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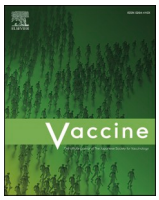

Short communication

Co-administration of a synthetic saccharide conjugate vaccine with BCG provides synergistic protection against murine tuberculosis

Socorro Miranda-Hernandez, Harindra D. Sathkumara, Guangzu Zhao, Louis Schofield [*,1], Andreas Kupz [*,1]

Australian Institute of Tropical Health and Medicine, James Cook University, Cairns & Townsville, Queensland, Australia

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ABSTRACT

Tuberculosis (TB) remains a major global health challenge claiming over 1 million lives annually. Bacillus Calmette-Guérin (BCG), the only licensed vaccine against TB, provides limited efficacy against pulmonary TB in adults and only partial protection against serious disease in children. Hence, the development of safer and more effective new vaccines against TB remains a global health priority. Here we show in a new approach that co-administration of BCG with a novel synthetic glycan conjugate vaccine, corresponding to conserved terminal mannose sequences in Phosphatidyl-*myo*-inositol-mannosides (PIMs) in the cell envelope of mycobacteria, results in reduced bacterial burden compared to BCG vaccination alone. This first *in vivo* efficacy study suggests that targeting conserved essential oligosaccharides of mycobacteria is an important factor in improving TB vaccine efficacy.

1. Introduction

Tuberculosis (TB) remains a leading cause of morbidity and mortality worldwide, especially in low- and middle-income countries, claiming about 1.3 million lives annually [1]. The only licensed TB vaccine, Bacillus Calmette-Guérin (BCG), has limited efficacy, particularly against pulmonary TB in adults, the most common and contagious form of the disease. Hence, the development of a BCG replacement vaccine and strategies to improve BCG efficacy remain a global health priority [1].

Phosphatidyl-*myo*-inositol mannosides (PIMs) are abundant glycolipids in the cell envelope of all *Mycobacterium* species, where they serve as essential structural components of the inner and outer membrane. PIMs also form the structural basis of lipomannan and lipoarabinomannan, critical molecules involved in host-pathogen interactions during TB [2]. PIM_6 of *Mycobacterium tuberculosis* (*Mtb*) elaborates the glycan structure *t*-α-Man*p*(1 → 2)-α-Man*p*(1 → 2)-α-Man*p*(1→6)-α-Man*p*(1 → 6)-α-Man*p*(1 → , attached to position 6 of the *myo*-inositol ring, in addition to a Man*p* residue present at position 2 of the *myo*-inositol [3].

Although saccharide-conjugate vaccines that induce Bcell effector responses have enormous public health utility against *Streptococcus pneumoniae, Salmonella enterica, Neisseria meningitides* and *Haemophilus influenzae* B [4], to date they have not been reported for efficacy against *Mtb* infection. Here we sought to determine if a synthetic saccharide-conjugate vaccine (SSCV; termed ST208-309), containing a sequence corresponding to the first 4 mannose residues of PIM_6, coupled to the generic protein carrier CRM_{197}, could increase protection against TB using the C57BL/6 mouse model of TB [5]. Furthermore, to explore whether the SSCV approach could offer added benefit in an aged host [6], where responses to traditional vaccines are often suboptimal, we investigated the effectiveness of co-vaccination with ST208-309 and BCG in aged mice.

2. Methods

2.1. ST208-309 production

A synthetic glycan of the sequence NH_2-CH_2-CH_2-PO_4-(Manα1-2)-6Manα1-2Manα1-6Manα1-4GlcNH_2, lacking inositol and lipids (ST208-309) was synthesized by CordenPharma (Boston) from protected building blocks using established solution-phase protocols. Structure and stoichiometry were confirmed by ^{1}H NMR, ^{13}C NMR, ^{31}P NMR and Mass Spectroscopy. Purity of 91.6 % was confirmed by HPLC-CAD. The

* Corresponding authors.
E-mail addresses: louis.schofield@saccharidetx.com (L. Schofield), andreas.kupz@jcu.edu.au (A. Kupz).
[1] Equally contributing senior author.

