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Mucosal Vaccination with a Self-Adjuvanted Lipopeptide Is Immunogenic and Protective against *Mycobacterium tuberculosis*

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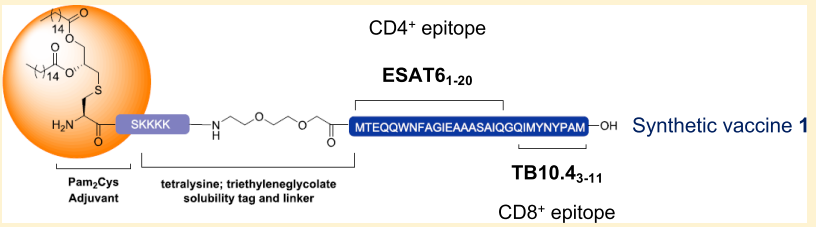
Supporting Information



ABSTRACT: Tuberculosis (TB) remains a staggering burden on global public health. Novel preventative tools are desperately needed to reach the targets of the WHO post-2015 End-TB Strategy. Peptide or protein-based subunit vaccines offer potential as safe and effective generators of protection, and enhancement of local pulmonary immunity may be achieved by mucosal delivery. We describe the synthesis of a novel subunit vaccine via native chemical ligation. Two immunogenic epitopes, ESAT6$_{1-20}$ and TB10.4$_{3-11}$ from *Mycobacterium tuberculosis* (Mtb), were covalently conjugated to the TLR2-ligand Pam$_2$Cys to generate a self-adjuvanting lipopeptide vaccine. When administered mucosally to mice, the vaccine enhanced pulmonary immunogenicity, inducing strong Th17 responses in the lungs and multifunctional peripheral T-lymphocytes. Mucosal, but not peripheral vaccination, provided substantial protection against Mtb infection, emphasizing the importance of delivery route for optimal efficacy.

INTRODUCTION

Tuberculosis (TB) has plagued human populations for a millennia and remains the leading cause of death from an infectious disease. The causative agent, *Mycobacterium tuberculosis* (Mtb), has the capacity to infect a healthy host and persist indefinitely without causing apparent disease, providing a reservoir of infection. Of the estimated 2 billion individuals colonized with Mtb, up to 10% will develop active disease in their lifetime.[1] In 2017, there were 1.6 million deaths and 10 million new cases of TB. Ten percent of these were children, indicating an ongoing active transmission.[2] The only available vaccine for TB, *Mycobacterium bovis* bacille Calmette−Guérin (BCG), is efficacious in reducing the risk of disseminated disease in infants, and its widespread use for TB protection is therefore likely to be continued into the future. However, BCG fails to prevent infection or provide long-term protection against the disease, and additionally is not considered suitable for use in immunocompromised individuals.[3] There is therefore an urgent need to explore alternative approaches for the prevention of TB, including novel, safe, efficacious, and easily deliverable vaccines for use as boosters or BCG adjuncts. This is of particular importance considering the growing number of drug-resistant TB cases worldwide.[4−6]

Recent clinical trials of peripheral vaccination with H4/IC31 or M72/AS01E, Mtb protein-based adjuvanted subunit vaccines that stimulate CD4$^+$ T-lymphocyte responses, demonstrated significant protection against new Mtb infection and pulmonary TB, respectively.[7,8] Formulation of vaccines for pulmonary delivery has been explored for other infectious diseases[9−11] and shows an opportunity to improve the immunogenicity and protective efficacy of TB vaccines by generating memory T lymphocytes in the lungs that may respond early to Mtb exposure.[12,13] Pulmonary vaccination has additional practical advantages, as it negates the need for sterile needles, containment of sharps waste and large numbers of clinically trained personnel, lowering cost.[12] The viral-vectored vaccine, MVA85A, which failed to provide protection from TB in clinical studies when given as a peripheral vaccine,[14] is now being examined as a pulmonary vaccine[15] (Identifier: NCT01954563), with a supporting preclinical evidence that this route provides greater efficacy.[16] Additionally, clinical trials are underway for an aerosol adenoviral-vectored TB vaccine (Ad85A; Identifier: NCT02337270) and aerosolized BCG (Identifier: NCT02709278). Safety concerns exist, however, regarding the use of live vaccines or viral vectors for pulmonary immunization, and repeat use may be limited due to immune responses to the viral backbone.[17,18] Protein- or peptide-based subunit vaccines may provide a safer alternative, can be used in

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immunocompromised individuals, and may be better suited for repeat use.

While peptide-based vaccines successfully administer the minimal amount of pathogenic material necessary to elicit an immune response, they often suffer from poor immunogenicity. Success in vivo is contingent upon co-administration of immune-stimulating adjuvants. It has been shown by us and others that covalent conjugation of adjuvants to peptide vaccines is an effective means of achieving enhanced immune responses compared to admixtures of individual components, as this minimizes the separation of adjuvant and antigen in vivo, potentiating immunogenicity.[19−26] When considering vaccines for pulmonary delivery, immune recognition receptors should be selected that may be accessed at the pulmonary mucosa and safely stimulated. Previous work in our laboratory demonstrated the successful use of powdered pulmonary vaccines, consisting of proteins from Mtb noncovalently associated with a TLR2-ligand, as a safe and effective immunization strategy for TB.[27] TLR2 agonizts may be particularly suitable for use in the pulmonary environment, as the receptor is expressed on multiple cell types, including both antigen-presenting cell subsets and pulmonary epithelial cells.[28−30] Of particular interest, a recent study demonstrated the protective potential of lipopeptide combinations from the Mtb protein antigen ESAT6 (Rv3875).[31] We reasoned therefore that covalent conjugation of peptide epitopes from Mtb to a TLR2-ligand would lead to an effective self-adjuvanted vaccine that may be more stable while providing a robust immunological response.

RESULTS AND DISCUSSION

Toward this end, we designed a self-adjuvanting vaccine candidate **1** (Figure 1) based on the covalent conjugation of

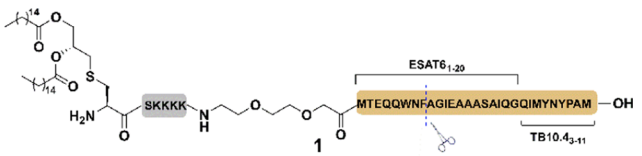
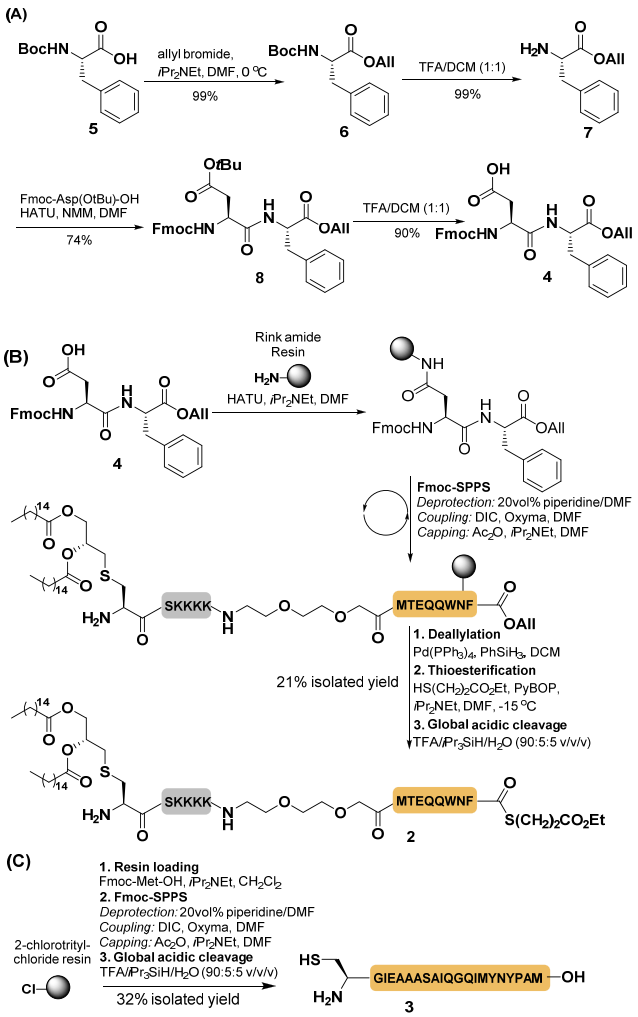


