Structure-function relationships of protein-lipopeptide complexes and influence on immunogenicity

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ABSTRACT

The lipopeptide, R₄Pam₂Cys, associates electrostatically with soluble protein antigens and significantly enhances their ability to induce protective humoral and cell-mediated responses. We demonstrate that antibody titers elicited by the antigen ovalbumin (OVA) associated with R₄Pam₂Cys are higher than those elicited by OVA in the presence of Alum and comparable to those elicited by OVA formulated with complete Freund's adjuvant (CFA). The hierarchy of anti-OVA antibody avidities was CFA > R₄Pam₂Cys = Alum. Each of the three adjuvants facilitated IgG class switching with significantly more IgG1 elicited by OVA when formulated with R₄Pam₂Cys. The effects of substituting naturally occurring L-stereoisomers of the cationic residues within R₄Pam₂Cys with D-stereoisomers revealed that substitution did not affect the ability of R₄Pam₂Cys to stimulate dendritic cell maturation or its ability to elicit antibody production when used as adjuvant. Minor detrimental effects were, however, observed in ensuing CD8⁺ T cell responses suggesting that the use of D-amino acids affects antigen processing and presentation pathways involved in generation of cell-mediated immunity at least when facilitated through TLR2. Both D- and L-forms were found to be resistant to digestion by trypsin indicating resistance of the branched structure to protease activity.

Keywords: lipopeptides, Toll-like receptors, antibody, CD8⁺ T cells

INTRODUCTION

Adjuvants are often administered with antigen to improve the quality and magnitude of ensuing immune responses. Alum, which has been used in human vaccines for more than 80 years, is appropriate for those vaccines where clearance of pathogens depends on the induction of *antibody* responses (Pasquale et al. 2015). Alum is not, however, known for its ability to elicit and maintain cell-mediated CD8⁺ T cell responses (Knudsen et al. 2016).

Vaccine delivery approaches using agonists of Toll-like receptors (TLRs) offer an advantage over Alum because they facilitate delivery of antigens to dendritic cells (DCs) improving antigen uptake leading to cell maturation which results in the production of pro-inflammatory cytokines that can lead to induction of antibody as well as cell-mediated immunity (CMI) particularly CD8⁺ T cell responses (Kastenmüller et al. 2011; Pufnock et al. 2011; Chua et al. 2011b; Chua et al. 2015; Goff et al. 2015). The induction of CMI is particularly important in providing protection against intracellular pathogens (Elemans et al. 2012).

Our laboratory has developed a lipopeptide-based vaccine delivery system containing the TLR2 agonist S-[2,3-bis(palmitoyloxy)propyl] cysteine (Pam₂Cys). A feature of this lipopeptide, R₄Pam₂Cys (Fig 1A), is its branched structure which is based on a scaffold of lysine residues with N-terminal arginine residues which impart an overall positive charge. This structure allows electrostatic association with oppositely charged regions of protein antigens under physiological conditions (Chua et al. 2011b). The non-covalent association between antigen and R₄Pam₂Cys overcomes the complexities associated with covalent conjugation of antigen and adjuvant. Formulation of antigens with R₄Pam₂Cys enhances their immunogenicity (Chua et al. 2011a; Chua et al. 2012; Chua et al. 2015) and is more effective at inducing CD8⁺ T cell responses when compared to other adjuvants (Chua et al. 2014).

Our previously reported (Chua et al. 2011b) method of synthesis of R₄Pam₂Cys was to couple *Fmoc*-Lys(*Mtt*)-OH to the solid support which allowed the branched lysine-based structure to be assembled from the α-amino group exposed following removal of the *Fmoc* protecting group (Fig. 1B, Method 1). Attachment of Pam₂Cys then takes place following removal of the *Mtt* group from the ε-amino group of the C-terminal lysine. While this method is effective, there remains a possibility that steric hindrance posed by the branched lysine structure may limit assembly of the Pam₂Cys moiety. We reasoned that improved yields may be attained if Pam₂Cys is assembled *before* the branched structure. We therefore used *Dde*-Lys(*Fmoc*)-OH in the C-terminal position which allows attachment of the lipid at the ε-amino group before beginning assembly of the branched structure at the α-amino group (Fig. 1B, Method 2). We then compared the quality and yield of R₄Pam₂Cys synthesized using Method 1 and Method 2 as well as investigating the biological activities of the products by examining their abilities to induce antibody and cell-mediated responses when formulated with the model antigen ovalbumin (OVA).

The use of synthetic peptides in clinical applications can be hampered by short biological half-lives due to their susceptibility to *in vivo* enzymatic degradation by proteases (Galati et al. 2003). Peptides composed of D-amino acids are, however, more protease-resistant while retaining biological activity (Hamamoto et al. 2002; Tugyi et al. 2005). We therefore examined the effects of substituting L-stereoisomers of component amino acids with the corresponding D-stereoisomers and evaluated if these alterations affected the susceptibility of R₄Pam₂Cys to protease-mediated hydrolysis as well as its ability to elicit antibody and CD8⁺ T cell responses.

MATERIALS & METHODS

Synthesis of R₄Pam₂Cys

Schematics summarizing the strategies employed for the assembly of R₄Pam₂Cys are shown in Figure 1B, Method 1 & 2.