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sulfhydryl linker 2-iminothiolane was introduced to the ethanolamine and the glycan coupled to maleimide-activated CRM_{197} (Inventprise Inc) in molar ratio 8:1. The conjugate vaccine was purified to 98 % homogeneity and lyophilized.

2.2. Bacterial cultures

BCG Pasteur and *Mtb* H37Rv were cultured in Middlebrook 7H9 (BD Difco) supplemented with ADC (BD BBL), 0.05 % Tween80 (Sigma), 0.4 % glycerol (Sigma). Bacteria were cultured until log phase at an OD_{600} of 0.8, harvested and stored at −80 °C for subsequent use.

2.3. Mice

Female C57BL/6 mice were purchased from the Animal Resource Centre (Perth, Australia) and kept in BSL 2 or 3 facilities under specific pathogen–free conditions at James Cook University, Australia. One cohort of mice was kept for 6 months prior to vaccination to allow ageing within the same facility. All experiments were conducted in accordance with the National Health and Medical Research Council guidelines and were approved by the animal ethics committee (A2346) of James Cook University.

2.4. Vaccinations

Frozen vials of BCG were thawed, washed and diluted in PBS. Mice were vaccinated subcutaneously with 1×10^6 BCG colony-forming units (CFU) in a volume of 100 µl. Lyophilized ST208-309 was diluted in PBS to 10 mg/ml, aliquoted and frozen at −80 °C. For vaccination with ST208-309, 333 µl of diluted vaccine was made up to 3 ml in PBS and mixed with 3 ml Incomplete Freund's Adjuvant (IFA) by vortexing, sonicating water bath and syringe passage. 200 µl of vaccine formulation was injected intraperitoneally. For the sham adjuvant and the BCG + IFA groups, IFA was mixed with equal volume of PBS or BCG respectively, and 200 µl were injected intraperitoneally. ST208-309 and IFA groups were boosted on day 20 and 40. BCG was only given once at day 0.

2.5. Mtb challenge

Mtb challenge was performed 60 days after initial vaccination. Frozen *Mtb* were thawed, diluted and treated in an ultrasound water bath. Infections were done using a Glas-Col inhalation exposure system. Lungs from 5 mice were collected 24 h after aerosol infections to determine the infectious dose. Mice were infected with a standard dose of 20–50 CFU *Mtb*.

2.6. Sample collection

Mice were sacrificed by cervical dislocation 45 days after *Mtb* infection. Blood was collected into *Z*-gel tubes (Sarstedt) and serum separated by centrifugation, filtered using 0.2 µm SpinX columns (Sigma) and stored at −20 °C. The right lung lobes and spleens were used for CFU enumeration, and the left lung lobes for histopathology.

2.7. CFU enumeration

Lungs and spleens were homogenized in sterile sample bags containing 1 ml of sterile PBS/0.05 % Tween80. CFU were determined on Middlebrook 7H11 agar plates supplemented with 0.2 % glycerol, 0.05 % Tween80, and 10 % OADC enrichment (BD Biosciences). Agar plates were incubated aerobically for 4–5 weeks at 37 °C. Total CFU per organ was calculated based on dilution factors. *Mtb* detection limits were 10 CFU in spleens and 20 CFU in lungs.

2.8. Histopathology

Left lung lobes were fixed overnight with 10 % formalin, transferred to 70 % ethanol and embedded in paraffin. Each lung lobe was sectioned into 2–3 slices, 4 µm thick, covering the entire lobe, transferred to glass slides, dewaxed and stained with H&E or Ziehl-Neelsen (ZN). One randomly selected stained section per lung was scanned with an Aperio CS2 (Leica) followed by analysis with Image Scope (Leica) and ImageJ (NIH). The percentage of lung damage was measured as described previously [7].

2.9. Cytokine and antibody measurements

Frozen serum samples were thawed and prepared according to the Bio-Plex Pro Mouse Cytokine 23-Plex assay (BioRad). Data was acquired on a MagPix (Luminex) instrument and analysed with xPONENT (Luminex) software. IgG levels were measured by ELISA as previously reported [8].