Figure 1. Self-adjuvanting vaccine candidate **1**, covalently conjugated Pam_2Cys as adjuvant and immunodominant T-lymphocyte epitopes from Mtb, $ESAT6_{1-20}$ and $TB10.4_{3-11}$.

the TLR2/TLR6 agonist Pam_2Cys via a small flexible triethylene glycol spacer to two antigenic peptides from the Mtb proteins ESAT6 and TB10.4 (Rv0288 or ESX-H). These peptides, $ESAT6_{1-20}$ and $TB10.4_{3-11}$, possess immunodominant $CD4^+$ and $CD8^+$ T-lymphocyte epitopes, respectively, in C57BL/6 mice. Initially, we attempted a linear synthesis of the vaccine construct using Fmoc-strategy solid-phase peptide synthesis (SPPS), but the lipophilicity of Pam_2Cys led to difficulties in purifying the target vaccine to homogeneity. We therefore turned to a native chemical ligation strategy for the convergent and chemoselective fusion of peptide fragments to generate larger constructs.[32] It was envisaged that a native chemical ligation strategy would permit initial synthesis of a shorter and easily purifiable lipopeptide thioester fragment, which could then be rapidly conjugated to the rest of the vaccine construct. To reduce the number of purification steps, 2,2,2-trifluoroethanethiol (TFET) was selected as a thiol additive to promote rapid ligation while enabling in situ radical desulfurization to access the target vaccine **1** (with a native alanine residue at ligation junction) in a one-pot manner.[33] With this synthetic strategy in mind, we chose to disconnect the vaccine between Phe8 and Ala9 of the ESAT6 epitope (Figure 1). This led to two synthetic targets, lipopeptide thioester **2**, bearing an N-terminal Pam_2Cys residue (Scheme 1B), and peptide **3**, possessing an N-terminal cysteine in place of native Ala9 in the ESAT6 epitope (Scheme 1C).

Scheme 1. (A) Synthesis of Dipeptide Building Block 4 from Boc-Phe-OH; (B) Synthesis of Lipopeptide Thioester Fragment 2 via Side Chain Anchoring, Fmoc-SPPS and Subsequent On-Resin Thioesterification; NB: Amino Acids within Resin-Bound Peptides Possess Standard Side-Chain-Protecting Groups Used in Fmoc-SPPS; (C) Synthesis of Peptide Fragment 3 via Fmoc-SPPS



Synthesis of lipopeptide thioester **2** was achieved via Fmoc-strategy SPPS on Rink amide resin using an on-resin thioesterification procedure.[34] Initially, we synthesized Fmoc-AspPhe-OAll **4** (Scheme 1A). This began by allyl ester protection of Boc-Phe-OH (**5**) to afford **6**, followed by Boc deprotection and coupling to Fmoc-Asp(O*t*Bu)-OH to afford dipeptide **8** in good yield. Deprotection of the side-chain *t*Bu ester on the aspartate residue then provided the target dipeptide **4**. With **4** in hand, it was next loaded via the condensation of the side-chain carboxylate of the aspartate residue to Rink amide resin (Scheme 1B). The polypeptide chain was then elongated using iterative Fmoc-SPPS, with

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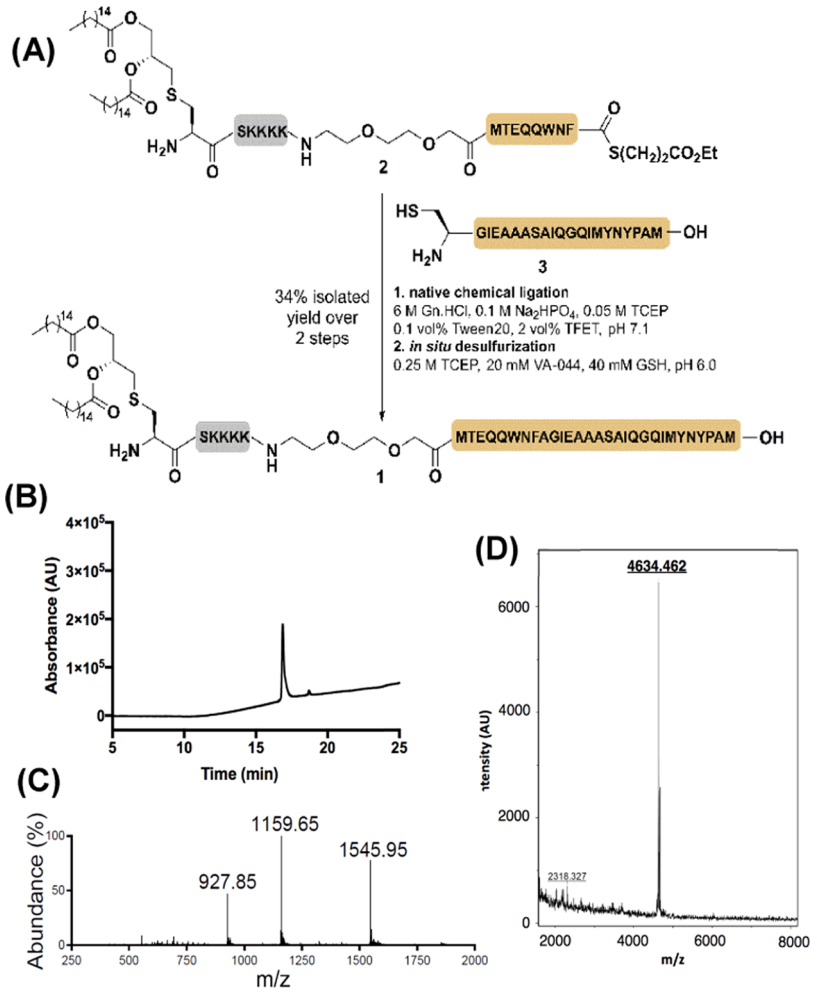
Scheme 2. (A) Synthesis of Self-Adjuvanting Tuberculosis Vaccine Candidate 1 via One-Pot Native Chemical Ligation and Desulfurization; (B) Analytical HPLC Chromatogram, (C) electrospray ionization mass spectrometry (ESI-MS), and (D) matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS Spectrum of Vaccine 1



amino acid, triethyleneglycolate, and Pam_2Cys couplings effected with *N*,*N*′-diisopropylcarbodiimide (DIC) and ethyl (hydroxyimino)cyanoacetate (Oxyma). At this point, the allyl ester-protected C-terminus of the peptide was unmasked by treatment with palladium *tetrakis*-triphenylphosphine and phenylsilane. Treatment with ethyl 3-mercaptopropionate in the presence of (benzotriazole-1-yloxy)-tripyrrolidinophosphonium hexafluorophosphate (PyBOP) and *N*,*N*-diisopropylethylamine at −15 °C[35,36] led to the formation of the C-terminal thioester with no detectable epimerization. Cleavage of the peptide thioester using an acidic cocktail comprising trifluoroacetic acid (TFA), triisopropylsilane, and water provided the crude lipopeptide thioester, which was purified via reverse-phase high-performance liquid chromatography (HPLC) to afford **2** in 21% yield (over 43 steps calculated from the initial resin loading). Peptide **3** was also accessed via Fmoc-strategy SPPS and isolated in 32% yield (over 63 iterative steps based on the initial resin loading) following reverse-phase HPLC (on C18 stationary phase) (Scheme 1C).

