLR2 activation and are capable of dendritic cell maturation.[27−29]

In this study, we aimed to assess the ability of lipopeptides with different structural arrangement of vaccine components and LAAs with different alkyl chain lengths to induce J14-specific mucosal IgA responses in mice. It was demonstrated that both the length of the LAA alkyl chain and the position of vaccine components had a significant effect on the IgA activity. The structure−activity relationship investigated identified a highly efficacious, promising GAS vaccine candidate and provided a rational basis for the development of a mucosally active lipopeptidic GAS vaccine candidate.

RESULTS AND DISCUSSION

Previous studies have shown that IgA in saliva and feces represents those immunoglobulins secreted into the mucus of the airways (a primary site for GAS infection) and intestines, enabling measurement of mucosal antibodies.[30] Following intranasal administration of lipopeptides **1**−**5** in inbred B10.BR mice, J14-specific saliva and fecal IgA titers were determined by enzyme-linked immunosorbent assay (ELISA). The selected lipopeptides represent different constructs which would provide information about the effect of varying epitope orientation (J14 and P25), lipid position, and alkyl chain length on mucosal immune responses. Their ability to induce systemic IgG response had been reported elsewhere following subcutaneous and intranasal administration.[31,32] Lipopeptide **1** induced significant J14-specific salivary and fecal IgA titers without additional adjuvant ($p < 0.01$ vs the negative control group PBS, Figure 3A,B). Highest saliva and fecal IgA titers ($p < 0.001$ vs PBS) were induced by the positive control group J14-DT+CTB (J14 conjugated to the carrier protein diptheria toxoid (DT), coadministered with the animal restricted cholera toxin B subunit (CTB)). DT may be an ideal carrier protein for GAS peptide epitopes and therefore selected as a positive control.[13] As no human approved mucosal adjuvant exists for the delivery of peptides, CTB represents a standard for mucosal adjuvanticity in comparison to our self-adjuvanting lipopeptides. While lower titers of IgA was observed for lipopeptide **1** than for J14-DT+CTB, it is more advantageous due to circumventing possible immune suppression resulting from carrier protein specific immune responses and negating use of CTB and its harmful side effects.

Lipopeptides **2**−**3** represented analogues of lipopeptide **1** with shorter alkyl chains for the lipid moiety and were compared to lipopeptide **1** to determine the effect of varying the alkyl chain length on the mucosal immune response. Lipopeptides **4**−**5** were compared to lipopeptide **1** to determine the effect of varying epitope/lipid moiety orientation on the mucosal immune response. Lipopeptides **2**−**3**, incorporating a shorter alkyl chain for the LAAs (C14 and C12, respectively), failed to induce statistically significant J14-specific saliva or fecal IgA titers ($p > 0.05$ vs the negative control PBS, Figure 3A,B). Lipopeptides **4**−**5** feature the same C16 LAAs as lipopeptides **1**, but these LAA are attached to the side chain of the central lysine residue instead of at the C terminus. The levels of J14-specific salivary and fecal IgA detected for lipopeptides **4**−**5** (Figure 3A,B) were not statistically significant ($p > 0.05$ vs PBS). The optimal GAS vaccine candidate featured a C-terminal lipid moiety with an alkyl chain length of 16 carbons, attached through a lysine residue to P25 at the N-terminus, and J14 on the lysine side chain (named as lipopeptide **1**). Interestingly, these constructs were all capable of eliciting systemic IgG response following subcutaneous and intranasal immunization.[31,32] These results indicated that lipopeptide **1** induced the desired mucosal immune response, unlike analogues with shorter alkyl chains, or with the epitopes/C16 lipid moiety in a different orientation. A reasonable explanation for the importance of the C16 LAAs in this study could be due inducing the highest TLR2 activation.[27] This is consistent with the results of others, who found that for TLR2-targeting lipid moieties, alkyl chains of C16 are optimal for immunogenicity and TLR2 activation.[33,34] However, it should be noted that repositioning the C16 LAAs from the C terminus (lipopeptide **1**) to the side chain of the central lysine (lipopeptides **4**−**5**) abolished mucosal activity. This observation suggests that mucosal activity is not solely related to TLR2 activation as lipopeptide **4** induced similar TLR2 activation to lipopeptide **1**.[29] These results clearly indicate the effect of the spatial arrangement of vaccine components (epitope and lipid position) on the mucosal immune response, highlighting this as an important aspect of rational mucosal vaccine design.

