Amino Acids (2010) 39:471–480
DOI 10.1007/s00726-009-0463-0

ORIGINAL ARTICLE

Structural requirement for the agonist activity of the TLR2 ligand Pam2Cys

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Received: 7 July 2009 / Accepted: 22 December 2009 / Published online: 21 January 2010

Abstract Synthetic lipopeptides have demonstrated great potential as a vaccine strategy for eliciting cellular and humoral immunity. One of the most potent lipid moieties used is *S*-[2,3-bis(palmitoyloxy)propyl]cysteine (Pam2Cys). Pam2Cys binds to and activates dendritic cells by engagement of Toll like receptor 2 (TLR 2). In this study, we have investigated the structural requirement of the agonist activity of Pam2Cys by varying the three structural elements of the core structure *S*-(2,3-dihydroxypropyl)-cysteine namely (1) the α-amino group of the cysteine residue (2) the sulphur atom of the cysteine residue and (3) the 2,3-dihydroxypropyl moiety. Four novel analogues of Pam2Cys were made and each of these analogues were incorporated into vaccine constructs and examined for immunogenicity. Our results demonstrate that (1) the potency of the peptide vaccine is least affected by removal of the amino group (2) substitution of the sulphur atom with an amide bond leads to significant reduction of biological activity (3) removal of the amino group and at the same time substitution of the sulphur with an amide bond significantly decreases the biological activity (4) in the two analogues in which the sulphur atom is replaced with an amide bond the analogue containing the 1,3-dihydroxypropyl moiety demonstrates higher activity than the one which contains 2,3-dihydroxypropyl. In conclusion, the results demonstrate strict structural requirements for agonist activity of the TLR2 ligand Pam2Cys.

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Keywords Vaccine · Peptide · TLR2 · Structure–activity relationship · Lipopeptide

Introduction

In recent years, we have demonstrated the potential of lipopeptides as prophylactic and therapeutic vaccines in a number of situations where they were shown to be capable of eliciting either $CD8^+$ T cell or antibody-mediated immune responses that mediate protection against viral (Alphs et al. 2008; Day et al. 2007; Deliyannis et al. 2006; Jackson et al. 2004; Lau et al. 2006) and bacterial (Batzloff et al. 2006; Jackson et al. 2004) infection, model tumours (Baz et al. 2008; Jackson et al. 2004) as well as abrogating reproductive function (Zeng et al. 2002, 2005, 2007).

Commonly used lipopeptide constructs consist of peptide epitopes of $CD4^+$ helper T cells and a target epitope of either $CD8^+$ T cells or B cells that are conjugated to a lipid moiety in a linear (Borges et al. 1994; Jung and Bessler 1995; Vitiello et al. 1995) or more recently in a branched (Zeng et al. 2002) configuration. One of the most potent lipid moieties used in such vaccines is *S*-[2,3-bis(palmitoyloxypropyl)]cysteine (Pam2Cys) which is derived from the lipid component of macrophage-activating lipopeptide 2 (MALP-2) present in Mycoplasma (Muhlradt et al. 1997, 1998). Lipopeptides containing Pam2Cys induce signalling and activation of cells of the innate immune system, particularly dendritic cells, through TLR2 in combination with TLR6 (Takeda et al. 2002) although it has been reported that some Pam2Cys-peptides can be recognised by TLR2 in a TLR6-independent manner (Buwitt-Beckmann et al. 2005a). A characteristic of the sequence of these peptides is that tetralysine is incorporated. Similar results have also

been obtained for some *N*-palmitoyl-*S*-[2,3-bis(palmitoyl-oxypropyl)]cysteine (Pam3Cys)-containing lipopeptides (Buwitt-Beckmann et al. 2006).

The structure of the Pam2Cys lipopeptide can be divided into three components: (1) two fatty acid components (2) the core structure *S*-(2,3-dihydroxypropyl)-cysteine to which the two fatty acids are attached and (3) the peptide component. The influence of the length of the fatty acid carbon chain on Pam2Cys activity has been previously investigated (Buwitt-Beckmann et al. 2005b; Chua et al. 2007) where it was demonstrated that immunogenicity, as measured by ability to activate cells expressing TLR2 (Buwitt-Beckmann et al. 2005b; Chua et al. 2007) and inducing specific antibody (Chua et al. 2007), is dependent on the length of the alkane chains with the hierarchy C16 = C18 > C12 > C8 (Buwitt-Beckmann et al. 2005b; Chua et al. 2007).

A number of reports have appeared concerning the structure–activity relationship of the core structure *S*-(2,3-dihydroxypropyl)cysteine of Pam2Cys. For example, the *R*-stereoisomer of lipopeptide MALP-2 (differing in the configuration around the asymmetric carbon atom of the 2,3-hydroxypropyl moiety) is 100 times more active than the *S*-stereoisomer (Takeuchi et al. 2000) and substitution of the sulphur atom by a methylene group leads to 77% reduction of biological activity (Metzger et al. 1991; Spohn et al. 2004). Finally, substitution of the two ester bonds, through which the two fatty acids are attached, by an amide bond also leads to abolition of TLR2 agonist activity (Schromm et al. 2007; Seyberth et al. 2006).

In this paper, we have studied the structural requirement for the agonist activity of Pam2Cys by varying three of its structural elements. To do this four analogues were constructed (Fig. 1) (1) in the first the amino group of the cysteine residue was removed (2) in the second analogue the sulphur atom of the cysteine residue was substituted with an amide bond (3) in the third analogue a 1,3-dihydroxypropyl group instead of 2,3-dihydroxypropyl was used (4) in the fourth analogue the amino group was removed and the sulphur atom was also replaced with an amide bond. Each of these analogues were then used in vaccine constructs and tested in vitro and in vivo for biological activity.

Materials and methods

Chemicals

Unless otherwise stated chemicals were of analytical grade or its equivalent. Acetonitrile, dichloromethane (DCM), diethylether, dicyclohexylcarbodiimide, diisopropylethylamine (DIPEA), 4-dimethylaminopyridine (DMAP), *N*,*N*′-dimethylformamide (DMF), diisopropylcarbodiimide (DICI), 1-hydroxybenzotriazole (HOBt), *N*-hydroxysuccinimide, isopropanol, O'benzotriazole-*N*,*N*,*N*′,N′-tetra methyl-uronium-hexafluorophosphate (HBTU), piperidine, tetrahydrofurane (THF), trifluoracetic acid (TFA) and triethylamine were obtained from either Auspep Pty Ltd (Melbourne, Australia) or Fluka (Buchs, Switzerland). Phenol, triisopropylsilane (TIPS), 3-amino-1,2-propandiol and serinol were from Aldrich (Milwaukee, USA). 3-Bromopropionic acid, 1-thioglycerol, succinic anhydride and trinitrobenzylsulphonic acid (TNBSA) were from Fluka (Buchs, Switzerland). 1,8-Diazabicyclo-[5.4.0] undec-7-ene (DBU) was obtained from Sigma. Fmoc-amino acids were obtained from either Auspep (Melbourne, Australia) or Merck Australia (Kilsyth, Australia).