With the two target fragments in hand, our attention turned to the key ligation−desulfurization assembly of vaccine **1**. Native chemical ligation is typically carried out in aqueous phosphate buffer (0.1 M, pH ∼7) containing tris-(carboxyethyl)phosphine (TCEP, 50 mM) as a reductant and guanidine hydrochloride (Gdn.HCl, 6 M) as a denaturing agent. However, lipopeptide thioester **2** was insoluble in this buffer system and, as a result, we supplemented the buffer with the nonionic surfactant Tween-20 (0.5% v/v) as a solubilizing detergent. Specifically, lipopeptide thioester **2** and peptide **3** were dissolved in this buffer and 2,2,2-trifluoroethanethiol (TFET, 2% v/v) was added.[33] Pleasingly, within 2 h, the reaction proceeded to completion to afford the ligation product. Without purification, in situ desulfurization of the ligation product was performed through treatment with the radical initiator 2,2′-azobis[2-(2-imidazolin-2-yl)propane]-dihydrochloride (VA-044) (20 mM) in the presence of reduced glutathione (40 mM) and TCEP (250 mM) to convert the cysteine to a native alanine residue at the ligation junction. HPLC purification using C18 reverse-phase HPLC furnished self-adjuvanting TB vaccine candidate **1** in 34% yield over two steps (Scheme 2).

The adjuvant activity of the synthesized conjugate vaccine **1** was first verified in vitro. Specifically, Pam_2Cys binding and activation of the TLR2 signaling pathway was assessed in a HEK-TLR2-reporter cell line. Conjugate vaccine **1** showed a strong activation of TLR2, comparable to stimulation with Pam_2Cys-SKKKK-PEG(OH) (Figure 2). The in vivo

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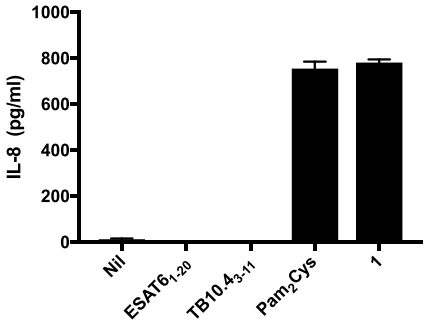

Figure 2. Vaccine candidate **1** activates TLR2 signaling in vitro. Stimulation of IL-8 released from HEK-TLR2 reporter cells by synthetic conjugate **1** is shown in comparison to stimulation with PBS (Nil), ESAT6$_{1-20}$ or TB10.4$_{3-11}$ peptides, or Pam$_2$Cys-PEG(OH) alone (at ~7 μM). Data are mean ± standard error of the mean (SEM) (n = 3) and are representative of two independent experiments.

immunogenicity induced by **1** was assessed in mice; three homologous vaccinations were given 2 weeks apart and responses were assessed 3 weeks after the final vaccination. Mice were immunized at either the pulmonary mucosa via intranasal (i.n.) instillation or peripherally by subcutaneous (s.c.) injection. The use of defined immunodominant peptide epitopes as antigens enabled quantitative and qualitative assessment of the CD4$^+$ and CD8$^+$ T-lymphocyte responses generated both locally and systemically. Lymphocytes from the lungs and spleens of immunized mice were restimulated with ESAT6$_{1-20}$ or TB10.4$_{3-11}$ peptides ex vivo prior to intracellular immunostaining and flow cytometry. Mucosal delivery of **1** induced substantial populations of ESAT6-specific CD4$^+$ T-lymphocytes in the lungs producing IL-17 and TNFα (Figure 3A). The multifunctionality of local T-lymphocytes were also increased, such that significant populations of IL-17$^+$ CD4$^+$ cells were observed that also expressed other key cytokines, including IFNγ, IL-2, and TNFα, indicative of a predominantly Th17-like phenotype (Figure S1). Notably, there was minimal detectable cytokine response in the lungs of mice that were vaccinated s.c. (Figures 3A and S1). The profile of cytokine-producing ESAT6-specific CD4$^+$ T-lymphocytes in the spleens of mucosally immunized mice was similar to that seen in the lungs, but additionally with enhanced IL-2 responses (Figures 3B and S2). A minor population of IL-2- and TNFα-producing cells were observed in the spleens of s.c. immunized mice; however, no other significant populations were identified (Figures 3B and S2). No significant cytokine-producing CD8$^+$ populations were seen in the lungs of any immunized group in response to TB10.4$_{3-11}$ (Figure S3A), but small populations of IFNγ- and TNFα-producing CD8$^+$ cells were seen in the spleens of s.c. immunized mice (Figure S3B). This may indicate that murine leukocytes were not able to efficiently process this epitope from **1** appropriately for presentation to CD8$^+$ T-lymphocytes. Overall, mucosal delivery of the vaccine candidate **1** substantially improved pulmonary immunogenicity and preferentially led to a strong Th17-like CD4$^+$ T-lymphocyte response.

Adjuvants for new vaccines should ideally be selected based on their ability to induce the type of immune response best correlated with protection against the pathogen. In the case of Mtb, there is strong evidence that a Th1-type CD4$^+$ T-lymphocyte response is required; however, this is not sufficient