2.10. Sample exclusion

Low dose aerosol infections of mice with *Mtb* sometimes carry the caveat of undetectable levels of *Mtb* [9]. Due to the absence of detectable CFU on agar plates and *Mtb* in ZN staining, 14 out of 140 mice were excluded from the analysis shown in Fig. 1 (Fig. S1). Results derived from all mice, including those with zero CFU are shown in Fig. S2A-C.

2.11. Statistics

Statistical analysis and graphs were generated using Prism version 10.1.2 (GraphPad) and R (2025.05.1 + 513). Comparisons were performed using Mann-Whitney test or one-way ANOVA, followed by Tukey's multiple comparison tests and Chi-square and Fisher's exact test. $p < 0.05$ was considered significant.

3. Results

3.1. Co-administration of ST208-309 and BCG provides synergistic protection against TB

To determine if co-administration of ST208-309 with BCG can reduce TB disease, six- to eight-week-old C57BL/6 mice were vaccinated subcutaneously with BCG, intraperitoneally with ST208-309 or both. Unvaccinated mice and mice receiving IFA only served as control groups. ST208-309- and IFA-containing groups were boosted at days 20 and 40 (Fig. 1A). As expected, BCG reduced the bacterial burden in lungs and spleens. Vaccination with ST208-309 or IFA had no effect on bacterial burden, indicating that ST208-309 by itself does not protect against TB. However, co-administration of BCG and ST208-309 significantly reduced lung bacterial burden compared to BCG vaccination alone by ~1 log (Fig. 1B). A similar pattern was observed in the spleen, with BCG/ST208-309 co-vaccination achieving the largest reduction in CFU burden (Fig. 1C) and the highest number of mice with no detectable CFU (Fig. S2E). Enumeration of lung pathology also showed that ST208-309 (mean infiltration 7.359 %) and IFA (mean 7.773 %) vaccination alone has no positive impact on histopathology compared to unvaccinated mice (mean 6.662 %). However, both BCG (mean 4.433 %) and BCG + ST208-309 (mean 2.926 %) reduced lung infiltration by 33.45 % and 56.08 %, respectively (Fig. 1D). Serum collected at 45 days after *Mtb* infection also showed that BCG + ST208-309 co-vaccination leads to an increase in circulating cytokines and chemokines, many of which have been associated with improved TB control (Fig. 1E). Notably, the BCG + ST208-309 group contained statistically significant increased levels of IL-3, IL-1β, TNF-α, IL-17, IL-1α, GM-CSF, IL-10, CCL4, CCL5 and CCL-11 (Fig. 1F). No differences in total serum IgG levels were observed (Fig. S2F). Collectively, those results suggest that co-administration of