A vaccine for human development will target a heterogeneous population, therefore immunogenicity in outbred mice was of interest. Following intranasal administration of lipopeptide **1** in outbred Swiss mice, statistically significant J14-

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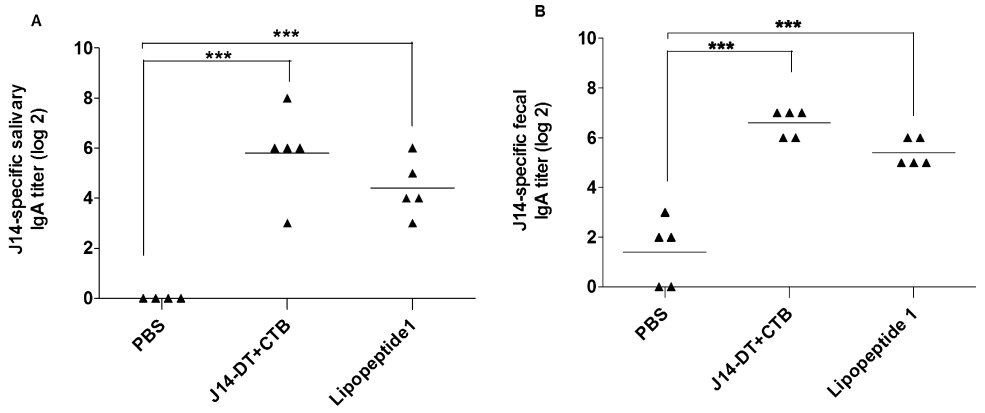

Figure 4. J14-specific IgA titer at day 47 following intranasal administration of lipopeptide to outbred Swiss mice. (A) Salivary titer. (B) Fecal titer. Mean J14-specific IgA titer is represented as a bar. Cohorts of Swiss mice (n = 5/group) received a primary immunization on day 0 followed by two further boosts on days 21 and 41. Samples were collected on days 27 and 47 after primary immunization. Statistical analysis was performed using a one-way ANOVA followed by the Tukey post hoc test (ns, $p > 0.05$; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

specific salivary and fecal IgA titers were observed ($p < 0.001$vs PBS, Figure 4A,B). The mucosal IgA titers elicited by lipopeptide **1** without additional adjuvant were comparable to the positive control group (J14-DT+CTB), which contained the mucosal adjuvant, CTB. These results highlight that lipopeptide **1** is a promising vaccine candidate, eliciting strong immune responses in both inbred and outbred mice. Outbred mice are genetically and phenotypically different, thus representing a more heterogeneous population. Theoretically, results from these mice can be better translated to similar studies in humans.

Mucosal vaccines administered intranasally have been shown to be effective in inducing antigen-specific immune responses in both systemic and mucosal sites. Significant J14-specific systemic IgG was also detected in serum samples obtained from outbred mice immunized with lipopeptide **1** ($p < 0.001$vs PBS, Figure 5). No significant difference was observed for J14-specific IgG titers induced by lipopeptide **1** in comparison to the positive control group (Figure 5, $p > 0.05$ for J14-DT+CTB vs lipopeptide **1**).

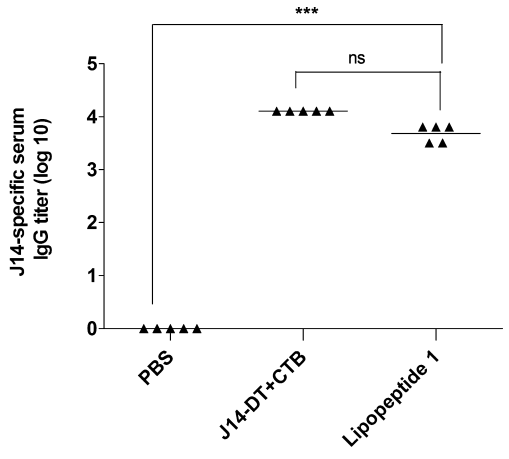

Figure 5. J14-specific serum IgG titer (log10) at day 60 elicited in response to intranasal immunization of Swiss mice. Mean J14-specific IgA titer is represented as a bar. Cohorts of Swiss mice (n = 5/group) received a primary immunization on day 0 followed by two further boosts on days 21 and 41. Serum was collected on days 20, 40, and 60 after primary immunization. Statistical analysis was performed using a one-way ANOVA followed by the Tukey post hoc test (ns, $p > 0.05$; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

