Influence of Physicochemical Properties of Lipopeptide Adjuvants on the Immune Response: A Rationale for Engineering a Potent Vaccine

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Physicochemical characteristics correlate with immunological responses of OVA-based lipopeptide self-adjuncting vaccine candidates. The vaccine candidate shown in the graphic contains a single OVA CD8 peptide epitope, two OVA CD4 peptide epitopes, and N-terminally positioned C16 lipids that provoke formation of long fibrillar, β -sheet particles. It also generated a robust cell-mediated immune response and DC activation.

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Biochemistry

adjuvant
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Adjuvant development and understanding the physicochemical properties of particles and interpreting the subsequent immunological responses is a challenge faced by many researchers in the vaccine field. We synthesized and investigated the physicochemical properties and immunogenicity of a library of multiple epitope self-adjuncting lipopeptides in a novel asymmetric arrangement. Vaccine candidates were synthesized using a combination of solid-phase peptide synthesis and copper-mediated click chemistry. In vivo studies showed that vaccine constructs containing a single OVA CD8⁺ T-cell epitope and two N-terminally located C16 lipid moieties were more effective at generating robust cellular immune responses compared to the same molecule containing multiple copies of the OVA CD8⁺ T-cell epitope with or without the C16 moieties. Furthermore, attachment of the two C16 lipids to the N-terminus provoked formation of long β -sheet fibrils and was shown to induce a higher CD8⁺ donor T-cell frequency and IFN- γ secretion, compared to vaccine constructs with an internal lipid placement. A regression analysis indicated that particle secondary structure had a significant impact on CD8⁺ donor T-cell frequency and cytolytic activity. In addition, IFN- γ

production was influenced significantly by particle shape. The findings of this research will impact the future design of a vaccine intended to elicit cellular immune responses.

Introduction

Adjuvant design and development are critical areas of research to improve the efficacy, safety, and specificity of vaccines.^[1] Induction of cell-mediated immune responses is a key requirement for the design and development of robust and effective vaccines against intracellular parasitic, bacterial, and viral pathogens.^[1b,^2] Amphiphilic lipopeptides that can form particles that display the antigens over their surface have been shown to function as both vaccine delivery vehicles and self-adjuvants.^[3] These biomaterials portray properties similar to virus-like particles and polymeric nanoparticles and protect peptide epitopes from degradation, prolonging antigen exposure to the immune system when compared to the peptide alone.^[4]

Modifying the number, length, position, and composition of the lipid and the orientation of the epitopes with respect to the lipid influences particle formation and allows them to suit a variety of applications.^[5] However, the impact of these factors and the relationship between the physicochemical properties of vaccine candidates and their effect on in vivo biological activity are largely unknown.^[6] Vaccine constructs with self-adjuvanting properties are advantageous to APCs by presenting unified antigen and lipid-based adjuvant(s) in a single construct simultaneously. This avoids the need for additional adjuvant(s) and their potential toxicity while improving the specified immune response.

Lipopeptide geometry has been reported to impact particle size, solubility and CD8⁺ T-cell responses.^[7] One of the most potent lipid moieties used in synthetic peptide vaccines is S-[2,3-bis(palmitoyloxy)propyl]cysteine (Pam2Cys). Pam2Cys was shown to have self-adjuvanting properties through its interaction with toll-like receptor (TLR) 2. Chua et al.[^] showed that lipopeptide vaccines against gonadotropin-releasing hormone had greater solubility when Pam2Cys was situated between the CD4⁺ T helper and the antigenic epitope(s) than when Pam2Cys was at the N-terminus.^[7b]

More recently, Skwarczynski et al. showed that inclusion of two C16 lipids internally between the promiscuous CD4⁺ T helper (P25, KLIPNASLIENCTKAEL) and Group A Streptococcus (GAS) J14 peptide epitopes (the conserved C-terminal GAS epitope) preserved the native α -helical conformation of the J14 epitope than structures that positioned lipids at the C-terminus.^[8] Furthermore, increasing the number of J14 peptide epitopes in a lipid core peptide (LCP) that contained three C12 lipids at the C-terminus preserved the native peptide conformation of J14.^[8] However, a systematic study to compare the impact of lipopeptide geometry within a library of vaccine constructs on the physicochemical properties and quality of CD8⁺ T-cell-mediated immune responses has not yet been reported. This study investigated synthesis and immunological evaluation of self-adjuvanting lipopeptide delivery systems that incorporate nonmicrobial lipoamino acids as a lipid adjuvant into a multiple antigenic peptide (MAP) system to display the ovalbumin (OVA)-derived peptide epitopes, OVA₃₂₃₋₃₃₉ (ISQAVHAAHAEINEAGR), recognized by antigen-specific CD4⁺ T cells in the context of MHC-II) and OVA₂₅₇₋₂₆₄ CD8 (SIINFEKL) recognized by antigen-specific CD8⁺ T cells in the context of MHC-I. We aimed to investigate the influence of lipid length and position, and the effect of increasing the number of OVA₂₅₇₋₂₆₄ CD8 epitopes, on the physicochemical properties of self-assembled lipopeptides. We also aimed to investigate whether these factors correlated with the ability of the vaccine constructs to induce cytolytic T-cell activity, level of donor T-cell frequency, tumor growth inhibition (TGI), and immunogenicity by cytokine secretion. To this end, we also evaluated the capacity of the vaccine constructs to induce activation of dendritic cell (DC) subsets to inform initiation of CD8⁺ T-cell responses. The peptide representing the OVA₂₅₇₋₂₆₄ CD8 epitope has been well characterized for its ability to stimulate cytotoxic T-cell activity via recognition in the context of MHC-I by C57BL/6 mice. The OVA₃₂₃₋₃₃₉ CD4 epitope, which is recognized by C57BL/6 mice in the context of MHC-II is also well known for its contribution to the development of effector CD8⁺ T cells.^[7a]