In Method 1, Fmoc-Lys(Mtt)-OH (Novabiochem, Hohenbrunn, Germany) was coupled to the solid support, PEG-SRAM (Rapp Polymere, Tübingen; Germany, substitution factor 0.24 mmol/g), in 4-fold molar excess in the presence of equimolar amounts of O-benzotriazole-N,N,N',N'-tetramethyl-uronium-hexa-fluoro-phosphate (HBTU; Protein technology, Inc, USA.), 1-hydroxyben-zotriazole (HOBt; CEM, USA), and a 6-fold molar excess of diisopropylethylamine (DIPEA; Sigma, Castle Hill, Australia) for 45 minutes. The conditions for this acylation step were also used for the coupling of other amino acid residues in this study or otherwise stated specifically. The α-amino group of this C-terminal lysine was then exposed following removal of the Fmoc group using 2.5% 1,8-diazobicyclo(5.4.0)undec-7-ene (DBU; Sigma-Aldrich, Missouri, USA) in N,N-Dimethylformamide (DMF; Merck, New Jersey, USA) for 10 minutes and a second lysine residue added using Fmoc-Lys(Fmoc)-OH (Novabiochem, Hohenbrunn, Germany). Removal of Fmoc protecting groups at the - and - amino groups of the second lysine using DBU then allowed a tier of 2 lysine residues to be added again using Fmoc-Lys(Fmoc)-OH. Following removal of the 4 Fmoc protecting groups using DBU and exposure of the 4 amino groups, Fmoc-Arg(Pbf)-OH (Novabiochem, Hohenbrunn, Germany) was then added to terminate the branched peptide structure with arginine residues. Following removal of the N-terminal Fmoc groups, the primary amino groups of the N-terminal arginine residues were protected by acylation with a 10-fold molar excess of di-*tert*-butyl dicarbonate (FlukaChemika, Buchs, Switzerland) in the presence of DIPEA. This step was necessary to prevent acylation of the -amino groups present on the arginine residues during the subsequent lipidation process.

Removal of the Mtt protective group attached to the ϵ -amino group of the C-terminal lysine was achieved by continual washing with 1% trifluoroacetic acid (TFA; Auspep, Parkville, Australia) in dichloromethane (DCM; Chem-supply, Adelaide, Australia) every 5 minutes over a period of 1 hour. Two serine residues, Fmoc-Ser(tBU)-OH (Novabiochem, Hohenbrunn, Germany), were then serially coupled to the exposed ϵ -amino group and a 4-fold molar excess of Fmoc-Cys-diol (manufactured according to Wiesmüller et al [Wiesmüller et al. 1983] and modified by Zeng et al [Zeng et al. 1996]) and HOBt together with a 6-fold molar excess of DICl dissolved in DCM coupled to the terminal serine residue. A 10-fold molar excess of palmitic acid (Acros Organics, New Jersey, USA), an equimolar amount of DMAP (Fluka, Steinheim, Germany) and a 12-fold molar excess of DICl in DCM was then added and the reaction allowed to proceed overnight at room temperature. The solid support then washed with DCM and a final de-protection step performed to remove the remaining Fmoc group from the cysteine residue.

Cleavage of the final product from the solid support was achieved by incubating the resin with 88% TFA, 5% phenol (Merck, New Jersey, USA), 2% triisopropylsilane (TIIPS; Merck, New Jersey, USA) and 5% water for 3 hours at room temperature (Chua et al. 2011b). Lipopeptides were then purified using reverse-phase (RP) HPLC with a Vydac C4 column (6 × 250mm) installed in a Shimadzu HPLC chromatography system (California, USA) with flow rate of 2.5ml/min using 0.1%TFA in water and 0.1%TFA in acetonitrile (VWR Chemicals, Philadelphia, USA) as the limit solvent.

In Method 2, Dde-Lys(Fmoc)-OH (Novabiochem, Hohenbrunn, Germany) was coupled to the solid support as the first amino acid. Following removal of the Fmoc protective group from the ϵ -amino group, the serine residues and Pam₂Cys lipid moiety were coupled as described above. The Dde protective group on the α -amino group of the C-terminal lysine was then removed by washing with 2% hydrazine hydrate (Fisons AAG, NSW, Australia) in DMF every 5 minutes for

10 minutes (Bycroft et al. 1993; Zeng et al. 2011) and the branched lysine structure assembled as described in Method 1.

Synthesis of R₄Pam₂Cys containing D-isomers

Synthesis of R₄Pam₂Cys containing D-isomers of lysine and arginine was performed using Method 2 except that Fmoc-D-Lys(Fmoc)-OH (P3 BioSystems, Louisville, USA) and Fmoc-D-Arg(Pbf)-OH (P3 BioSystems, Louisville, USA) were used to assemble the branched structure.

Characterization of lipopeptides

The purity of each product was analyzed by reversed phase high performance liquid chromatography (RP-HPLC) using a Vydac Protein C4 column (4.6mm x 250mm) installed in a Shimadzu HPLC chromatography system using a flow rate of 1 mL/min and 0.1 % trifluoroacetic acid (TFA) in H₂O and 0.1 % TFA in acetonitrile as the limit solvent. Peptide content was determined by amino acid analysis using Waters AccQTag derivatisation reagents according to the manufacturer's instructions and analysis using a Waters Acquity UPLC System using an AccQTag ultra column (2.1×100mm). The mass of each lipopeptide was determined using an Agilent 1100 Series LCM/MSD Ion Trap mass spectrometer.

