Mucosal delivery of a multistage subunit vaccine promotes development of lung-resident memory T cells and affords interleukin-17-dependent protection against pulmonary tuberculosis

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The development of effective vaccines against bacterial lung infections requires the induction of protective, pathogen-specific immune responses without deleterious inflammation within the pulmonary environment. Here, we made use of a polysaccharide-adjuvanted vaccine approach to elicit resident pulmonary T cells to protect against aerosol Mycobacterium tuberculosis infection. Intratracheal administration of the multistage fusion protein CysVac2 and the delta-inulin adjuvant Advax (formulated with a TLR9 agonist) provided superior protection against aerosol M. tuberculosis infection in mice, compared to parenteral delivery. Surprisingly, removal of the TLR9 agonist did not impact vaccine protection despite a reduction in cytokine-secreting T cell subsets, particularly CD4+ IFN-γ+ IL-2+ TNF-α multifunctional T cells. CysVac2/Advax-mediated protection was associated with the induction of lung-resident, antigen-specific memory CD4+ T cells that expressed IL-17 and RORγT, the master transcriptional regulator of Th17 differentiation. IL-17 was identified as a key mediator of vaccine efficacy, with blocking of IL-17 during M. tuberculosis challenge reducing phagocyte influx, suppressing priming of pathogen-specific CD4+ T cells in local lymph nodes and ablating vaccine-induced protection. These findings suggest that tuberculosis vaccines such as CysVac2/Advax that are capable of eliciting Th17 lung-resident memory T cells are promising candidates for progression to human trials.

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INTRODUCTION

Tuberculosis (TB) remains a major cause of morbidity and mortality worldwide, with 10 million new cases and 1.5 million deaths per year1. Mycobacterium bovis bacillus Calmette–Guérin (BCG) is currently the only licensed vaccine against TB, however, its efficacy varies greatly, especially against the adult pulmonary form of the disease2. The 2015 WHO End TB Strategy identified the development of a more effective and easily administered vaccine for controlling TB and halting the global epidemic3. In recent decades, extensive research has resulted in many new TB vaccine candidates, 14 of which are currently in human vaccine trials and are reviewed in detail elsewhere4. Recently, a Phase Ib clinical trial of the fusion protein vaccine M72/AS01E showed protective efficacy of 50% in M. tuberculosis-infected adults after 3 years5,6. Although promising, vaccines with higher efficacy are considered necessary to reduce TB incidence to the targets outlined in the End TB Strategy objectives7.

One of the major limitations of current vaccination strategies is that the administration route may not be optimal for the induction of memory immune responses at the site of pathogen entry, i.e., the lung. Pulmonary vaccine delivery has been hindered by the fact that most adjuvants are either unable to induce sufficient mucosal immunity or are too toxic to be administered to the lung8. However, recent evidence suggests that the mucosal vaccination may provide superior protection against respiratory M. tuberculosis infection over parenteral vaccination. For example, lung-resident CD4+ memory T cells (T RM) induced after pulmonary vaccination with a recombinant influenza virus expressing M. tuberculosis antigens provided protection in the lung in the absence of circulating memory cells9. T RM induction has also been proposed as the possible mechanism of protection in macaques that demonstrate sterilizing immunity after intravenous vaccination with BCG and subsequent M. tuberculosis infection10. When administered through the mucosal route, BCG induced better protection compared to the intradermal immunization, which was linked to lung T RM and Th17 polarization of the CD4+ T cells10. Th17 responses have been associated with the influx of neutrophils with bactericidal activity11 and increased CD4+ T cell recruitment to the lung after M. tuberculosis infection12. Vaccines inducing high levels of pulmonary IL-17 have demonstrated efficacy against M. tuberculosis in different animal models13,14, although balancing the protective and pathogenic roles of IL-17 in the lung is a critical consideration15.