Synthesis of the derivatives for preparation of the Pam2Cys analogues (Scheme 1)

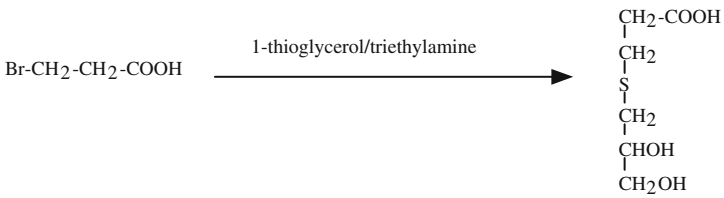
Synthesis of 3-S-(1,2-dihydroxypropyl)-propionic acid (Dhp-OH, 1a)

To 1.53 g (10 Mmol) of 3-bromopropionic acid in 6 ml of acetonitrile was added 1 ml of 1-thioglycerol (11.52 Mmol) and 1.6 ml (21.77 Mmol) of triethylamine. The solution was left at 37°C overnight. The reaction mixture was evaporated in vacuo to dryness. The residue was washed by adding acetone and dried in vacuo. The product was used without further purification.

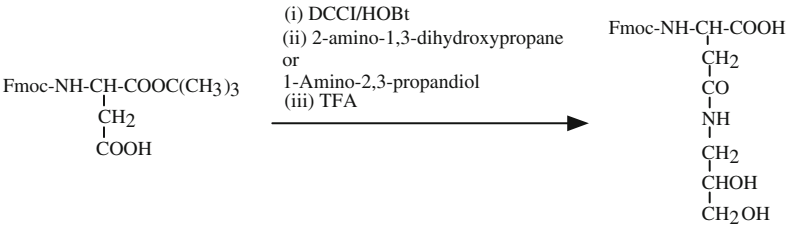
Synthesis of N^{α}-Fmoc-β-1,2-dihydroxylpropylamide aspartic acid (Fmoc-1,2-Dha-OH, 1b) and N^{α}-Fmoc-β-1,3-dihydroxylpropylamide aspartic acid (Fmoc-1,3-Dha-OH, 1c)

To 500 mg of Fmoc-Asp-OtBu (1.21 Mmol) in 4 ml of tetrahydrofurane (THF) was added 140 mg of *N*-hydroxysuccinimide (1.21 Mmol) and 280 mg of dicyclohexylcarbodiimide (1.34 Mmol). The reaction mixture was left at room temperature (RT) overnight. The urea was filtered off and the filtrate evaporated in vacuo to leave a residue. This residue was dissolved in 4 ml of acetonitrile and to this solution was added 110 mg (1.21 Mmol) of 3-amino-1,2-propandiol in 1 ml of isopropanol. Finally 168 µl (1.21 Mmol) of triethylamine was added slowly. After 3 h the solution was evaporated in vacuo to dryness. The residue was dissolved in 5 ml of TFA and left at RT for 90 min and the TFA was then evaporated under a stream of nitrogen and the product precipitated with cold diethylether. The product (Fmoc-1,2-Dha-OH, *1b*) eluted as a single peak using reverse-phase chromatography and had a mass of 429.0 Da (theoretical mass 429.4 Da). The same procedure was used for the synthesis of Fmoc-1,

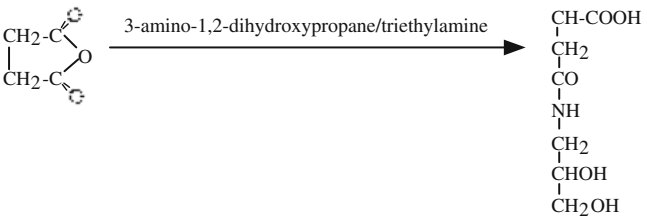
Scheme 1 Schematic representation of the synthesis of the derivatives for preparation of Pam2Cys-analogues



3-S-[2,3-Dihydroxypropyl]-propionic acid, **Dhp-OH, 1a**



Nα-Fmoc-N-[2,3-dihydroxypropyl]-aspartic acid, **Fmoc-1,3-Dha-OH, 1b** or Nα-Fmoc-N-[1,3-dihydroxypropyl]-aspartic acid, **Fmoc-2,3-Dha-OH, 1c**



N-(2,3-dihydroxypropyl)-succinic acid, **Dhs-OH, 1d**

3-Dha-OH (*1c*). The only change was that serinol (2-amino-1,3-propandiol) was used instead of 3-amino-1, 2-propandiol.

Preparation of N-(1,2-dihydroxypropyl)-succinic acid (Dhs-OH, 1d)

To a solution of 1.07 g (11.74 Mmol) of 3-amino-1, 2-propanediol in 4 ml of isopropanol and 1.63 ml (22.18 Mmol) of triethylamine was added slowly 1.18 g (11.79 Mmol) of succinic anhydride in 4 ml acetonitrile. The reaction was left at RT for 30 min and then evaporated in vacuo to dryness. The product (*Dhs-OH, 1d*) was used without further purification.

Synthesis of the lipopeptide containing Pam2Cys and Pam2Cys-analogues (Scheme 2)

A schematic of the lipopeptide construct into which Pam2Cys and each of the four analogues was incorporated is shown in Fig. 1. The T helper cell epitope [(T)] used throughout the study is from the light chain (HA2) of influenza virus hemagglutinin and has the sequence GALNNRFQIKGVELKS (Jackson et al. 1995). The target epitope is a truncated form (Zeng et al. 2007) of the luteinising hormone releasing hormone (LHRH) with the sequence of SYGLRPG.

