

## Group A streptococcal vaccine delivery by immunization with a self-adjuvanting M protein-based lipid core peptide construct

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**Background & objectives:** To develop a broad strain coverage GAS vaccine, several strategies have been investigated which included multi-epitope approaches as well as targeting the M protein conserved C-region. These approaches, however, have relied on the use of adjuvants that are toxic for human application. The development of safe and effective adjuvants for human use is a key issue in the development of effective vaccines. In this study, we investigated the lipid polylysine core peptide (LCP) system as a self-adjuvanting GAS vaccine delivery approach.

**Methods:** An LCP-GAS construct was synthesised incorporating multiple copies of a protective peptide epitope (J8) from the conserved carboxy terminal C-repeat region of the M protein. B10.BR mice were immunized parenterally with the LCP-J8 construct, with or without conventional adjuvant, prior to the assessment of immunogenicity and the induction of serum opsonic antibodies.

**Results:** Our data demonstrated immunogenicity of LCP-J8 when coadministered in complete Freund's adjuvant (CFA), or administered in the absence of conventional adjuvant. In both cases, immunization led to the induction of high-titre J8 peptide-specific serum IgG antibody responses, and the induction of heterologous opsonic antibodies that did not cross-react with human heart tissue proteins.

**Interpretation & conclusion:** These data indicated the potential of a novel self-adjuvanting LCP vaccine delivery system incorporating a synthetic GAS M protein C-region peptide immunogen in the induction of broadly protective immune responses, and pointed to the potential application of this system in human vaccine development against infectious diseases.

**Key words** GAS vaccine delivery - lipid core peptides - M protein - synthetic peptide vaccines

The development of a vaccine against group A streptococci (GAS) the etiologic agents of rheumatic fever and rheumatic heart disease has focused largely on the bacterial surface anti-phagocytic M protein<sup>1,2</sup>. The M protein is a major virulence factor in GAS infection and consists of a variable amino terminal region which defines the GAS serotype (over 100 serotypes are known), and a highly conserved carboxy terminal C-repeat region<sup>1</sup>. Protective immunity to GAS infection has been associated with type-specific opsonic antibodies

against M protein<sup>1,2</sup> although opsonic antibodies specific to the C-region have been demonstrated in humans<sup>3</sup>, and mice immunized with C-region peptides<sup>4</sup>, and are also important in eliciting protective immunity to GAS<sup>5</sup>. The variability in M proteins and the potential for the induction of autoimmunity due to antigenic molecular mimicry between GAS M protein and heart antigens<sup>6-9</sup>, represent significant hurdles in the development of a broad strain coverage vaccine. Multivalent M protein constructs containing epitopes from several type-specific regions

of different M proteins<sup>5, 10, 11</sup> and those based on the conserved C-region<sup>5,12-14</sup>, have shown promising results in animal trials. The efficacy of the GAS vaccine constructs, however, required the use of adjuvants that are not suitable for use in humans due to their toxicity. The development of novel synthetic adjuvants offers the possibility of vaccine delivery without the need for additional, toxic, adjuvant.

Synthetic lipid-based compounds provide an attractive alternative for vaccine delivery. Lipopeptide compounds utilising a synthetic analog of the N-terminal moiety of bacterial lipoprotein from *Escherichia coli* (tripalmitoyl-S-glyceryl cysteine, Pam3cys) as a lipidic anchor moiety<sup>15</sup>, were found to be potent immunogens with self-adjuncting properties, eliciting humoral and cellular responses irrespective of the route of administration<sup>16-19</sup>. The lipidic polylysine core peptide (LCP) system<sup>20</sup>, has also been described using a lipidic anchor moiety in conjunction with the multiple antigenic peptide system<sup>21</sup>, and is uniquely designed to incorporate antigen, carrier and adjuvant in a single molecular entity. Furthermore, lipopeptide compounds represent potentially safe vaccines for human application<sup>22</sup>. LCP-based vaccine candidates incorporating variable domains of *Chlamydia trachomatis* outer membrane protein have been shown to significantly enhance peptide immunogenicity when compared to peptide monomers given alone in adjuvant<sup>23</sup>, and an LCP compound incorporating a foot-and-mouth disease viral peptide was immunogenic resulting in the induction of anti-peptide antibodies in the absence of additional adjuvant<sup>24</sup>.