The recoveries of R₄Pam₂Cys synthesized using Method 1 or Method 2, are expressed as a percentage of the total theoretical yield (Y) determined using the formula:

$$Y = \frac{[(R_4Pam_2Cys)mg * 100]}{S * s * Mr}$$

where:

(R₄Pam₂Cys)mg yield in mg of R₄Pam₂Cys (determined by amino acid analysis)

S weight of solid support used for the synthesis

s substitution factor of the solid support

Mr molecular weight of R₄Pam₂Cys

Lipopeptide–antigen binding assay

10 µg of ovalbumin (OVA, 0.22 nmol; InvivoGen, California, USA) was mixed with a 3-fold molar excess of R₄Pam₂Cys synthesized by Method 1 or Method 2 in a total volume of 50 µL of saline in a microtube. The size distribution and polydispersity of particles were measured in a 4µl disposable cyclic olefin co-polymer cuvette installed in a DynaPro NanoStar dynamic light scattering (DLS) instrument (Wyatt Technology, Santa Barbara, USA). The size of particles is reported as radius in nm and percent polydispersity as the ratio (base-line width of size distribution/mean size distribution) in solutions N.B. Solutions with particles that are >30% poly-dispersed can be considered to be heterogeneous in their size distribution (Geers et al. 2016).

Up-regulation of surface MHC Class II expression on D1 cells

Immature murine dendritic, D1, cells, were cultured from C57BL/6 mice as previously described (Chua et al. 2008). After 21 days of culture, D1 cells were shown to display surface MHC class II and CD11c expression by staining with FITC conjugated anti-murine I-A/I-E and PE-conjugated CD11c antibodies (both from Biolegend, San Diego, USA), respectively for 30 min at 4°C. Cells (2×10^5) were incubated with 0.02, 0.2, or 2 ng (0.44, 4.4, or 44 fmol) of OVA pre-mixed with a 3-fold molar excess of lipopeptide in a total volume of 500 µL in a 48-well plate. Cells were also incubated with 0.02, 0.2, or 2 ng OVA alone or maintained untreated. After an incubation period of 14 hours, cells were stained with anti-I-A/I-E-FITC. Samples were analyzed by flow cytometry using a FACSCalibur (BD Biosciences, USA) and data analyzed using FlowJo software (Tree star, Inc, USA).

Intracellular cytokine staining (ICS) assay

For measurement of antigen-specific cell-mediated immune responses following inoculation with OVA, 6–8wk-old C57BL/6 mice (4 mice per group) were inoculated via the subcutaneous (s.c.) route at the base of the tail with 25 µg (0.55 nmol) of OVA alone or OVA formulated with a 3-fold molar excess of R₄Pam₂Cys in a total volume of 100µl of saline. The spleens of vaccinated animals were removed 7 days after inoculation and incubated with the H-2K^b restricted, OVA-derived peptide epitope SIINFELK (OVA₂₅₇₋₂₆₄; 1 mg/ml) in RPMI-1640 medium (Invitrogen, USA) supplemented with 10% heat inactivated FCS, gentamicin (12 mg/ml), 2 mM glutamine, 1 mM sodium pyruvate, penicillin (100 IU/ml), streptomycin (100 mg/ml) and 55 mM 2-mercaptoethanol, Brefeldin A (1 mg/ml) in the form of BD GolgiPlug from the Cytofix/Cytoperm Plus Kit (Becton Dickinson) and recombinant IL-2 (10 U/ml; Roche, Mannheim, Germany) at 37°C and 5% CO₂. After 11 hours, lymphocytes were washed with FACS Wash Buffer (1% FCS 5 mM EDTA in PBS) and stained with a PerCP-conjugated rat anti-mouse CD8 antibody (Clone 53-6.7; Becton Dickinson). Fixation and permeabilization of cell membranes was then performed for 20 min at 4°C using Cytofix/Cytoperm solution (Cytofix/Cytoperm Plus Kit, Becton Dickinson) according to the manufacturer's instructions. Cells were washed once with Perm/Wash solution and stained with a FITC-conjugated rat anti-mouse IFN-γ antibody (CloneXMGI.2; Becton Dickinson) before flow cytometric analysis using a FACs Canto II (BD Biosciences, USA). Data analysis was performed using FlowJo software (Treestar, Ashland, OR).

