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

Anneliese S. Ashhurst,†,* David M. McDonald,†,* Cameron C. Hanna,† Vicki A. Stanojevic,†
Warwick J. Britton,*†§ and Richard J. Payne*†∥

1School of Chemistry, ‡Tuberculosis Research Program Centenary Institute, and §Faculty of Medicine and Health, The University of Sydney, Camperdown, NSW 2050, Australia

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, ESAT61−20 and TB10.44−11, from Mycobacterium tuberculosis (Mtbb), were covalently conjugated to the TLR2-ligand Pam2Cys to generate a self-adjuvanted 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 Mtbb 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 (Mtbb), 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 Mtbb, 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–Guerin (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.4 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, Mtbb protein-based adjuvanted subunit vaccines that stimulate CD4+ T-lymphocyte responses, demonstrated significant protection against new Mtbb infection and pulmonary TB, respectively.7 Formulation of vaccines for pulmonary delivery has been explored for other infectious diseases8−11 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 Mtbb 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 vaccine15 (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
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. 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. TLR2 agonists 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. Of particular interest, a recent study demonstrated the protective potential of lipoprotein combinations from the Mtb protein antigen ESAT6 (Rv3875). 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 the TLR2/TLR6 agonist Pam3Cys 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, and TB10.4 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 Pam3Cys 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. 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.

With this synthetic strategy in mind, we chose to disconnect the vaccine between Phe8 and Ala9 of the ESAT6 epitope (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 Thiosterification; 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

Figure 1. Self-adjuvanting vaccine candidate 1, covalently conjugated Pam3Cys as adjuvant and immunodominant T-lymphocyte epitopes from Mtb, ESAT6, and TB10.4.
135 amino acid, triethyleneglycolate, and Pam2Cys couplings
136 effected with N,N′-diisopropylcarbodiimide (DIC) and ethyl
137 (hydroxyimino)cyanoacetate (Oxyma). At this point, the allyl
138 ester-protected C-terminus of the peptide was unmasked by
139 treatment with palladium tetrakis-triphenylphosphine and
140 phenylsilane. Treatment with ethyl 3-mercaptopropionate in
141 the presence of (benzotriazole-1-yloxy)-
142 tripyrrolidinophosphonium hexafluorophosphate (PyBOP)
143 and N,N′-diisopropylethylamine at −15 °C35,36 led to the
144 formation of the C-terminal thioester with no detectable
145 epimerization. Cleavage of the peptide thioester using an acidic
146 cocktail comprising trifluoroacetic acid (TFA), triisopropylsili-
147 lane, and water provided the crude lipopeptide thioester, which
148 was purified via reverse-phase high-performance liquid
149 chromatography (HPLC) to afford 2 in 21% yield (over 43
150 steps calculated from the initial resin loading). Peptide 3
151 was also accessed via Fmoc-strategy SPPS and isolated in 32% yield
152 (over 63 iterative steps based on the initial resin loading)
153 following reverse-phase HPLC (on C18 stationary phase)
154 (Scheme 1C).

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

The adjuvant activity of the synthesized conjugate vaccine 1
176 was first verified in vitro. Specifically, PamCys binding and
177 activation of the TLR2 signaling pathway was assessed in a
178 HEK-TLR2-reporter cell line. Conjugate vaccine 1 showed a
179 strong activation of TLR2, comparable to stimulation with
180 Pam2Cys-SKKKK-PEG(OH) (Figure 2). The in vivo
181
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 ESAT61−20 or TB10.43−11 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 TB10.43−11 (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 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.
for sterilizing immunity. 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. TLR2 stimulation, which leads to the production of TNF and IL-6, is critical for the activation of antigen-presenting cells 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, as well as recruitment of IFNγ-producing cells to the site of infection by induction of CXCL10 expression. This is suggested to be one of the mechanisms by which Th17 cells may promote the immune response to Mtb. While the role of vaccine-induced IL-17 in protection against Mtb is controversial, 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. 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.