This study was carried out to investigate the LCP system as a novel self-adjuncting GAS vaccine delivery approach. An LCP construct was synthesised incorporating multiple copies of a GAS M protein conserved C-region protective conformational peptide epitope, referred to as J8, which lacks potentially deleterious T-cell autoepitopes<sup>25</sup>. Mice were immunized parenterally with the LCP-J8 construct prior to the assessment of immunogenicity and the induction of serum opsonic antibodies.

## Material & Methods

*Synthesis and purification of group A streptococcal J8 peptide:* The sequence of the J8 peptide is

QAEDKVKQSREAKKQVEKALKQLEDKVVQ corresponding to amino acid residues 344-355 inclusive of the conserved carboxy terminal C-region of the M protein of the M1 GAS strain. The J8 peptide used is a chimeric peptide that contains 12 amino acids from the M protein C-region (shown in bold) and is flanked by yeast-derived GCN4 sequences which was necessary to maintain the correct helical folding and conformational structure of the peptide<sup>26</sup>. J8 peptide was synthesised by manual solid phase peptide synthesis using Boc (*tert*-butoxycarbonyl) chemistry<sup>27</sup>, and peptide purification was carried out on a Waters HPLC system (model 600 controller, 490 E UV detector, 60 F pump).

*Synthesis of LCP-J8 construct:* Boc (*tert*-butoxycarbonyl)-L-amino acids and 4-methylbenzhydrylamine (MBHA) resin (Novabiochem, Läufelfingen, Switzerland) were used to synthesise the LCP-J8 construct (Fig. 1). Racemic lipoamino acids were synthesized with Boc protection according to Gibbons *et al*<sup>28</sup>. For the synthesis of the LCP-J8 construct containing four copies each of J8 GAS peptide, preactivated Boc-Gly-OH was coupled to the MBHA resin. The next two cycles were carried out with Boc-lipoamino acids containing 12 carbon atoms (C12: -HN-CH[(CH<sub>2</sub>)<sub>9</sub>CH<sub>3</sub>]-OH) which was followed sequentially by the coupling of Boc-Gly-OH, Boc-C12-OH and Boc-Lys(Boc)-OH. After deprotection of the lysine  $\alpha$ - and  $\epsilon$ - amino groups, a four-branch system was formed by coupling of Boc-Lys(Boc)-OH to the free amino groups. After deprotection, four identical peptide J8 sequences were synthesised directly onto each of the  $\alpha$ - and  $\epsilon$ - amino groups of each lysine of the branched lysine core with the appropriate protecting groups applied on the side chains of the amino acids. Thus, the lipophilic anchor of the LCP-J8 construct contained three 2-amino-dodecanoic lipoamino acids attached to the polylysine core, with glycine spacers employed; one between the resin and the first lipoamino acid and another between the second and third lipoamino acids. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel electrophoresis resulted in a band for LCP-J8 of the expected size. Determination of the mass spectrum of LCP-J8 resulted in a calculated molecular weight of 14,158.45 daltons.

*Mice and immunization:* All protocols were approved by the Bancroft Centre Research Ethics Committee, and

were carried out according to Australian National Health and Medical Research guidelines for the care and use of laboratory animals. Female (4-6 wk old) B10.BR mice (Animal Resource Centre, Perth, WA, Australia) were used (n=10 per group) in two separate experiments for subcutaneous immunization at the tail base on day 0 with 30 µg LCP-J8, either emulsified 1:1 with complete Freund's adjuvant (CFA) (Sigma, USA) (LCP-J8/CFA), or given alone in a total volume of 50 µl sterile-filtered PBS (LCP-J8/PBS). After three weeks, mice received a further four (exp 1) or five boosts (exp 2) at weekly intervals with 3 µg LCP-J8/PBS prior to the collection of blood. Controls received either 30 µg J8 GAS peptide or pepM1 (amino terminal half of M protein) in CFA with boosts of 3 µg in PBS, or CFA/PBS alone.

*Collection of sera:* Blood was collected one week after the last immunization from each mouse by the tail artery and allowed to clot at 37°C for 1 h followed by the removal of clots by centrifugation at 3000 rpm for 10 min. Sera were then stored at -20°C.