Measurement of antigen-specific antibody responses

Groups of female 6–8-wk-old BALB/c (4-5 mice per group) were inoculated by the s.c. route at base of tail on day 0 and again on day 21 with 25 µg of OVA alone or OVA formulated with a 3-fold molar excess of lipopeptide in a total volume of 100µl of saline. Sera were obtained from blood taken 3 weeks after the primary inoculation and also 2 weeks after the secondary inoculation. Flat-bottom-well polyvinyl plates (96 wells/plate) were coated with OVA antigen (5µg/mL) in PBS for 18–20 hours at room temperature in a humidified atmosphere. Unbound antigen was discarded and BSA (10 mg/ml) in PBS was added as a blocking solution for 1 hour before washing with PBS containing v/v 0.05% tween-20. Serial dilutions of sera obtained from immunized mice were added to wells and held overnight at room temperature. After washing, antigen-bound antibody was detected using horse-radish peroxidase (HRP)-conjugated rabbit anti-mouse IgG antibodies (Dako Agilent Pathology Solutions, Denmark) or anti-rat mouse isotype specific antibodies (SouthernBiotech, Birmingham, USA) for 1 hour. The enzyme substrate (0.2 mM 2,29-azino-bis 3- ethylbenzthiazoline-sulfonic acid in 50 mM citric acid containing 0.004% hydrogen peroxide) was then added and left to develop for 15 minutes before addition of 50mM sodium fluoride (NaF; 50µL/well) to stop the reaction. Antibody titers were expressed as the reciprocal of the highest dilution of serum required to achieve an OD of 0.2.

Analysis of antisera-antigen binding

Analysis of antisera-antigen binding was performed by surface plasmon resonance using a Bio-Rad ProteOn (Hercules, CA). OVA was diluted in 10mM sodium acetate (pH 4.5) and immobilized on the solid phase of a GLC Sensorchip (Bio-Rad) flow cell using standard amine coupling chemistry as described by the manufacturer. An adjacent flow cell, to which protein was not introduced, was activated and quenched in the same manner to serve as a negative control. Mouse sera were diluted 1:10 in running buffer (PBS with 3mM EDTA and 0.05%

Tween 20). All experiments were performed at 25°C using a flow rate of 30 L/min running buffer with an injection time of 120 seconds and dissociation time of 600 seconds. The data was collected as described by Real-Fernandez et al (Real-Fernández et al. 2015). The surface was regenerated using Glycine-HCl (pH 2.5). Binding levels of antibody were expressed as the mean of the maximum response (Rmax) attained.

Tryptic digestion

R₄Pam₂Cys (15 g) containing L- or D-isomers of lysine and arginine were pre-treated with trypsin versene (60 g, Department Microbiology and Immunology, University of Melbourne) in a final volume of 150 L for 10 minutes at 37°C. The mixtures were centrifuged at 13,000rpm for 1 minute and 100 L of each supernatant analyzed by RP-HPLC using a Vydac C4 column (4.6mm x 250mm) installed in a Shimadzu HPLC system as described above. Eluting material was detected by measuring the optical absorbance at 214 nm. As a positive control for the activity of trypsin, Pam₂CysSK₄ (synthesized in-house) was pre-treated with trypsin in a similar manner.

Statistical analysis

Analysis of variance and p-values were determined using one-way analysis of variance (ANOVA) non-parametric test and Tukey's multiple comparison test performed using the Prism 6 software package (Graphad Software, La Jolla, CA, USA).

RESULTS

Antigen binding properties of R_4Pam_2Cys synthesized using Method 1 or Method 2

R_4Pam_2Cys assembled by Methods 1 and 2 and analyzed by HPLC each revealed a single major species (Figure 2A) which each exhibited a mass similar to the expected value (Figure 2B).

Comparison of yields from three batches synthesized by each method demonstrated that Method 2 provided an almost two-fold average improvement in recovery of product compared with Method 1 (Figure 2C). Furthermore, more consistent quantities were recovered using Method 2.

We have previously shown (Chua et al. 2011b) that OVA, which has a net negative charge of -11, forms complexes when mixed with R_4Pam_2Cys . To investigate whether the different methods of assembling R_4Pam_2Cys affects its ability to bind to OVA, we analyzed the sizes of particles formed after mixing lipopeptide and antigen by dynamic light scattering (DLS). The results (Figure 3) show that solutions containing OVA alone or lipopeptide alone contain heterogeneously sized particles with dominant populations exhibiting average radii <20nm. Association of OVA and R_4Pam_2Cys synthesized using Method 1 or Method 2 yielded larger, similarly sized complexes with the majority being ~350-380nm in radius. The percentage polydispersity of complexes formed between OVA and either lipopeptide were within the range (<30%) that would be considered mono-dispersed and indicative of uniform size. This is in contrast to the broader distribution of particle sizes present in either OVA and the individual lipopeptides alone.

Antibody and $CD8^+$ T cell responses induced by antigen formulated with R_4Pam_2Cys synthesized by Method 1 or Method 2

We evaluated antibody and CD8⁺ T cell responses induced by antigen formulated with R₄Pam₂Cys in BALB/c and C57BL/6, respectively. Our justification for using these two strains is based on many studies reporting differences in their immune response bias. BALB/c mice are widely known to induce better Th2-type bias and hence are more suitable for inducing and studying antibody responses whereas C57BL/6 mice are better at inducing a Th1-type bias and thus CD8⁺ T cell responses (Heinzel et al. 1989; Chen et al. 2005; Mourglia-Ettlin et al. 2016). The use of each strain therefore provides the best measure of the type of immune response examined. C57BL/6 mice were inoculated with OVA alone or OVA formulated with R₄Pam₂Cys assembled by Method 1 or Method 2 and CD8⁺ T cell responses in the spleen measured 7 days later using an ICS assay. The results show that inoculation of mice with OVA formulated with either lipopeptide induced higher levels of CD8⁺ T cells secreting IFN- γ than did OVA alone (Figure 4A). No differences in responses were observed with either one or the other forms of R₄Pam₂Cys used indicating that the different methods of synthesis do not affect the lipopeptides abilities to induce CD8⁺ T cell responses.

