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In Vivo Evaluation of Pam_2Cys-Modified Cancer-Testis Antigens as Potential Self-Adjuvanting Cancer Vaccines

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ABSTRACT

Peptide-based vaccines, formulated with an appropriate adjuvant, offer a versatile platform for targeted cancer immunotherapy. While adjuvants are usually coadministered for nucleic acid and protein vaccines, synthetic peptide antigens afford a more effective opportunity to covalently and regioselectively graft immunostimulatory motifs directly onto the antigen scaffold to yield *self-adjuvanting* vaccines. Herein, we explore the synthesis of two tissue-restricted cancer-testis antigens (CTAs); New York oesophageal cell carcinoma 1 (NY-ESO-1) and B melanoma antigen 4 (BAGE4), both carrying the toll-like receptor (TLR) agonist, Pam_2Cys. These constructs were evaluated in vivo along with a lipid nanoparticle (LNP) preparation of the underexplored BAGE4 melanoma antigen.

1 | Introduction

Immunotherapy has emerged in recent years as an alternative cancer treatment that can be administered without the drawbacks of more conventional approaches such as chemotherapy, radiotherapy and surgery [1]. The production of tumour-associated antigens (TAAs) provides an opportunity to direct the patient's immune system to target cancer via vaccination [1]. Cancer-testis antigens (CTAs), a class of highly tissue-restricted TAA, expressed in male germ-line cells and aberrantly expressed across a broad range of cancer types, have been explored as promising targets for vaccine development [2–4]. Due to the existence of the blood-testis barrier, and the lack of human leukocyte antigen (HLA) class I expression on the surface of germ cells, CTAs do not activate the autoimmune response; a process that may hinder the viability of cancer immunotherapy treatments [5]. The most promising CTA candidate to-date is New York oesophageal cell carcinoma 1 (NY-ESO-1) [6]; this TAA demonstrates strong spontaneous humoral and cell-mediated immune responses [2]. Clinical trials have been conducted for this antigen employing a range of strategies, including peptide (epitope), protein, nucleic acid (DNA and mRNA), dendritic cell (DC) and whole-tumour cell therapy via viral, bacterial and lipid nanoparticle (LNP) delivery vectors [7–10]. While these trials have produced promising initial results, many have not progressed to Phase III. Unfortunately, the results of those trials that were progressed to completion have been ultimately disappointing [2, 11].

Despite the robust immune response TAAs such as NY-ESO-1 can elicit, few antigens produce a strong enough response to challenge cancer without coadministration of an adjuvant—a substance that recruits antigen-presenting cells (APCs), increasing the delivery of antigens to APCs or activating APCs

to produce cytokines and trigger T-cell responses [12]. In both academic and clinical settings, CTAs have been coadministered with several effective adjuvants such as incomplete Freund's adjuvant (IFA), ISCOMATRIX, cytokines and toll-like receptor (TLR) agonists such as monophosphoryl lipid A and CpG oligonucleotides (ODN) [12]. TLR agonists in particular have been shown to be effective adjuvants for a range of vaccines [13]. Ten TLR variants are present in humans; the TLR family display affinities for particular pathogen-associated molecular patterns (PAMPs), that is, bacterial and viral components [14, 15]. TLR2 (activated with TLR6) recognises lipopeptides derived from the bacterial cell wall. Pam_2Cys, a synthetic analogue of the lipid component of macrophage-activating lipopeptide-2 (MALP-2), is a potent TLR2/6 agonist [14, 15]. Pam_2Cys (as well as Pam_3Cys) are among the most common lipid moieties used in the production of lipopeptide vaccines [16], several of which have advanced to human clinical trials, showing a high level of protection with little to no side effects reported [17–19]. This adjuvant can be effectively coadministered as PEG-Pam_2Cys [20] or as the palmitoylated cysteine residue along with other lipids (and cholesterol) formulated into a LNP as a delivery vehicle for DNA/mRNA vaccines [21].

While coadministration of the adjuvant has proven to be effective, the ability to covalently graft the adjuvant moiety onto the antigen scaffold enables the preparation of a *self-adjuvanting* vaccine. Such multicomponent constructs may circumvent undesired immune responses and have been demonstrated to be promising immunotherapy tools [22]. In this regard, peptide-based vaccines [23] (sequences that represent highly immunogenic epitopes of protein antigens) are ideal antigen scaffolds. Noncanonical amino acids bearing adjuvant groups can be incorporated into the peptide sequence during solid-phase synthesis. Alternatively, chemo- and regio-selective chemical modification of the synthesised peptide using established bioconjugate chemistry can be applied. Using the former strategy, multiple-component Pam_2Cys-peptide vaccines have been prepared and shown to produce robust immune responses against (among others) cancer [24], *Mycobacterium tuberculosis* [25] and SARS-CoV-2 [26].

Due to the small size of many CTAs (several under 100 amino acids in length), it is possible to produce the whole CTA synthetically via solid-phase peptide synthesis (SPPS), coupled with native chemical ligation if required [27]. Recently, Brimble and co-workers employed an NY-ESO-1 epitope bearing Pam_2Cys and relevant analogues to illustrate the importance of the Pam_2Cys stereochemistry (following on from earlier studies demonstrating that the *R* stereoisomer in the glyceryl moiety is significantly more active than the *S*) [28] and ester linkages, using an in vitro reporter system [17–19]. These structure–activity relationship studies demonstrated that the C16 chain is optimal for activity; although homologues of Pam_2Cys are still active, short-chain fatty acid alternatives induce a very weak response [19]. Replacement of the fatty acid chains with unsaturated groups, polyether or polyamine functionalities also decreases potency [19]. Additionally, the ester linkage has been shown to be essential for activity [17]. While the NY-ESO-1 epitope carrying Pam_2Cys was shown to be the most efficacious of the analogues, this product was not evaluated in vivo during these studies.

Due to the initial promise of NY-ESO-1 as a vaccine candidate, the lack of in vivo evaluation of the palmitoylated analogue of this antigen, and the lack of in vivo data for many other CTAs, including the B melanoma antigen 4 (BAGE4) sequence [29], further evaluation of the self-adjuvanting efficacy of these scaffolds warrants additional exploration. Herein, we describe the synthesis, formulation and in vivo evaluation in healthy models of two CTA epitope peptides covalently attached to the lipid adjuvant, Pam_2Cys; $BAGE4_{18–39}$ [29] a small (22 amino acid, excluding the signal peptide) underexplored melanoma antigen, which has yet to be evaluated in vivo [29], and the NY-ESO-1 epitope, NY-ESO-$1_{157\text{-}165}$ (SLLMWITQC) [30]. Three approaches were explored for the preparation of the Pam_2Cys modified antigens **1**–**3** (Figure 1); (1) alkylation of antigen peptide bearing an N-terminal cysteine (Cys) residue with a bespoke iodinated ester moiety; (2) palmitoylation of glycerol installed onto the Cys sidechain, on-resin; (3) synthesis and coupling of the Fmoc-Pam_2Cys-OH (**4**) building block into the peptide sequence, on-resin.