Results and Discussion

Vaccine candidates were prepared from a library of 1) azido-functionalized lipopeptide building blocks that contained peptides representing two OVA CD4⁺ T-cell

epitopes (Figures, Schemes, Sections 1--4, Supporting Information) and 2) alkyne-functionalized peptide building blocks that contained one (**5**) or four (**5'**) copies of the OVA CD8⁺ T-cell epitope (Figure 1A and Supporting Information, Sections 1--6). The building blocks were prepared according to standard solid-phase peptide synthesis (SPPS) techniques by using 9-fluorenylmethyl carbamate (Fmoc) coupling chemistry. The C12 or C16 lipoamino acids were incorporated at the N- or C-terminus of the OVA CD4 building blocks as depicted in Sections 1--2 and 3--4, Supporting Information, respectively (Figure 1A). Peptide building blocks were released from the resin by treatment with trifluoroacetic acid (TFA, 95%), and purified and characterized by reverse-phase HPLC (RP-HPLC) and electrospray ionization mass spectrometry (ESI-MS) with yields of 19--49% and greater than 95% purity.^[9] Conjugation of azido-functionalized lipopeptide building blocks (**1--4**) to alkyne-functionalized peptide building blocks (**5** or **5'**) was achieved using a copper(I)-catalyzed Huisgen cycloaddition reaction to give multivalent synthetic vaccine candidates (**6--9** and **6'-9'**, Figures 1A and B, Experimental Section) with 33--49% yields and greater than 95% purity (Supporting Information, Figures S1 and S2). All constructs **6--9** contained a

CD8⁺ T cell epitope monomer and **6'--9'** contained a tetrameric CD8⁺ T-cell epitope antigen.

Constructs **CTRL-1** and **2** were prepared as experimental controls (Supporting Information, Figure S3). Detailed synthesis and characterization of constructs **1--9** have previously been reported by the authors.^[9]

Self-assembly of vaccine constructs **6--9** and **6'--9'** in PBS was investigated using TEM and far-ultraviolet CD spectroscopy. Results showed that increasing the lipid alkyl chain from 12 to 16 carbon atoms had a significant impact on the particle shape and secondary structure of the vaccine constructs (Figures 2A,B, and Table S1, Supporting Information). For example, construct **6** self-assembled into spherical particles (110±50 nm) with a random coiled secondary structure, while construct **7** formed long fibrils (15±2.3 nm in diameter) with a β -sheet secondary structure. However, variation in lipid length when

multiple OVA CD8 epitopes were present (6' and 7') did not alter their particle shape or secondary structure. This is also attributed to the higher strength of the hydrogen-bond interactions between CD8 dendrimers compared to the strength of hydrophobic interactions among lipid moieties (Figure² A).

A comparison between the topological features of lipopeptide constructs that contained one (7) or four (7') OVA CD8 peptide epitopes by using AFM showed an improved packing between particles, resulting in the dense outer shell of the assembled lipopeptides in 7' (Figure² B). This is consistent with the report by Cavallo and Fraternali on poly(propylenimine) dendrimers.^[10]

Changing the position of the lipid moiety from the N-terminus (6--7 and 6'--7') to an internal position (8--9 and 8'--9') induced self-assembly into a mixture of both spherical and short fibril particles with helical secondary structures (Figures² A and C, and Table^{S1}, Supporting Information). The length of these particles ranged from 0.3--0.9 μm . Variation in the morphology of these particles compared to lipids placed at the N-terminus can be attributed to the weak interactions between the corona of the spherical aggregates as observed by Yu et al..^[11]

Furthermore, increasing the length of the lipid in this position from C12 (8 and 8') to C16 (9 and 9') induced the formation of larger spherical (10--90 nm) and cylindrical particles (0.8--0.9 μm).^[12] Constructs 8, 8', and 9 formed α -helical conformations (Figure² C). Notably, construct 9' showed a CD graph with more tendency to a β -sheet conformation, which was attributed to improved lateral association of cylindrical particles and produced elongated cylinders (Figure² A, C, and Table^{S1}, Supporting Information).

Al-Jamal et al., have also observed this in their research on lipophilic dendrimers.^[12] The CD spectrum of 9' in Figure² C showed a signal at 215 nm indicating that vaccine 9' has tendency to fold as a β -sheet structure. In this instance, an extra signal close to 200 nm was also observed that could be associated with a weak association of spherical particles as depicted in Figure² A. We have previously reported that OVA CD8

has a random coil secondary structure^[15] and the reported structure for the OVA CD4 peptide antigen forms an alpha helix.^[13]

CD analysis of vaccine constructs showed that by increasing the number of OVA CD8 epitopes within a single construct (e.g. construct **6** compared with construct **6'**), altered the secondary structure from random coiled to beta sheet. Additionally, the position of the lipid moieties was also shown to impact the level of interaction among these CD4 and CD8 epitopes. Consequently, this study supports previous research in which the formation of an alpha helix and or β -sheets in vaccine constructs is unlikely to be influenced by peptide epitopes alone, and that the lipids are also likely to play a key role. Hence, it is important to review the impact of peptide design and self-assembly conditions on the final peptide (or lipopeptide) structure and their biological efficacy.^[14]

Overall, construct geometries have a critical contribution to particle distribution in the body and the potential to generate and influence immunological responses.^[14a,^15]

Additionally, activation of CD8⁺ T cells was shown to be particle shape-dependent in a study using artificial antigen presenting cells with Sunshine and co-workers.^[16] In this respect, spherical particles 20--200^{^^}nm in diameter mimicked viruses by having prolonged circulation times and T-cell activation, whereas fibril particles activated cytotoxic T-cell responses and expanded T cells ex vivo more efficiently than the spherical particles.^[15a,^16]