To further characterize the antigen-specific immune response elicited by the mucosally active GAS vaccine candidate, antibody isotypes were evaluated by ELISA. IgG isotype expression can give information on the prevailing cytokine environment. All four IgG isotypes were observed for the J14-DT+CTB immunized group (Figure 6). This is indicative of a

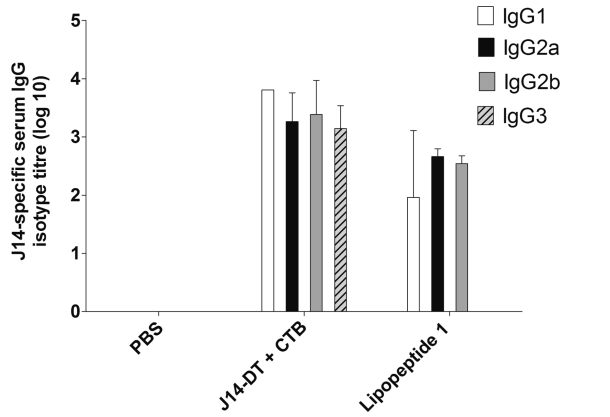

Figure 6. J14-specific serum IgG isotype titer (log10) at day 60 response to intranasal immunization of Swiss mice. Error bars represent standard deviation. Cohorts of Swiss mice (n = 5/group) received a primary immunization on day 0 followed by two further boosts on days 21 and 41. Serum was collected on days 20, 40, and 60 after primary immunization.

mixed T helper type 1 (Th1)/Th2 response.[35] The mucosally active lipopeptide **1** elicited IgG1, IgG2a, and IgG2b isotypes (mixed Th1/Th2 response).[35] A mixed Th1/Th2 response is optimal for our lipopeptides because this is associated with differentiation of B cells committed to produce IgA and for the transport of secretory IgA to effector sites.[36]

J14 is a chimeric peptide containing the minimal GAS B cell epitope from p145 flanked by nonstreptococcal sequences. The antibodies induced by vaccination with lipopeptide **1** were tested for reactivity against the minimal GAS B cell epitope from p145 using ELISA. The systemic IgG and mucosal IgA antibodies elicited by lipopeptide **1** in Swiss mice were capable of significantly binding to p145 (Figure 7A,B, $p < 0.05$ vs PBS).

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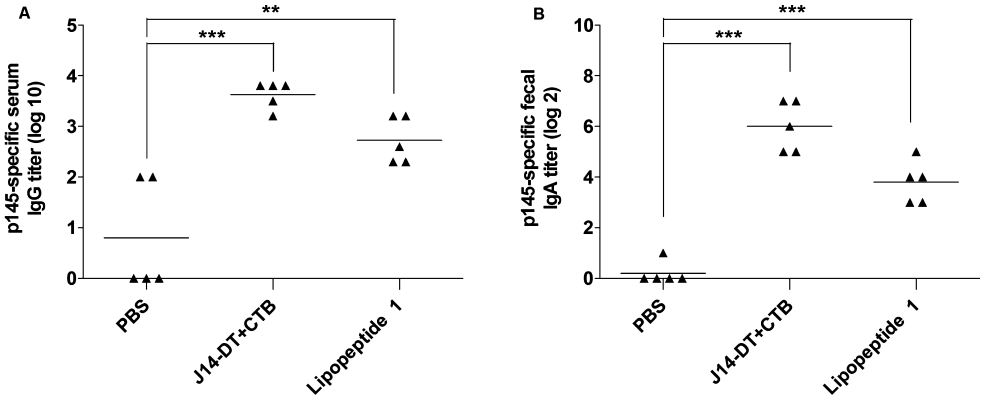