Peptide and branched lipopeptides containing Pam2Cys were synthesized in house using Fmoc chemistry throughout; details of the synthetic procedures are to be found elsewhere (Zeng et al. 2005). The same protocol was adapted for the synthesis of the branched lipopeptides incorporating the Pam2Cys analogues. Briefly, as shown in Scheme 2, a contiguous peptide containing both the target epitope and the T helper cell epitope was assembled with an Fmoc lysine derivative, Fmoc-Lys(Mtt)-OH, inserted between the two epitopes. At the end of synthesis, and with the peptides still attached to the solid support, the epsilon amino protecting group Mtt present on the lysine situated between the two epitopes was selectively removed by

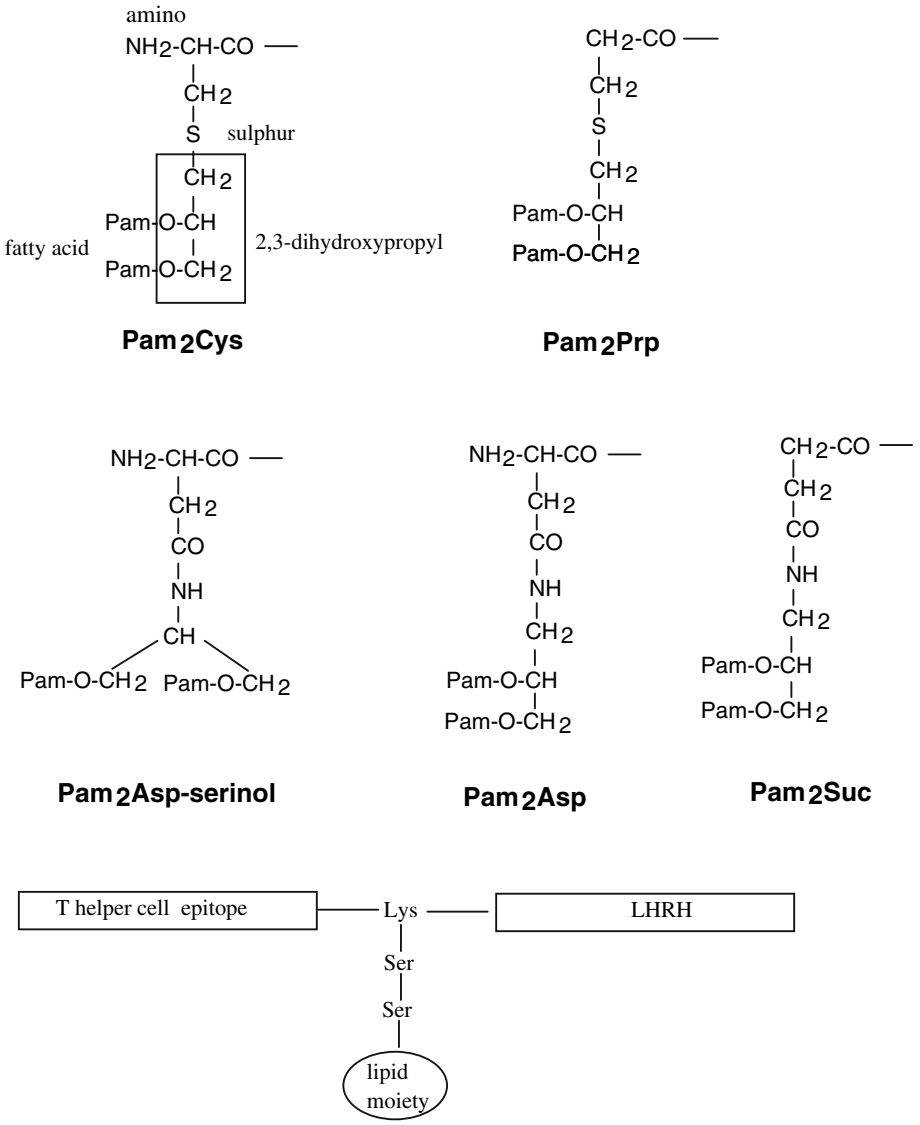
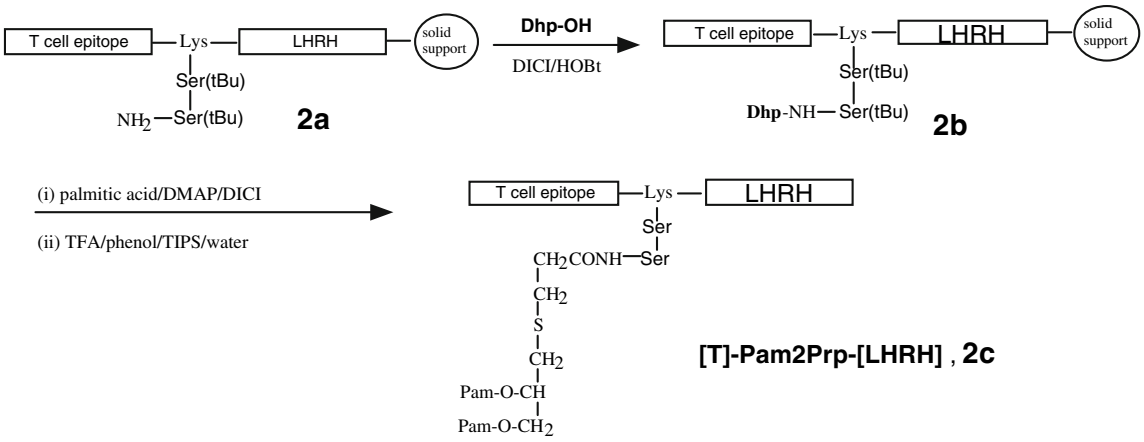


Fig. 1 Schematic representation of Pam2Cys, its four analogues and the lipopeptide vaccine construct into which the lipid moieties were incorporated

treating the peptide solid support with 1% TFA in dichloromethane for 60 min. To the exposed epsilon amino group two serine residues were then added.

For the synthesis of Pam2Prp-containing, Dhp-OH (*1a*) was coupled to the terminal serine residue using HOBt and DICI as activators and the two hydroxy groups were then acylated overnight with palmitic acid (10 equiv.) using DICI (10 equiv.) and DMAP (1 equiv.) as activator. The lipopeptide [T]-Pam2Prp-[LHRH] (*2c*, Scheme 2) was cleaved from the solid support by treatment with Reagent B (88% TFA containing 5% phenol, 5% water and 2% triisopropylsilane) and purified by semipreparative HPLC.