The objective of an effective CD8⁺ T-cell vaccine is to generate a memory T-cell population. The size and function of the effector CD8⁺ T-cell pool generated after vaccination is taken to be a good indicator of the potential memory response.^[17] To test the effect of changing the lipid position and number of CD8⁺ T-cell epitopes on the adjuvanting properties of the vaccine candidates (**6--9** and **6'--9'**), we utilized an adoptive transfer model to assess the expansion of TCR transgenic T cells engineered to recognize the CD8 epitope OVA₂₅₇₋₂₆₄ CD8 (SIINFEKL) and the CD4 epitope OVA₃₂₃₋₃₃₉ (ISQAVHAAHAEINEAGR). C57BL/6 mice were adoptively transferred with naïve OT-I CD8⁺ T cells and OT-II T cells MACS purified from the spleens and lymph nodes of OT-I mice and OT-II mice crossed with congenic PTP^{Prca} mice expressing the congenic CD45.1 allele to allow easy tracking of the adoptively transferred cells. Immunization and an experiment timeline plan are depicted in

Figure 3A. Constructs with lipids present at the N-terminus (6--7 and 6'--7') induced a strong increase of donor CD8⁺ OT-I T cells when compared to the PBS group ($p < 0.001$, Figure 2B). Additionally, construct 7' induced a variable effect on OT-I and OT-II frequency. Furthermore, constructs 8' and 9' with internally placed lipids but a tetrameric CD8⁺ T-cell epitope (Figure 1B) induced significant increases in the frequency of OT-I and OT-II transferred cells when compared to the PBS (Figure 3B). In contrast, constructs 8 and 9, which contain internally placed lipids, and CTRL-1 (no lipids) showed no significant increase of either OT-I or OT-II T-cell frequency when compared to the PBS group. Analysis of the kinetics of CD8⁺ OT-I transferred cells frequency over the course of the experiment showed significantly higher OT-I T-cell frequency following the second boost by construct 7 when compared to the prime and first boost immunization ($p < 0.05$, Figure 3C). This was not significantly different to the positive control (OVA peptides+Cholera toxin, CT) indicating a robust effector response was induced by construct 7, which was consistent with a previous report for nanofibers of OVA CD8.^[18] Post-boost, changes in the level of donor CD4⁺ and CD8⁺ cells for all other groups was not significant when compared with the initial vaccination (Figure S7, Supporting Information).

A limited frequency of CD4⁺ OT-II T cells compared to an extensive level of CD8⁺ OT-I T cells was observed (Figure 3B) that may be attributed to the responsive roles of CD4⁺ and CD8⁺ T cells as regulators and effector cells, respectively, in the development of an effective adoptive immune response as previously reported.^[19]

The vaccine construct's ability to induce cytolytic T lymphocyte (CTL) responses (effector function of activated OT-I cells) was evaluated using an in vivo CTL assay. On day 51 (Figure 3A), specific lysis was evaluated by flow cytometry and showed that vaccine candidates that contained N-terminal C12 or C16 lipids (6--7 and 6'--7'), or four copies of the CD8 peptide with an internal C16 lipid (9') all induced very robust cytolytic activity (73--96%) with no significant difference between the constructs and the positive control (Figure 3D). Previously, Baz et al., showed lipopeptide geometries of constructs that contained a single OVA CD4 and OVA CD8 epitope

and an internally positioned lipidic moiety (Pam2Cys) induced stronger and sustained CTL activity than N-terminal placement. However, the particulate nature of those constructs was not studied.^[7a] In contrast, Tsunoda et al., reported that the solubility of an OVA-conjugated influenza lipopeptide vaccine was influenced by the length of the lipid chain and this was associated with the robustness of the CTL response.^[6c]

Our findings for the constructs that contained longer lipids (7, 7', and 9') are consistent with those results. In our study, we investigated whether there was a correlation between the physicochemical properties (including particle diameter, particle shape, and secondary

structure as detailed above) of vaccine constructs 6--9 and 6'--9' and their immune responses (CTL, CD8⁺ donor T-cell frequency, and TGI, Figure³ E).

Construct 6 which has a spherical shape and a random coiled secondary structure, and constructs 7, 6', and 7' which formed long β -sheet fibrils showed higher levels of CD8⁺ transferred cells frequency and effector function when compared to 8--9, and 8' groups (Figure² E). Furthermore, construct 7 (long β -sheet fibrils) stood out amongst all groups taking into account all immune response readouts. Moreover, at the second boost, CD8⁺ transferred cells frequency improved (13.5 to 25.4%, Figure³ C) and CD8 cytolytic activity and TGI increased (96.2 and 99.1%, respectively, Figure³ E).

A regression analysis statistical model (D-optimal design) was used to analyze the correlation between these physicochemical variables and the immune responses. Particle secondary structure was found to have a significant effect on CD8⁺ transferred cells frequency and cytolytic activity, with β -sheet conformation ($p < 0.05$, Tables^{S2} and S3, Supporting Information) having the highest influence. The impact of β -sheet secondary structure on cytolytic activity could be attributed to a sustained antigen release from β -sheet particles resulting in improved CTL priming by antigen presenting cells (APCs), as noted by Maji et al.^[20] Furthermore, the strong CTL response observed for 6 (Figure³ E) can be attributed to the size of its nanoparticles (<200 nm), as previously described by Fifth et al.,^[21] who showed that their antitumor vaccines had size-

dependent immunogenicity. More recently, our group showed previously that OVA CD8 lipopeptide vaccines with spherical particles of 50--70^{nm} diameter size elicited a strong CTL effect in a similar system.^[22]