Figure 7. p145-specific serum IgG and fecal IgA titer (log10) at day 60 elicited in response to intranasal immunization of Swiss mice. (A) Serum IgG titer. (B) Fecal IgA titer. Mean p145-specific IgG/IgA titer is represented as a bar. Cohorts of Swiss mice (n = 5/group) received a primary immunization on day 0 followed by two further boosts on days 21 and 41. Serum was collected on days 20, 40, and 60 after primary immunization. Statistical analysis was performed using a one-way ANOVA followed by the Tukey post hoc test (ns, $p > 0.05$; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

Systemic and mucosal p145-specific antibody titers from the positive control (J14-DT+CTB) were the highest (Figure 7A,B) ($p < 0.001$ vs PBS). The ability of IgG and IgA antibodies elicited by lipopeptide **1** to recognize p145 suggests that the immune response is directed against the native, GAS derived sequence in J14, supporting the possibility of immunized mice recognizing GAS during infection.

In a separate experiment from the immunogenicity studies, a larger group (n = 15) of B10.BR mice were administered lipopeptide **1** and the nonmucosally active lipopeptide **4** intranasally with the same dose and immunization protocol described above. Then 56 days after primary immunization, mice were challenged intranasally with a virulent M1 GAS strain. Throat swabs obtained on days 1, 2, 3, 6, 9, and 15 after challenge were monitored for bacterial growth (Figure 8). Lipopeptide **1** was able to reduce throat colonization after GAS challenge; only 5 out of the 15 mice were swab positive on day 1 (Figure 8). In comparison, 12 and 10 out of 15 mice were swab positive on day 1 for the PBS and lipopeptide **4** groups, respectively (Figure 8). All surviving mice (75%) immunized with lipopeptide **1** were swab negative after day 3, which was similar to results achieved in the positive control group administered CTB with J14 (CTB+J14) (Figure 8). Lipopeptide **1** reduced GAS throat colonization, with a faster rate of GAS clearance than the mucosally inactive lipopeptide **4** (Figure 8). This observation coincides with the exceptional ability of lipopeptide **1** to induce mucosal IgA response in comparison to the mucosally inactive lipopeptide **4** as previously mentioned (Figure 3). Using a mucosal animal model system, our data showed a high degree of GAS colonization in the throats of B10.BR mice administered PBS and the mucosally inactive lipopeptide **4** following challenge with the virulent M1 GAS strain. In contrast, GAS colonization of the throat cleared more quickly and effectively in mice administered the mucosally active lipopeptide **1**. These data indicated that antidisease and transmission blocking immunity to GAS infection can be evoked following intranasal administration of lipopeptide **1**. A degree of lethality from virulent GAS challenge (75% protection) was still observed for mice immunized with lipopeptide **1** despite induction of an IgA and possibly due to lower level of IgG response in comparison to other GAS vaccine candidates.[13,32]

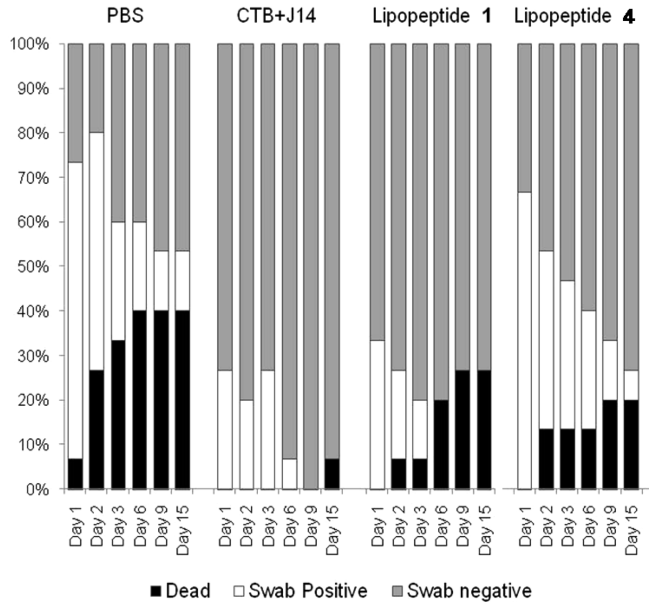

Figure 8. Percentage of dead, throat-swab-positive/-negative mice during the two weeks after challenge with virulent GAS M1 strain. Cohorts of B10.BR mice (n = 15/group) received a primary immunization on day 0 followed by two boosts on days 21 and 41. Mice were challenged intransally with virulent GAS M1 strain 56 days post primary immunization. Mice immunized with lipopeptide **1** were compared with PBS group (negative control group), lipopeptide **4**, and mice immunized with J14 coadministered CTB (CTB+J14; positive control group).