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. Pam2Cys 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. After 6 weeks, the mice were challenged with aerosol Mtb H37Rv (100 CFU). Alternatively, the mice received 5 × 10^6 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).

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 × 10^6 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).

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 Pam2Cys 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
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 Mt. 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 Mt b 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 CH2Cl2 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).

isolation 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 CDC13 on a Bruker DRX 300 or DRX 400 NMR spectrometer at frequencies of 300 MHz or 400 MHz for 1H NMR and 75 or 100 MHz for 13C NMR, respectively.

Chemical shifts are reported in parts per million (ppm) and coupling constants in Hertz (Hz). Residual solvent was used as internal standards (CDCl3: δH = 7.26, δC = 77.16). 1H NMR data are reported as chemical shift values (ppm). Mass spectrometry with electrospray ionization (ESI) was performed on a Shimadzu 2020 ES/MS (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 Autotof 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. 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 measured on a PerkinElmer 341 polarimeter at a wavelength of 589 nm.

**Amino acid coupling:** A solution of Fmoc-SPPS was performed on the side-chain-loaded resin using the general procedure for Fmoc-SPPS. For the coupling of Pam2Cys, a solution of Fmoc-Pam2Cys-OH was used for HPLC purification. Fmoc deprotection with 20% piperidine/DMF (2:1 v/v) was added to the resin. The resin was washed with CH2Cl2 (3 mL), capped with Ac2O/PyBOP (4 equiv) in DMF (5 mL), and washed with CH2Cl2 (5 mL), and DMF (5 mL) was added to the resin at rt for 2 h. The resin was filtered, then washed with DMF (5 × 417 mL), CH2Cl2 (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), CH2Cl2 (5 × 3 mL), and DMF (5 × 3 mL). The resin-bound H-ESAT6 was purified 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 H2O; 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 H2O:MeCN:PrOH (8:1:1 v/v/v); solvent B: 0.1% TFA in MeCN: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. TFE (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 alamine-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) was followed by lyophilization afforded pure vaccine candidate 1 as a white solid (1.5 mg, 34% yield). Analytical HPLC: RI, 16.9 min (symmetry C4 column, 300 Å, 5 μm, 4.6 mm × 250 mm, gradient: 20–100 B over 25 min (solvent A: H2O + 0.1% TFA, solvent B: MeCN + 0.1% TFA); MS: calcd: [M + 3H]⁺ = 1545.48; [M + 4H]⁺ = 1159.36; [M + 5H]⁺ = 927.69; found (ESI+): 1545.95, 1159.65, 927.85. HRMS calcd for [M + 4H]⁺: [M + 4H]⁺ = 4634.466; found (MALDI-TOF): 4634.462.