For the synthesis of Pam2Asp-containing lipopeptide [T]-Pam2Asp-[LHRH], Fmoc-1,2-Dha-OH (*1b*) was coupled to the terminal serine residue using HOBt and DICI as activators. The two hydroxy groups were subsequently acylated overnight with palmitic acid using DMAP and DICI as activators. The same conditions were used to prepare [T]-Pam2Asp-serinol-[LHRH] in which Fmoc-1,



Scheme 2 Schematic representation of the synthesis of Pam2Cys- and Pam2Cys-analogue-containing lipopeptide

Table 1 HPLC elution and mass characteristics of the peptide constructs incorporated with Pam2Cys and its four analogues

Peptide constructs	Retention time (min)[a]	Expected mass (Da)	Experimental determined mass (Da)[b]
[T]-Pam2Cys-[LHRH]	40.4	3460.3	3459.8
[T]-Pam2Prp-[LHRH]	43.1	3445.4	3444.8
[T]-Pam2Asp-[LHRH]	41.0	3470.3	3470.7
[T]-Pam2Asp-serinol-[LHRH]	40.8	3470.3	3470.7
[T]-Pam2Suc-[LHRH]	40.5	3455.2	3455.0

[a] RP chromatography was carried out on Vydac C4 column (4.6 × 250 mm) installed in a Waters HPLC system and developed at a flow rate 1 ml/min using 0.1% TFA in H2O and 0.1% TFA in CH3CN as the limit solvent

[b] Mass spectrometric measurements were carried out using a Agilent 1100 Series LC/MS Trap operated with electrospray ionisation in the positive ion mode

3-Dha-OH (*1c*) instead of Fmoc-1,2-Dha-OH was used. At the end of the synthesis the Fmoc group was removed by treating the peptide resin in 2.5% DBU in DMF twice for 5 min and the peptide was cleaved from the solid support and purified semipreparative HPLC.

For the synthesis of [T]-Pam2Suc-[LHRH] lipopeptide, Dhs-OH (*1d*) was used to couple to the terminal serine residue using HOBt and DICI as activators and the two hydroxy groups were then acylated overnight with palmitic acid as described above. The peptide was cleaved from the solid support and purified semipreparative HPLC.

Analytical HPLC was carried out using a Vydac C4 column (4.6 × 300 mm) installed in a Waters HPLC system. Chromatograms were developed at a flow rate of 1 ml/min using 0.1% TFA in H_2O and 0.1% TFA in acetonitrile as the limit buffer. Purification of peptide constructs was carried out using a semipreparative Vydac C4 column (10 × 300 mm) installed in a Waters HPLC system using the same solvent system as for analytical HPLC except that a flow rate of 2.5 ml/min was used. All peptides eluted as single major peaks by analytical HPLC. Mass analysis was carried out using an Agilent 1100 Series Capillary LC system in-line with an Agilent 1100 Series LC/MSD ion-trap mass spectrometer. The mass spectrometer was operated with electrospray ionisation configured in the positive ion mode. All purified peptides and lipopeptides had the expected mass (Table 1). The final quantitation of the immunogens was determined by measurement of UV absorption at 280 nm exploiting the presence of tyrosine residues in the constructs. The molar extinction coefficient for tyrosine $e = 1.1 \times 10^3$ was used in the calculation.

Flow cytometric analysis of D1 cells to detect upregulation of surface MHC Class II expression of dendritic cells

This assay was carried out as described previously (Jackson et al. 2004; Zeng et al. 2002). Briefly, mouse spleen-derived immature dendritic cells (DC) were prepared according to the method adapted from (Winzler et al. 1997). The prepared D1 cells (1×10^5 cells per sample) were then seeded in a Petri dish with 1 ml of DC media and incubated with 10 or 100 pmol of lipopeptide. After overnight incubation the cells were then exposed to

FITC-conjugated 14-4-4S monoclonal antibody ($IgG_{2a,}$ anti-I-$E^{k,d}$) (Becton-Dickinson, USA), for 30 min on ice. FITC-labelled mouse IgG2a (Becton-Dickinson, USA) as an isotype control. Flow cytometric analysis was performed using a FACSCalibur flow cytometer (BD Immunocytometry Systems) and the data analysed by FlowJo sofware (Tree Star, Inc., San Carlos,CA, USA).

NF-κB reporter gene assay to determine the ability of lipopeptides to signal through TLR2

This assay was carried out as described previously (Jackson et al. 2004; Lau et al. 2006; Sandor et al. 2003). The HEK293 cells were cultured in 96-wells plate at 2×10^4 per well and transfected 24 h later with 100 ng of an NF-κB luciferase reporter gene, 50 ng of TK-Renilla-luciferase expressing plasmid (Promega corporation, Madison, USA) with or without 5 ng TLR2-expressing plasmid in the presence of 0.8 µl Fugene 6 (Roche Diagnostic) per well. Lipopeptides were added to the wells 24 h later at concentrations of 0.025 or 0.25 nmol/ml, respectively in PBS. Cell lysates were then prepared 5 h after stimulation using reporter lysis buffer (Promega Corporation, Madison, USA). Luciferase activities in the cell lysates were determined using a reagent kit (Promega Corporation, Madison, USA) and a TopCount NXT (Perkin–Elmer Life and Analytical Sciences Inc., Boston, USA). The NF-κB-dependent firefly luciferase activity was normalised with NF-κB-independent renilla luciferase activity. The relative stimulation was calculated as the ratio of the stimulated to non-stimulated samples.

Enzyme-linked immunosorbent assays (ELISAs)

Groups of five female BALB/c mice, 6–8 weeks old, were inoculated subcutaneously on day 0 with a dose of 40 nmol vaccine formulated in saline. Mice received a second similar dose of vaccine on day 28. Animals were then bled 4 weeks after the primary inoculation and 2 weeks following the secondary inoculation. Sera were prepared from the blood and used for ELISAs. ELISA assays were carried out on serum samples as described previously (Zeng et al. 2005) using LHRH as the coating antigen. Serial dilutions of antisera were prepared and incubated overnight on antigen-coated plates. Antibody titres were expressed as the reciprocal of the highest dilution of serum achieving an optical density of 0.2. This value represents approximately five times the optical absorbance observed in the absence of anti-peptide antibody.

Fertility studies in mice

Fertility studies were initiated 2 weeks after the second inoculation of vaccine candidate and were carried out as described previously (Zeng et al. 2002). Briefly, female mice were examined for their ability to drop litters following inoculation with peptide immunogens and subsequent co-habitation with untreated male mice. A male mouse was introduced to either two or three female mice 2 weeks after females had received the second dose of vaccine. Males were rotated between each group of females in order to expose each female to every male. Males and females were kept together for a total of 3 weeks at the end of which time males were removed and the females kept under observation. A group of female mice inoculated with non-lipidated peptide in saline was used as a negative control.

Statistical analysis

The statistical analyses of the antibody data was carried out using two-tailed Student's *t* test calculated using Prism software. Each of the resultant *P* values for a particular comparison is shown in the appropriate text or in the figure legend.