Protection from GAS infection at systemic sites has previously been correlated with systemic IgG with opsonic activity toward GAS.[13] The capacity of lipopeptide **1** induced systemic IgG to opsonize live M1 GAS was determined by an in vitro, indirect bactericidal assay. Antibodies from mice immunized with J14-DT+CTB and lipopeptides **1** had statistically significantly higher levels of GAS opsonization when compared with the PBS group (Figure 9). The average percentage opsonization of the virulent M1 GAS strain by J14-DT+CTB antisera was 72% (Figure 9). The average percentage opsonization by lipopeptide **1** antisera was 33% (Figure 9). The bactericidal assay demonstrated that the systemic IgG antibodies elicited by lipopeptide **1** were opsonic toward a virulent

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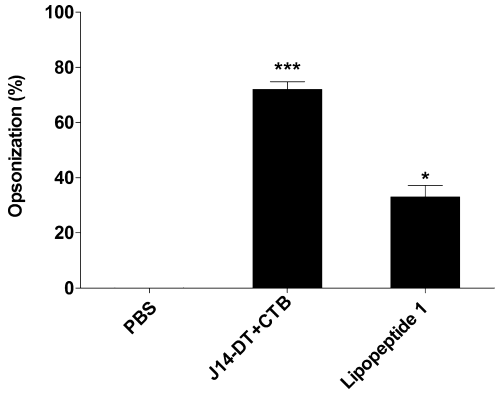

Figure 9. Average percent opsonization of M1 GAS strain by serum taken at day 60 postprimary immunization of Swiss mice. Statistical analysis was performed using a one-way ANOVA followed by Tukey's multiple comparison test (ns, $p > 0.05$; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

GAS strain, an important correlation of protection against GAS infection at systemic sites.[13]

Because of their amphipathic structure, lipopeptides can self-assemble into particles of distinct sizes in aqueous solutions.[37] Particle size is an important parameter for vaccine design that plays a crucial role in eliciting an immune response by affecting antigen uptake, processing, and biodistribution.[38] The size distribution of lipopeptides **1**–**5** was measured with a particle size analyzer (nanosizer) to determine if the ability of these lipopeptides to self-assemble and form particles of specific sizes could be correlated with mucosal immune response observed for lipopetide **1**. To visually define particle size, transmission electron microscopy (TEM) measurements of lipopeptides were also performed (Figure 10A–E). Analysis of the size distribution by intensity showed one peak at 12 nm (size at highest peak point) and two other peaks at 35 nm and 393 nm for lipopeptide **1**. These values coincided well with the TEM image of lipopeptide **1** (Figure 10A). Particle size by intensity for lipopeptide **2** showed three peaks at 10, 270, and 5560 nm. However, analysis of lipopeptide **2** by volume distribution revealed the 5560 nm aggregates were not present. This most likely occurred because not many particles are present at the larger size (5560 nm) and are only apparent by an intensity distribution. Large particles scatter more laser light than small particles, resulting in a higher intensity value for that size distribution. The TEM image of lipopeptide **2** (Figure 10B) correlated well with the two smaller sizes (10 and 270 nm). Lipopeptide **3** (Figure 10C) showed three peaks at 9, 30, and 163 nm. Lipopeptide **4** (Figure 10D) showed three peaks at 11, 64, and 266 nm. Three peaks at 11, 112, and 310 nm were observed for lipopeptide **5** (Figure 10E). All lipopeptides displayed a heterogeneous size distribution. As such, no direct correlation could be established between size and the different immunogenicity of the lipopeptides.