Vaccine constructs with and without a peptide solubility tag (SK_4) [31] were successfully synthesised, and the constructs Pam_2Cys-$BAGE4_{18–39}$ **1**, Pam_2Cys-SK_4-$BAGE4_{18–39}$ **2** and Pam_2Cys-SK_4-NY-ESO-$1_{157\text{-}165}$ **3** were evaluated in vitro. In vivo evaluation of the immune response elicited by candidates **2** and **3** was then conducted by measuring the T-cell responses to these epitopes in healthy mice. Furthermore, LNPs formulated from BAGE4 candidate **2** were compared to LNPs carrying the BAGE4 peptide conjugated to the outer envelope of the LNP via in vivo evaluation.

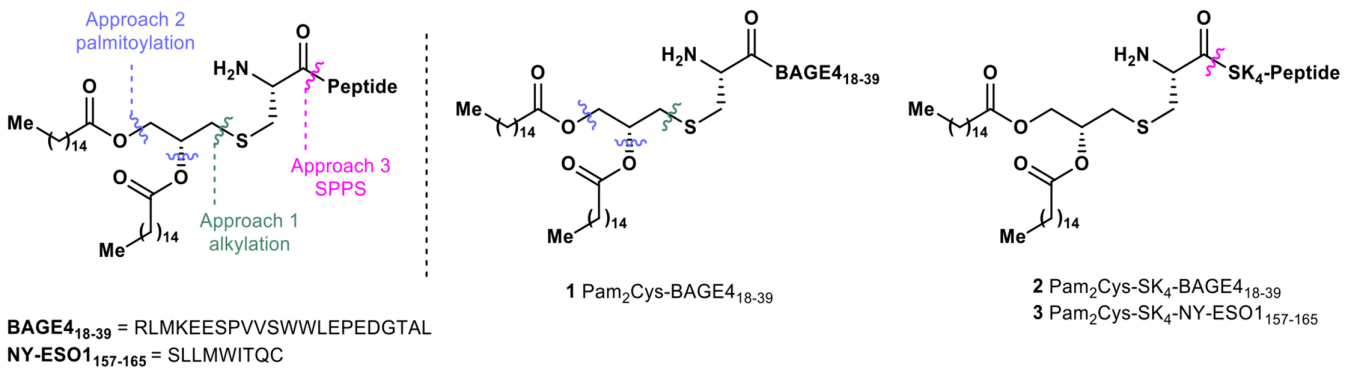


FIGURE 1 | Approaches to the construction of vaccine-adjuvant conjugates **1**–**3**.

At the outset of our investigation, Pam_2Cys-CTAs had not been explored as vaccine candidates. However, during the course of our studies, Brimble et al. described the synthesis of Pam_2Cys-NY-ESO peptide via approach 1 and conducted in vitro testing of the human and mouse TLR2 agonistic activities of lipopeptide homologues of Pam_2Cys using HEK293 cells [17]. Approaches 2 and 3 to Pam_2Cys-functionalised peptides have been previously described by Jackson et al. and Brimble et al., respectively [19, 32].

2 | Materials and Methods

2.1 | General Methods, Reagents and Chemical Synthesis

General methods for all chemical syntheses are included in the Supporting Information. All chemicals were of commercial quality and were used without additional purification. All commercially available reagents and reagent-grade solvents were purchased from Merck, Fluorochem or Fisher and used as received unless otherwise stated. Amino acids, coupling reagents and resins were obtained from Novabiochem, Fluorochem or GL Biochem. Antibodies were purchased from Sigma Aldrich; DSPC and DOTAP were purchased from Avanti Polar Lipids Inc. Experimental procedures for the synthesis, purification and characterization of the novel maleimide lipid (MalLipid **5**) are described in the Supporting Information and in earlier reported work [33]. All aqueous solutions were prepared using deionised water. Dry solvents were used when indicated in the procedure.

2.2 | Formulation of LNP With Vaccine Construct 2 (2-LNP)

The constituent lipids (DSPC and DOTAP) and cholesterol were dissolved in chloroform to a conc. of 2 mM; Pam_2Cys-SK_4-$BAGE4_{18–39}$ **2** was dissolved in methanol to a conc. of 2 mM. The lipid and cholesterol components were mixed in a DSPC:DOTAP:cholesterol:**2** ratio of 40:15:35:10. Solvents were evaporated in vacuo to form a continuous lipid film, which was dried in vacuo overnight. Milli-Q water (0.9 mL) was added to hydrate the film with vortexing at 55°C. The resulting mixture was sonicated for 10 min, no pulsing, amplitude at 90% (SONICS Vibra-Cell, CPX 130) in an ice bath and 0.1 mL of 10× PBS buffer added to the formulation to give a final conc. of 2 mM.

2.3 | Formulation of Antigen-Conjugated LNP (CysBAGE4-MalLNP)

The constituent lipids (DSPC, DOTAP, MalLipid **5**) and cholesterol were dissolved in chloroform to a conc. of 2 mM. The lipid and cholesterol components were mixed in a DSPC:DOTAP:MalLipid **5**:cholesterol ratio of 40:15:10:35. The solvent was evaporated in vacuo to form a continuous lipid film, which was dried in vacuo for 4 h. PBS was added to hydrate the film at a final conc. of 2 mM with vortexing at 55°C for 1–2 min. The resulting mixture was sonicated for 10 min, no pulsing, amplitude at 90%, using an ice bath; 1200 μL of 2 mM MalLNP formulation (containing 0.24 μmol MalLipid **5**) was conjugated to Cys-$BAGE4_{18–39}$ (0.48 μmol, 2.0 eq.) in the presence of an excess of TCEP (2 eq. over peptide), at pH 7. The reaction mixture was agitated at rt for 3 h. The sample was loaded into Slide-A-Lyzer Dialysis Cassette (10K MWCO) then placed in dialysate buffer (PBS), 500× the volume of loaded sample. The dialysate buffer was changed after 2 h, 3 times, and then allowed to dialyse overnight. The sample was recovered and analysed by UV–Vis spectroscopy at 280 nm.

2.4 | LNP Characterization

CysBAGE4-MalLNP and **2**-LNP formulations were analysed using DLS, circular dichroism (CD), and TEM. DLS analysis: CysBAGE4-MalLNP and **2**-LNP formulations were diluted with Milli-Q water and transferred to disposable cuvettes before measurement at 25°C with a 173° light scattering angle, wavelength range: 180–280 nm. CD analysis: CysBAGE4-MalLNP and **2**-LNP formulations were diluted with Milli-Q water and analysed using a quartz cell with a 0.01 mm path length. Spectra were measured using a 0.5 nm step, 1 nm bandwidth and 1 s collection time per step. TEM analysis: CysBAGE4-MalLNP and **2**-LNP formulations were diluted in Milli-Q water and applied to glow-discharged carbon-coated copper 200 mesh grids and negative-stained with 2% uranyl acetate.

2.5 | ELISA and Competitive ELISA Assays

General methods for the ELISA and competitive ELISA assays can be found in the Supporting Information.