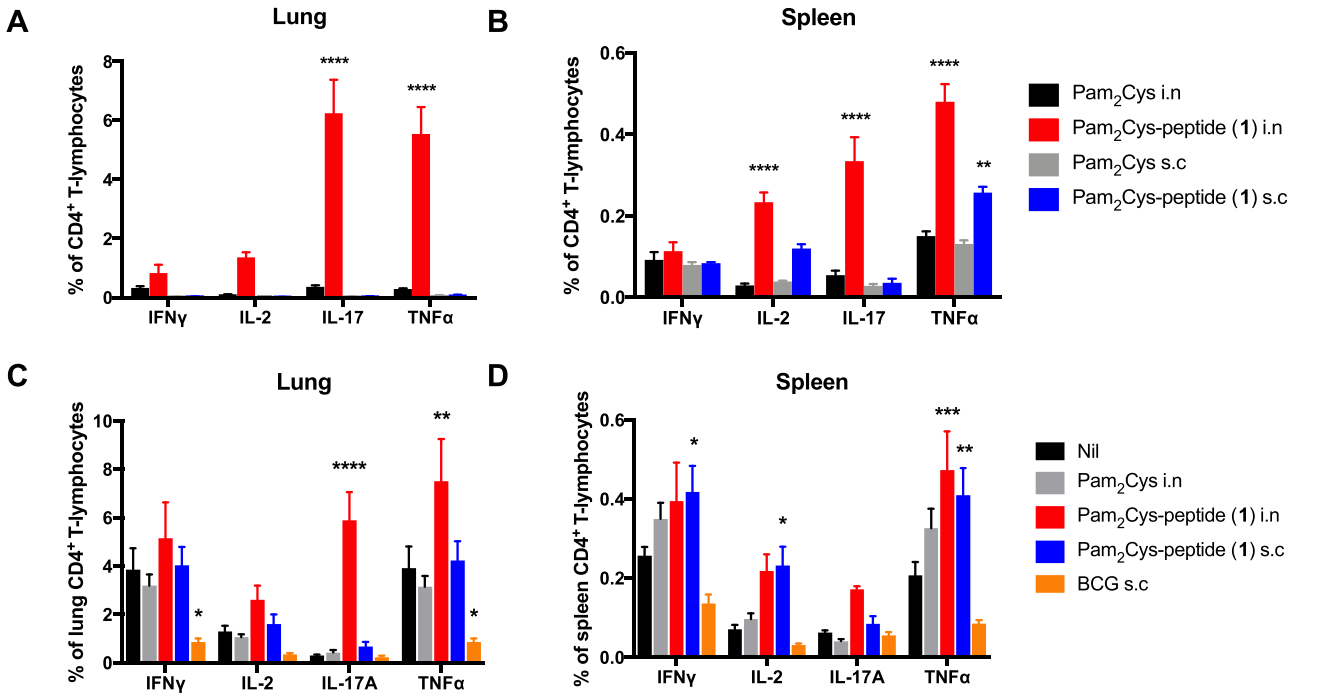

Figure 3. Mucosal, but not peripheral, delivery of **1** induces a strong antigen-specific pulmonary IL-17 and TNFα responses that are maintained post-Mtb challenge. Frequency of cytokine-producing CD4$^+$ T-lymphocytes in the (A) lungs and (B) spleen 3 weeks following final immunization (n = 3) and (C) lungs and (D) spleen 4 weeks post-Mtb challenge (n = 6). Epitope-specific cells were detected by intracellular immunostaining and flow cytometry after recall with ESAT6$_{1-20}$ (10 μg/mL). Data are mean ± SEM. Statistically significant differences (A, B) to relevant Pam$_2$Cys control by analysis of variance (ANOVA) with Tukey's multiple comparison test, and (C, D) to Nil control, by ANOVA with Dunnett's multiple comparison test (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001).

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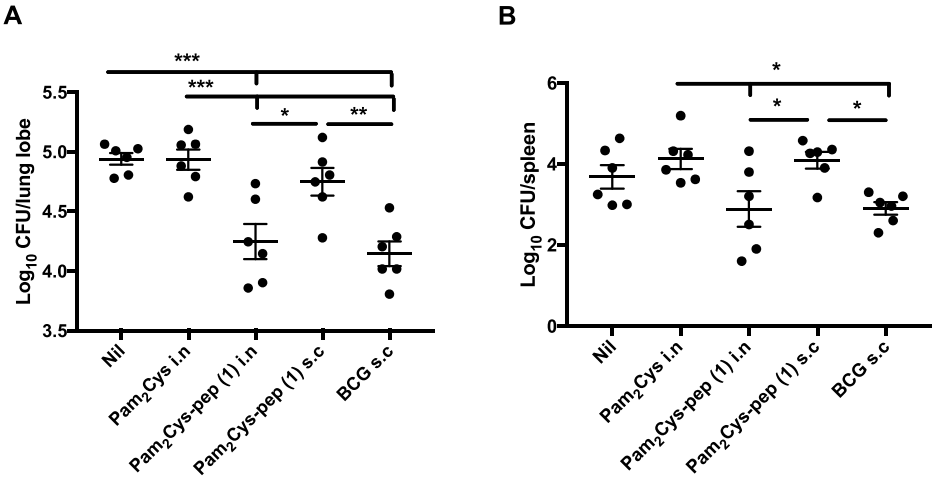

Figure 4. Protective efficacy against Mtb infection was induced by mucosal immunization with **1**. Mice (n = 6) were immunized with **1** (10 μg peptide component), or an equivalent amount of Pam2Cys, three times at two-weekly intervals. Six weeks later, the mice were challenged with aerosol Mtb H37Rv (100 CFU). Alternatively, the mice received 5 × 10^5 CFU BCG s.c. 10 weeks before challenge. After 28 days, the bacterial loads in (A) lungs and (B) spleen were enumerated. Data are mean ± SEM and are representative of two independent experiments. Statistical significance was determined by ANOVA with Tukey's multiple comparisons test (*p < 0.05, **p < 0.01, ***p < 0.001).

for sterilizing immunity.[37] Immunization at the pulmonary mucosa tends to drive a Th17-based response to peptide antigens, likely due to the induction of IL-1, IL-6, and TGFβ expression.[38,39] TLR2 stimulation, which leads to the production of TNF and IL-6, is critical for the activation of antigen-presenting cells[28] and are also inducers of Th17 polarization, characterized by IL-17, IL-21, and IL-22 production. IL-17 may promote granuloma formation and neutrophil recruitment by inducing the pro-inflammatory cytokines and chemokines G-CSF, IL-6, and IL-8,[40,41] as well as recruitment of IFNγ-producing cells to the site of infection by induction of CXCL10 expression.[42] This is suggested to be one of the mechanisms by which Th17 cells may promote the immune response to Mtb.[43] While the role of vaccine-induced IL-17 in protection against Mtb is controversial,[44,45] several studies have indicated the plasticity of vaccine-induced Th17 cells in mice, and their capacity to revert after exposure to Mtb to expression of the classical Th1-associated cytokine IFNγ, even after long-term residency as memory cells in the lungs.[46,47] It is therefore possible that mucosal immunization with **1**, despite inducing primarily strong Th17 responses, may provide a pool of lung resident $CD4^+$ T-lymphocytes that can function as Th1 or Th17 as needed and provide protective responses against the pathogen.[12,13]

Having demonstrated robust pulmonary immunogenicity from mucosal administration of **1**, we next tested the self-adjuvanting vaccine candidate in an aerosol Mtb infection. C57BL/6 mice received three homologous immunizations 3 weeks apart with i.n. Pam_2Cys alone or **1** by i.n. instillation or s.c. injection. A separate group of mice received BCG s.c., considered the gold-standard comparison in murine models of Mtb challenge.[48] After 6 weeks, the mice were challenged by aerosol with Mtb and the postchallenge immune response to the vaccine epitopes were assessed 4 weeks later. Mice immunized i.n. with **1** had maintained significantly upregulated populations of ESAT6-specific $CD4^+$ T-lymphocytes producing IL-17 and TNFα in the lungs, which were not seen in peripherally immunized mice (Figure 3C). The diversity of multifunctional cytokine-producing populations in the lungs of mucosally immunized mice was maintained postchallenge (Figure S4). Populations of a similar phenotype were also seen in the spleen (Figures 3D and S5), consistent with the immunogenicity pre-Mtb challenge. At this postchallenge stage, mice immunized s.c. had increased ESAT6-specific IFNγ^+, IL-2^+, and TNFα^+ $CD4^+$ T-lymphocyte populations in the spleen (Figures 3D and S5). There were minimal vaccine-induced responses seen to the $TB10.4_{3-11}$ peptide post-challenge, and it is likely that these populations reflect responses to the Mtb challenge itself (Figure S6).