CONCLUSION

In this study, we have undertaken structure–activity investigations with lipopeptide constructs to develop a rationally designed GAS vaccine based on a lipopeptide platform technology. A promising GAS vaccine candidate was identified, and the efficacy of this vaccine candidate was further investigated by evaluating the immune responses via in vivo and in vitro assays. Lipid length and the position of vaccine components had a significant effect on the desired mucosal immune response. Our study highlighted the impact of the spatial arrangement of the vaccine components and choice of LAA alkyl chain length on the induction of mucosal immunity.

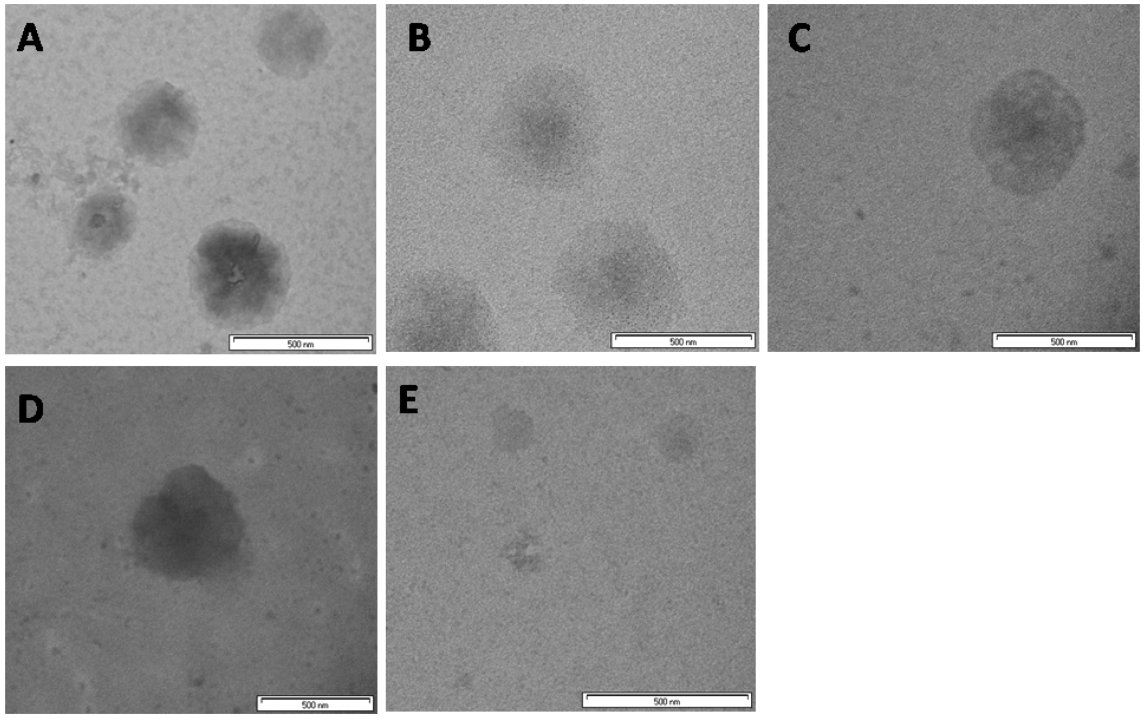

Figure 10. Transmission electron microscopy images of lipopeptides. Images are of a 2 mg/mL concentration of lipopeptides dissolved in PBS, representing the concentration administered for immunological studies. (A) Lipopeptide **1**. (B) Lipopeptide **2**. (C) Lipopeptide **3**. (D) Lipopeptide **4**. (E) Lipopeptide **5**. Scale bars: 500 nm.

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The leading GAS vaccine candidate featured a C-terminal lipid moiety with an alkyl chain length of 16 carbons, attached through a lysine residue to P25 at the N-terminus, and J14 on the lysine side chain. The structure−activity study provided a rational mucosal vaccine design, and consequent in vivo and in vitro studies identified the leading lipopeptide as a promising and efficacious GAS vaccine candidate. The GAS vaccine candidate elicited IgA, which correlated with reduction in GAS colonization the upper respiratory tract. Optimal GAS vaccine requires induction of systemic IgG responses for protection against lethality and ability of systemic IgG induced by the vaccine candidate to opsonize GAS was demonstrated. IgG isotyping provided an insight into the cytokine responses elicited by the lipopeptide and particle size analysis demonstrated lipopeptides self-assembled to form aggregates that were heterogeneous in their size distribution.