*ELISA:* Enzyme-linked immunosorbent assay (ELISA) was performed for the measurement of total IgG antibody titres in sera, essentially as previously described<sup>4</sup>. Serum samples were assayed using two-fold dilutions of a 1 in 100 dilution of sera. For IgG isotyping, horse radish peroxidase-conjugated sheep anti-mouse IgG1, IgG2a, IgG2b and IgG3 antibodies (The Binding Site, Birmingham, UK) were used as the secondary antibodies. Antibody titres were defined as the lowest dilution that gave an optical density (OD) reading at 450 nm of more than three standard deviations above the mean OD of control wells containing normal mouse sera (obtained from mice immunized with CFA in PBS).

*Bactericidal assay :* Serum opsonic antibodies against the M1, 8830, NS27 and BSB24 GAS strains were detected using an indirect bactericidal assay, as previously described<sup>3,4</sup>. Briefly, GAS were incubated end-over-end at 37°C for 3 h in the presence of non-opsonic human donor blood and either heat-inactivated (60°C for 10 min) mouse immune or control normal mouse sera. GAS were then plated in duplicate on 2 per cent (v/v) horse blood agar plates and colonies counted after 24 h of incubation. The percentage of GAS opsonized was determined by counting the number of colonies growing after incubation with test immune sera and comparing this with the

number of colonies growing after incubation with control sera. The opsonic activity (% reduction in mean cfu) was calculated as  $[1 - (\text{cfu in the presence of immune sera}) / (\text{mean cfu in the presence of normal mouse sera})] \times 100$ .

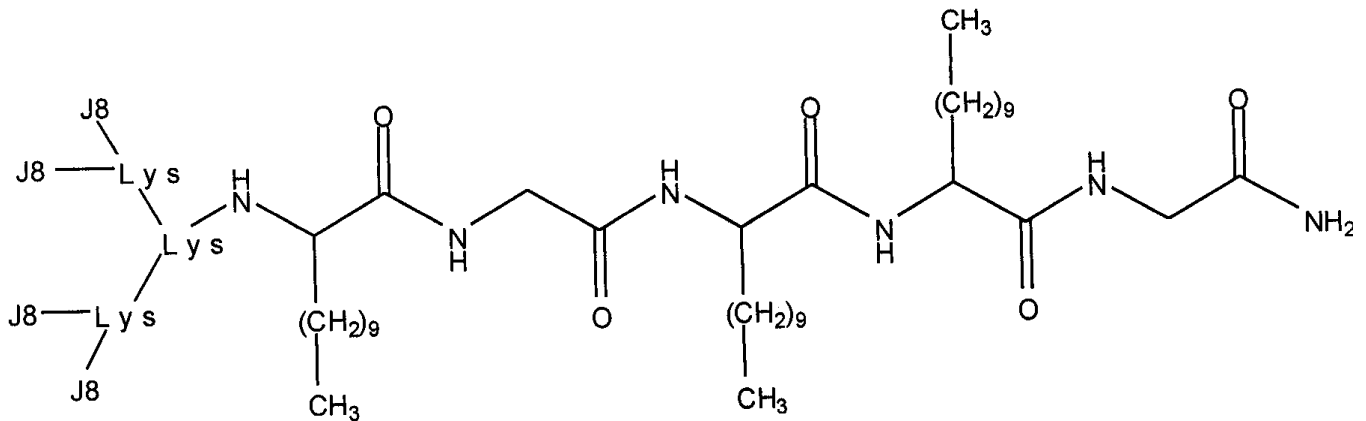
*Peptide inhibition bactericidal assay :* Peptide inhibition bactericidal assay was performed essentially as described above except that sera were initially incubated for 30 min at room temperature with 100 µg peptide, or no peptide, prior to incubation with GAS and non-opsonic donor blood. Peptides used were J8 GAS peptide, and a non-specific control peptide from *Schistosoma* (EGKVSTLPLDIQIIAATMSK). The percentage inhibition of opsonisation was calculated as  $(\text{immune sera with peptide cfu} - \text{immune sera with no peptide cfu}) / \text{normal mouse sera with no peptide cfu} \times 100$ .

*Western blot analysis:* Mouse LCP-J8 antisera were tested for cross-reactivity to four different human heart preparations (two heart and two mitral valve extracts) obtained from patients undergoing heart transplant surgery at The Prince Charles Hospital with ethical approval from The Prince Charles Hospital Foundation, porcine heart myosin (Sigma, USA) and porcine muscle tropomyosin (Sigma), using standard SDS-PAGE and Western blot analysis<sup>4</sup>. The J8 GAS peptide was conjugated to diphtheria toxoid (DT) and included as a positive control. Pre-stained kaleidoscope protein size markers were used (Biorad, Hercules, CA, USA). Antiserum was used as the primary antibody at a dilution of 1: 1,500 and the secondary sheep anti-mouse IgG antibody (Silenus Pty Ltd., Melbourne, VIC, Australia) was used at a dilution of 1: 1,500.