To assess if the T-lymphocyte responses generated by **1** were functionally beneficial, the Mtb bacterial burden in the lungs and spleen were enumerated to determine the protective efficacy of the vaccine. We were pleased to observe that when **1** was delivered mucosally, it provided substantial protection in both the lungs and spleen, similar to that provided by BCG (Figure 4). Consistent with the immunological data, while there was a trend toward protection in the lungs after subcutaneous vaccination, this did not reach statistical significance in all experiments (Figure 4A) and no protection was seen in the spleen (Figure 4B). These data strongly support the model that vaccination strategies promoting a pulmonary immune response offer more robust protection from Mtb. Taken together, this study provides an exciting proof of principle data that synthetic self-adjuvanting peptide vaccines can provide substantial pulmonary immunogenicity and protection against virulent Mtb.

CONCLUSIONS

In summary, we have successfully produced a synthetic lipopeptide conjugate vaccine candidate **1** by one-pot native chemical ligation−desulfurization chemistry and assessed the delivery by pulmonary or peripheral routes to induce protective immune responses to Mtb. Mucosal delivery offered increased immunogenicity and protective efficacy. The use of this vaccine in humans will depend on future toxicology and careful evaluation of the adjuvant Pam_2Cys in phase I clinical studies. It will be important in future studies to assess whether pulmonary vaccination with constructs such as **1** may be used to boost the protective efficacy of BCG or improve the long-term protective efficacy against Mtb. Pulmonary boosting may

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have the additional benefit of drawing memory T-lymphocytes generated by subcutaneous BCG into the lungs, creating a further reservoir of memory cells that may act quickly to combat future encounter with Mtb. Further, these conjugated lipopeptides provide the means to assess the impact that the type of conjugation between antigen and adjuvant has on immunogenicity and protective efficacy. The ligation technology employed in this study is applicable to the conjugation of diverse peptide or protein antigens to different adjuvants that stimulate a variety of pattern recognition receptors. Exploring pulmonary administration to enhance efficacy and ease of delivery with a range of different adjuvants and antigens from Mtb may contribute novel constructs to the TB vaccine pipeline and will be the subject of continued work in our laboratories.

EXPERIMENTAL SECTION

General Methods. Unless otherwise stated, reactions were carried out under an argon atmosphere and at room temperature (rt) (22 °C). Reactions carried out at 0 °C employed a bath of water and ice. Anhydrous CH_2Cl_2 and dimethylformamide (DMF) was obtained using a PureSolv solvent purification system (water < 10 ppm). Reactions were monitored by thin layer chromatography (TLC) on aluminum-backed silica plates (Merck Silica Gel 60 F254). Visualization of TLC plates was undertaken with an ultraviolet light at λ = 254 nm and staining with solutions of vanillin or phosphomolybdic acid, followed by exposure of the stained plates to heat. Silica gel 60 40−63 μm was used for flash column chromatography. NMR spectroscopy was performed in $CDCl_3$ on a Bruker DRX 300 or DRX 400 NMR spectrometer at frequencies of 300 MHz or 400 MHz for ^{1}H NMR and 75 or 100 MHz for ^{13}C NMR, respectively. Chemical shifts are reported in parts per million (ppm) and coupling constants in Hertz (Hz). Residual solvent was used as internal standards ($CDCl_3$: δ_H = 7.26, δ_C = 77.16). ^{13}C NMR data are reported as chemical shift values (ppm). Mass spectrometry with electrospray ionization (ESI) was performed on a Shimadzu 2020 (ESI) mass spectrometer operating in a positive mode. High-resolution mass spectra were recorded on a Bruker-Daltronics Apex Ultra 7.0T Fourier transform (FTICR) mass spectrometer. MALDI-TOF mass spectrometry was performed on a Bruker Autoflex Speed MALDI-TOF mass spectrometer operating in reflectron mode using a matrix of 10 mg/mL α-cyano-4-hydroxycinnamic acid in water/acetonitrile containing 0.1% TFA (1:1 v/v). Optical rotations were measured on a PerkinElmer 341 polarimeter at a wavelength of 589 nm. IR spectra were recorded on a Bruker Alpha FT-IR spectrometer using a diamond ATR unit. The purity of all compounds was >97% as judged by NMR spectroscopy and HPLC.

General Procedure for Fmoc-SPPS. Fmoc-SPPS was performed on a Biotage Initiator+ Alstra microwave peptide synthesizer using the following conditions. Amino acid coupling: A solution of Fmoc-protected amino acid (4 equiv), DIC (4 equiv), and Oxyma (4 equiv) in DMF (Labscan, final amino acid concentration 0.1 M) was added to the resin. After 30 min at 50 °C, the resin was washed with DMF (5 × 5 mL). Deprotection: The resin was treated with 20% piperidine/DMF (2 × 3 mL, 3 min) at rt and then washed with DMF (5 × 5 mL). Capping: Ac_2O/pyridine (1:9 v/v) was added to the resin (3 mL). After 3 min, the resin was washed with DMF (5 × 5 mL).

General Procedure for Resin Cleavage. Peptides were cleaved from resin (with concomitant side-chain deprotection) by treatment with an acidic cocktail containing TFA/triisopropylsilane/water (90:5:5 v/v/v) with gentle shaking for 2 h. The cleavage solution was filtered and the resin washed with TFA (2 × 2 mL). The solution was concentrated and then precipitated with cold diethyl ether. The mixture was centrifuged and the supernatant was discarded.

Preparative and Semipreparative Reverse-Phase HPLC. HPLC purification was performed using a Waters 600 Multisolvent Delivery System and Waters 500 pump with a Waters 490E programmable wavelength detector operating at 214, 230, or 280 nm. Peptide **3** was purified by preparative HPLC on a Waters Sunfire C18 column (5 μm, 19 × 150 mm) at a flow rate of 7 mL min^{-1} (solvent A: 0.1% TFA in H_2O; solvent B: 0.1% TFA in MeCN). Lipopeptides **1** and **2** were purified by semipreparative HPLC on a Phenomenex Luna C18(2) column (5 μm, 10 × 250 mm) in a column heater at 40 °C and a flow rate of 7 mL min^{-1} (solvent A: 0.1% TFA in H_2O:MeCN:*i*PrOH (8:1:1 v/v/v); solvent B: 0.1% TFA in MeCN:*i*PrOH (9:1 v/v)).

Synthesis of Vaccine 1 via One-Pot Native Chemical Ligation Desulfurization. Lipopeptide thioester **2** (2.5 mg, 0.96 μmol) and peptide **3** (2 equiv, 4.2 mg, 1.92 μmol) were dissolved in aqueous phosphate buffer (0.1 M, pH = 7.4, 192 μL, 5 mM with respect to thioester **2**) containing guanidine hydrochloride (6 M), TCEP (50 mM), and Tween-20 (0.5% v/v). The pH of the reaction mixture was adjusted with aqueous NaOH (1 M) to pH 7.2. TFET (2% v/v) was added and the reaction was incubated at 37 °C for 2 h. The reaction was followed by ultraperformance liquid chromatography-tandem mass spectrometry (UPLC-MS). After consumption of the thioester (as measured by UPLC-MS analysis, 2 h) the reaction was degassed under a stream of argon, and one volume of degassed aqueous phosphate buffer containing guanidine hydrochloride (6 M), TCEP (500 mM), reduced glutathione (80 mM), and the radical initiator VA-044 (40 mM) was added. The reaction was allowed to proceed at 37 °C for 8 h, at which time the ligation product bearing cysteine at the ligation junction was fully converted to the corresponding alanine-containing product (as judged by UPLC-MS analysis). Semipreparative HPLC purification (0−60% B over 40 min, 7 mL min^{-1}, 40 °C, Phenomenex Luna C18(2), 5 μm, 100 Å, 10 × 250 mm) followed by lyophilization afforded pure vaccine candidate **1** as a white solid (1.5 mg, 34% yield). Analytical HPLC: R_t 16.9 min symmetry C4 column, 300 Å, 5 μm, 4.6 mm × 250 mm, gradient: 20−100 B over 25 min (solvent A: H_2O + 0.1% TFA, solvent B: MeCN + 0.1% TFA); MS calcd: $[M + 3H]^{3+}$ = 1545.48, $[M + 4H]^{4+}$ = 1159.36, $[M + 5H]^{5+}$ = 927.69; found (ESI^+): 1545.95, 1159.65, 927.85. HRMS calcd for $C_{214}H_{348}N_{47}O_{58}S_4$: $[M + H]^+$ = 4634.466; found (MALDI-TOF): 4634.462.