We next tested the ability of the vaccine constructs to inhibit tumor growth in vivo.^[23] At 14^{days} after the last of three vaccinations (Figure³ A), mice were challenged by subcutaneous (s.c.) injection with B16 melanoma cells that express OVA₂₅₇₋₂₆₄ (which contains the CD8 epitope). At 21^{days} post challenge, mice were sacrificed, tumors excised and weighed. Mice vaccinated with PBS (negative control) presented with a large tumor growth (Figure³ F). In contrast, vaccination with constructs **6--7** and **6'--9'** induced strong tumor growth inhibition ($99.1 \pm 0.11\%$). Groups that showed protection from tumor growth contained lipids at the N-terminus (constructs **6** and **7**) and/or multiple copies of the OVA CD8 epitope (constructs **6'--9'**). Tumor growth was inhibited in mice treated with **CTRL-2** (containing multiple OVA CD8 epitopes but no lipid), which confirmed the significance of OVA CD8 multiplicity in TGI.^[24] Our study showed the important role lipids play in TGI when a single OVA CD8 epitope is present in the constructs while a low TGI percentage for **CTRL-1** was observed. In this regard, particle shape, the number of OVA CD8 peptide epitopes and their display was crucial for a strong tumor growth inhibition.^[25]

Tumor growth inhibition effects of the vaccine constructs were consistent with their impact on early expansion of CD4⁺ and CD8⁺ T cells. Moreover, **6--7** and **6'--9'** vaccines consistently showed Th2-biased cytokine specially increased IL-6 response (Figure⁴ B and Figure^{S8}, Supporting Information). There are controversial studies on the necessity of CD4⁺ T-cell epitopes for the development of antitumor immune responses, although various parameters such as particle size and particle shape have been implicated in contributing to the antitumor effect.^[26] The data in Figure⁴ B were generated from pooled supernatant from 5^{wells} and the data in Figures⁴ A,B represents three experimental replicates from one experiment, hence statistical analysis was not performed.

To confirm the significance of β -sheet conformation in the induction of cell-mediated immunity and the influence of physicochemical properties on cytokines that polarize the

Th1/Th2 responses, we next assessed splenocytes of vaccinated mice for their ability to secrete interferon (IFN)- γ using an IFN- γ ELISpot assay, and Th1/Th2 cytokine polarization using a cytometric bead array (Figure⁴ A,B). On day 77, splenocytes were restimulated with OVA CD4, OVA CD8, or OVA CD4+OVA CD8 peptides in vitro (Figures⁴ A, B and S3, Supporting Information). Cytokines essential for the maturation of APCs to achieve a biased Th1 response include IFN- γ , interleukin (IL)-2 and tumor necrosis factor (TNF)- α and production of a Th2-biased response stimulates B cell proliferation and antibody production, include IL-4, IL-5, IL-6, IL-10, and IL-13.^[27]

This study showed that the number of antigen-specific effector cells that secreted IFN- γ after stimulation with constructs 7 and 7' was significantly higher than the negative control (Figure⁴ A). Furthermore, both these constructs increased the secretion of IFN- γ , IL-2 and TNF- α after restimulation with both OVA CD4 and CD8 peptides when compared with the PBS control group (Figure⁴ B). Interestingly, constructs 8--9 presented with the lowest Th1-linked cytokine production. A regression analysis model (D-optimal design) investigated the correlation between the physicochemical parameters of the vaccine constructs and the number of cells that secreting IFN- γ in response (Figure⁴ C).

It was shown that particle shape had a significant effect on IFN- γ response with the highest influence from long fibril constructs ($p < 0.05$, Tables²S2 and S3, Supporting Information). This is consistent with the report by Rudra and co-workers for peptide constructs comprised of the OVA CD8 epitope that presented with fibril morphologies. Those constructs stimulated robust primary and recall CD8 responses, in addition to increased IFN- γ production.^[18] Interestingly, in our study, the same relationship trend for IL-2 and TNF- α cytokine secretion and particle shape was observed (Figure⁴ B). Furthermore, restimulating of splenocytes from the vaccinated mice with the OVA CD4 peptide induced a greater effect on the generation of IFN- γ than restimulation with the OVA CD8 peptide (Figure⁸S8, Supporting Information). This might be attributed to the intervention of CD4⁺ T helper cells and generation of IFN- γ cytokine observed by Janssen et al.^[28] In fact, CD4⁺ T helper cells are known to release IFN- γ activating APCs, contributing to increased antigen presentation to CTLs.^[29]

Constructs **6'--7'** and **8'--9'** which contained four copies of the OVA CD8 antigen, elicited higher secretion of IL-5, IL-6, IL-10, and IL-13 cytokines compared with constructs **6--7** and **8--9** following restimulation with OVA CD4 or the pooled peptides (Figures⁴ B and S3, Supporting Information). In relation to this, the increased packing density of the particles that contained multiple copies of the OVA CD8 peptide epitope (**6'--9'**) was shown to have induced Th2 cytokine activation, regardless of lipid length or placement. Furthermore, lipopeptide fibrils (**7**) that contained a lower packing density of particles induced production of cytokines associated with both Th1 and Th2 activation (Figure⁴ B). This difference in response could possibly be attributed to the peptide-MHC binding and its recognition by T-cell receptors as noted by Fahmy et al.^[30]

We next assessed the relative abilities of the vaccine constructs to generate antibody responses, for which the total IgG of vaccinated mice was analyzed on day⁵⁰ by enzyme-linked immunosorbent assay (ELISA) against the native OVA protein (Figure⁴ D). Constructs **6**, **7**, **6'--9'**, and **CTRL-2** induced high antibody responses with a significant difference observed when compared with the PBS group. In this respect, the small particle size of construct **6**, or multivalence of the CD8 epitopes in constructs **6'--9'** and **CTRL-2** for which a repetitive display of epitopes was shown to enhance antibody responses. Lipopeptides have been shown to induce antibody responses.^[31]