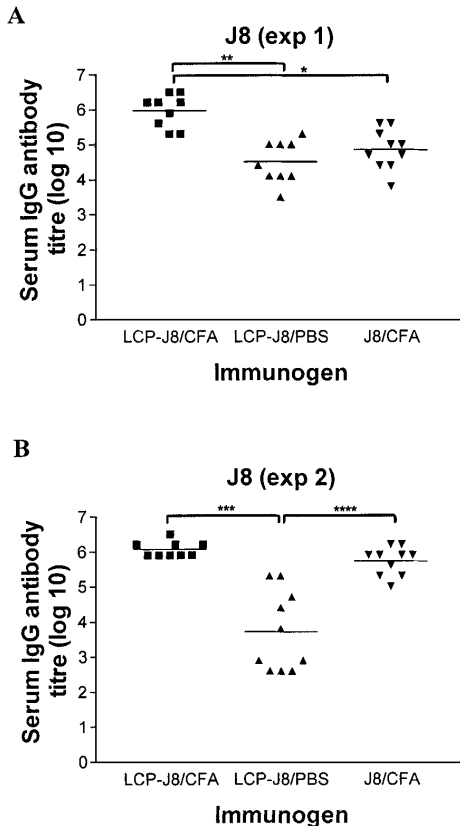
*Statistical analysis:* Analysis of variance (ANOVA) with Tukey's post-hoc method for multiple comparisons was used for the comparison of antibody titres and percentage opsonisation levels between the different groups. SPSS Release 10.0 was used for statistical analysis.  $P < 0.05$  was taken as statistically significant.

## Results

*Serum IgG antibody response in B10.BR mice immunized with LCP-J8 :* In the first experiment (Fig. 2A), in which mice received a primary immunization