Results

Synthesis of the Pam2Cys analogues

Four analogues were constructed as shown in Fig. 1: in the first, Pam2Prp**,** the amino group was removed; in the second, Pam2Asp**,** the sulphur atom was substituted with an amide bond; in the third, Pam2Asp-serinol, the 1,3-dihydroxypropyl group was substituted with 2,3-dihydroxypropyl and in the fourth, Pam2Suc, the amino group was removed and the sulphur atom was also replaced with an amide bond. The syntheses of each of these analogues and their incorporation into lipopeptides are shown in Schemes 1 and 2. As an example, in order to prepare the Pam2Prp analogue, 3-*S*-[2,3-dihydroxypropyl]-propionic acid (Dhp-OH**,** *1a*) was firstly prepared by reacting thioglycerol with bromopropionic acid in the presence of triethylamine. The peptide portion consisting of the T helper cell epitope [T] [derived from the light chain of influenza virus hemagglutinin and the B cell target epitope (luteinizing hormone release hormone (LHRH)], was synthesized as a contiguous sequence with a single lysine residue with its epsilon amino group protected with the methyltrityl group (Mtt) inserted between the two epitopes. Selective removal of the Mtt group with 1% trifluoroacetic acid (TFA) and subsequent coupling of two serine residues then generated *2a*. Reaction of Dhp-OH *1a* with *2a* in the presence of 1-hydroxybenzotriazole (HOBT) and diisopropylcarbodiimide (DICI) and subsequent esterification of the two hydroxyl groups in the presence of palmitic acid,

dimethylamino pyridine (DMAP) and DICI produced *2b*. Cleavage of *2b* generated the final product *2c,* [T]-Pam2Prp-[LHRH]. In a similar manner, reaction of *2a* with Fmoc-1,3-Dhd-OH (*1b*), Fmoc-2,3-Dhd-OH (*1c*) and Dhs-OH (*1d*) allowed the production of [T]-Pam2Asp-serinol-[LHRH], [T]-Pam2Asp-[LHRH] and [T]-Pam2Suc-[LHRH] lipopeptides, respectively. A summary of their characteristics, conducted by analytical RP-HPLC and mass spectrometry, is presented in Table 1. Each of these lipopeptide constructs was then tested in vitro and in vivo for biological activity.

Upregulation of surface MHC Class II expression on dendritic cells

Previous reports have demonstrated that the immunogenicity of the Pam2Cys-containing peptide-based vaccine [T]-Pam2Cys-[LHRH] correlates with its ability to mature dendritic cells by upregulating surface MHC class II expression (Zeng et al. 2002). Here, using a similar approach, we compared the ability of the four Pam2Cys-analogue-containing lipopeptide constructs to cause dendritic cell maturation. The results (Fig. 2) showed that at a concentration of 10 nM the peptide construct containing Pam2Cys caused a similar number of cells to express high levels of MHC class II molecules on their surface as did lipopolysaccharide (LPS) which is used here as a positive control. With the possible exception of [T]-Pam2Prp-[LHRH] none of other three Pam2Cys analogues caused a significant higher level of maturation than did medium alone.

At a concentration of 100 nM (Fig. 2), there was little change in the ability of the [T]-Pam2Cys-[LHRH] to activate DC. However, at this concentration [T]-Pam2Prp-[LHRH] and [T]-Pam2Asp-serinol-[LHRH] did cause significant upregulation of MHC class II.

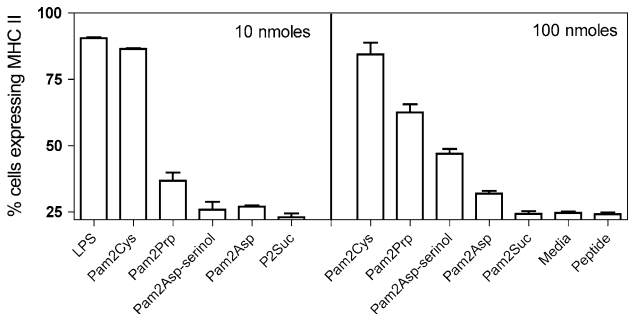


Fig. 2 Up-regulation of MHC Class II expression on the surface of D1 cells exposed to Pam2Cys- and Pam2Cys-analogue-containing peptides. D1 cells were incubated with 10 or 100 pmol of lipopeptide for 24 h. Lipopolysaccharide purified from *E. coli* serotype O111:B4 (Difco, Detroit, Michigan, USA) was used at 5 μg/ml as a positive control. Data are expressed as the percentage of cells showing high levels of MHC class II molecule expression

Ability of lipopeptides to signal through TLR2

Using a TLR2-dependent NF-kB activation assay it has been demonstrated that Pam2Cys-containing peptides signal through TLR2 (Jackson et al. 2004; Lau et al. 2006). Here, we have also compared the ability of the Pam2Cys-analogue-containing peptides to stimulate NF-kB gene activation in a TLR2-dependent manner. Lipopeptides at two different concentrations (0.25 and 0.025 nmol/ml), were incubated with HEK293 cells that were transfected with a TLR2-encoding plasmid and an NF-kB luciferase reporter gene and the induced luciferase activities determined (Jackson et al. 2004; Sandor et al. 2003).

The results (Fig. 3a, b) show that [T]-Pam2Cys-[LHRH] could stimulate NF-kB-dependent gene activation in TLR2-transfected cells to a significantly higher extent at both concentrations than any of the analogues. At a concentration of 0.025 nmol/ml (Fig. 3a) little or no significant stimulatory activities was observed with the analogue-containing lipopeptides or the non-lipidated peptide. At a concentration of 0.25nmol/ml (Fig. 3b), [T]-Pam2Prp-[LHRH] and [T]-Pam2Asp-serinol-[LHRH] activated NF-kB to a level which was significantly higher than either [T]-Pam2Suc-[LHRH] or the non-lipidated peptide. [T]-Pam2Asp-[LHRH] shows the lowest activity at both concentrations.

Induction of anti-LHRH antibody

Luteinizing-hormone releasing hormone is a peptide hormone secreted by the hypothalamus amd initiates a cascade of endocrine events which lead to the control of reproduction in female and male of most mammals in which the sequence is conserved. Previous studies have shown that a peptide construct containing [T]-Pam2Cys-[LHRH] induced a strong anti-LHRH antibody response which is similar to if not better than, that elicited by mice receiving the non-lipidated peptide in the presence of complete Freund's adjuvant and the presence of the high titres of anti-LHRH antibody rendered mice infertile (Zeng et al. 2002).

Here, we compared the ability of the Pam2Cys-containing peptide construct and the four lipid analogue-based vaccines to induce a specific anti-LHRH antibody response. Groups of 5 BALB/c mice received two doses of 40 nmol of lipopeptide vaccine candidates on days 0 and 21. Animals were bled on days 21 and 35 and anti-LHRH antibody titres in sera determined by ELISA.