Low antibody titers were detected in all BALB/c mice receiving a single dose of antigen (Figure 4B) but after two doses, much higher titers of antibody were present in mice that received OVA formulated with R₄Pam₂Cys synthesized by Method 1 or Method 2 than mice inoculated with OVA alone. Again, no significant differences in antibody titers were detected between animals vaccinated with R₄Pam₂Cys synthesized using Method 1 or Method 2. We conclude that Method 2 improves yield without impacting biological activity of the product.

Comparison of antibody responses induced by OVA formulated with R₄Pam₂Cys M2, Alum and CFA

Formulation of OVA with R₄Pam₂Cys M2 elicits a secondary antibody response with significantly higher antibody titers than OVA formulated with Alum and titers which are comparable to antibody titers obtained with a single dose of OVA formulated with complete Freund's adjuvant (Figure 5A).

IgG1 was the dominant antibody isotype elicited by OVA formulated with each of the adjuvants but no IgG1 was detected when OVA was inoculated in the absence of adjuvant (Figure 5B). The titers of IgG1 isotype induced by R₄Pam₂Cys M2 were higher than those induced by CFA or Alum. In addition, IgM was present in sera of mice vaccinated with all formulations with the highest titer detected in mice inoculated with the CFA-OVA combination.

The avidities of binding between antibodies and OVA, measured by surface plasmon resonance, (Figure 5C) demonstrated that there was little or no interaction between OVA and sera obtained from animals inoculated with OVA alone. In the case of sera from animals vaccinated with OVA formulated with R₄Pam₂Cys M2, Alum or CFA, however, resonance signals indicative of association of serum antibodies with antigen were detected. A comparison of R_{max} values indicate that antibodies obtained from animals inoculated with OVA in the presence of CFA exhibited the strongest avidity while antibodies obtained from animals inoculated with OVA and Alum or OVA and R₄Pam₂Cys synthesized by Method 2 had similar avidity (Figure 5D).

Association of R₄Pam₂Cys stereoisomers with antigen and biological activity of the complexes

To examine if different isomeric forms of R₄Pam₂Cys differ in biological activity, we used Method 2 to assemble R₄Pam₂Cys in which the D stereoisomers of lysine and arginine were used and compared the activities of this stereoisomer (R₄Pam₂Cys^D) with the stereoisomer containing the usual, L-isomers of lysine and arginine (R₄Pam₂Cys). The amino acids present in the

Pam₂Cys moieties of each lipopeptide viz. serine and cysteine, were retained as the L-stereoisomers to avoid lack of recognition by and signalling through TLR2.

The results (Fig. 6A) of the experiments demonstrate that the sizes of each lipopeptide stereoisomer are similar with perhaps a slight skewing of size and size distribution of R₄Pam₂Cys^D to the right (Figure 5A). Nevertheless, the average sizes of each stereoisomer were similar (Figure 6B).

Because of the key role played by DCs in inducing immune responses, we compared the effectiveness of lipopeptides to stimulate DC maturation by determining the expression of surface MHC class II following incubation of spleen-derived D1 cells with R₄Pam₂Cys or R₄Pam₂Cys^D-OVA complexes. At none of the concentrations tested was OVA alone able to induce significant up-regulation of MHC class II expression (Figure 7A) whereas formulation of OVA with either R₄Pam₂Cys or R₄Pam₂Cys^D was able to activate these cells with similar efficiency confirming that the *in vitro* biological activity of R₄Pam₂Cys is independent of the stereochemistry of the lysine and arginine residues present in the branched structure.

Antibody and CD8⁺ T cell responses induced by antigen formulated with R₄Pam₂Cys^D

The results of experiments in which the ability of R₄Pam₂Cys^D and R₄Pam₂Cys when formulated with OVA to induce antigen-specific CD8⁺ T cell response were compared (Figure 7B) demonstrate that although the difference is not statistically significant, somewhat fewer CD8⁺ T cells secreting IFN- γ were induced by R₄Pam₂Cys^D.

Much better antibody titers were achieved with each lipopeptide when formulated with OVA than were achieved with OVA alone and a slight trend was apparent in the ability of R₄Pam₂Cys^D to elicit antibody when compared with R₄Pam₂Cys (Figure 7C).

Tryptic digestion of R₄Pam₂Cys^D

In light of reports that peptides composed of D-amino acids are protease-resistant [12, 13], we compared the stability of R_4Pam_2Cys and $R_4Pam_2Cys^D$ when exposed to trypsin. This protease was chosen because it hydrolyses peptide bonds at the carboxyl side of arginine and lysine unless followed by proline (Simpson 2006).