Synthesis of Lipopeptide Thioester 2. Rink amide resin (25 μmol, 0.3 mmol/g loading) was initially washed with CH_2Cl_2 (5 × 3 mL) and DMF (5 × 3 mL), followed by Fmoc deprotection with 20% piperidine/DMF (2 × 5 min). The resin was washed with DMF (5 × 3 mL), CH_2Cl_2 (5 × 3 mL), and DMF (5 × 3 mL). PyBOP (4 equiv) and *N*-methylmorpholine (NMM) (8 equiv) were added to a solution of Fmoc-AspPhe-OAll **4** (4 equiv) in DMF. After 5 min of preactivation, the mixture was added to the resin and then shaken at rt for 2 h. The resin was filtered, then washed with DMF (5 × 3 mL), CH_2Cl_2 (5 × 3 mL), and DMF (5 × 3 mL), capped with Ac2O/pyridine (1:9 v/v; 2 × 3 min), and washed with DMF (5 × 3 mL), CH_2Cl_2 (5 × 3 mL), and DMF (5 × 3 mL). The resin-bound H-SKKKK-triethyleneglycolate-ESAT6(1−8) peptide was synthesized on the side-chain-loaded resin using the general procedure for Fmoc-SPPS. For the coupling of Pam_2Cys, a solution of Fmoc-Pam_2Cys-OH (1.2 equiv), DIC (1.2 equiv), and HOAt (1.5 equiv) in DMF was added to the resin, and the reaction was shaken at rt for 16 h. The resin was next treated with a solution of $Pd(PPh_3)_4$ (1 equiv) and $PhSiH_3$ (40 equiv) in dry CH_2Cl_2. The resin was shaken for 1 h and the procedure was repeated. Afterward, the resin was washed with CH_2Cl_2 (10 × 5 mL), DMF (5 × 5 mL), and CH_2Cl_2 (5 × 5 mL). A solution of ethyl-3-mercaptoproprionate (4 equiv) and PyBOP (4 equiv) in DMF (2−3 mL) was added to the resin at −15 °C in a salt−ice−water bath. Subsequently, ^{i}Pr$_2$NEt (4 equiv) was added to the resin. The resin was left at −15 °C for 3 h. Afterward, the resin was washed with CH_2Cl_2 (10 × 5 mL), DMF (5 × 5 mL), and CH_2Cl_2 (5 × 5 mL). The peptide was cleaved from resin and worked up as described in the general resin cleavage procedure. The dried pellet was dissolved in water containing 40% MeCN + 0.1% TFA, purified by preparative RP-HPLC (50−100% B over 40 min, 4 mL min^{-1}, 40 °C, Phenomenex Luna C18(2), 5 μm, 100 Å, 10 × 250 mm) and lyophilized to afford the desired lipopeptide thioester as a white solid (13.5 mg, 21% yield). Analytical HPLC: R_t 16.0 min (50−100% B

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over 30 min, 0.2 mL min^{-1}, Phenomenex Luna C18(2), 5 μm, 2.1 × 150 mm, λ = 214 nm). MS calcd: $[M + 2H]^{2+}$ = 1300.24, $[M + 3H]^{3+}$ = 867.17, $[M + 4H]^{4+}$ = 650.62; found (ESI^+): 1300.80, 867.45, 650.75.

Synthesis of Peptide 3. 2-Chlorotrityl chloride resin (50 μmol, 1.22 mmol/g loading) was swollen in dry CH_2Cl_2 for 30 min then washed with CH_2Cl_2 (5 × 3 mL). A solution of Fmoc-Met-OH (0.5 equiv relative to resin functionalization) and $^iPr_2NEt$ (2.0 equiv relative to resin functionalization) in CH_2Cl_2 (final amino acid concentration 0.1 M of amino acid) was added and the resin was shaken at rt for 16 h. The resin was washed with DMF (5 × 3 mL) and CH_2Cl_2 (5 × 3 mL). The resin was treated with a solution of $CH_2Cl_2/CH_3OH/^iPr_2NEt$ (17:2:1 v/v/v, 3 mL) for 1 h and washed with DMF (5 × 3 mL), CH_2Cl_2 (5 × 3 mL), and DMF (5 × 3 mL). The ESAT6(9−20)-TB10.4(3−11) peptide was synthesized via Fmoc-strategy solid-phase peptide synthesis as outlined in the general methods section. The peptide was cleaved from resin and worked up via the general resin cleavage procedure. The dried pellet was dissolved in water containing 30% MeCN + 0.1% TFA and purified by preparative HPLC (0−60% B over 40 min, 7 mL min^{-1}, Sunfire C18, 5 μm, 100 Å, 19 × 150 mm) to afford the desired peptide as a white solid (35 mg, 32% yield). Analytical HPLC: R_t 13.1 min (0−100% B over 30 min, 0.2 mL min^{-1}, 0.1% TFA, Sunfire C18, 5 μm, 100 Å, 2.1 × 150 mm, λ = 214 nm). MS calcd: $[M + 2H]^{2+}$ = 1102.50, $[M + 3H]^{3+}$ = 735.33; found (ESI^+): 1102.60, 734.45.

Fmoc-L-Asp-L-Phe-OAll (4). The protected dipeptide **8** (872 mg, 1.42 mmol) was treated with TFA in CH_2Cl_2 (10 mL, 1:1 v/v). The reaction mixture was stirred at room temperature for 1 h, after which the TFA/CH_2Cl_2 solvent mixture was removed under a gentle stream of nitrogen. The residue was dissolved in CH_2Cl_2 and then washed with water (2 × 30 mL), saturated aqueous $NaHCO_3$ solution (2 × 30 mL), and brine (30 mL). The organic phase was dried with anhydrous $MgSO_4$, concentrated in vacuo, and purified by flash column chromatography to afford the target compound **4** as a yellow oil (728 mg, 90% yield). R_f = 0.42 (EtOAc/hexane: 1:2 v/v) ^1H NMR ($CDCl_3$, 400 MHz) δ (ppm): 7.77 (d, J = 7.41 Hz, 2H), 7.54 (m, 2H), 7.39 (t, J = 7.4 Hz, 2H), 7.29 (t, J = 7.4 Hz, 2H), 7.20−7.08 (m, 6H), 5.97 (d, 8.1 Hz, 1H), 5.82 (ddt, J = 5.5, 10.5, 16.5 Hz, 1H), 5.25 (dd, J = 17.9, 10.5 Hz, 2H), 4.85 (dd, 6.3, 6.8 Hz, 1H), 4.56 (m, 3H), 4.42−4.34 (m, 2H), 4.18 (t, J = 7.0 Hz, 1H), 3.12−3.06 (m, 3H), 2.70 (m, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ (ppm): 170.8, 156.1, 143.6, 141.3, 141.2, 135.5, 131.2, 129.2, 128.5, 128.5, 127.8, 127.2, 127.1, 125.1, 120.0, 119.1, 67.5, 66.2, 53.6, 50.7, 47.0, 37.7, 35.9; HRMS: (+ESI) Calc. for 542.2053 $[M + Na]^+$, found: 542.2058 $[M + Na]^+$; IR (ATR): ν_{max} = 3300, 3065, 3030, 2924, 2854, 1710, 1661, 1532, 1449, 1248, 1211, 1049, 738, 701 cm^{-1}; $[\alpha]_D$: +21° (c 1.0, CH_2Cl_2).