Our findings are an important step toward overcoming current obstacles in the development of a GAS vaccine to prevent infection at mucosal sites. No human approved mucosal adjuvant exists for the delivery of peptides and the mucosal self-adjuvanticity of the lipopeptide-based delivery system overcomes this obstacle. Furthermore, the study demonstrates how several features of a lipopeptidic vaccine construct (epitope and lipid position/structure) can collectively induce the desired mucosal immune responses to combat infection. This work is the first to report on a self-adjuvanting, LAA-based, mucosally active GAS vaccine candidate. This strategy could be a useful approach in the development of peptide-based mucosal vaccines against other pathogenic organisms.

EXPERIMENTAL SECTION

Peptide Synthesis. Lipopeptides **1**−**5** were synthesized, purified, and characterized as described elsewhere.[31] Lipopeptides was synthesized on *p*MBHA resin (0.4 mmol NH_2/g; 0.5 mmol scale) using manual stepwise solid-phase peptide synthesis, 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate and *N*,*N*-diisopropylethylamine in situ neutralization, and Boc-chemistry.[39] After the synthesis of each lipopeptide, resins were cleaved with anhydrous HF. Following HF removal under reduced pressure, the peptides were precipitated in ice-cold diethyl ether, filtered, and dissolved in 40% aqueous acetonitrile (MeCN) containing 0.1% trifluoroacetic acid (TFA) and lyophilized. The lyophilized products were then purified by preparative RP-HPLC on C4 column using a gradient of 10% solvent B (90% MeCN/0.1% TFA/H_2O) to 100% solvent B over 60 min. The fractions were analyzed by ESI-MS, SDS-PAGE, and analytical RP-HPLC and where appropriate combined to give pure product (>95%).

Immunological Assessment of Vaccine Candidates. *Animals.* Four−six-week-old female B10.BR ($H\text{-}2^K$) or outbred ARC-Swiss mice were used in this study (The Animal Resource Centre, Perth, Western Australia). All animal protocols used were approved by the Institute's ethics committee (Griffith University Research Ethics Review Board for Animal-Based Work, GU ref no. BDD-06-10-AEC) in accordance with National Health and Medical Research Council (NHMRC) of Australia guidelines.

Immunization of Mice. Cohorts of 5 B10.BR or 5 ARC-Swiss mice were intranasally administered 60 μg of diastereomeric lipopeptides dissolved in 30 μL (15 μL/nare) of sterile phosphate-buffered saline (PBS), followed by similar booster doses on days 21 and 41. Similarly, a negative control was administered 30 μL of PBS and a positive control received 30 μg of J14 conjugated to diptheria toxoid (J14-DT) coadministered with 10 μg of CTB in a total volume of 30 μL of PBS. Mice were anesthetized with a mixture of xylazine and ketamine (1:1:10 mixture of xylazine:ketamine:H_2O; Provet, Sydney, Australia) prior to immunization.

Preparation of Antigen-Specific Immune Serum, Saliva, and Fecal Samples. Serum was collected on days 20, 40, and 60 postprimary immunization to determine the level of J14- or p145-specific systemic antibodies. Blood was collected from mice via the tail artery and allowed to clot for at least 30 min at 37 °C. Serum was then collected after centrifugation for 10 min at 1000*g*, heat inactivated for 10 min at 56 °C, and stored at −20 °C. Two fecal and saliva samples were collected six days after each booster immunization for measurement of mucosal antibodies. Mice were administered 50 μL of a 0.1% solution of pilocarpine (Ioquin) to induce salivation. Saliva was then collected and mixed with 2 μL of 50 mmol/L phenylmethylsulfonylfluoride (PMSF) protease inhibitor (Sigma, United States). Particulate matter was separated by centrifugation for 10 min at 13000*g*, and samples were stored at −70 °C.

Six to ten fresh fecal pellets were collected from individual mice, frozen at −70 °C, and lyophilized. The dry weight of fecal solids was determined before they were resuspended by vortexing in 5% nonfat dry milk, 50 mmol/L EDTA, 0.1 mg/mL soyabean trypsin inhibitor (Sigma, United States), and 2 mmol/L PMSF (20 mL/mg of dry weight). Solid matter was separated by centrifugation for 10 min at 13000*g*.