We confirmed the activity of trypsin by incubation with the Pam_2Cys agonist, Pam_2CysSK_4 , which contains L-isomeric forms of lysine residues arranged in *linear* sequence. Analysis by RP-HPLC (Figure 8A) demonstrated that Pam_2CysSK_4 presents as a single major peak which elutes at 40 minutes whereas the trypsin preparation used presented two major peaks eluting at approximately 22 and 32 minutes. Exposure of Pam_2CysSK_4 to trypsin resulted in significant reduction in the size of the major peak and the appearance of 4 smaller peaks with retention times clustered around 40 minutes. Analysis of the components under these peaks by mass spectrometry revealed species with masses corresponding to intact Pam_2CysSK_4 and truncated forms with losses of 1-2 lysine residues (data not shown). These results indicate that the trypsin used is effective at degrading a Pam_2Cys -based lipopeptide containing L-amino acids.

In contrast to these results, exposure of R_4Pam_2Cys or $R_4Pam_2Cys^D$ to trypsin showed little or no indication of degradation as indicated by the similarities of the HPLC chromatogram profiles obtained before and after exposure to trypsin (Figure 8B). These results indicate that irrespective of isomer, the branched arrangement of lysine and arginine residues within R_4Pam_2Cys confers resistance to proteolytic digestion.

DISCUSSION

We have shown that the manufacture of R_4Pam_2Cys can be improved, without affecting its ability to enhance immunogenicity of antigens with which it is formulated, simply by reversing

the order in which Pam₂Cys and the branched moiety are assembled into the product. Addition of the lipid moiety prior to addition of the branched structure provided a more consistent record of manufacture than when the lipid moiety is added after the branched structure has been put in place. Although there was little statistical significance in the yields produced by the two methods, which could be attributed to the variability observed using Method 1, the trend for higher and more consistent yields was apparent using Method 2. If it is assumed that the coupling efficiency at each acylation cycle is ~90%, it becomes apparent that the overall yield for coupling 10 amino acids, 1 Cys-diol, and 2 palmitic acid residues will be ~25%. Therefore an improvement of 2-4% in addition to the consistency of manufacture is highly significant and advantageous particularly when economies of scale are considered.

The improvement in synthesis efficiency observed using Method 2 could be attributed to a reduction in steric hindrance due to absence of the large branched peptide structure when the lipid is added. A similar observation has been reported for the synthesis of a large branched and cyclic peptide from the Fc region of IgG Fc used as an immunomodulatory ligand for FcγRI where reduced yields were explained by steric hindrance caused by the presence of the branched peptide and can be overcome if its addition is preceded by cyclization of the linear portion of the peptide (Sheridan et al. 1999).

A second advantage of Method 2 is in the use of Dde-Lys(Fmoc)-OH instead of Fmoc-Lys(Mtt)-OH used in Method 1; it takes much less time to remove the Dde protecting group than it takes to remove the Mtt protecting group. Two 5 minute washes with 2% hydrazine in DMF is sufficient to remove Dde (Bycroft et al. 1993) whereas continual washing with 1% TFA in DCM every 5 minutes for 1 hour is required to remove Mtt. In addition, exposure of the peptidyl resin to DCM can result in loss of synthesis product during de-protection due to partial cleavage from the resin (Bourel et al. 2000), a factor which may account for the lower yields of R₄Pam₂Cys

obtained using Method 1. Finally, the efficiency of removal of the Mtt protecting group can be affected by the amino acid sequence flanking this residue (Bourel et al. 2000). It is clear that use of Method 2 could save considerable manufacturing time and also decrease solvent consumption almost certainly reducing production costs. Importantly, the similarity in antigen-binding profiles as well as the *in vitro* and *in vivo* biological activity of R₄Pam₂Cys produced by Method 2 indicates adoption of Method 2 for manufacturing purposes

Similarities in biological activities were observed between R₄Pam₂Cys containing either D isomers or L-isomers of lysine and arginine although there appeared to be a propensity for the D isomer-containing compound to elicit fewer CD8⁺ T cells and slightly lower titers of antibody. Although these differences were not statistically significant and therefore in general agreement with a previous report (Makimura et al. 2006) in which L and D isomers of TLR2 ligand-containing immunogens were compared, these authors did not examine the biological activity of their TLR2 agonists as they related to the induction of antibody or CD8⁺ T cells when used in the context of an antigen adjuvant. These results therefore lead us to speculate that D-isomers of amino acids may affect antigen processing and presentation pathways involved in the generation of immune responses at least when initiated using the TLR2 compartment. We also found that unlike Pam₂CysSK₄, which contains a linear tetra-lysine sequence, the D- and L-isomers of R₄Pam₂Cys are stable to the actions of trypsin. Although it has been reported that substituting L-amino acid residues of a linear peptide with their D-amino forms increases resistance against trypsin (Hamamoto et al. 2002; Tugyi et al. 2005), our results and those of others demonstrate that a peptide with a branched arrangement is resistant to protease activity (Tam et al. 2002; Bracci et al. 2002) and more stable under physiological conditions (Tam et al. 2002; Dewan et al. 2009).

Although trypsin is a protease predominantly found in the digestive system of many vertebrates and our compounds are not delivered orally, we note however, that the presence and activity of trypsin and trypsin-like proteases in other tissues including the brain (Wang et al. 2008), muscle (Bossola et al. 2008), blood (Lefkowitz et al. 2010) and interstitial fluids (Xie et al. 2016) have also been reported in many studies. Together with its ability to specifically cleave the type of peptide bonds found in our lipopeptides, we therefore believe that its use in our experiments bears physiological relevance and demonstrates that these design features overcome some of the issues that limit the clinical use of peptide-based therapeutic agents (Liu and Nussenzweig 2010).