2.6 | In Vivo Evaluation

Animal experiments were carried out with ethical approval from University of Nottingham ethical review board and under a Home Office approved project license (PP2706800). Mice were dosed with 10 nmol of material in 50 μL at Days 1, 8 and 15 ($n = 3$). IFNϒ ELISpot assays on splenocytes were conducted on termination at Day 21. General methods for the ELISpot can be found in the Supporting Information. Preparation of media and buffers and steps performed on Days 1 and 2 post-termination; the ELISpot assays were performed using a laminar flow cabinet and aseptic techniques to ensure the sterility of media, reagents and plates at stages before development of the ELISpot. Development of the ELISpot on Day 4 posttermination was performed on the laboratory bench.

3 | Results and Discussion

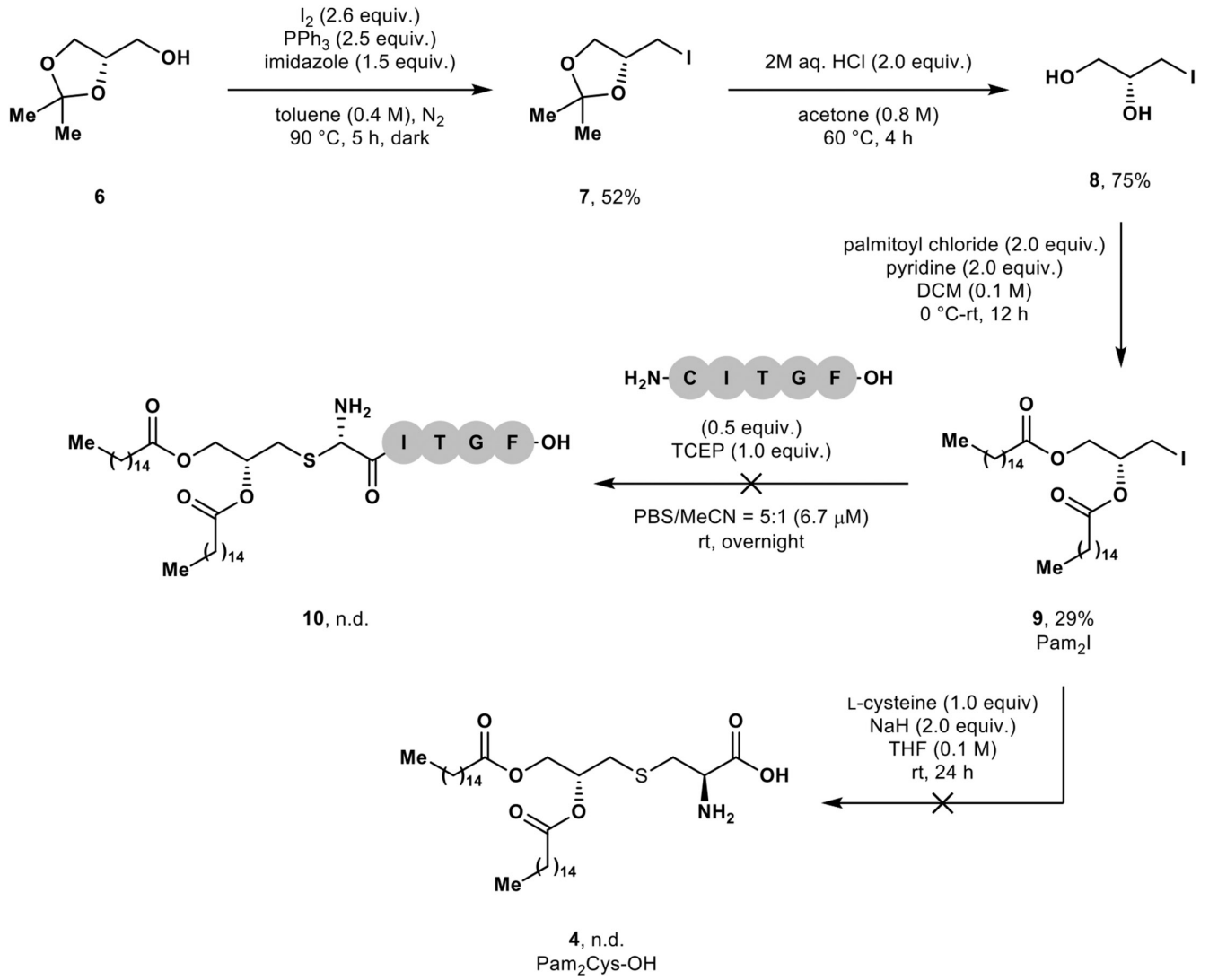
3.1 | Synthesis of Pam_2Cys-Modified CTAs (1–3)

Two CTA epitopes; NY-ESO-$1_{157-165}$ and $BAGE4_{18–39}$ were employed as the antigenic components in our studies. NY-ESO-$1_{157-165}$ is spontaneously immunogenic and able to bind to HLA-A2, expressed by a wide range of cancers [34–36]. This epitope is known to reactivate T-cell responses (CD8+ cells) in models vaccinated against NY-ESO-1 [34–36]. $BAGE4_{18–39}$ represents the entire BAGE4 CTA minus the signal peptide; this

antigen is expressed in 22% of melanomas and 30% of infiltrating bladder carcinomas [29]; to our knowledge, it has not been evaluated in vivo. Inclusion of the SK_4 solubility tag within our vaccine constructs enables formulation of the Pam_2Cys peptides in buffer for administration [31, 37].

In light of Brimble's SAR studies on Pam_2Cys, and to avoid possible compromises in activity, no modifications or deviations from the Pam_2Cys moiety were explored, and the essential ester linkage was used in the construction of palmitoylated antigens. Native (*R*)-stereochemistry has been retained throughout the routes applied as the (*S*)-analogue of the Pam_2Cys adjuvanting moiety exhibits a hundredfold decrease in potency compared to the (*R*)-analogue [19]. An additional benefit of using palmitoyl functionality is the ability of the fatty acid chains to be incorporated into nanoparticles via self-assembly, which we have utilised in this study. The three synthetic approaches explored (Figure 1) enable the effective on-resin synthesis of the vaccine (approaches 2 and 3) as well as investigations into the use of an electrophilic moiety that could be employed to introduce the Pam_2Cys adjuvant into peptide and protein antigens via late-stage conjugation (approach 1).

Our initial approach towards the target vaccine constructs involved the synthesis of alkylating agent, Pam_2I **9**, outlined in Scheme 1. Briefly, Pam_2I **9** was prepared from solketal **6** in three steps, beginning with the preparation of alkyl iodide **7** under Garegg–Samuelsson conditions [38]. The acetal protecting group was then removed under acidic conditions to furnish diol **8**, which was palmitoylated to yield Pam_2I **9** in an 11% yield over three steps. Compound **9** could potentially be a powerful reagent for the installation of the Pam_2Cys adjuvant into peptides and proteins carrying an N-terminal Cys residue. Unfortunately, the alkylation of model peptide H-CITGF-OH was unsuccessful; no conversion of the starting peptide was observed. This is attributed to the mismatch in solubility between alkyl iodide **9** and the peptide in both organic and organic/aqueous solvent mixtures. Attempts to prepare key building block **4** via this route were also unsuccessful. In addition to the recalcitrant nature of the conjugation reaction, isomerisation of the alkyl iodide, akin to that observed by Brimble et al. for similar substrates [17], cannot be ruled out, which would further complicate the utilisation of building block **9**. Any production of the *S* isomer of the glyceryl unit either in the peptide conjugate or amino acid **4** would diminish the activity of the vaccine construct [19, 28].