In contrast, our results suggest that lipids play no role in the antibody response observed when four OVA CD8 epitopes were present in the vaccine constructs (**6'--9'** and **CTRL-2**). Rather, constructs with N-terminal lipids (**6--7**) or multiple CD8 epitopes (**6'--9'**, **CTRL-2**) were found to produce high amounts of IL-2 and IL-6 and induced high TGI and antibody responses (Figures³ F and 4 B, D). It has been shown that IL-2 plays a central role in priming Th2 responses, B lymphocyte activation, and IL-4 production which are required for antibody responses. In addition, IL-2 has significant role in Th1 activation and TGI effects.^[32] Mechanistically, vaccines that initiate CD8⁺ T-cell responses are likely to act via dendritic cells (DCs).^[33] Our results suggest that the studied lipopeptides induced immunogenicity through a DC activation pathway, and several of the lipid moieties utilized are known to act via TLR2 and TLR4^[34] which are also known to be expressed on DCs and on the DC2.4 cell line.^[35] Injected vaccines are taken up directly by DCs with an adjuvant

included in the vaccine construct to induce DC activation.^[36] Thereafter, DCs process the antigen and present it to CD8⁺ T cells in complex with MHC-I molecules (classically known as signal¹).^[37] DCs must also express the correct co-stimulatory molecules (signal²) to achieve appropriate T-cell activation and proliferation. To better understand the activity mechanism of our vaccine constructs, we asked whether the constructs could directly and differentially affect DC activation. Accordingly, vaccine constructs were incubated overnight in vitro with DC2.4 cells, after which the expression levels of activation and co-receptor molecules were measured by flow cytometry (Figure⁵ ^A).^[38]

Construct 7 and the positive control (OVA peptides + CT) displayed a strong ability to induce upregulation of important co-receptor molecules including, CD252, CD86, CD80, CCR7, CD40, and CD44. We next tested the ability of DCs exposed to the vaccine constructs to directly activate CD8⁺ T cells. Accordingly, vaccine constructs were incubated overnight in vitro with DC2.4 cells, after which the expression levels of activation and co-receptor molecules were measured by flow cytometry (Figure⁵ ^A).^[38] Construct 7 and the positive control (OVA peptides + CT) displayed a strong ability to induce upregulation of important co-receptor molecules including, CD252, CD86, CD80, CCR7, CD40, and CD44.

We next tested the ability of DCs exposed to the vaccine constructs to directly activate CD8⁺ T cells. In this experiment, DC2.4 cells were incubated overnight with each vaccine construct, and then washed and cultured for three days in the presence of OT-I CD8⁺ T cells, after which the live OT-I cells were enumerated (Figure⁵ ^B). We noticed a striking correlation between the number of OT-I cells recovered from the in vitro DC assay and the frequency of cells observed in the peripheral blood following the third vaccination ($p=0.043$, Figure⁵ ^C). Taken together, these data suggested that when given as vaccines these constructs exerted their task by inducing activation of DCs and may induce antigen-specific CD8⁺ T-cell expansion by acting via DCs; however, we cannot rule out the possibility that other antigen-presenting cell types, such as macrophages also participate in this process.^[39]

Conclusions

In this study, the impact of structural arrangement in lipopeptide vaccine constructs containing varying lengths and placement of lipids as well as a differing number of CD8⁺ T-cell epitopes was investigated for impact on induction of potent cell-mediated immune response. Results showed that robust CD8⁺ donor T-cell induction, cytolytic activity, and IFN- γ secretion were highly correlated with the secondary structure and shape of the lipopeptide adjuvant, rather than the number of CD8 peptide epitopes and particle diameter size; this was confirmed using a regression analysis model. We showed that the CD8⁺ T-cell response was most impacted by a structure of β -sheet long fibril particles. Our results suggest that the studied lipopeptides induced immunogenicity via a DC activation pathway. Interestingly, vaccine construct 7 (which contained two OVA CD4⁺ T-cell epitopes and two N-terminally positioned C16 lipidic moieties) provoked formation of long β -sheet fibrils and presented the highest CD8⁺ T-cell mediated immunity and DC activation responses. Further studies to investigate the interaction of the vaccine constructs and DC membrane and its intracellular processing, peptide-MHC binding and T-cell interaction are required to inform on the vaccine's molecular functionality.^[16,^40] Also, our studies included the use of adoptively transferred antigen-specific T cells to monitor vaccine efficiency, but future studies could be performed without cell transfer and utilize tetramer identification of responding cells to eliminate any possible aberrations induced by higher than normal antigen-specific precursor T-cell numbers. This would also allow the utilization of different antigens, such as the Influenza NP₃₆₆ peptide.

Overall, our studies establish the relation between size, shape, and secondary structure of vaccine constructs and T-cell activation by engineering novel lipopeptide vaccine candidates. This is the first systematic investigation of lipopeptide vaccines that contain multiple peptide epitopes designed to induce CD8⁺ T-cell responses that correlate the physicochemical properties of the vaccine constructs with immune response. The correlation between epitope number and arrangement in lipopeptide vaccine design identified herein would have important implications in the design and development of vaccines to treat prevalent diseases.

Experimental Section

Synthesis of vaccine constructs 6'--9'

Constructs 6--9 and 6'--9' were synthesized by dissolving OVA CD4 lipopeptide azides (1--4, 0.35^{mmol}, 1^{equiv}) in separate vials containing a solution of OVA CD8 peptide-alkyne (5, 0.7^{mmol}, 2^{equiv} or 5', 0.53^{mmol}, 1.5^{equiv}) in DMSO:DMF (1:1, 1^{mL}; for synthetic protocols and characterization of building blocks 1--5 and 5' see the Supporting Information, Sections¹⁻⁻⁵). Copper wires (60^{mg}) were added and the solution was protected from light with aluminum foil and stirred under an atmosphere of nitrogen at 50^{°C}. The reaction was monitored by analytical HPLC (Shimadzu) on a reverse-phase (RP) C4 column (Vydac, Grace) with a gradient of 45--50% acetonitrile (90%) in water containing 0.1% TFA (solvent^B) over 40^{min}. Following reaction completion, copper wires were removed by filtration and washed with DMSO (1^{mL}). Samples were diluted with Millipore endotoxin-free water (50^{mL}), lyophilized, and crude products were purified using semi-preparative RP-HPLC on a C4 column (Grace Vydac) using a gradient of 45--50% solvent^B over 60^{min}. Vaccine constructs 6--9 and 6'--9' molecular weights were verified by EIMS. Materials greater than 95% purity were lyophilized and stored below <M>20^{°C} until use.