ELISA. ELISA was performed for J14- and p145-specific antibody determination as essentially described elsewhere.[17] Serial 2-fold dilutions of samples were produced in 0.5% skim milk/PBS-Tween 20 buffer, starting at a concentration of 1:100 for sera and 1:1 for saliva and fecal samples. The titers were described as the lowest dilution that gave an absorbance of >3 standard deviation (SD) above the mean absorbance of control wells (containing normal mouse serum immunized with PBS). Statistical significance ($p < 0.05$) was determined using a one-way analysis of variance (ANOVA) with Tukey post hoc test. For IgG isotype determination, horse radish peroxidase-conjugated sheep antimouse IgG1, IgG2a, IgG2b, and IgG3 antibodies were used as the secondary antibodies.

Indirect Bactericidal Assay. Sera from immunized mice were assayed for their ability to opsonize GAS in vitro as essentially described elsewhere.[17] M1 GAS strain was incubated end over end at 37 °C for 3 h in the presence of nonopsonic human donor blood (prescreened prior to the assay ensuring that it could support GAS growth by at least 32 times the inoculums) and either heat-inactivated (60 °C for 10 min) individual mouse immune serum or control normal mouse serum. GAS was then plated in duplicate on 2% (v/v) Todd−Hewitt agar plates, and colonies were counted after 24 h of incubation at 37 °C. The percentage of GAS opsonized was determined by counting the number of colonies growing after incubation with test immune serum and comparison to the number of colonies growing after incubation with control serum. Opsonic activity was calculated (percent reduction in mean colony forming units (cfu)) as [1 − (cfu in the presence of antipeptide sera)/(mean cfu in the presence of normal mouse sera)] × 100. Statistical significance ($p < 0.05$) was determined using a one-way ANOVA with Tukey post hoc test.

Intranasal GAS Colonization Experiment. Cohorts of 15 B10.BR mice were intranasally administered 60 μg of lipopeptides dissolved in 30 μL (15 μL/nare) of sterile phosphate-buffered saline (PBS), followed by similar booster doses on days 21 and 41. Similarly, a negative control was administered 30 μL of PBS and a positive control received three doses of 30 μg of J14 peptide coadministered with 10 μg of CTB (CTB+J14) in a total volume of 30 μL of PBS . Mice were challenged intransally with a predetermined dose of the virulent GAS M1 strain 56 days post primary immunization. Throat swabs were obtained from mice on days 1, 2, 3, 6, 9, and 15 after challenge to determine GAS colonization. Throat swabs were streaked out on Todd−Hewitt agar plates containing 2% horse blood and incubated overnight at 37 °C. Swabs yielding one or more GAS cfu were considered positive.

Measurement of Lipopeptide Particle Size. Lipopeptides were dissolved in PBS at the same concentration for in vivo studies. The average particle size (nm) of each lipopeptide in solution was taken at 25 °C using a Nanosizer (Zetasizer Nano Series ZS, Malvern Instruments, United Kingdom) with disposable capillary cuvettes.

Sizes were analyzed using a noninvasive backscatter system, and measurements taken with a scattering angle of 173°. Correlation times were based on 10 s per run, and a total of three runs were made per measurement. The results are shown as the average of triplicate measurements. Results were analyzed using Dispersion Technology Software (Malvern Instruments, United Kingdom).

Transmission Electron Microscopy (TEM). Samples of the lipopeptide solution used for the Nanosizer experiments were added to carbon coated 200 mesh grids. After 3 min, the excess liquid was wicked off with filter paper. Pictures were taken from a JEM-1010 transmission electron microscope (JEOL Ltd., Japan) operated at 80 kV.

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The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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ABBREVIATIONS USED

Boc, *tert*-butoxycarbonyl; CFA, complete Freund's adjuvant; CTB, cholera toxin B-subunit IgA/IgG, immunoglobulin A/G; LAA, lipoamino acid; PBS, phosphate buffered saline; *p*MBHA, *p*-methylbenzhydrylamine; SPPS, solid-phase peptide synthesis; TEM, transmission electron microscopy; TLR2, Toll-like receptor 2

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