SCHEME 1 | Attempted synthesis of peptide-adjuvant conjugate **10** via alkylation approach.

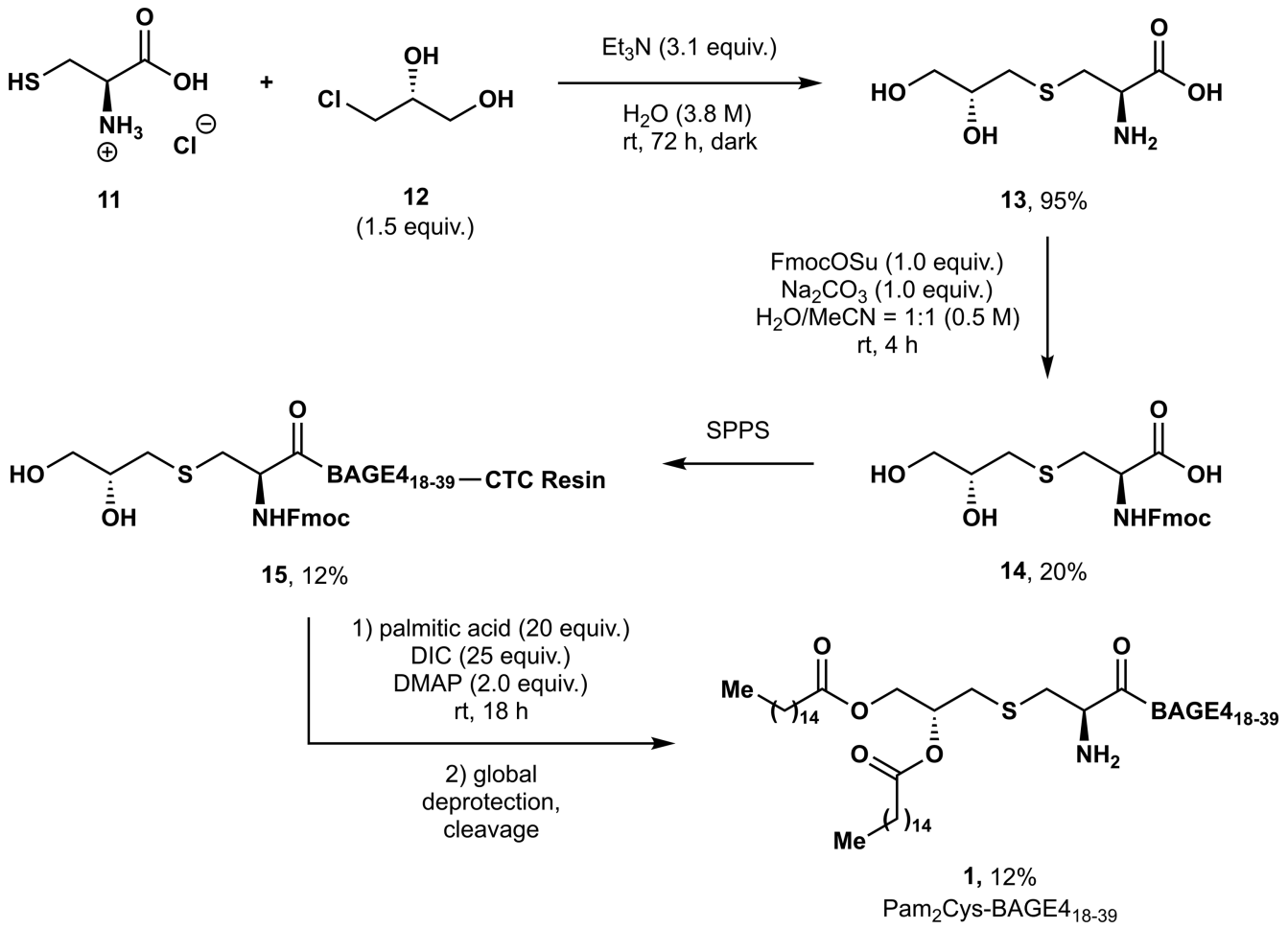
An alternative approach to the synthesis of the palmitoylated antigen targets via late-stage esterification on-resin was also considered (Scheme 2). Alkylation of cysteine hydrochloride **11** with α-chlorohydrin **12** yielded diol **13** in an excellent yield of 95%, which was taken to the next step without further purification. Treatment of **13** with Fmoc-OSu produced Fmoc-protected amino acid **14**, which was then installed at the N-terminus of antigenic peptide $BAGE4_{18–39}$ via standard SPPS (**15**). Installation of the palmitoyl chains was completed using a Steglich esterification between the resin-bound peptide and palmitic acid [39]. The adjuvant-peptide conjugate was cleaved from the resin and globally deprotected to furnish the desired product $Pam_2Cys\text{-}BAGE4_{18–39}$ **1** in a 12% yield over the final coupling and esterification steps.

This successful route to $Pam_2Cys\text{-}BAGE4_{18–39}$ **1** was applied to the synthesis of adjuvant-vaccine conjugates **2** and **3**, both of which carry the solubility sequence SK_4; however, the final palmitoylation step tended to yield mixtures of the desired *bis*-palmitoylated product with impurities consistent with the formation of *mono*-palmitoylated products. To avoid this complication, targets **2** and **3** were prepared via the installation of palmitoylated amino acid Fmoc-Pam_2Cys-OH **4**. Since the synthesis of **4** using Pam_2I **9** was unsuccessful, an alternative route was sought (Scheme 3).