Alum has been widely used in human vaccines for decades and has an excellent safety profile and efficacy record when long-lasting Th2 type antibody responses are sought (Lindblad 2004; Reed et al. 2009). The inability of Alum to induce Th1 type cell responses or cytotoxic T cell responses, however, restricts its use in cases where cellular-mediated responses are needed (Gupta 1998). Moreover, complete Freund's adjuvant has no translational application for use in human vaccines as it is far too reactogenic and is prohibited from use in non-experimental animals (Stills 2005).

In this study, we found that the antibody titers elicited using R₄Pam₂Cys to be comparable if not better than Alum in some animals but less effective than CFA at inducing antibody. The heightened antibody response in CFA-treated animals may be attributed to the presence of higher levels of IgM-isotype antibodies which, although known to exhibit low antigen binding affinities (Boes 2000), bind to more antigenic sites by virtue of their pentavalent structure which explains the higher binding avidities observed (Muthana et al. 2015). Nonetheless, all adjuvanted formulations were able to facilitate class-switching to IgG-and in this context, R₄Pam₂Cys was the most effective at promoting isotype switching.

Taken altogether and given the ability of R₄Pam₂Cys to induce CD8⁺ T cell responses in animal models, this adjuvant appears to be more effective than Alum (Chua et al. 2014) and also exhibits a better safety profile than CFA. Our findings also highlight the benefit of using R₄Pam₂Cys as a single adjuvant to achieve balanced Th1- and Th2-type immune responses that can be usually achieved through combined use of TLR5 and TLR9 agonists (Bargieri et al. 2008) or TLR4 and TLR7 agonists (Goff et al. 2015).

FIGURES

Figure 1. Diagrammatic representation of synthetic branched cationic lipopeptide R₄Pam₂Cys and schematic of the synthesis strategies used in assembly. (A) Schematic structure of R₄Pam₂Cys. The molecule is assembled as a two-tiered branching scaffold composed of lysine (K) residues to which are attached arginine (R) residues. The lipopeptide contains the TLR2 agonist Pam₂Cys which is attached to **one of the two serine residues (S₂) coupled** to the ε-amino group of the C-terminal K residue. (B) R₄Pam₂Cys was synthesized using solid phase peptide synthesis methodologies using two different synthetic routes. Method 1 (left); Fmoc-Lys(Mtt)-OH was coupled to the solid support (grey sphere) which allows assembly of the branched lysine

structure at the α -amino group of the C-terminal K residue(s) following removal of the Fmoc group. Arginine residues are then ligated to the ϵ and α -amino groups of the N-terminal K residues. The moiety is then assembled at the exposed ϵ -amino group of the C-terminal K residue following removal of the Mtt protecting group. Method 2 (right); Dde-Lys(Fmoc)-OH is attached to the solid support followed by removal of the Fmoc group and assembly of the Pam₂Cys moiety at the exposed ϵ -amino group of the C-terminal K residue. Removal of the Dde group then allows assembly of the branched lysine structure at the N-terminal α -amino group of the C-terminal K residue as described in Method 1.

Figure 2. Characterization and yield of R_4Pam_2Cys synthesised using Method 1 (M1) or Method 2 (M2).

(A) RP-HPLC was performed on a C4 column using 0.1% TFA in ddH₂O and 0.1% TFA in acetonitrile as the limit solvent at a flow rate of 1 mL/min. Eluting material was detected by measuring the optical absorbance at 214nm. (B) Mass analysis was carried out with electrospray ionization configured in the positive ion mode. The spectra contain signals due to various protonation states of the samples and the m/z information was used to measure the mass of the uncharged peptide construct. (C) The percentage recovery of R_4Pam_2Cys synthesised was determined by comparing the yield of purified product from each of 3 batches relative to the theoretical yield based on the amount and substitution factor of solid support used. Each point represents the percentage recovery of a batch with the mean depicted as the horizontal bar ($\pm$ SD).

Figure 3. Particle size distribution of OVA formulated with R₄Pam₂Cys synthesized using either Method 1 (M1) or Method 2 (M2). (A) Solutions containing 10 µg (0.22 nmoles) of OVA, 0.67 nmoles of R₄Pam₂Cys M1 or M2, or OVA formulated with R₄Pam₂Cys synthesised by M1 or M2 at a 1:3 molar ratio in a total volume of 50 µl of saline were analyzed by dynamic light scattering. The size distribution (radius in nm) of particles formed are shown. Each profile is representative of a sample from each group (n=3). The average particle size of individual populations within each histogram is indicated as is the percentage polydispersity (Pd). (B) Bar graphs depict the average size (±SD) of all particles in each sample (n = 3).

Figure 4. Immune responses induced by R₄Pam₂Cys assembled by Method 1 (M1) or Method 2 (M2) associated with OVA.