The results (Fig. 4) show that two doses of the lipopeptide vaccine [T]-Pam2Cys-[LHRH] elicited a strong anti-LHRH antibody response. The other lipopeptide candidates also elicited significantly higher specific secondary antibody responses than that obtained with non-lipidated

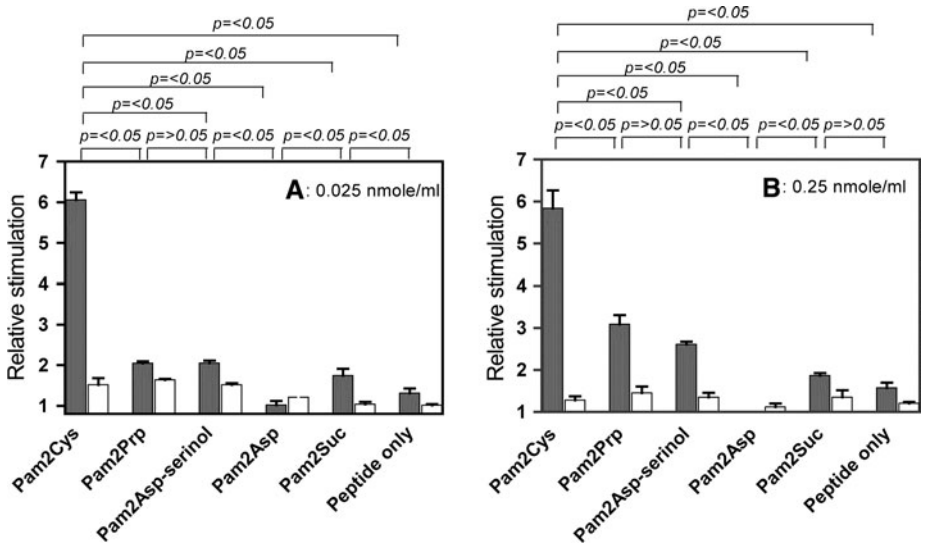


Fig. 3 NF-kB reporter gene assay to determine the ability of lipopeptides to signal through TLR2. The target epitope in these constructs is LHRH. HEK293 cells were transfected transiently using an NF-κB luciferase reporter gene system with or without a TLR-2-expressing plasmid using FuGENE. Twenty-four hours after transfection, Pam2Cys-, Pam2Prp-, Pam2Asp-, Pam2Asp-serinol- or Pam2Suc-containing lipopeptide were added to individual wells at a concentration of 0.025, 0.25 nmol/ml for an additional 5 h. Cell lysates were then prepared using lysis buffer and luciferase activities in the cell lysates were determined. Data are expressed as mean stimulatory activities and SD was determined from triplicate samples. *Closed bars* show the results of samples treated with the TLR2 plasmid and open bars show those without the TLR2 plasmid

peptide inoculated in saline. Of the responses elicited by the analogues, [T]-Pam2Prp-[LHRH] and [T]-Pam2Asp-serinol-[LHRH] gave the highest titres, a hierarchy that correlates well with the results obtained in the two in vitro assays described above.

The results (Fig. 4) of the fertility trials carried out to determine the functionality of the antibodies elicited by each of the lipopeptides indicate that [T]-Pam2Cys-[LHRH] rendered all five mice infertile while the group that received non-lipidated peptide in saline all produced litters. Although significant antibody titres were detected in the sera obtained from the mice that received [T]-Pam2Prp-[LHRH] and [T]-Pam2Asp-serinol-[LHRH], some mice in each group remained fertile. Incidences of pregnancy were also observed in the groups that received the other two lipidated peptides. These results appear to correlate with the antibody levels observed.

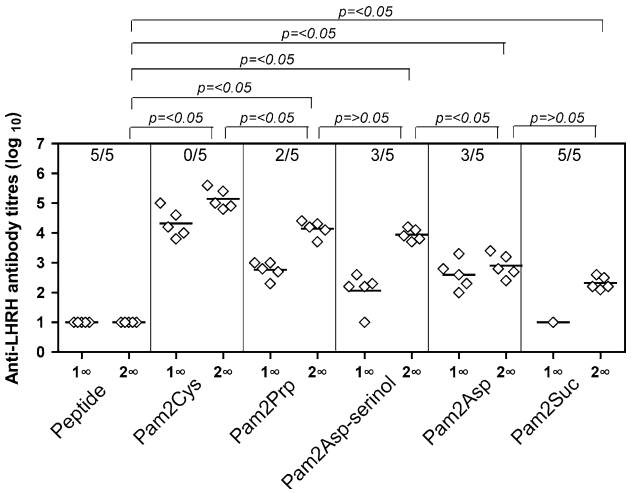


Fig. 4 Induction of anti-LHRH antibody responses by Pam2Cys-containing lipopeptide and Pam2Cys-analogue-containing lipopeptides. The target epitope in these constructs was LHRH. Groups of five BALB/c mice received two doses (40 nmol) of lipidated peptides in saline on days 0 and 21. Non-lipidated peptide administered in saline was also included as a control. Mice were bled on days 21 and 35 and sera prepared. The anti-LHRH antibody response was determined by ELISA using LHRH as coating antigen. Antibody titres were expressed as the reciprocal of the highest dilution of serum achieving an optical density of 0.2. The numbers on the top of each panels refer to incidence of pregnancy during the course of fertility studies carried out 2 weeks following the second inoculation

Discussion

Pam2Cys is a synthetic analogue of the lipid moiety of MALP2 lipopeptide which activates the innate immune system promoting adaptive immunity following stimulation with specific antigens. One of the main receptors responsible for the functional recognition of the lipopeptide is Toll like receptor 2, a member of the Toll family which are prominent pattern recognition receptors of the innate immune system and which recognise conserved features present on various pathogens. The TLRs differ not only in ligand specificity but also in their expression pattern on different cells of the innate immune system. In addition the

recognition events experienced by distinct TLRs result in the expression of different sets of target genes (Heine and Lien 2003).

In this study, four novel analogues of Pam2Cys designed around its three structural elements (1) the α-amino group (2) the sulphur atom and (3) the 2,3-dihydroxypropyl moiety were prepared and incorporated into vaccine candidates and then tested in vitro and in animals for immunogenicity.