**Synthesis of Lipopeptide Thioester 2.** Rink amide resin (25 mg, 0.3 mmol/g loading) was initially washed with CH2Cl2 (5 × 410 mL) and DMF (5 × 3 mL), followed by Fmoc deprotection with 20% piperidine/DMF (2 × 5 min). The resin was washed with DMF (5 × 412 mL), CH2Cl2 (5 × 3 mL), and DMF (5 × 3 mL), capped with Ac2O/PyBOP (4 equiv) in DMF (5 × 3 mL), and washed with CH2Cl2 (5 × 3 mL), capped with Ac2O/Pyridine (1:9 v/v), 2 × 3 min, and washed with DMF (5 × 3 mL), CH2Cl2 (5 × 3 mL), and DMF (5 × 3 mL). The resin-bound H-ESAT6 was purified 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 H2O; 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 H2O:MeCN:PrOH (8:1:1 v/v/v); solvent B: 0.1% TFA in MeCN:PrOH (9:1 v/v)).
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 (EST): 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 CH₂Cl₂ for 30 min then washed with CH₂Cl₂ (3 × 3 mL). A solution of Fmoc-Met-OH (5 μmol, 2.1 mmol) was treated with TFA in CH₂Cl₂ (10 mL, 1:1 v/v). The reaction mixture was stirred at room temperature for 1 h, after which the solvent was removed under a stream of nitrogen. The residue was dissolved in CH₂Cl₂ and then washed with (EtOAc/hexane, 3:7 v/v). The reaction mixture was stirred at room temperature for 16 h. The reaction mixture was diluted with EtOAc (40 mL), and then washed with (H₂O, 2 × 30 mL), 2 M HCl (20 mL), saturated aqueous NaHCO₃ solution (20 mL), and brine (30 mL). The organic phase was dried with anhydrous MgSO₄, concentrated in vacuo, and purified by flash column chromatography to afford the target compound S₃ as a yellow oil (2.10 g, 98% yield). HRMS: (+ESI) Calc. for 598.2679 [M + Na]⁺, found: 598.2681 [M + Na]⁺; IR (ATR): v_max = 3301, 3060, 3030, 2924, 2954, 1711, 1661, 1532, 1449, 1248, 1211, 1050, 738, 701 cm⁻¹; [α]D = +6.25° (c = 1.0, CH₂Cl₂).

**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 that secretes IL-8 upon TLR activation (kindly provided by A/P Dr. Ashley Mansell, Monash University), were used as a reporter for TLR4 stimulation by Pam2Cys in the vaccine construct. Cells were grown in Dulbecco’s modified Eagle’s medium (Gibco, MA) with 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% CO₂.

**Mice and Immunization Procedures.** All murine experiments were conducted with the approval of the Sydney Local Health District and Animal Welfare Committee (protocol numbers 2013/054, 2013/075, and 2016/044), in full compliance with local and institutional guidelines. Female C3H/HeN 6- to 8-week-old mice were obtained from Animal BioResources (Moss Vale, NSW, Australia). The mice were housed in the Centenay Institute animal facility under SPF conditions. For i.n. administration, mice were anesthetized by intraperitoneal injection of ketamine/xylazine solution (50 mg/0.625 mg/kg). Vaccine (14.2 μg equivalent to 10 μg peptide) in phosphate-buffered saline (PBS; 50 μL final volume) was also used as a stimulus. Supernatants were collected and IL-8 secretion determined as a measure of stimulation of TLR4 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 and Animal Welfare Committee (protocol numbers 2013/054, 2013/075, and 2016/044), in full compliance with local and institutional guidelines. Female C3H/HeN 6- to 8-week-old mice were obtained from Animal BioResources (Moss Vale, NSW, Australia). The mice were housed in the Centenay Institute animal facility under SPF conditions. For i.n. administration, mice were anesthetized by intraperitoneal injection of ketamine/xylazine solution (50 mg/0.625 mg/kg). Vaccine (14.2 μg equivalent to 10 μg peptide) in phosphate-buffered saline (PBS; 50 μL final volume) was also used as a stimulus. Supernatants were collected and IL-8 secretion determined as a measure of stimulation of TLR4 by ELISA (Biolegend), according to manufacturer’s instructions. Data shown are representative of two independent experiments.
vaccinations 2 weeks apart and proceeded to either immunogenicity
study at 3 weeks after last vaccination, or Mtbb challenge at 6 weeks
after last vaccination. Mice receiving BCG were vaccinated s.c. once
only with 5 X 10^5 CFU, 10 weeks prior to Mtbb challenge.

**Bacterial Strains and Growth Conditions.** *Mangora bovis* BCG
Pasteur 1173P2 and Mtbb H37Rv (BEI Resources, NIAID, NIH, NR-
13648) were cultured at 37 °C in Middlebrook 7H9 (Difco) broth
supplemented with albumin-dextrin-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-dextrin-catalase (10% v/v)
and glycerol (0.5% v/v) and incubated at 37 °C for up to 21 days.