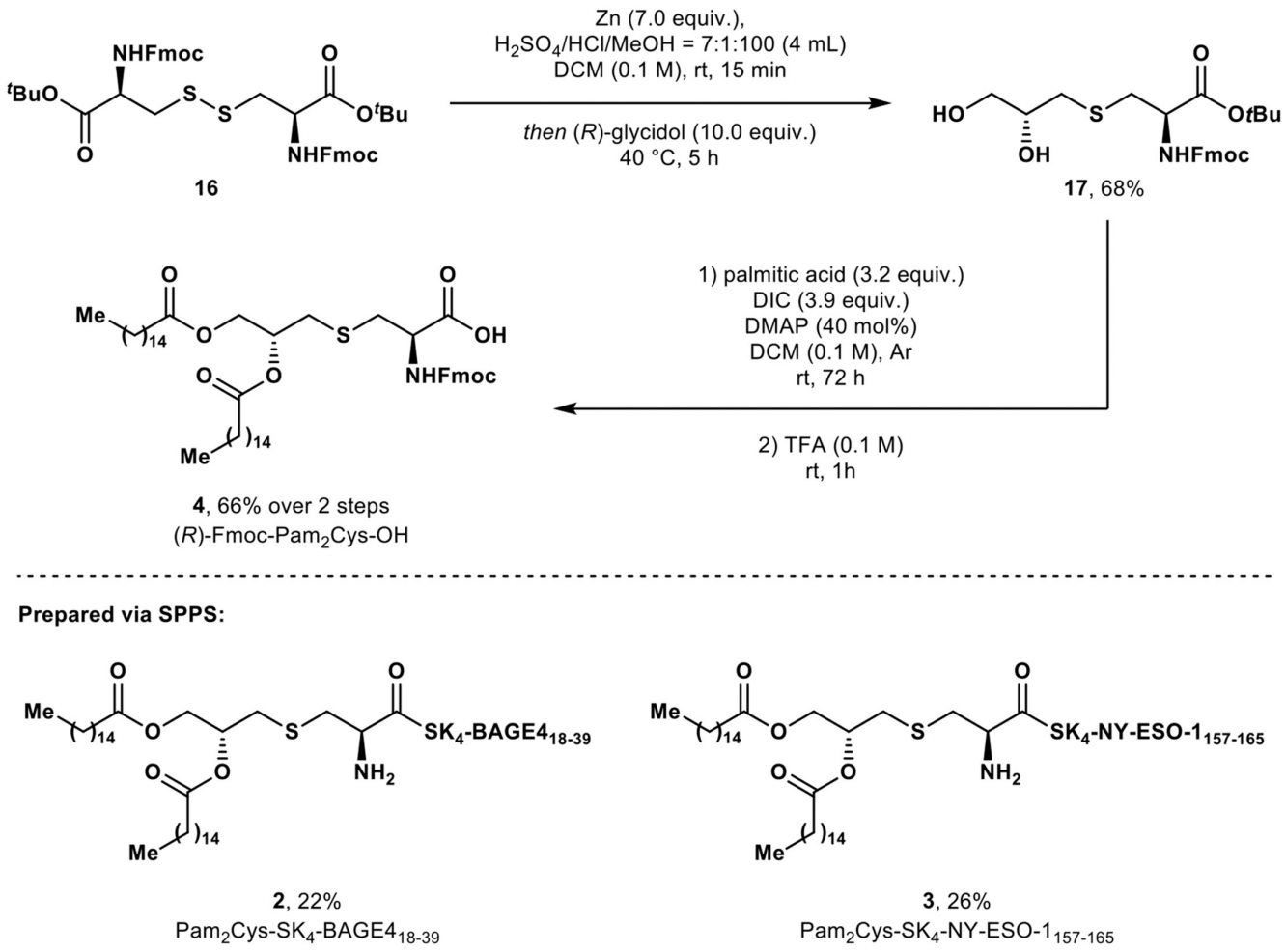
The preparation of (*R*)-Fmoc-Pam_2Cys-OH **6** was completed in three steps, beginning with *S*-alkylation of Fmoc-Cys-O^{t}Bu **16** with (*R*)-glycidol to yield diol **17**. Palmitoylation and deprotection of **17** afforded the desired product **4** in a 45% yield over three steps. Although routes to Fmoc-Pam_2Cys-OH **4** have been reported from Fmoc-Cys-OH [19, 40], the chosen route from commercially available Fmoc-Cys-O^{t}Bu **16** shortens the synthetic route by two steps. Using amino acid **4**, two vaccine-adjuvant constructs were prepared—$Pam_2Cys\text{-}SK_4\text{-}BAGE4_{18–39}$ **2** and $Pam_2Cys\text{-}SK_4\text{-NY-ESO-}1_{157–165}$ **3**. Rink amide resin (yielding a C-terminal primary amide) was used in the synthesis of vaccine-adjuvant constructs **2** and **3**, after improved overall yields for SPPS were achieved compared to synthesis on 2-chlorotrityl chloride (2-CTC) resin (yielding the carboxylate). Moreover, C-terminal amidation, a common posttranslational modification (PTM) observed widely across the proteome, confers enhanced stability in vivo due to resistance to enzymatic degradation and, in many cases, enhances binding affinity [41].

3.2 | Evaluation of Vaccine Secondary Structure and Fibril Formation

Interrogation of the structure of peptide-adjuvant constructs **2** and **3** was performed using CD spectroscopy as well as modelling studies (PEP-FOLD) [42, 43]. The CD spectra (Figures S12 and S13) show that both compounds form α-helical structures, however, NY-ESO-$1_{157–165}$ **3** exhibits significantly more α-helical character ($f_\alpha = 0.41$) than $Pam_2Cys\text{-}SK_4\text{-}BAGE4_{18–39}$ **2** ($f_\alpha = 0.07$),



SCHEME 2 | Synthesis of $Pam_2Cys\text{-}BAGE4_{18–39}$ **1**.



SCHEME 3 | Top: Synthesis of (*R*)-Fmoc-Pam$_2$Cys-OH **4**. Bottom: vaccine-adjuvant conjugates **2** and **3** prepared from amino acid **4**.

which PEP-FOLD indicates has a disordered C-terminal domain (see Supporting Information for details). SAXS and cryo-TEM studies (Figures S16–S18) show that both constructs form fibrils (Supporting Information). Fibril formation by α-helical peptides is typically observed due to lateral association of coiled-coils [44]. Here, this may play a role, although lateral interaction of the hydrophobic alkyl chains is likely to be the essential driver for the fibril formation. The fibril core radius 14.0–18.5 Å from SAXS (Table S1) is consistent with the length of an extended Pam lipid chain.

3.3 | In Vitro Evaluation of BAGE4$_{18–39}$ Antigen and Pam$_2$Cys-SK$_4$-BAGE4$_{18–39}$ (2)

To confirm antibody recognition of the BAGE4$_{18–39}$ antigen when incorporated into the vaccine construct **2** the primary antibody to this antigen (anti-BAGE4 antibody, produced in rabbit; Sigma Aldrich SAB4301150) was incubated with Pam$_2$Cys-SK$_4$-BAGE4$_{18–39}$ **2** and peptide BAGE4$_{18–39}$ at 11 different concentrations between 1000 and 0.98 ng/mL (PBST as the negative control). Antibody–antigen complexes were then added to 384-well plates which were precoated with 10 ng/mL of antigen. The secondary antibody, specific to the primary and conjugated to horseradish peroxidase (HRP) enzyme (produced in rabbit), was added, followed by TMB for colour development. The binding affinity of Pam$_2$Cys-SK$_4$-BAGE4$_{18–39}$ **2** gave an IC$_{50}$ value of 8.04 μM; BAGE$_{18–39}$ alone gives an IC$_{50}$ value of 11.41 μM (Figure 2). Thus, we can be confident that the covalent grafting of the Pam$_2$Cys adjuvant does not affect recognition of the antigen.