Our results showed that although each of the four analogues showed reduced biological activities compared with Pam2Cys, there was nevertheless a hierarchy of activities observed. Two of the analogues, Pam2Prp and Pam2Asp-serinol, were consistently more potent than Pam2Asp and Pam2Suc at activating NF-kB in a TLR2-dependent manner, inducing DC maturation and also at eliciting specific antibody responses. Given that Pam3Cys, which does not possess a primary amino group, is a strong stimulator of splenocytes (Metzger et al. 1995) and macrophages (Muhlradt et al. 1997, 1998) it is perhaps not surprising that Pam2Prp which lacks the primary amino group has the highest activity among the four analogues. Furthermore earlier reports have shown that the length of the carbon chain of the N-terminal amide-bound fatty acid has minimal or no effect on the TLR2-mediated effect; no significant differences in the release of IL-8 from cells stimulated with lipopeptides containing N-bound fatty acyl residues ranging from hexanoic acid with 6 carbon to palmitic acid with 16 carbon atom chains has been observed (Buwitt-Beckmann et al. 2005b). Nevertheless, Pam2Prp is much less potent than Pam2Cys indicating the importance of the primary amino group. The crystal structure of TLR2 in complex with a Pam2Cys-containing peptide has recently been reported. The exact interaction of the amino group of Pam2Cys with either TLR2 or TLR6 or solvent, however, is yet to be defined (Jin et al. 2007; Jin and Lee 2008a, b).

An earlier report (Metzger et al. 1991) has shown that replacement of sulphur with a $–CH_2–$ group (i.e. $CH_2–CH_2–CH_2$ instead of $CH_2–S–CH_2$) reduces activity to about 23%. We extended this finding by substitution of the sulphur with an amide bond. Our results show that this alteration led to significant loss of biological activities as in the case of the Pam2Asp analogue. Simultaneous removal of the primary amino group as in the case of the Pam2Suc analogue also generated reduced activities. These results demonstrate the importance of the presence of the thioether bond between the 1,2-dihydroxypropyl group and the cysteine residue.

Although replacement of the thioether bond with an amide bond (Pam2Asp and Pam2Asp-serinol analogues) leads to significantly lower biological activity the lipopeptide construct incorporating Pam2Asp-serinol demonstrated consistently higher biological activity than did Pam2Asp. The difference between these two analogues is the substitution position of the fatty acids in the dihydroxypropyl moiety. In Pam2Asp the two palmitoyl groups are vicinal (1,2-substitution) the same as in Pam2Cys and also in other TLR2 ligands such as lipoteichoic acid (Kumagai et al. 2008; Lehner et al. 2001; Schwandner et al. 1999), but in Pam2Asp-serinol the two 1,3-palmitoyl groups are separated by a carbon atom. It remains to be determined if the replacement of 1,2-dihydroxypropyl moiety with 1,3-dihydroxypropyl in Pam2Cys would lead to an increase in its biological activity. On the other hand the higher immunogenicity of Pam2Asp-serinol may be due to a synergistic effect caused by simultaneous replacement of thioether bond with an amide bond and the 1,2-dihydroxypropyl group with the 1,3-dihydroxypropyl group.

We have also replaced the core structure of Pam2Cys with (+)-glyceric acid, (−)-glyceric acid and shikimic acid and assessed their biological activities in assays similar to those described in this paper. The results (data not shown) indicate that these changes also lead to significantly reduced biological activity.

In conclusion, our results show the importance of the intact core structure as well as the sulphur atom and the free amino group of the cysteine residue of Pam2Cys in causing the activation of dendritic cells which play a central role in mediating both innate and adaptive immune responses (for a review see Belz et al. 2004). The finding that even in the relative absence of dendritic cell activation, significant antibody responses can be detected with some of the analogues does, however, indicate that an adaptive immune response is being initiated perhaps raising the possibility that these compounds can act as ligands for TLR2 without causing all of the downstream signal transduction events associated with full occupancy of TLR2 with Pam2Cys. The biological consequences of occupancy without full activation are currently under examination.

Acknowledgments This work was supported by grant from the National Health and Medical Research Council of Australia.

References

Alphs HH, Gambhira R, Karanam B, Roberts JN, Jagu S, Schiller JT, Zeng W, Jackson DC, Roden RB (2008) Protection against heterologous human papillomavirus challenge by a synthetic lipopeptide vaccine containing a broadly cross-neutralizing epitope of L2. Proc Natl Acad Sci USA 105:5850–5855

Batzloff MR, Hartas J, Zeng W, Jackson DC, Good MF (2006) Intranasal vaccination with a lipopeptide containing a conformationally constrained conserved minimal peptide, a universal T cell epitope, and a self-adjuvanting lipid protects mice from group A streptococcus challenge and reduces throat colonization. J Infect Dis 194:325–330

Baz A, Buttigieg K, Zeng W, Rizkalla M, Jackson DC, Groves P, Kelso A (2008) Branched and linear lipopeptide vaccines have different effects on primary CD4+ and CD8+ T-cell activation but induce similar tumor-protective memory CD8+ T-cell responses. Vaccine 26:2570–2579

Belz G, Smith C, Bharadwaj M, Rice A, Jackson D (2004) DCs as targets for vaccine design. Cytotherapy 6:88–98

Borges E, Wiesmuller KH, Jung G, Walden P (1994) Efficacy of synthetic vaccines in the induction of cytotoxic T lymphocytes. Comparison of the costimulating support provided by helper T cells and lipoamino acid. J Immunol Methods 173:253–263

Buwitt-Beckmann U, Heine H, Wiesmuller KH, Jung G, Brock R, Akira S, Ulmer AJ (2005a) Toll-like receptor 6-independent signaling by diacylated lipopeptides. Eur J Immunol 35:282–289

Buwitt-Beckmann U, Heine H, Wiesmuller KH, Jung G, Brock R, Ulmer AJ (2005b) Lipopeptide structure determines TLR2 dependent cell activation level. Febs J 272:6354–6364

Buwitt-Beckmann U, Heine H, Wiesmuller KH, Jung G, Brock R, Akira S, Ulmer AJ (2006) TLR1- and TLR6-independent recognition of bacterial lipopeptides. J Biol Chem 281:9049–9057

Chua BY, Zeng W, Lau YF, Jackson DC (2007) Comparison of lipopeptide-based immunocontraceptive vaccines containing different lipid groups. Vaccine 25:92–101

Day EB, Zeng W, Doherty PC, Jackson DC, Kedzierska K, Turner SJ (2007) The context of epitope presentation can influence functional quality of recalled influenza A virus-specific memory CD8+ T cells. J Immunol 179:2187–2194

Deliyannis G, Kedzierska K, Lau YF, Zeng W, Turner SJ, Jackson DC, Brown LE (2006) Intranasal lipopeptide primes lung-resident memory CD8+ T cells for long-term pulmonary protection against influenza. Eur J Immunol 36:770–778