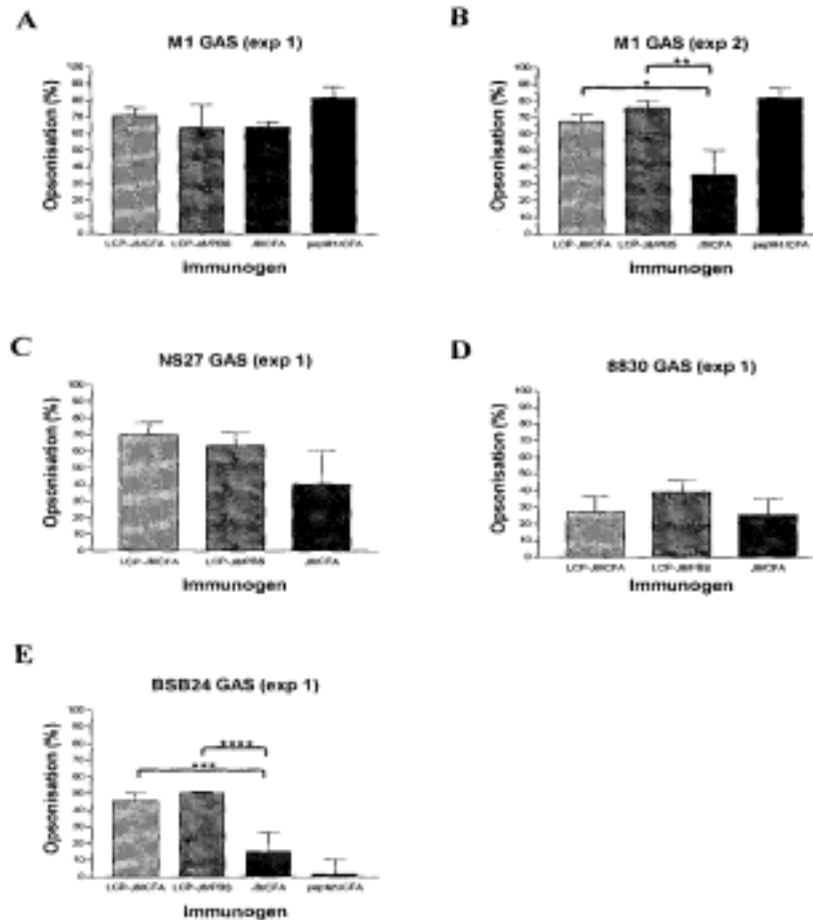


**Fig.1.** Chemical structure of the LCP-J8 construct. Four identical peptide J8 sequences were synthesised directly onto the two amino groups of each lysine (Lys) of a branched polylysine core. The lipophilic anchor of the LCP-J8 construct contains three 2-amino-dodecanoic lipopeptides (shown as branched alkyl side chains) attached to the polylysine core, with a glycine amino acid spacer between the resin and the first lipopeptide, and a second between the second and third lipopeptides.



**Fig.2.** Serum IgG antibody response in B10.BR mice immunized parenterally with the LCP-J8 construct in the presence or absence of CFA, for experiment 1 (A) and experiment 2 (B). Antibody titres to the J8 peptide for individual mice are shown, with the average titre (geometric mean) represented as a bar. Antibody titres to J8 are shown for individual control mice that were immunized with J8 in CFA. Antibody titres were significantly greater in mice immunized with LCP-J8/CFA when compared to those immunized with J8 alone in CFA (exp 1; \* $P < 0.001$ ). Significant differences in antibody titres were also observed as indicated by \*\* $P < 0.001$ , \*\*\* $P < 0.001$  and \*\*\*\* $P < 0.001$ .

and four boosts each of the same immunogen, J8-specific antibodies were detected in all mice three weeks after the primary immunization with LCP-J8 in CFA, and J8 in CFA giving a final average antibody titre after four boosts of  $1.5 \times 10^6$  and  $1.4 \times 10^5$ , respectively (exp 1; Fig.2A). J8-specific antibodies were not detected at three weeks post-immunization, in mice immunized with LCP-J8 without adjuvant. However, after one boost of immunogen, six of the nine mice had J8-specific antibodies and after the third boost, antibodies to J8 were detected in all mice. After the final boost (boost number four) the average J8 antibody titre in mice immunized with the LCP construct without adjuvant was  $4.4 \times 10^4$  (exp 1). In the second experiment (Fig.2B), in which mice received a primary immunization and five boosts each of the same immunogen, the final average J8-specific serum IgG antibody titres were approximately  $1.3 \times 10^6$ ,  $5.0 \times 10^4$  and  $7.5 \times 10^5$  for mice immunized with LCP-J8/CFA, LCP-J8/PBS and J8/CFA, respectively. IgG isotyping demonstrated strong J8-specific IgG1, IgG2b and IgG2a antibody responses with lower titres or no J8-specific antibodies detected for IgG3 in LCP-J8/CFA and J8/CFA-immunized mice. In mice immunized with LCP-J8 alone, there was a strong IgG1 response and IgG3 was only detected in one mouse. Of these mice, 50 per cent demonstrated an IgG2a response (exp 2) and IgG2b was barely detected in either experiment. No IgG2a was detected in experiment 1. Our data showed that the LCP-J8 construct was more immunogenic in CFA when compared to monomeric J8 peptide given in CFA, and was also immunogenic when delivered in the absence of adjuvant.



**Fig.3.** Opsonisation of GAS by LCP-J8/CFA, LCP-J8/PBS and control (J8/CFA and pepM1/CFA) B10.BR mouse antisera for experiment 1 (A,C-E) and experiment 2 (B). The average percentage opsonisation for each individual group is shown with the standard error of the mean represented as a bar. Opsonisation against M1 and BSB24 GAS was significantly greater in mice immunized with LCP-J8/CFA and LCP-J8/PBS when compared to those immunized with J8 alone in CFA (exp 2,  $*P=0.051$  and  $**P=0.008$ , respectively for M1; exp1,  $***P=0.031$  and  $****P=0.013$ , respectively for BSB24).