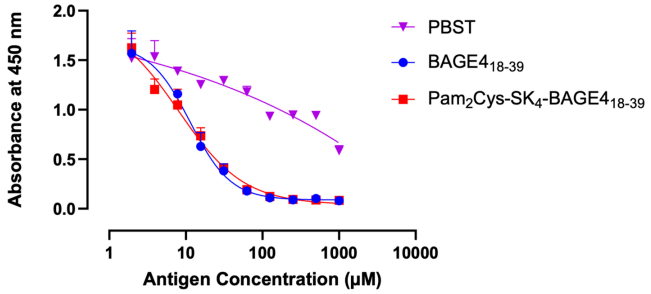


FIGURE 2 | ELISA data to compare the binding affinity of BAGE4$_{18–39}$ antigen IC$_{50}$ = 11.41 μM +/−3.52, and Pam$_2$Cys-SK$_4$-BAGE4$_{18–39}$ **2** IC$_{50}$ = 8.04 μM +/−6.04, PBST buffer with 0.05% Tween 20 as negative control, absorbance measured at 450 nm.

3.4 | In Vivo Evaluation of BAGE4 Peptide-Adjuvant Construct

To compare the T-cell responses induced by the $BAGE4_{18–39}$ peptide (administered with IFA as the adjuvant) and Pam_2Cys-SK_4-$BAGE4_{18–39}$ **2**, groups of healthy mice were immunized on three occasions (1, 8 and 15 days) with 10 nmol of each conjugate via s.c injection (0.2 mM dose concentration). The response was measured by count of peptide-specific IFNγ-secreting T-cells by ELISpot assay. Since no studies to date have addressed T-cell responses to BAGE4 in conventional or HLA-A2 (HHDII/DR1) transgenic mice, conventional mice were initially selected. The H-2d haplotype (BALB/c) strain was selected for this study as the epitope predictions for peptides that bind to MHC I and II are effective for this strain (IEDB Analysis Resource). Splenocytes from the immunised mice were tested against three predicted epitope peptides from $BAGE4_{18–39}$, which should reactivate T-cell responses in this strain of mice when immunised with $BAGE4_{18–39}$ vaccine candidates. Lipopolysaccharide (LPS; 5 μg/mL) was used as the positive control (non-specific stimulus of the immune system).

The $BAGE4_{18–39}$ sequence was observed to be immunogenic in conventional BALB/c mice, stimulating T-cell responses that recognise BAGE4 with good responses to the whole native $BAGE4_{18–39}$ sequence (median = 511) and slightly lower responses to the $BAGE4_{18–32}$ peptide (Figure 3). There is almost no response to $BAGE4_{22–31}$; a one-way ANOVA statistical test was carried out and confirmed a significant effect for $BAGE4_{18–39}$ ($p = 0.9999$, q = 0.02932, DF = 8) and $BAGE4_{18–32}$ ($p = 0.0009$, q = 6.142, DF = 8), while $BAGE4_{22–31}$ shows no significance ($p = > 0.9999$, q = 0.02932, DF = 8).

Unfortunately, mice dosed with Pam_2Cys-SK_4-$BAGE4_{18–39}$ **2** reacted negatively and were culled on Day 1. There was significant bleeding and darkening at the injection site and the liver appeared pale and patchy coloured. Repeating the experiment using a 20-fold decrease in concentration of **2** produced the same result. Since the negative in vivo reaction upon administration of **2** was apparent within 24 h of vaccine administration, immune-mediated toxicity is unlikely which typically takes longer than 24 h to manifest effects [45, 46]. The $BAGE4_{18–39}$ antigen **8** alone does not show any toxicity and Pam_2Cys is a well-studied and safe adjuvant; [45, 46] thus, further investigation is required to elucidate the mechanism(s) behind this unanticipated toxicity.

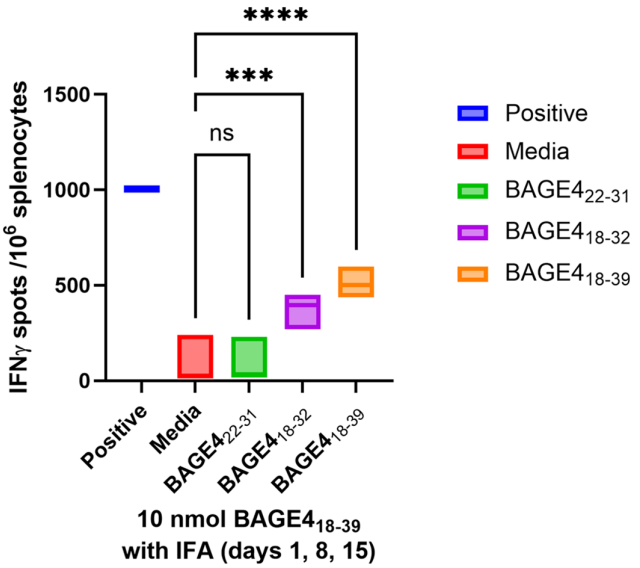


FIGURE 3 | Immune responses induced in BALB/c mice immunized with $BAGE4_{18–39}$ peptide mixed with IFA. Isolated splenocytes from the immunised mice tested against three peptides from the $BAGE4_{18–39}$ peptide and measured in IFNγ ELISpot assay.

3.5 | Formulation of Antigen-Loaded LNPs

In addition to the synthesis and evaluation of the palmitoylated antigens administered alone, we also explored the formulation of the vaccine construct **2** into LNPs and the conjugation of the BAGE4 CTA onto the outer envelope of a LNP for in vivo delivery, a method often employed for targeted nucleic acid delivery (Figure 4) [47, 48]. To date, most reported LNP vaccines have been formulated by antigen entrapment [49]. However, antigen-entrapped liposomes and surface-coupled antigens of liposomes are reported to induce different types of immune responses [50]. Antigen-entrapped liposomes have been shown to induce antigen-specific IgE antibody production [51], while antigens coupled to the surface of liposomes induced substantial IgG antibody production with a minimal amount of IgE antibody production as shown by ovalbumin-liposome [52], tetanus toxoid [53] or Shiga-like toxin [54], coupled to the outer envelope of LNPs. Antigen-LNP conjugates are therefore considered to be suitable vaccine candidate strategies that cause minimal allergic reaction [55]. Nanoparticles were formulated using either covalent linkage of Cys-$BAGE4_{18–39}$ to maleimide-containing LNPs (CysBAGE4-MalLNP, using a bespoke maleimide lipid (**5**)), or self-assembly of LNPs from Pam_2Cys-SK_4-$BAGE4_{18–39}$ **2** (**2**-LNP), and in vivo assays were conducted.