Characterization of vaccine constructs 6'--9'

Constructs were dissolved in room temperature HCl (0.01^M, pH²) until completely solubilized. The pH was then increased to 7.4 by using PBS (20^{mM}) in Milli-Q water. Particles were allowed to equilibrate in an incubator at 37^{°C} for at least 12^h and were then analyzed within 24^h.

TEM was performed on a JEM-1010 TEM (JEOL Ltd., Japan) operated at 100^{kV}. Images were taken and analyzed using AnalySIS software (Soft Imaging Systems, Megaview III, Munster, Germany). The samples were dropcast onto the 200-mesh carbon-coated grids for 2^{min}. Excess liquid was wicked away using filter paper. Dried samples were imaged within 24^h. For AFM, constructs were self-assembled in PBS (50^{μL}, 20^{Mm}) and placed on freshly cleaved mica and allowed to dry overnight. The mica was washed once with Milli-Q water and excess solution was wicked away before the mica was dried under a gentle stream of nitrogen. Samples were analyzed immediately with a Nanoscope IIIa controller and "E" scanner in tapping mode. Olympus high aspect ratio, etched silicon probes with a spring constant of 20--80^{Nm^{<M>1}} were used.

CD spectroscopy was measured on a JASCO (Tokyo, Japan) J-710 spectropolarimeter using a quartz cuvette of 1^{mm} path length at 23^{°C}.

In-vivo immune responses

Mice: Female C57BL/6 (CD45.2⁺) and B6.SJL/J-Ptprc mice (4--6^{weeks} old) were purchased from the Animal Resources Centre (Perth, Australia). OT-I (243.2) and OT-II (H425.2) TCR-transgenic mice were bred at the QIMR Berghofer Institute of Medical Research (Brisbane, Australia) animal facility. All mice experiments were approved by the QIMR Berghofer Animal Ethics Committee and were conducted in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (2004).

T-cell preparation and adoptive transfer

OT-I and OT-II cells were isolated and purified from lymph nodes and spleens of OT-I and OT-II mice and used as a source of CD8⁺ and CD4⁺ T cells, respectively. Single suspensions of the T cells in Dulbecco's PBS with 2% fetal calf serum (FCS) were isolated by passing the tissues through a cell sieve (100^{μm}, BD Falcon) followed by a Ficoll-Paque density gradient (Amersham Bioscience, Australia) to isolate lymphocytes. T cells were enriched by positive selection using a MACS column CD8a (Ly-s) or CD4⁺ (L3T4) microbeads (Miltenyi Biotec, North Ryde, Australia) diluted in cold MACS buffer (1×PBS, 0.5% FCS, 2^{mM} EDTA). Cell purity was determined using flow cytometry (LSRFortessa flow cytometer) and was determined to be >98% for OT-I (CD8+V α 2+V β 5+) and >82% for OT-II (CD4+V α 2+V β 5+) cells (Supporting Information, Section⁸). C57BL/6 mice received 0.5×10⁶ OT-I and 0.5×10⁶ OT-II cells by tail-vein injection 2^h prior to initial immunization.

Immunization, OT-I and OT-II transferred cells frequency study

This experiment was designed to measure the frequency of the OT-I and OT-II transferred cells to the CD8⁺ and CD4⁺ T-cell population, respectively. For this, adoptively transferred C57BL/6 mice were immunized with vaccine constructs (6--9, 6'--9', 30^{μg}, 100^{μL}/mice), CTRL-1 and 2, OVA peptides+cholera toxin (CT) (30^{μg} OVA CD8+30^{μg} OVA CD4+1^{μg} CT, 100^{μL}/mice), or PBS alone (100^{μL}/mice) by subcutaneous injection (SC) at the base of the tail on days⁰, 21, and 42. Eight days post each immunization, blood samples were collected and diluted in MAX buffer to

prevent clotting. RBCs were lysed using lysis buffer (0.168^M NH₄Cl). Cells were stained using pooled antibodies (Supporting Information, Section⁸) and analyzed on a LSRFortessa flow cytometer. Data were presented as the proportion of transferred cells in the total CD8⁺ or CD4⁺ population.

In vivo CTL assay

To assess the cytotoxic capacity of the OT-I cells activated during the vaccination study, on day⁵⁰ splenocytes from naïve C57BL/6 mice were isolated and purified using a Ficoll-Paque density gradient method as described above. Cells were equally divided (4.75 × 10⁶ cells in each). One population was incubated with the OVA CD8 peptide (10^{−6} μg/mL) diluted in Dubecco's-PBS 2% FCS media, and the other incubated in media alone. Cells were incubated for 1 h at 37°C and 5% CO₂. Groups were stained with a high (5 μM) or low (2.5 μM) concentration of VPD 450 (Invitrogen, CA) as pulsed or unpulsed splenocytes, respectively. The two groups were mixed using an equal number of cells, and administered to the mice through tail vein injection (9.5 × 10⁶ cells/mice). Blood was collected 17 h post injection and specific cell lysis was determined using flow cytometry. Cells were stained with pooled antibodies (Supporting Information, Section⁸) and analyzed on the Fortessa flow cytometer. CTL analysis was performed based on the cells' VPD level and analyzed using Equation⁽¹⁾. The other stains also allowed the analysis of the OT-I and OT-II cells at that time.

$$\text{Specific}^{\text{cell}}\text{lysis}^{\%} = \left[\left(1 - \frac{\frac{\% \text{VPD}_{\text{low}}}{\% \text{VPD}_{\text{high}}} \text{ in naive mice}}{\frac{\% \text{VPD}_{\text{low}}}{\% \text{VPD}_{\text{high}}} \text{ in immunized mice}} \right) \right] \times 100 \quad (1)$$