*Induction of opsonic antibodies in B10.BR mice immunized with LCP-J8:* Opsonic activity of serum IgG antibodies elicited after parenteral delivery of LCP-J8 to the homologous M1 GAS strain was assessed (Fig. 3). The average percentage opsonisation of M1 GAS by J8/CFA antisera generated in experiments 1 and 2 was 64 and 36 per cent, respectively, and the pepM1 positive control gave an average percentage opsonisation of 82 per cent for each experiment. The average percentage opsonisation for LCP-J8/CFA antisera against M1 was 71 per cent (exp 1) and 68 per cent (exp 2); 64 per cent (exp 1) and 76 per cent (exp 2) for LCP-J8/PBS antisera. To confirm the specificity of the opsonic antibodies induced in LCP-J8-immunized mice, a peptide inhibition bactericidal assay was performed. The opsonisation of M1 GAS by pooled LCP-J8/CFA and LCP-J8/PBS antisera in the absence

of peptide was 37 and 55 per cent, respectively. Pre-incubation of LCP-J8/CFA antisera with J8, however, led to the complete inhibition of opsonisation. There was a 91 per cent inhibition of opsonisation for LCP-J8/PBS antisera pre-incubated with J8. The non-specific peptide was shown to inhibit opsonisation of LCP-J8/CFA and LCP-J8/PBS antisera by 12 and 27 per cent, respectively. Incubation of pepM1 antisera with J8 also had little effect on opsonisation (23% inhibition of opsonisation), consistent with the fact that M protein represents the amino terminal half of M protein.

The opsonic ability of LCP-J8 antisera against another three GAS strains- NS27, 8830 and BSB24 was assessed (Fig.3). LCP-J8/CFA and LCP-J8/PBS antisera opsonised NS27 GAS with an average percentage opsonisation of 70 and 64 per cent, respectively. Lower opsonisation was

observed against 8830 GAS (27% for LCP-J8/CFA antisera and 39% for LCP-J8/PBS antisera). In the case of BSB24, LCP-J8/CFA and LCP-J8/PBS antisera opsonised GAS with an average percentage opsonisation of 46 and 50 per cent, respectively. J8/CFA antisera opsonised NS27, 8830 and BSB24 GAS with an average percentage opsonisation of 40, 26 and 15 per cent, respectively. Antisera to pepM1 was shown not to opsonise the heterologous GAS strain, BSB24.

### Discussion

In this study we report on the synthesis, immunogenicity and protective potential of a novel LCP-GAS vaccine candidate incorporating a non-host cross-reactive M protein conserved C-region protective peptide epitope. Our data showed that mice immunized with the LCP-J8 construct elicited high-titre serum J8-specific IgG antibodies when the construct was coadministered with CFA or administered alone. Further, opsonisation of the homologous M1 GAS strain by LCP-J8/CFA and LCP-J8/PBS antisera indicated that serum opsonic antibodies were induced in mice following immunization with the LCP-J8 construct. Moreover, the induction of opsonic antibodies was not dependent on the presence of conventional adjuvant, supporting the efficacy of the LCP system as a self-adjuncting GAS vaccine delivery modality. The ability of the J8 peptide to inhibit the opsonisation of M1 GAS by LCP-J8 antisera indicates that LCP-J8 induced serum opsonic antibodies specifically directed against the M protein conserved C-region J8 peptide epitope on GAS, and that these antibodies may potentially be important in protective immunity against GAS.

This prompted us to determine whether serum IgG antibodies induced to LCP-J8 could opsonise other GAS strains, and secondly whether heart cross-reactive antibodies were elicited. We assessed the opsonic ability of the LCP-J8 antisera against another three GAS strains- NS27, 8830 and BSB24. Both NS27 and 8830 contain an identical sequence to J8 in the C-region whereas BSB24 has disparities in three amino acids (underlined) with the sequence SREAKKKVEADL. LCP-J8/CFA and LCP-J8/PBS antisera opsonised all three GAS strains to varying degrees, with the highest opsonisation observed against NS27. The induction of serum heterologous opsonic antibodies against different GAS strains following immunization of mice with LCP-

J8 was supportive of the efficacy of a broadly protective GAS vaccine based on the conserved C-region of the GAS M protein. Moreover, no cross-reactivity of LCP-J8 antisera was observed to heart proteins in human heart and mitral valve extracts. These data further highlighted the potential of LCP-J8 in the induction of broadly protective immune responses in the absence of conventional adjuvant. These results supported our earlier findings demonstrating the efficacy of the LCP system as a vaccine delivery approach for enhancing the immunogenicity of various synthetic peptide immunogens<sup>23,24</sup>, including viral and bacterially-derived antigens.

In conclusion, the present findings indicated the potential of the LCP system in the delivery of a self-adjuncting synthetic peptide GAS vaccine, with a view to the development of a mucosal-based vaccine for human application.

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