Boc-L-Phe-OAll (6). Boc-L-Phe-OH **5** (799 mg, 3.0 mmol) was dissolved in DMF (10 mL) and cooled to 0 °C in an ice bath. $^iPr_2NEt$ (1.57 mL, 9 mmol) was added dropwise, followed by allyl bromide (280 μL, 3.3 mmol). The solution was allowed to warm to rt and stirred for 16 h. The reaction mixture was diluted with EtOAc (40 mL), and then washed with water (2 × 30 mL) and brine (30 mL). The organic phase was dried with anhydrous $MgSO_4$, concentrated in vacuo, and purified via flash column chromatography to afford allyl ester **6** as a yellow oil (905 mg, 99% yield). ^1H NMR ($CDCl_3$, 400 MHz) δ (ppm): 7.33−7.13 (m, 5H), 5.88 (ddt, J = 5.7, 11.0, 16.2 Hz, 1H), 5.32 (dq, J = 2.0, 17.3 Hz, 1H), 5.26 (dd, J = 2.0, 10.5 Hz, 1H), 4.62 (dt, J = 1.5, 6.0 Hz, 2H), 3.18−3.05 (m, 2H), 1.44 (s, 9H). ^{13}C NMR ($CDCl_3$, 100 MHz) δ (ppm): 171.6, 155.1, 135.9, 131.5, 129.4, 128.5, 127.2, 118.9, 79.9, 65.9, 54.5, 38.4, 28.3. These data are in agreement with those previously reported by Lang et al.[49]

L-Phe-OAll (7). Boc-L-Phe-OAll (**6**) (885 mg, 2.90 mmol) was treated with TFA in CH_2Cl_2 (10 mL, 1:1 v/v). The reaction mixture was stirred at room temperature for 1 h, after which the TFA/CH_2Cl_2 solvent mixture was removed under a gentle stream of nitrogen. The reaction mixture was diluted into CH_2Cl_2 (30 mL) and then washed with water (2 × 30 mL) and brine (30 mL). The organic phase was dried with anhydrous $MgSO_4$ and then concentrated in vacuo to afford **7** as a yellow oil (592 mg, 99% yield). ^1H NMR ($CDCl_3$, 400 MHz) δ (ppm): 7.27−7.07 (m, 5H), 5.70 (ddt, J = 5.7, 11.0, 16.2 Hz, 1H), 5.20−5.16 (m, 2H), 4.49 (d, J = 6.1 Hz, 2H), 4.20 (t, J = 7.0 Hz, 1H), 3.24−3.11 (m, 2H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ (ppm): 168.6, 132.9, 130.2, 129.3, 128.2, 120.2, 67.4, 54.3, 36.2. These data are in agreement with those previously reported by Lang et al.[49]

Fmoc-L-Asp(OtBu)-L-Phe-OAll (8). A solution of Fmoc-L-Asp-(OtBu)-OH (1.21 g, 2.91 mmol), HATU (1.11 mg, 2.90 mmol), and N-methylmorpholine (NMM, 1 mL, 9.0 mmol) in DMF (final amino acid concentration 0.1 M) was added to a reaction vessel containing H-L-Phe-OAll **7** (533 mg, 2.6 mmol). The reaction was stirred for 2 h, after which the solvent was removed under a stream of nitrogen. The residue was dissolved into CH_2Cl_2 (30 mL), washed with water (2 × 30 mL), 2 M HCl (20 mL), saturated aqueous $NaHCO_3$ solution (20 mL), and brine (30 mL). The organic phase was dried with anhydrous $MgSO_4$, concentrated in vacuo, and purified by flash column chromatography to afford the target compound **S3** as a yellow oil (1.14 g, 74% yield); R_f = 0.40 (EtOAc/hexane, 3:7 v/v). ^1H NMR ($CDCl_3$, 300 MHz) δ (ppm): 7.72 (d, J = 7.9 Hz, 2H), 7.53 (d, J = 7.5 Hz, 2H), 7.36 (dd, J = 7.1, 7.4 Hz, 2H), 7.30−6.96 (m, 7H), 5.90 (d, J = 7.6 Hz, 1H), 5.79 (ddt, J = 6.0, 11.2, 16 Hz, 1H), 5.22 (dd, J = 18, 10.6 Hz, 2H), 4.80 (dd, J = 13.1, 6.2 Hz, 1H), 4.50 (m, 1H), 4.37−4.27 (m, 2H), 4.17 (dd, J = 14.1, 7.1 Hz, 1H), 3.07 (dd, J = 6.2, 5.7 Hz, 2H), 2.84 (dd, 17, 4.0 Hz, 1H), 2.56 (dd, 17, 6.6 Hz, 1H), 1.40 (s, 9H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ (ppm): 171.2, 170.6, 170.2, 155.9, 143.7, 141.3, 135.6, 131.4, 129.3, 128.5, 127.7, 127.1, 125.1, 120.0, 119.0, 81.9, 67.3, 66.0, 53.6, 50.9, 47.0, 37.8, 28.5; HRMS: (+ESI) Calc. for 598.2679 $[M + Na]^+$, found: 598.2681 $[M + Na]^+$; IR (ATR): ν_{max} = 3301, 3066, 3030, 2924, 2854, 1711, 1661, 1532, 1449, 1248, 1211, 1050, 738, 701 cm^{-1}; $[\alpha]_D$: +6.25° (c 1.0, CH_2Cl_2).

In Vitro Assessment of Vaccine Adjuvant Activity. Human Embryonic Kidney 293 (HEK293; ATCC CRL-1573) cells, stably transfected with a plasmid expressing YFP-TLR2 fusion protein[50] that secretes IL-8 upon TLR activation (kindly provided by A/Prof Ashley Mansell, Monash University), were used as a reporter of TLR2 stimulation by Pam_2Cys in the vaccine construct. Cells were grown in Dulbecco's modified Eagle's medium (Gibco, MA) with D-glucose (4.5 g/L), L-glutamine (3.996 mM), penicillin−streptomycin (100 U/mL; Gibco), geneticin (0.5 mg/mL; Gibco), and 10% heat-inactivated fetal calf serum (FCS) at 37 °C and 5% CO_2. After seeding 2 × 10^5 cells/well of a 96-well flat-bottom plate (Corning) and allowing cells to adhere, the cells were stimulated in triplicate for 16−18 h with vaccine candidate **1** (33.5 μg/mL; such that Pam_2Cys-SKKKK-triethyleneglycolate was at 10 μg/mL, the optimal concentration for stimulation of this cell line as determined by previous experiments, $ESAT6_{1-20}$ ∼15 μg/mL and $TB10.4_{3-11}$ ∼8 μg/mL). As a comparison, Pam_2Cys-SKKKK-triethyleneglycolate (10 μg/mL), $ESAT6_{1-20}$ peptide (15 μg/mL), or $TB10.4_{3-11}$ peptide (Genscript; 8 μg/mL) was also used as a stimulus. Supernatants were collected and IL-8 secretion determined as a measure of stimulation of TLR2 by ELISA (Biolegend), according to manufacturer's instructions. Data shown are representative of two independent experiments.