Tumor protection

Mice were inoculated SC on the lower abdomen with B16 melanoma cells expressing OVA (106 cells, ATCC) on day 56. Mice were sacrificed on day 77 and tumors were excised and weighed. Tumor growth inhibition (TGI) % was calculated using Equation⁽²⁾:

$$\text{TGI}^{\%} = \left(1 - \frac{\text{Tumor}^{\text{weight}}^{\text{test}}}{\text{Tumor}^{\text{weight}}^{\text{control}}} \right) \times 100 \quad (2)$$

IFN-γ enzyme-linked immunosorbent spot-forming cell (ELISpot) assay

To enumerate IFN-γ secreting cells, on day⁷⁷ vaccinated mice were sacrificed and their splenocytes isolated. Briefly, MSIPS4510 MultiScreen HTS IP 96 plates (Millipore, Ireland) were coated with

anti-mouse IFN- γ ($10^{\mu\text{g mL}^{-1}}$, clone R4-6A2, BD Pharmingen, USA) diluted in PBS. Wells were washed and then blocked with full media (modified DMEM containing $50^{\mu\text{M}}$ 2-mercaptoethanol, $216^{\text{mg L}^{-1}}$ L-glutamine, and 10% heat-inactivated FCS) for 3 h at 37°C . Splenocytes from immunized mice (0.5×10^6 cells/well) were stimulated with peptide antigens ($10^{\mu\text{g mL}^{-1}}$, OVA CD4, OVA CD8, or OVA CD4+OVA CD8) at 37°C in an atmosphere of 5% CO_2 . Concanavalin A (ConA, $5^{\mu\text{g mL}^{-1}}$) or complete media were used as positive and negative controls, respectively. After 30 h incubation, plates were washed and then incubated with biotinylated anti-mouse IFN- γ ($4^{\mu\text{g mL}^{-1}}$ (BD 551216) diluted in PBS containing 0.5% bovine serum albumin (Sigma A2153) for 3 h at 37°C . Plates were washed and then incubated with streptavidin-HRP (BD557630, BD, Australia) at $1^{\mu\text{g mL}^{-1}}$ diluted in PBS, for 1 h at room temperature, then washed and developed using please clarify steps the AEC substrate set (BD Pharmington, Australia) according to the manufacturer's instructions. The number of IFN- γ secreting cells was determined using an automated ELISpot AID iSpot reader (Autoimmun Diagnostika GmbH, Strassberg, Germany). Results were subtracted from media-only (background) wells. All plate washes were done using PBS with 0.05% Tween 20 (PBS-T).

Cytometric bead array assay

Cytokine secretion was measured from the stimulated splenocytes in vaccinated mice. On day 77, mice were sacrificed and their splenocytes (1×10^6 cells/well) were cultured with peptide antigens (OVA CD4, OVA CD8 or OVA CD4+OVA CD8, $10^{\mu\text{g mL}^{-1}}$ of each) in full media in 96-wells flat-bottomed plates (Corning Incorporated, Corning, NY, USA). Phorbol 12-myristate 13-acetate (PMA): Ionomycin ($5:500^{\text{ng mL}^{-1}}$) or full media alone was used as positive and negative controls, respectively. After 72 h, cultured supernatants from individual mice were collected and pooled for each group of mice and cytokines (IL-2, IL-4, IL-5, IL-6, IL-10, IL-13, IFN- γ and TNF- α , BD Bioscience, San Jose, USA) were analyzed using Cytometric Beads Array flex kits (BD Biosciences, San Jose, USA) according to the manufacturer's instructions. Analysis was performed on a FACSarray cytometer equipped with CellQuest Pro and CBA software (BD Bioscience, San Jose, USA).

In-vitro analysis

DC2.4 assay: DC2.4 cells (a gift of Dr Kenneth L. Rock, University of Massachusetts Medical School and Dana-Farber Cancer Institute, USA) were cultured in 96-well flat bottom plates overnight with a mid-log titration (over 2.5 logs) of the vaccine constructs starting at $30 \mu\text{g mL}^{-1}$ in media and controls included CT, OVA CD8, and OVA CD4 peptides (starting at $1 \mu\text{g}$ and $20 \mu\text{g mL}^{-1}$, respectively) and full media only. DC2.4 cells were grown in full media. The cells were then harvested with trypsin, transferred to 96-well V-bottom plates, washed and stained on ice for 15 min with FC-blocking antibody (2.4G2 supernatant) followed by a panel of fluorochrome-conjugated antibodies to anti-mouse CD252-AF 647 (Biolegend 108809), anti-CD70- PE (BD 555286), anti-H-2Kb MHC I-FITC (Biolegend 116506), anti-CD86-AF700 (Biolegend 105024), anti-CD197 (CCR7)- PE/Cy5 (Biolegend 120114), Rat Anti-Mouse I-A/I-E -V500 (BD 562366).

The mix was incubated on ice for 10 min, washed, and re-suspended in MACS buffer with Sytox blue or Pi (1/1000, ThermoFisher, S34857) for dead cell exclusion.^[33] The samples were run on an LSRFortessa flow cytometer and analyzed using FlowJo (Treestar Inc). Data was excluded when DC2.4 viability was below 50%. Data shown represents cells taken from the highest concentration of the titration in which cell viability was >50%.

Frequency of OT-I cells

DC2.4 cells were cultured overnight (as described above), harvested, washed, and cultured for 3 days with naïve OT-I CD8⁺ T cells. The OT-I cells frequency was measured after 3 days by staining with antibodies expressed on all OT-I cells but not DC2.4 cells (anti-CD8-APC-Cy7 (BioLegend, 100712), anti-mouse V α 2-APC (BioLegend 127810). Stained cells were analyzed on a LSRFortessa flow cytometer using a HTS plate loading system. Dead cells were excluded by (Pi) uptake. The number of live cells acquired within a set sample volume was reported.