Heine H, Lien E (2003) Toll-like receptors and their function in innate and adaptive immunity. Int Arch Allergy Immunol 130:180–192

Jackson DC, Fitzmaurice C, Sheppard RC, McMurray J, Brown LE (1995) Antigenic and immunogenic properties of synthetic peptide-based T-cell determinant polymers. Biomed Pept Proteins Nucleic Acids 1:171–176

Jackson DC, Lau YF, Le T, Suhrbier A, Deliyannis G, Cheers C, Smith C, Zeng W, Brown LE (2004) A totally synthetic vaccine of generic structure that targets Toll-like receptor 2 on dendritic cells and promotes antibody or cytotoxic T cell responses. Proc Natl Acad Sci USA 101:15440–15445

Jin MS, Lee JO (2008a) Structures of the toll-like receptor family and its ligand complexes. Immunity 29:182–191

Jin MS, Lee JO (2008b) Structures of TLR-ligand complexes. Curr Opin Immunol 20:414–419

Jin MS, Kim SE, Heo JY, Lee ME, Kim HM, Paik SG, Lee H, Lee JO (2007) Crystal structure of the TLR1-TLR2 heterodimer induced by binding of a tri-acylated lipopeptide. Cell 130:1071–1082

Jung G, Bessler WG (1995) Lipopeptides as adjuvant and intergratd adjuvanticity. In: Zegers ND, Boersma WJA, Claassen E (eds) Immunological recognition of peptides in medicine and biology. CRC Press, Boca Raton, pp 159–168

Kumagai Y, Takeuchi O, Akira S (2008) Pathogen recognition by innate receptors. J Infect Chemother 14:86–92

Lau YF, Deliyannis G, Zeng W, Mansell A, Jackson DC, Brown LE (2006) Lipid-containing mimetics of natural triggers of innate immunity as CTL-inducing influenza vaccines. Int Immunol 18:1801–1813

Lehner MD, Morath S, Michelsen KS, Schumann RR, Hartung T (2001) Induction of cross-tolerance by lipopolysaccharide and highly purified lipoteichoic acid via different Toll-like receptors independent of paracrine mediators. J Immunol 166:5161–5167

Metzger J, Jung G, Bessler WG, Hoffmann P, Strecker M, Lieberknecht A, Schmidt U (1991) Lipopeptides containing 2-(palmitoylamino)-6, 7-bis(palmitoyloxy) heptanoic acid: synthesis, stereospecific stimulation of B-lymphocytes and macrophages, and adjuvanticity in vivo and in vitro. J Med Chem 34:1969–1974

Metzger JW, Beck-Sickinger AG, Loleit M, Eckert M, Bessler WG, Jung G (1995) Synthetic *S*-(2, 3-dihydroxypropyl)-cysteinyl peptides derived from the N-terminus of the cytochrome subunit of the photoreaction centre of Rhodopseudomonas viridis enhance murine splenocyte proliferation. J Pept Sci 1:184–190

Muhlradt PF, Kiess M, Meyer H, Sussmuth R, Jung G (1997) Isolation, structure elucidation, and synthesis of a macrophage stimulatory lipopeptide from Mycoplasma fermentans acting at picomolar concentration. J Exp Med 185:1951–1958

Muhlradt PF, Kiess M, Meyer H, Sussmuth R, Jung G (1998) Structure and specific activity of macrophage-stimulating lipopeptides from Mycoplasma hyorhinis. Infect Immun 66:4804–4810

Sandor F, Latz E, Re F, Mandell L, Repik G, Golenbock DT, Espevik T, Kurt-Jones EA, Finberg RW (2003) Importance of extra- and intracellular domains of TLR1 and TLR2 in NFkappa B signaling. J Cell Biol 162:1099–1110

Schromm AB, Howe J, Ulmer AJ, Wiesmuller KH, Seyberth T, Jung G, Rossle M, Koch MH, Gutsmann T, Brandenburg K (2007) Physicochemical and biological analysis of synthetic bacterial lipopeptides: validity of the concept of endotoxic conformation. J Biol Chem 282:11030–11037

Schwandner R, Dziarski R, Wesche H, Rothe M, Kirschning CJ (1999) Peptidoglycan- and lipoteichoic acid-induced cell activation is mediated by toll-like receptor 2. J Biol Chem 274:17406–17409

Seyberth T, Voss S, Brock R, Wiesmuller KH, Jung G (2006) Lipolanthionine peptides act as inhibitors of TLR2-mediated IL-8 secretion. Synthesis and structure-activity relationships. J Med Chem 49:1754–1765

Spohn R, Buwitt-Beckmann U, Brock R, Jung G, Ulmer AJ, Wiesmuller KH (2004) Synthetic lipopeptide adjuvants and Toll-like receptor 2—structure–activity relationships. Vaccine 22:2494–2499

Takeda K, Takeuchi O, Akira S (2002) Recognition of lipopeptides by Toll-like receptors. J Endotoxin Res 8:459–463

Takeuchi O, Kaufmann A, Grote K, Kawai T, Hoshino K, Morr M, Muhlradt PF, Akira S (2000) Cutting edge: preferentially the R-stereoisomer of the mycoplasmal lipopeptide macrophage-activating lipopeptide-2 activates immune cells through a toll-like receptor 2- and MyD88-dependent signaling pathway. J Immunol 164:554–557

Vitiello A, Ishioka G, Grey HM, Rose R, Farness P, LaFond R, Yuan L, Chisari FV, Furze J, Bartholomeuz R et al (1995) Development of a lipopeptide-based therapeutic vaccine to treat chronic HBV infection. I. Induction of a primary cytotoxic T lymphocyte response in humans. J Clin Invest 95:341–349

Winzler C, Rovere P, Rescigno M, Granucci F, Penna G, Adorini L, Zimmermann VS, Davoust J, Ricciardi-Castagnoli P (1997) Maturation stages of mouse dendritic cells in growth factor-dependent long-term cultures. J Exp Med 185:317–328

Zeng W, Ghosh S, Lau YF, Brown LE, Jackson DC (2002) Highly immunogenic and totally synthetic lipopeptides as self-adjuvanting immunocontraceptive vaccines. J Immunol 169:4905–4912

Zeng W, Gauci S, Ghosh S, Walker J, Jackson DC (2005) Characterisation of the antibody response to a totally synthetic immunocontraceptive peptide vaccine based on LHRH. Vaccine 23:4427–4435

Zeng W, Pagnon J, Jackson DC (2007) The C-terminal pentapeptide of LHRH is a dominant B cell epitope with antigenic and biological function. Mol Immunol 44:3724–3731