Optimization of the lipid and cholesterol ratios allows the size distribution and stability of the LNP formulations to be 'tuned' [56]. Nanoparticles of diameter 100–200 nm, PDI < 0.3 and zeta potentials (ζ) of > ±10 mV were deemed desirable, and formulations containing 35%–40% cholesterol were used throughout. A cationic liposome formulation was selected, as anionic liposomes quickly engage with the biological system after becoming opsonized by circulating protein [57, 58], resulting in the rapid uptake by the reticuloendothelial system (RES) and toxic effects such as pulmonary hypertension, dyspnea and a drop in circulating platelets and leukocytes [59]. A range of lipid compositions that would yield stable cationic LNPs within the appropriate diameter range were evaluated; the optimum composition was found to be 40 mol% DSPC, 15 mol% DOTAP, 35 mol% cholesterol and either 10 mol% MalLipid **5** [33] or 10 mol% Pam_2Cys-SK_4-$BAGE4_{18–39}$ **2**. These LNPs were formulated in Milli-Q water via the thin film hydration method [60], and 10 vol% (final volume) of 10x PBS solution was added after sonication to afford the LNPs in PBS (2 mM); the samples were then analysed by TEM and DLS. The MalLNP sample (including repeats to ensure consistency) gave average particle diameters of 70 nm (PDI 0.236) and a zeta potential (ζ) of +30 mV. Storage of the solution for 36 days at 4°C and reanalysis after this time showed little deviation from these values indicating acceptable stability.

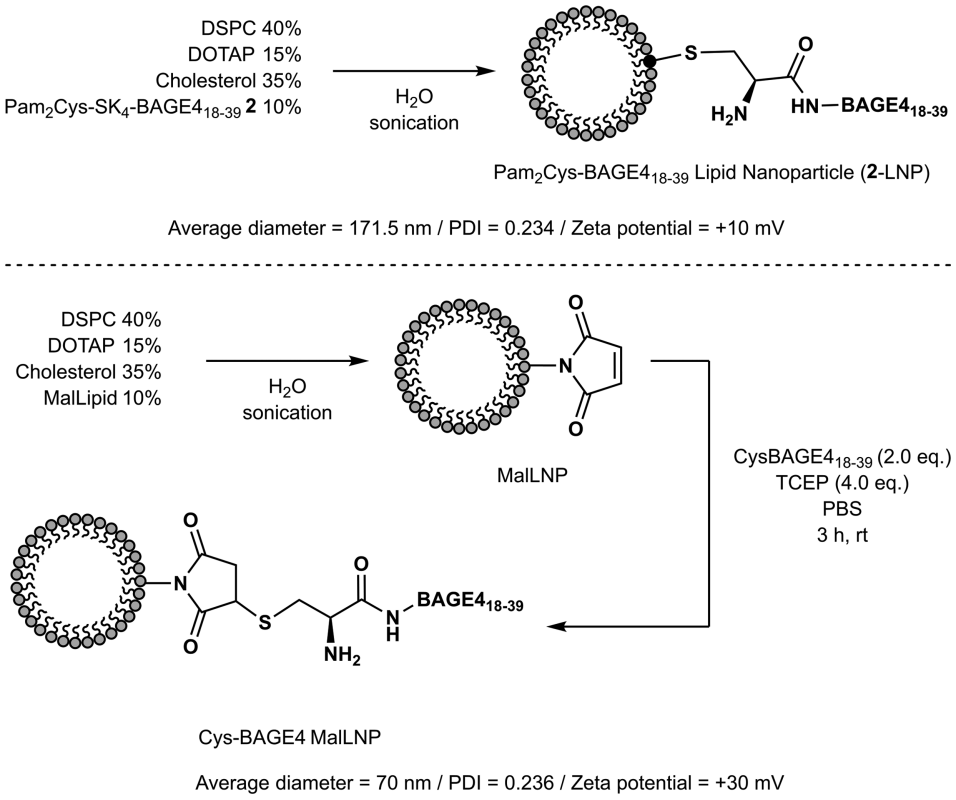


FIGURE 4 | Formulation of nanoparticles $Pam_2Cys\text{-}BAGE4_{18-39}$ LNP (**2**-LNP) and Cys-BAGE4-MalLNP used in this study. DSPC = 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine. DOTAP = 1,2-dioleoyl-3-trimethylammonium propane. LNPs formulated as multilamellar vesicles.

A control formulation, which omitted the MalLipid **5** (45 mol% DSPC, 15 mol% DOTAP, 40 mol% cholesterol; non-MalLNP), was also prepared (diameter 83 nm, PDI 0.26, zeta potential +35 mV). The formulation made up with 10 mol% $Pam_2Cys\text{-}SK_4\text{-}BAGE4_{18-39}$ **2** (**2**-LNP) afforded particles with an average diameter of 165 nm (PDI 0.3) and a zeta potential of +10 mV.

To prepare CysBAGE4-MalLNPs, a solution of MalLNP (100 μL of a 2 mM formulation containing 0.02 μmol of MalLipid **5**), 5 equiv. of $CysBAGE4_{18-39}$ and 10 equiv. of TCEP was agitated for 3 h then purified via dialysis. UV–Vis analysis showed the successful loading of 0.06 μmol of peptide, while the control reaction using NonMal-LNPs demonstrated 0.02 μmol of loading (see Supporting Information for details). Due to the cationic nature of the MalLNPs (+ 30 mV) and the net negative charge of the $CysBAGE4_{18-39}$ peptide at pH 7.0, electrostatic association of the peptide with the LNPs was anticipated; however, significantly more loading was observed for the MalLNPs than expected considering the NonMal-LNP control. Thus, to minimise electrostatic loading, 2 equiv. of $CysBAGE4_{18-39}$ (in the presence of 2 equiv. of TCEP relative to peptide) was employed for the CysBAGE4-MalLNP samples intended for in vivo evaluation.

Competitive ELISA for the CysBAGE4-MalLNP conjugate shows reduced competition relative to the $BAGE4_{18-39}$ antigen ($IC_{50} = 151.4\,\mu M$ and $IC_{50} = 11.41\,\mu M$, respectively, Figure S26), comparable with the negative control. This result may be explained by ineffective antigen presentation on the surface of the particles due to the formation of higher order multilayered liposomes (i.e., the antigen is buried within the lipid (bi)layers) [61], or ineffective coating of the plates with the LNP sample.

3.6 | In Vivo Evaluation of Antigen-Loaded LNPs

In vivo evaluation of the antigen-loaded LNPs was conducted as described in Section 3.4. The CysBAGE4-MalLNP formulation will serve as an informative control, demonstrating the response of the peptide and nanoparticle without adjuvant. The immune response for this peptide-LNP conjugate was somewhat varied (385, 139, 323, with median = 282). A one-way ANOVA statistical test was carried out and confirmed no significant effect for $BAGE4_{18-39}$ ($p = 0.5013$, q = 1.359, DF = 8), $BAGE4_{18-32}$ ($p = 0.9165$, q = 0.6330, DF = 8) & $BAGE4_{22-31}$ ($p > 0.9999$, q = 0.08425, DF = 8) due to high variability between repeats (Figure 5).

The muted immune response to the delivery of CysBAGE4-MalLNP was to be expected as the bespoke MalLipid **5** is unlikely to act as an effective adjuvant due to deviation in chemical structure relative to Pam_2Cys [62]. As was the case with dosing of $Pam_2Cys\text{-}SK_4\text{-}BAGE4_{18-39}$ **2**, mice dosed with $Pam_2Cys\text{-}SK_4\text{-}BAGE4_{18-39}$ self-assembled liposomes (**2**-LNP) reacted negatively and were culled on Day 1 of administration.