In this study we sought to determine if the candidate TB vaccine, CysVac2/Advax16, is effective as a mucosal vaccine to protect against M. tuberculosis. CysVac2 is a fusion protein of two M. tuberculosis antigens; the immunodominant Ag85B and CysD, a component of the sulfur assimilation pathway that is over-expressed in chronic stages of infection17. Advax is a particulate

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poly saccharide adju vant with a low inflammatory profile that has proven to be safe and a strong inducer of vaccine immunogenicity in humans, thus making it an ideal candidate for mucosal administration. Advax was recently shown to provide safe and effective enhancement of influenza vaccine immunity when administered via the intrapulmonary route in different animal models. Notably, it is currently being used in Phase I clinical trial as part of COVAX-19, a candidate vaccine for COVID-19 (NCT04453852).

We report here that intrapulmonary administration of CysVac2/Adavax-induced greater protection in mice than parenterally administered vaccine, with the vaccine promoting the accumulation of antigen-specific, IL-17-secreting CD4+ T cells in the lungs. Furthermore, IL-17 was essential for the protective efficacy afforded by the intrapulmonary CysVac2/Adavax vaccine, thus defining a crucial role for this cytokine in vaccine-mediated control of TB.

RESULTS

Pulmonary administration of CysVac2/AdvaxCpG provides superior protection against M. tuberculosis challenge than parenteral vaccination

Previous studies of intramuscular (i.m.) vaccination of mice with CysVac2/AdvaxCpG demonstrated substantially enhanced systemic CD4+ T cell responses composed of multifunctional Th1 polarized cells, which correlated with protection against aerosol M. tuberculosis infection. In this study, we evaluated if delivery to the lung by intratracheal (i.t.) instillation of this vaccine candidate could improve the level of protection induced by this vaccine. Mice were vaccinated by either the i.t. or i.m. routes with CysVac2/AdvaxCpG three times, 2 weeks apart (Fig. 1a). When the vaccine-specific T cell response was examined in the blood prior to M. tuberculosis challenge (following the gating strategy outlined in Supplementary Fig. 1), a higher level of circulating polyfunctional CD4+ T cells expressing IFN-γ was present after i.m. vaccination (Fig. 1b and Supplementary Fig 2a), with the most prominent phenotype identified as multi-cytokine-secreting CD4+CD4+ T cells (Fig. 1c). After i.t. vaccination with CysVac2/AdvaxCpG, PBMC-derived CD4+ T cells expressing either IL-2, TNF, or IL-17 were more prominent when compared to the i.m. route (Fig. 1b). Both vaccination regimens induced similar proportions of T-bet expression in circulating CD4+ T cells, however, i.t. vaccination induced a higher proportion of cells expressing RORγT (Fig. 1d).

The pattern of CD4+ T cells immune responses pre-M. tuberculosis challenge was compared to that after M. tuberculosis infection. In i.m. vaccinated mice, the greatest frequency of CD4+ T cells observed were those secreting IFN-γ or TNF (Fig. 1e) and this was dominated by cells with polyfunctional Th1 responses (IFN-γ+IL-2+TNFα, Fig. 1f). I.t. vaccination with CysVac2/AdvaxCpG resulted in a high frequency of CD4+ T cells secreting either IL-17 or TNF (Fig. 1e) or both cytokines (Supplementary Fig 2b); these T cell subsets were not observed after i.m. vaccination (Fig. 1e and Supplementary Fig. 2). Further, the frequency of CD4+ T cells expressing RORγT was significantly enhanced after i.t. vaccination compared to unvaccinated or i.m. vaccinated mice (Fig. 1g). Therefore, i.t. vaccination of mice with CysVac2/AdvaxCpG results in a Th17-polarized T cell response post-M. tuberculosis exposure, which was not observed after i.m. immunization.