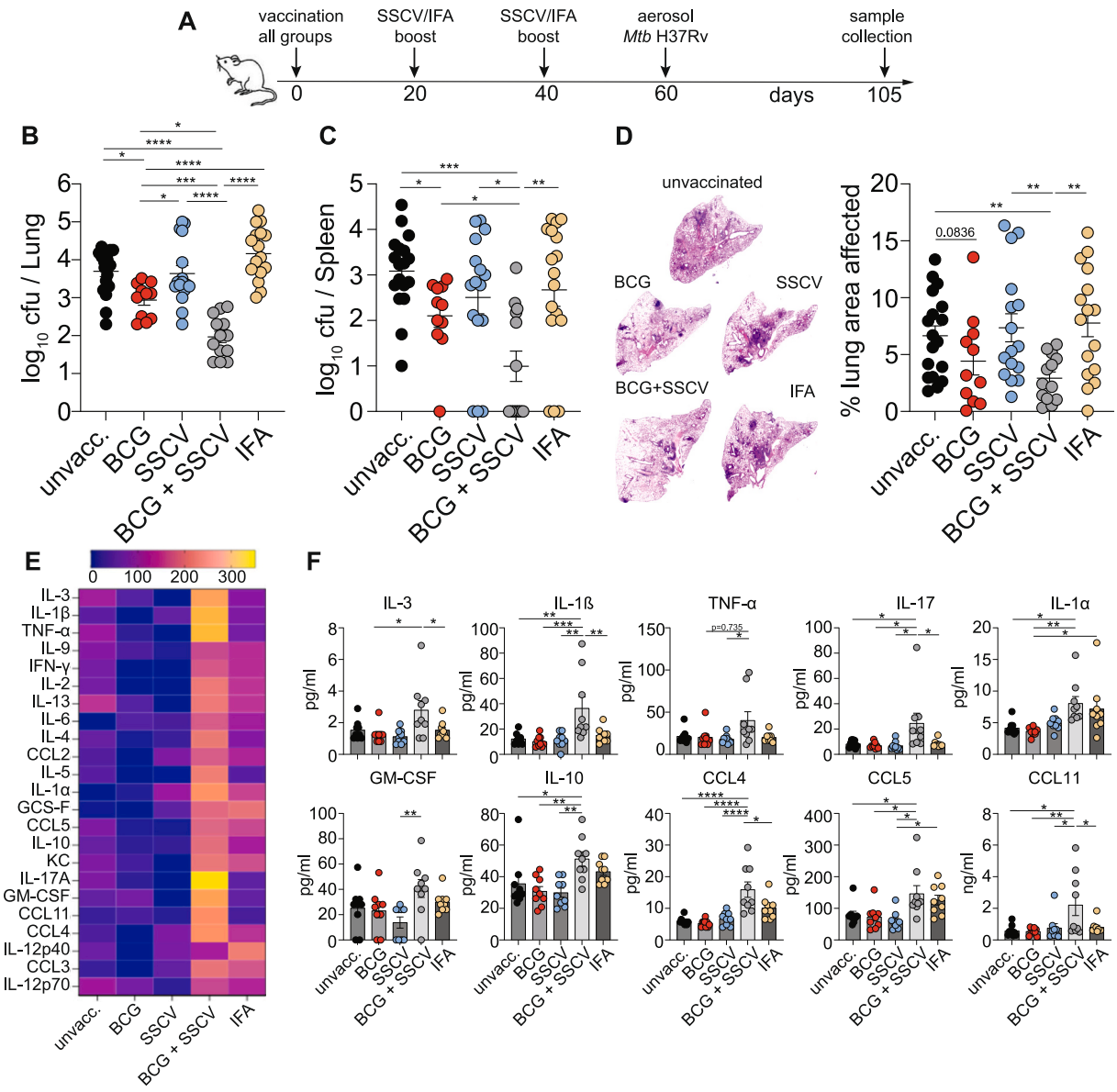

Fig. 1. Co-administration of ST208-309 and BCG provides improved protection against murine tuberculosis. C57BL/6 mice were vaccinated and subsequently challenged with *Mtb* (A). *Mtb* CFU in lung (B) and spleen (C), lung histopathology (D), and cytokine levels in serum (E, F) were assessed 45 days post-challenge. Results are presented as individual data points ± SEM (B, C, D, F), representative images (D) and data means heatmap (E) from 2 pooled independent experiments (n = 12–18 mice per group). Statistical analyses: One-way ANOVA followed by Tukey's multiple comparison test and Mann-Whitney U test; significant differences are indicated by asterisks: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

ST208-309 enhances the protective capacity of BCG in the C57BL/6 model of TB.

3.2. ST208-309-based enhancement of BCG efficacy wanes in aged mice

In humans, BCG efficacy often wanes with age [6,10]. To assess whether ST208-309 co-vaccination could offer added benefit in an aged host, where responses to traditional vaccines are often suboptimal, we repeated the experiment in mice that had been left to age for 6 months prior to vaccination (Fig. 2A). In line with the results obtained in young mice, neither ST208-309 nor IFA vaccination alone reduced bacterial burden in lungs (Fig. 2B) and spleens (Fig. 2C). BCG vaccination alone reduced CFU by ~1 log as expected. Co-administration of IFA with BCG had no beneficial effect on BCG efficacy. Interestingly, the positive impact of ST208-309 on BCG vaccination seen in young mice was not observed in older mice, with CFU levels in lungs and spleens not being significantly different between the BCG and the BCG + ST208-309 groups (Fig. 2B, C; Fig. S3B, C). Similarly, while all groups containing BCG showed the lowest level of lung histopathology, there was only a non-statistically significant improvement observed in the BCG + ST208-309 group (48.47 % reduction in infiltration) compared to BCG alone (35.6 % reduction in infiltration) and BCG + IFA (27.79 % reduction in infiltration) (Figs. 2D; S3D). Collectively, these results suggest that ST208-309 co-administration with BCG may be more beneficial at a younger age.