3.7 | In Vivo Evaluation of Pam_2Cys-NY-ESO-1 Peptide-Adjuvant Construct

In vivo evaluation of $Pam_2Cys\text{-}SK_4\text{-}NY\text{-}ESO\text{-}1_{157-165}$ **3** in healthy HLA-A2 transgenic mice demonstrated specific but weak

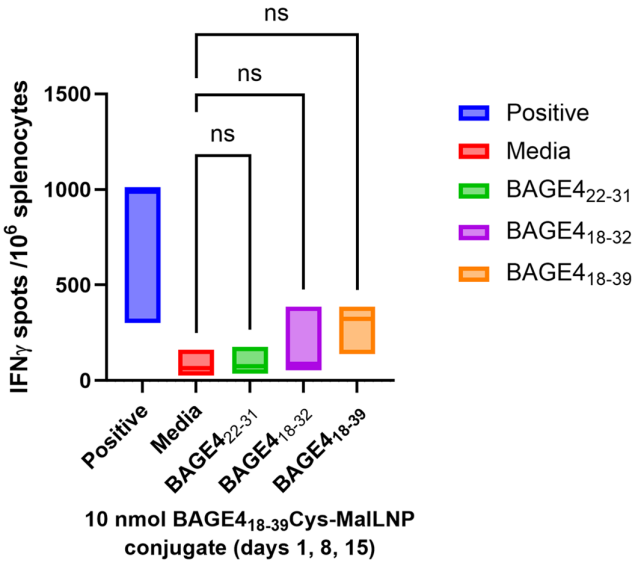


FIGURE 5 | Immune responses induced in BALB/c mice immunized with CysBAGE4-MalLNPconjugate. Isolated splenocytes from the immunised mice tested against three peptides from the $BAGE4_{18-39}$ peptide and measured in IFNγ ELISpot assay.

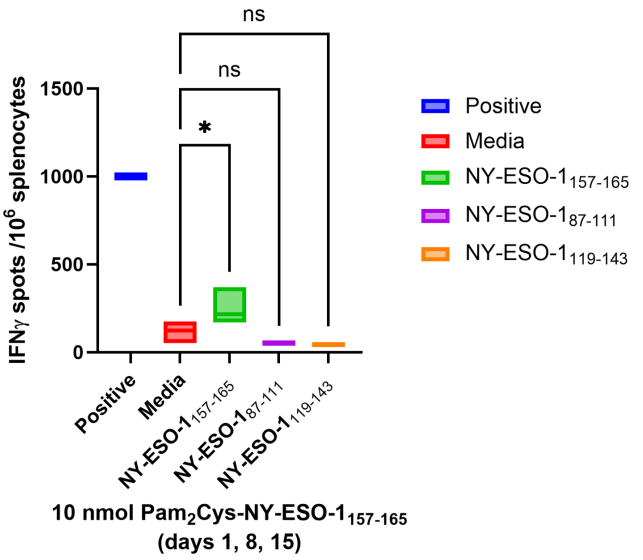


FIGURE 6 | Immune responses induced in HHDII/DR1 mice immunized with the $Pam_2Cys\text{-}SK_4\text{-}NY\text{-}ESO\text{-}1_{157\text{-}165}$ **3** vaccine candidate. Isolated splenocytes from the immunised mice tested against three peptides from the NY-ESO-1 protein, $NY\text{-}ESO\text{-}1_{157\text{-}165}$, $NY\text{-}ESO\text{-}1_{87\text{-}111}$ & $NY\text{-}ESO\text{-}1_{119\text{-}143}$, and measured in IFNγ ELISpot assay.

responses for **3** over the media-only negative control (Figure 6). A one-way ANOVA statistical analysis was carried out to assess the significance of the effect of $Pam_2Cys\text{-}SK_4\text{-}NY\text{-}ESO\text{-}1_{157\text{-}165}$ **3** conjugate on the immune response of mice and confirmed a significant effect for $NY\text{-}ESO\text{-}1_{157\text{-}165}$ ($p < 0229$, $q = 3.567$, $DF = 8$), while the data for both $NY\text{-}ESO\text{-}1_{87\text{-}111}$ ($p = 0.3280$, $q = 1.704$, $DF = 8$) and $NY\text{-}ESO\text{-}1_{119\text{-}143}$ ($p = 0.2293$, $q = 1.969$, $DF = 8$) were not significant.

Confirmation that $Pam_2Cys\text{-}SK_4\text{-}NY\text{-}ESO\text{-}1_{157\text{-}165}$ **3** is weakly immunogenic is a promising development towards the construction of a synthetic vaccine based on this antigen and adjuvant combination. However, further engineering of the vaccine is required to increase the potency of this candidate before proceeding to cancer models.

4 | Conclusion

Herein, we report the synthesis of peptide-adjuvant constructs $Pam_2Cys\text{-}BAGE4_{18-39}$ **1**, $Pam_2Cys\text{-}SK_4\text{-}BAGE4_{18-39}$ **2** and $Pam_2Cys\text{-}SK_4\text{-}NY\text{-}ESO\text{-}1_{157\text{-}165}$ **3** and demonstrate that $Pam_2Cys\text{-}SK_4\text{-}NY\text{-}ESO\text{-}1_{157\text{-}165}$ **3** shows activity as a self-adjuvanting vaccine candidate in the first in vivo studies of this scaffold. LNPs were formulated via self-assembly of constituent lipids, which included $Pam_2Cys\text{-}SK_4\text{-}BAGE4_{18-3}$ **2**, and via conjugation of $Cys\text{-}BAGE4_{18-39}$ to the outer envelope of LNPs containing a maleimide lipid (MalLipid **5**), and explored as a delivery tool, antigen display scaffold and adjuvant (**2**-LNP). The unexpected in vivo toxicity of both $Pam_2Cys\text{-}SK_4\text{-}BAGE4_{18-39}$ **2** and **2**-LNP is of interest, considering the components of these vaccines are non-toxic when administered separately. Immune-mediated toxicity is an unlikely factor due to the rapid onset of symptoms in these models. Whilst fibril formation was observed for $Pam_2Cys\text{-}SK_4\text{-}BAGE4_{18-39}$ **2**, $Pam_2Cys\text{-}SK_4\text{-}NY\text{-}ESO\text{-}1_{157\text{-}165}$ **3** also formed fibrils and was found to be non-toxic. Localization of the peptide is likely to be dramatically altered by palmitoylation, which may result in toxicity. However, further clarity regarding the activity of BAGE4 (a secreted peptide with evidence at transcriptional level only) would be required to draw any substantive conclusions. Due to the promising results obtained from the $Pam_2Cys\text{-}SK_4\text{-}NY\text{-}ESO\text{-}1_{157\text{-}165}$ **3** scaffold, future work will involve the integration of additional components into this vaccine (such as T-helper epitopes) to further enhance the potency of this self-adjuvanting vaccine construct.

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Conflicts of Interest

Lindy Durrant is the director of Scancell; Victoria Brentville and Peter Symonds are employees of Scancell.

Data Availability Statement

Data for this article including experimental details, compound and particle characterization data, in vitro and in vivo evaluation data are available in the ESI.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.