Statistical analysis

Assessment of statistical significance was performed using a Prism 6.00 software package (GraphPad, San Diego, USA). Regression analysis was performed using Stat-ease, D-Optimal design with significant level $p < 0.05$ (Design-Expert 9, Minneapolis, USA).

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Conflict of interest

The authors declare no conflict of interest.

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Figure¹ Synthesis and physicochemical examination of lipopeptide vaccine constructs.

A) Synthetic strategy for the preparation of vaccine constructs **6--9'** using the copper-catalyzed azide--alkyne cycloaddition reaction. Azide-functionalized building blocks (**1--4**) are conjugated with alkyne-functionalized building block **5'** generating vaccine constructs **6--9'**, respectively. B) Vaccine construct library. Synthesis of vaccine constructs **6--9** were reported.^[9] Constructs **6**, **6'**, **8**, and **8'** contain C12 ($n=9$) lipoamino acids, and constructs **7**, **7'**, **9**, and **9'** contain C16 ($n=13$) lipoamino acids. OVA323-339 CD4 is ISQAVHAAHAEINEAGR and OVA257-264 CD8 is SIINFEBL.

Figure² A) TEM images of **6--9** and **6--9'** (unstained) assembled in PBS (bar 200 nm, larger sized images were depicted in Figures^{S4} and ^{S5}; TEM images of **CTRL-1** and **CTRL-2** constructs were shown in Figure^{S6}, Supporting Information. B) AFM images of **7** and **7'** assembled in PBS (bar 100 nm). C) CD spectra of **6--9** and **6--9'** in PBS 20 mM.

Figure³ In vivo studies of vaccine constructs and correlation between cell-mediated immune readouts and physicochemical properties. A) Vaccination and tumor challenge

schedule. B) Effect of the vaccine constructs on OT-I and OT-II cell frequency 8 days post prime compared with the negative control (PBS). Results were analyzed using a Mann-Whitney U test. C) OT-I CD8⁺ T-cell frequency kinetic during the course of the study. Results compared to the prime immunization by two-way ANOVA, Tukey's HSD post-hoc. D) Cytolytic activity (CTL) of effector CD8⁺ T cells. Presented as the percentage of OVA CD8 specific lysis, Results analyzed using one-way ANOVA Tukey's HSD post-hoc. E) Correlation between the physicochemical properties (Figure 1) and immune readouts. Left y-axis represents particle diameter (nm, for the constructs generating mixture of spherical and cylindrical particles, diameter of non-spherical particles were compared as main component), and the right y-axis represents the OT-I CD8⁺ T-cell frequency, CTL and tumor growth inhibition (TGI) percentage. The secondary structure for each construct, as determined using CD spectroscopy, is displayed on the graph. F) Tumor growth inhibition measured by tumor weight of immunized mice on day 77. Constructs 6-7 and 6'-9' are statistically significant when compared with the PBS group by one-way ANOVA with Dunnett's post-hoc test. In all cases, the data are presented as mean $\pm$ SEM from five mice per group (* $p < 0.05$, ** $p < 0.01$). CTRL-1 and CTRL-2 represent control peptides without lipids (Figure S3, Supporting Information). OVA peptides+CT and PBS are positive and negative controls, respectively. β : β -sheet, α : α -helix.

Figure 4 Immunogenicity of vaccine constructs, control peptides via cytokine secretion and antibody response. A) IFN- γ -ELISpot assay determined the number of IFN- γ secreting cells (spot forming cells (SFCs) per million splenocytes) following restimulation with OVA CD4+CD8 peptides for constructs 7 and 7' compared with the PBS group are statistically significant. B) IFN- γ , IL-2, and TNF- α cytokines correlate with a Th1-type response, and IL-4, IL-5, IL-6, IL-10, and IL-13 cytokines correlate with a humoral immune response (Th2) measured via cytometric bead array assay on pooled supernatants of restimulated splenocytes with OVA CD4+CD8 peptides. C) Correlation between the physicochemical properties (Figure 1) and Th1 cytokine levels for vaccine constructs. Left y-axis represents particle diameter (nm, for the constructs generating mixture of spherical and cylindrical particles, diameters of nonspherical particles were compared as main component), and the

right y-axis represents the cytokine level. D) Antibody responses 8 days post the second boost against OVA native protein and compared with PBS group are statistically significant ($*p<0.05$; $**p<0.01$, $***p<0.001$; $****p<0.0001$) by one-way ANOVA with Tukey HSD post-hoc test. In all cases, the data are presented as mean $\pm$ SEM from five mice per group. CTRL-1 and CTRL-2 are control peptides without lipid, OVA peptides+CT and PBS are positive and negative controls, respectively. β^* : β -sheet, $\alpha^!$: α -helix.

Figure 5 In-vitro dendritic cell activation induced by the vaccine constructs. DC2.4 cells were incubated overnight with the vaccine constructs and the expression of activation and co-receptor antigens were assessed by flow cytometry. A) The median fluorescence intensity of each sample was normalized to DC2.4 cells grown in media alone and is shown as relative fluorescence intensity (RFI). SUM is the sum of all RFIs less the value of the media control. B) DC2.4 cells incubated overnight with vaccine constructs, control peptides without lipid (CTRL-1 and CTRL-2), and positive control (OVA peptides+CT) were cultured for 3 days with naïve OT-I CD8⁺ T cells and the number of live OT-I cells were counted. In graphs A and B, the data are presented as mean $\pm$ SEM from triplicate experiments. C) Correlation of cell recovery from Figure 4B with the frequency of OT-I cells in the peripheral blood following three vaccinations (as in Figure 2B). Data are reported as the mean for each group and are log10 normalized. Correlation has been calculated using Prism V6.05 with Pearson correlation and two-tailed test, $r=0.617$, $p=0.043$.