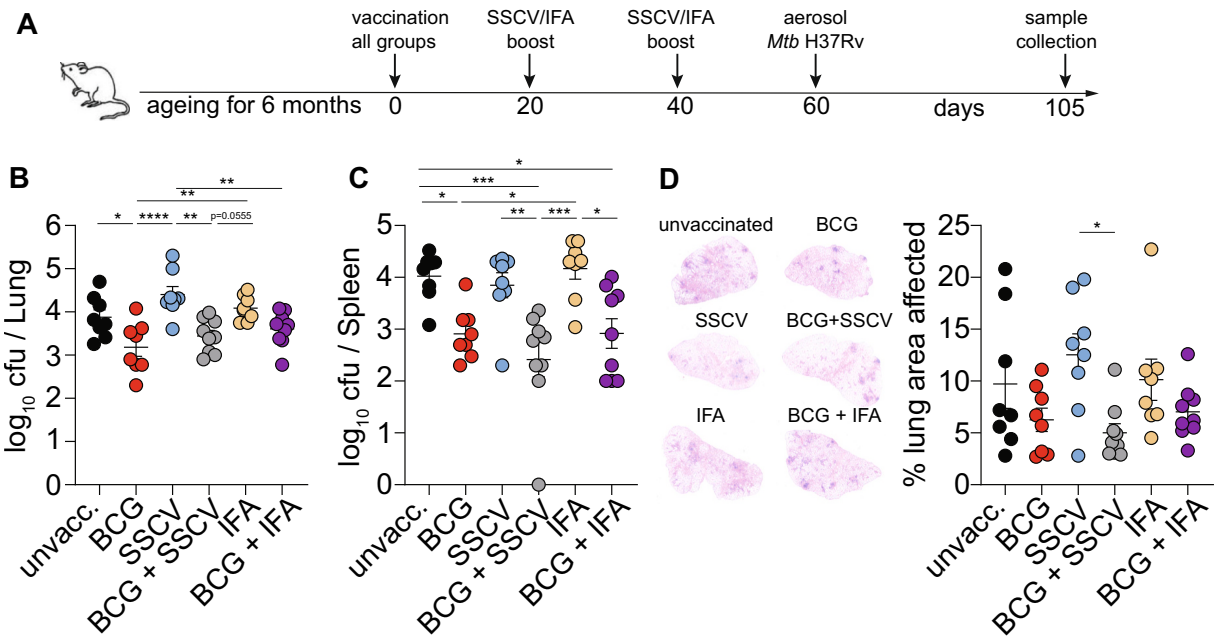

Fig. 2. ST208-309-based enhancement of BCG efficacy wanes in aged mice. C57BL/6 mice were left to age for 6 months. Subsequently, mice were vaccinated and challenged with *Mtb* (A). *Mtb* CFU in lung (B) and spleen (C), and lung histopathology (D) were assessed 45 days post-challenge. Results are presented as individual data points ± SEM (B, C, D) and representative images (D) from 2 pooled independent experiments (n = 8–9 mice per group). Statistical analyses: One-way ANOVA followed by Tukey's multiple comparison test; significant differences are indicated by asterisks: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

4. Discussion

It is widely acknowledged that the TB vaccine pipeline needs diversification and expansion. This may include alternative approaches to improve BCG efficacy [8]. Here we tested the hypothesis that a synthetic saccharide conjugate vaccine could improve protective immunity against *Mtb*. Our data reveal a significant reduction in bacterial burden when ST208-309 is co-administered with BCG, suggesting an augmented protective effect against TB. This is a significant finding, considering the limitations of BCG and the difficulties in developing a better TB vaccine [11]. The synergistic reduction in *Mtb* CFU of approximately 1 log following ST208-309 co-administration is on par with or even better than the results obtained in the C57BL/6 model of TB by some of the most advanced TB vaccine candidates, such as MTBVAC, VPM1002 and H107 [12,13]. It is important to note that CFU levels observed in this study are on the lower end of what is typically observed with H37Rv challenges in C57BL/6 mice, suggesting a comparatively low virulence of our *Mtb* H37Rv isolate. While signs of murine TB, including lung histopathology, were reduced in co-vaccinated young mice, the enhanced protection waned in mice that had been left to age for six months prior to vaccination. These results suggest that BCG/ST208-309 co-vaccination could be beneficial in a paediatric and perhaps adolescent population, but the diminished effect in aged mice questions the translatability to older populations.