(A) Groups of C57BL/6 mice ($n = 4$ per group) were vaccinated s.c. at the base of the tail with 25 μ g of OVA alone (0.55 nmol) or OVA formulated with a 3-fold molar excess of R₄Pam₂Cys assembled using Method 1 (M1) or Method 2 (M2) in a total volume of 100 μ L/dose. Spleens of vaccinated mice were removed 7 days later and IFN- γ secretion by OVA₂₅₇₋₂₆₄-specific CD8⁺ T cells determined in an intracellular cytokine-secretion assay. The bar graph shows the mean of the total numbers ($\pm$ SD) of OVA-specific CD8⁺ T cells elicited by the various treatments. (B) Groups of BALB/c mice ($n = 5$ per group) were inoculated on day 0 and again on day 21 with the same inocula as indicated above. Sera were obtained from blood taken 20 days after the primary (1°) and 14 days after the secondary inoculations (2°). Antibody responses were then determined by ELISA. The antibody titres of individual animals is presented with the mean value shown as the horizontal bar. * $p < 0.01$ as determined by one-way ANOVA.

Figure 5. OVA-specific antibody titers and binding avidities induced by different adjuvants

(A) Groups of BALB/c mice ($n = 5$ per group) were vaccinated s.c. at the base of the tail on day 0 and again on day 21 with 25 μ g of OVA alone or OVA formulated with Alum, CFA or with a 3-fold molar excess of R₄Pam₂Cys in a total volume of 100 μ L on day 0 and again on day 21. Sera were obtained from blood taken 20 days after the primary (1°) and 14 days after the secondary inoculations (2°) and antibody responses then determined by ELISA. (B) Sera taken after the second dose of antigen were also tested for antibody isotypes IgM, IgG1, IgG2a, IgG2b

and IgG3. **(C)** Surface plasmon resonance (SPR) was used to measure the binding avidities of antibodies. Sera from vaccinated mice ($n=4-5$) were diluted (1/10) and injected at a flow rate of 30 μ L/min over immobilised OVA on a GLC sensor chip for 120 seconds. Each line on the sensorgram shows the response for an individual mouse. Results are presented in resonance units (RU) after subtraction of the baseline values. Solid vertical lines at 0 and 120 seconds indicate injection start and stop, respectively. **(D)** Levels of antibody binding are summarised as the mean ($\pm$ SEM) of the maximum SPR response (R_{max}). * $p<0.05$, ** $p<0.01$, as determined by one-way ANOVA for (A) and (C) or two-way ANOVA for (B).

Figure 6. Particle size distribution of OVA formulated with a different isomeric form of R₄Pam₂Cys. (A) Solutions containing 10 µg of OVA (0.22 nmoles), 0.67 nmoles of R₄Pam₂Cys or R₄Pam₂Cys^D, or OVA formulated with R₄Pam₂Cys or R₄Pam₂Cys^D at a 1:3 molar ratio in a total volume of 50 µl of saline were analyzed for their dynamic light scattering properties. Formed particle size distributions within these solutions are presented as the radius in nm with the average particle size of populations within each histogram indicated as is the percentage polydispersity (Pd). (B) Average size data representative of triplicate samples: mean particle size (±SD).

Figure 7. Ability of $R_4Pam_2Cys^D$ - and R_4Pam_2Cys -associated with OVA to induce DC maturation and induce immune responses

(A) D1 dendritic cells (2×10^5) were incubated with 0.02 ng, 0.2 ng or 2 ng (0.44, 4.4, or 44 fmol) of OVA alone or OVA formulated with a 3-fold molar excess of R_4Pam_2Cys or $R_4Pam_2Cys^D$ in a total volume of 500 μ L in a 48-well plate. Untreated cells were kept as controls. Surface MHC class II expression was determined 14 hours later and the percentage of mature cells in culture presented as the mean ($\pm$ SEM). (B) C57BL/6 mice ($n = 4$ per group) were vaccinated s.c. at the base of the tail with 25 μ g of OVA alone (0.55nmol) or OVA formulated

with a 3-fold molar excess of R₄Pam₂Cys or R₄Pam₂Cys^D. Spleens of vaccinated mice were removed 7 days later and IFN- γ secretion by OVA₂₅₇₋₂₆₄-specific CD8⁺ T cells determined in an intracellular cytokine-secretion assay. (C) BALB/c mice ($n = 5$ per group) were similarly inoculated on day 0 and again on day 21 and antibody responses measured by ELISA using sera obtained from blood taken 20 days after the primary (1°) and 14 days after the secondary inoculations (2°). * $p < 0.01$ as determined by one-way ANOVA.

Figure 8. Stability of *L* and *D* isomers of branched *R*₄Pam₂Cys to trypsin digestion.

Solutions of saline (150μl) containing **(A)** Pam₂CysSK₄, **(B)** R₄Pam₂Cys or R₄Pam₂Cys^D were incubated either alone or with trypsin (60μg) for 10 minutes at 37°C and then centrifuged at

13,000 rpm for 1 minute and analyzed by RP- HPLC (RP-HPLC). The chromatograms were developed using 0.1% trifluoroacetic acid (TFA) in ddH₂O and 0.1% TFA in acetonitrile as the limit solvent at a flow rate of 1mL/min. Eluting material was detected by measuring the optical absorbance at 214nm. Species within the dashed boxes were analyzed by mass spectrometry.

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Ethics approval: All animal experimentation was performed with approval from The University of Melbourne's animal ethics committee.

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