Mice and Immunization Procedures. All murine experiments were conducted with the approval of the Sydney Local Health District Animal Welfare Committee (protocol numbers 2013/054, 2013/075, and 2016/044), in full compliance with local and institutional guidelines. Female C57BL/6 6- to 8-week old mice were obtained from Animal BioResources (Moss Vale, NSW, Australia). The mice were housed in the Centenary Institute animal facility under SPF conditions. For i.n. administration, mice were anesthetized by intraperitoneal injection of ketamine/xylazine solution (50 mg/6.25 mg/kg). Vaccine (14.2 μg, equivalent to 10 μg peptide) in phosphate-buffered saline (PBS; 50 μL final volume, such that the vaccine reached the deep lung) was applied to the nares and mice allowed to inhale the solution. Mice receiving subcutaneous vaccines (14.2 μg for protection studies and 56.8 μg for immunogenicity studies, equivalent to 10 or 40 μg peptide respectively; in PBS, 200 μL final volume) were anesthetized with gaseous isoflurane (4%) and injected at the base of tail. No adverse effects were observed in mice receiving this vaccine by either mucosal or subcutaneous routes. Mice received 3

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vaccinations 2 weeks apart and proceeded to either immunogenicity study at 3 weeks after last vaccination, or Mtb challenge at 6 weeks after last vaccination. Mice receiving BCG were vaccinated s.c. once only with 5×10^5 CFU, 10 weeks prior to Mtb challenge.

Bacterial Strains and Growth Conditions. *Mangora bovis* BCG Pasteur 1173P2 and Mtb H37Rv (BEI Resources, NIAID, NIH, NR-13648) were cultured at 37 °C in Middlebrook 7H9 (Difco) broth supplemented with albumin-dextrose-catalase (ADC; 10% v/v), Tween-80 (0.05% v/v), and glycerol (0.2% v/v). To enumerate, cultures were plated onto Middlebrook 7H10 or 7H11 (Difco) agar, supplemented with oleic-acid-albumin-dextrose catalase (10% v/v) and glycerol (0.5% v/v) and incubated at 37 °C for up to 21 days.

Experimental Mtb Infection. At 6 weeks after 3 immunizations, mice received a low-dose aerosol infection (100 CFU) in an inhalation exposure system (Glas-Col, Terre Haute, IN). At 4 weeks after challenge, serial dilutions of lung and spleen homogenates were plated to enumerate the bacterial loads.

Collection and Processing of Murine Organs for Leukocyte Isolation. Mice were sacrificed via CO_2 asphyxiation, the tissues removed aseptically and maintained at 4 °C. Circulating blood was removed from the lung lobes by perfusion with an injection of PBS and heparin (20 U/mL; Sigma) into the right atrium of the heart. For isolation of leukocytes, diced tissue was digested with collagenase type 4197 (50 U/mL; Sigma) and DNAse I (13 μg/mL; Sigma) at 37 °C for 45 min, followed by homogenization and multiple filtration steps through a 70 μm sieve. The spleen, mediastinal lymph node (MLN) of i.n. immunized mice or inguinal lymph nodes (ILN) of s.c. immunized mice was homogenized through a 70 μm sieve and the leukocytes pelleted by centrifugation. Erythrocytes were removed by ACK lysis buffer, then viable leukocytes enumerated by hemocytometer or using a BD Countess, with Trypan Blue (0.04%) exclusion.

Polyfunctional T-Lymphocyte Responses to Immunization. Epitope-specific cytokine production by T-lymphocytes was enumerated by antigen-recall, intracellular immunostaining, and flow cytometry. Single-cell suspensions of up to 4×10^6 lymphocytes were stimulated for 1−2 h (37 °C, 5% CO_2) with $ESAT6_{1-20}$ or $TB10.4_{3-11}$ peptides (10 μg/mL; Genscript), or with antimouse CD3 (1452C11, 5 μg/mL) and antimouse CD28 (37.51, 5 μg/mL; BD Pharmingen) or media alone as controls. Brefeldin A (10 μg/mL; Sigma) was added and further incubation (16 h, 37 °C, 5% CO_2) allowed intracellular accumulation of cytokine. After washing with FACS wash (PBS with 2% FCS), Fc receptors were blocked with antimouse CD16/CD32 (2.4G2; BD Biosciences). Surface markers were labeled with an antimouse CD8-APCCy7 (53-6.7; BD Pharmingen, San Jose, CA), CD4-PECy7 (RM4-5; BD Pharmingen), CD3-PerCPCy5.5 (17A2; Biolegend, San Diego, CA), and Live/Dead fixable blue dead cell stain (Invitrogen, CA) in FACS wash, then the cells were washed thoroughly. Cells were fixed using BD Cytofix/perm, followed by thorough washing with BD Perm/Wash. To label intracellular cytokines, cells were incubated with antimouse IFNγ-FITC (XMG1.2; BD Pharmingen), IL-17A-PB (TC11-18H10.1; Biolegend), TNFα-PE/APC (MP6-XT22; Biolegend), and IL-2-APC/PE (JES6-5H4; Biolegend) prepared in BD Perm/Wash buffer and then washed. Compensation controls were prepared by immunostaining BD CompBeads with the same antibody utilized in the experimental panel, except for live dead staining, in which case murine leukocytes were labeled in the same manner as experimental samples. Immunostained cells or beads were fixed in 10% neutral buffered formalin prior to the acquisition of the data using an LSRFortessa or LSRII SL flow analyser (BD Biosciences) and analysis using FlowJo (Tree Star Inc.).

Statistical Analysis. Statistical analysis was performed using GraphPad Prism 6 or 7 software (GraphPad Software, La Jolla, CA), as detailed in figure legends. Differences between two groups were analyzed by Students *t*-test, or between multiple groups by one- or two-way analysis of variance (ANOVA) with Tukey's or Dunnett's multiple comparisons test and were considered significant when p values were ≤ 0.05 ($^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$).

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jmedchem.9b00832.

NMR spectra, MALDI-TOF mass spectrum of **1**, and immunological data (PDF)

Molecular formula strings (CSV)

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Author Contributions

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Notes

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■ ABBREVIATIONS

BCG, *Mycobacterium bovis* bacille Calmette−Guérin; DIC, *N*,*N*′-diisopropylcarbodiimide; i.n., intranasal; Mtb, *Mycobacterium tuberculosis*; Oxyma, ethyl (hydroxyimino)cyanoacetate; PyBOP, (benzotriazole-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate; SPPS, solid-phase peptide synthesis; TCEP, tris(2-carboxyethyl)phosphine hydrochloride; TFET, 2,2,2-trifluoroethanethiol; Th, T-helper; VA-044, 2,2′-azobis[2-(2-imidazolin-2-yl)propane]dihydrochloride

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