While it was beyond the scope of this study, our results call for a deeper exploration of the immunological mechanisms underlying the observed protective effects of ST208-309 co-administration in young mice. It is very likely that the conserved mannose sequence in ST208-309 triggers a robust humoral immune response against *Mtb* PIMs. Although ST208-309 contains 4 mannoses corresponding to the 4 terminal mannoses of PIM_6, it is also possible that such putative antibodies cross-react with the 3 terminal mannoses of the immediate precursor molecule PIM_5. As PIM_5 and PIM_6 are the only structures known from *Mycobacterium spp.* that express homology with the synthetic glycan used in vaccination, it follows that PIM_5 and PIM_6 may be legitimate vaccination targets using synthetic glycans. Both PIM_5 and PIM_6 additionally elaborate the structure Manα1-6Manα1-6*myo*-inositol-1,2-cyclic-PO_4 in a 1–6 linkage to the terminal mannose residues targeted by ST208-309, and inclusion of this latter structure or variants thereof may increase the efficacy of synthetic glycan vaccination.

Pure *Mtb* PIMs containing lipids have been trialled as vaccine antigens to target donor-unrestricted T cells (DURTs) in humans, cattle and guinea pigs [14,15]. While we did not investigate if DURTs recognise conserved structures between PIMs and ST208-309, this would be unlikely as ST208-309 lacks lipids which are required for presentation of glycolipids to T cells.

It remains unclear why BCG efficacy wanes in adolescence, and both increased distance from the equator and less exposure to environmental mycobacteria have been linked to longer lasting protective efficacy [6]. It has been suggested that the age-related decline in BCG efficacy could also be due to immunosenescence, a gradual deterioration of immune function with age [16]. But given that BCG efficacy often drops in early adulthood, a period of peak immune fitness, age-related deterioration of the immune system is unlikely to be a major driver. On the other hand, puberty, driven by changing levels in sex hormones, has a profound impact on the immune system. It is well established that certain autoimmune diseases, such as multiple sclerosis, predominantly occur after puberty, and that the functional capacity of many immune cell subsets changes in puberty [17]. It is therefore conceivable that waning immunity of BCG in adolescence could be a consequence of sex hormone-related changes to the immune system. Understanding the mechanisms behind the decline in BCG efficacy with age is crucial for optimizing vaccine strategies for older populations and should remain a major research priority for TB vaccine development.

The promising results shown here present an intriguing case for an integrated vaccination approach against TB. The composition and formulation of ST208-309 is likely to offer both comparatively low cost-of-goods and a much simpler production. Due to the structural similarities between mycobacterial PIMs and glycosylphosphatidylinositol, a malaria parasite toxin that is highly conserved across malaria species and life stages, it could also be worth exploring if SSCV-mediated boost of BCG immunity provides some cross-protection against malaria infection. Collectively, our study calls for further exploration of synthetic saccharide-based vaccines and co-vaccination approaches to

achieve more effective control of TB.

CRediT authorship contribution statement

Socorro Miranda-Hernandez: Writing – review & editing, Investigation, Formal analysis, Data curation. **Harindra D. Sathkumara:** Writing – review & editing, Visualization, Investigation, Formal analysis. **Guangzu Zhao:** Formal analysis, Investigation, Methodology, Visualization, Writing – review & editing. **Louis Schofield:** Writing – review & editing, Writing – original draft, Resources, Methodology, Funding acquisition, Formal analysis, Conceptualization. **Andreas Kupz:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

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Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Andreas Kupz reports financial support was provided by National Health and Medical Research Council. Louis Schofield reports financial support was provided by Australian Tropical Medicines Commercialization. Louis Schofield reports a relationship with Saccharide Therapeutics that includes: consulting or advisory and equity or stocks. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2025.127912.

Data availability

Data will be made available on request.

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