**Experimental Mtbb Infection.** At 6 weeks after 3 immunizations,
mice received a low-dose aerosol infection (100 CFU) in the
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 CO₂ 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 l.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 hemocy-
tometer 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 enum-
ERATED 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% CO₂) with ESAT6_1-20 or
TB10.4_1-11 peptides (10 µg/mL; Genscript), or with antitoxine CD3
1452C11, 5 µg/mL) and antitoxine CD28 (451, 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% CO₂)
allowed intracellular accumulation of cytokine. After washing with
FACS wash (PBS with 2% FCS), Fc receptors were blocked with
antitoxine CD16/CD32 (2.4G2; BD Biosciences). Surface markers
were labeled with an antitoxine CD8-APC.Cy7 (53-67; BD
Pharmingen, San Jose, CA), CD4-PE.Cy7 (RM4-5; BD Pharmingen,
CD3-PerCPC5.5 (17A2; Biologend, 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 antitoxine IFNγ-
FITC (XMG12.2; BD Pharmingen), IL-17A-APC (TC11-18H11.01;
Biologend), TNF-PE/APC (MP6-XT22; Biologend), and IL-27-
PE.CP (JES6-5H4; Biologend) 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 5L 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**

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

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

Molecular formula strings (CSV)

**AUTHOR INFORMATION**

**Corresponding Authors**

*E-mail: w.britton@centenary.org.au (W.J.B.).
E-mail: richard.payne@sydney.edu.au (R.J.P.).

**ORCID**

Richard J. Payne: 0000-0002-3618-9226

**Author Contributions**

A.S.A. and D.M.M. contributed equally to this work.

**Author Contributions**

All authors have given approval to the final version of the
manuscript.

**Funding**

This work was supported by the National and Medical
Research Council of Australia Project Grant (APP1044343),
Centre for Research Excellence in Tuberculosis Control
(APP1043225), and the NSW Government through its
infrastructure grant to the Centenary Institute. A.S.A.,
D.M.M., and C.C.H. each received an Australian Postgraduate
Award.

**Notes**

The authors declare no competing financial interest.

**ACKNOWLEDGMENTS**

We thank Prof J Triccas, the University of Sydney, for
provision of BCG stocks, Dr B Saunders, the University of
Technology Sydney, for provision of antimouse CD3 antibody
and A/Prof A Mansell, Monash University, for provision of
HEK-TLR2 reporter cell line stocks. We thank Dr G
Nagalingam, Dr D Quan, Dr L Lin, Dr M Flórido, and Dr S
Rudrawar, the Centenary Institute Animal Facility and Sydney
Cytometry, for technical assistance and advice.

**ABBREVIATIONS**

BCG, *Mycobacterium bovis* bacille Calmette–Guérin; DIC, 689
N,N′-disopropylcarbodiimide; i.n., intranasal; Mtb, *Mycobac-
terium tuberculosis*; Oxyma, ethyl (hydroxyimino)cyanoacetate;
PyBOP, (benzotriazole-1-yl)oxytripyrrolidinophosphonium
hexafluorophosphate; SPPS, solid-phase peptide synthesis;
TCEP, tris(2-carboxyethyl)phosphine hydrochloride; TFET, 687
2,2,2-trifluorooethanethiol; Th, T-helper; VA-044, 2,2′-azobis-
[2-(imidazolin-2-yl)propane]dihydrochloride

**REFERENCES**


2. Global Tuberculosis Report; World Health Organization: Geneva,
2018.

3. Andersen, P.; Doherty, T. M. The success and failure of BCG-
implications for a novel tuberculosis vaccine. *Nat. Rev. Microbi-

4. Marais, B. J.; Sintchenko, V. Epidemic spread of multidrug-

H

DOI: 10.1021/acs.jmedchem.9b00832
J. Med. Chem. XXXX, XXX, XXX–XXX
Robust immune responses elicited by a fully synthetic three-component vaccine.