Considering the differential pattern of immune responses induced by varying the route of administration of CysVac2/AdvaxCpG, we next determined if this had an impact on protective efficacy. I.t. vaccinated mice challenged with low dose aerosol M. tuberculosis, demonstrated significantly enhanced lung protection when compared to i.m.-vaccinated or unvaccinated mice (Fig. 1h). A similar result was observed in the spleen, suggesting that i.t. vaccination might improve protection against disseminated infection (Fig. 1i). Taken together, these results demonstrate that pulmonary vaccination with CysVac2/AdvaxCpG induces superior protection compared to i.m. vaccination and this is associated with an enhanced generation of Th17 cells in the circulation and in the lung.

CpG is dispensable for protection generated by pulmonary vaccination with CysVac2/Advax

CpG oligonucleotides are TLR9 agonists that help drive Th1 immune responses. While the CpG component was shown to be important to the protection obtained after CysVac2 i.m. immunization, we were interested whether a simplified formulation of Advax without the CpG component would still generate protective pulmonary immunity. CysVac2/Advax or CysVac2/AdvaxCpG were delivered by the i.t. route, and the mice challenged with aerosol M. tuberculosis. Both vaccines resulted in the generation of IL-17-producing CD4+ T cells following antigen re-stimulation ex vivo (Fig. 2a), however, all inflammatory cytokines (IFN-γ, IL-17, TNF) were reduced in Advax-immunized as compared to AdvaxCpG-, vaccinated mice (Fig. 2a). Strikingly, the removal of the CpG component resulted in the loss of multifunctional CD4+ T cells with a triple cytokine-secreting profile (IFN-γ+IL-2+TNFα) (Fig. 2b and Supplementary Fig. 3). However, both vaccinated groups displayed equivalent expression of the transcription factors T-bet (Fig. 2c) and similar protection against M. tuberculosis in the lungs (Fig. 2c) and spleen (Fig. 2d). This indicates that CysVac2/Advax is sufficient for protection and this protection does not correlate with the presence of multifunctional T cells secreting high levels of inflammatory cytokines.

Intrapulmonary CysVac2/Advax stimulates antibody formation and iBALT formation in the lung

We further sought to define the immunogenic profile of CysVac2/Advax by defining the generation of humoral responses after pulmonary vaccination. Analysis of serum antibody titers revealed CysVac2-specific IgG1 antibodies at all time points examined, that was at least 1 log higher in the CysVac2/Advax group than mice vaccinated with antigen alone (Fig. 3a). IgG2c levels were comparably lower than that IgG1, particularly early after vaccination, but significantly greater at week 4 post-vaccination in CysVac2/Advax-vaccinated (Fig. 3b). We were also able to detect low levels of IgA in the serum of mice vaccinated i.t. with CysVac2/Advax, but not in other groups (Fig. 3c). Immunofluorescence staining of lung sections at 4 weeks post-infection identified the presence of agglomerates of T and B cells in peribronchial areas of the lung in mice vaccinated with CysVac2/Advax but not in mice vaccinated with PBS or CysVac2 alone, suggesting the formation of inducible Bronchial associated lymphoid tissue (iBALT) structures by the vaccine (Fig. 3d). Overall these observations indicate that mucosal vaccination with CysVac2/Advax is able to induce the secretion of CysVac2-specific antibodies and the formation of iBALT structures in the lung.

Intrapulmonary CysVac2/Advax generates lung-resident, antigen-specific CD4+ T cells

To more precisely define the vaccine-specific cellular responses after immunization with CysVac2/Advax, Ag85B:I-Ab tetramer staining was employed to identify CD4+ T cells specific for the p25 epitope of Ag85B, an antigenic component of CysVac2. Ag85B tetramer-positive cells in the lungs were only detected after i.t. delivery of CysVac2/Advax and not after CysVac2 antigen alone, confirming the critical role of Advax in inducing antigen-specific T cell expansion (Fig. 4a). Ag85Btet+ cells were present in significantly greater numbers in CysVac2/Advax-vaccinated samples at all time points examined, although numbers contracted by 8 weeks post-vaccination (Fig. 4b). CysVac2/Advax-vaccinated mice showed enrichment of lung parenchymal-residing (intravascular negative IV') CD4+ T cells,
which expressed CD69 and CD44 with a low level of expression of the lymphoid homing receptor, L-selectin (CD62L) (Fig. 4d). This population of CD4^+CD44^hiCD62L^lowCD69^+ IV^- were defined as TRM-like cells\(^2^3\) and were significantly greater at all times points post-vaccination in CysVac2/Advax-vaccinated mice, comprising approximately 25% of total CD4^+ T cells in the lung following vaccination (Fig. 4c). The majority of Ag85B-tet^+ cells detected at 8 weeks post-vaccination showed a TRM-like phenotype and were present within the parenchyma of the lung (Fig. 4e). Along with TRM markers, this subset expressed high levels of the integrin
CD11a and the cell surface receptor PD-1, with low levels of expression of CD103 and KLRG-1 (Fig. 4e). Taken together, these data indicate that CysVac2/Advax vaccination induces a population of antigen-specific CD4⁺ T cells in the lung with a T RM phenotype, and these are detectable through 8 weeks post-vaccination.

We further characterized the ability of the CD4⁺ T cells generated in the lung to produce cytokines in response to re-stimulation with vaccine antigen at time points after vaccination (pre-challenge) as well as at 4 weeks post-challenge with M. tuberculosis. Intratracheal CysVac2/Advax vaccination induced a distinct cytokine profile that was relatively consistent across all time points prior to M. tuberculosis infection. While a marginal increase in IFN-γ production was observed at 4 weeks post-vaccination, this was not apparent at any other time point (Fig. 4a). Indeed, following infection with M. tuberculosis, a lower percentage of IFN-γ-producing CD4⁺ T cells

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**Fig. 1** Pulmonary vaccination with CysVac2/AdvaxCpG demonstrates improved protection against M. tuberculosis infection compared to parenteral administration. C57BL/6 mice (n = 5–6) were vaccinated by either the i.m. or i.t. route with CysVac2(CV2)/AdvaxCpG (three times, 2 weeks apart). One week after last vaccination mice were bled for vaccine immunogenicity assessment. Six weeks after last immunization mice were challenged with H37Rv by aerosol (~100 CFU) and 4 weeks later culled to enumerate the bacterial burden and the T cell phenotype in the lung (a). PBMCs from tail blood of vaccinated mice (b–d) or cells from lung of infected mice (e–g) were restimulated ex vivo with CysVac2, and the production cytokines (IFN-γ, IL-2, IL-17, TNF), or transcription factors (TF; T-bet, RORγt) by CD4⁺ T cells was determined by flow cytometry using the gating strategy described in Supplementary Fig. 1. Data are represented as the percentage of cytokine-producing or transcription-factor-positive CD4⁺ T cells ± SEM. Bacterial load was assessed in the lungs (h) and in the spleen (i) and presented as log₁₀ of the mean CFU ± SEM. Data are pooled from three independent experiments. The significance of differences between the groups was determined by ANOVA (*p < 0.05; **p < 0.01; ***p < 0.001).

**Fig. 2** CpG is dispensable for protective immunity induced by CysVac2/Advax. C57BL/6 mice (n = 5–10) were vaccinated by the i.t. route with CysVac2/AdvaxCpG or CysVac2/Advax (three times, 2 weeks apart). Six weeks after last immunization mice were challenged with M. tuberculosis H37Rv by aerosol (~100 CFU) and four weeks later culled to enumerate the bacterial burden and the T cell phenotype in the lung. Cells from the lung of infected mice were restimulated ex vivo with CysVac2 and the production of cytokines (IFN-γ, IL-2, IL-17, TNF (a, b)) or transcription factors (T-bet, RORγt; c) by CD4⁺ T cells was determined by flow cytometry using the gating strategy described in Supplementary Fig. 1. Data are represented as the percentage of cytokine-producing CD4⁺ T cells ± SEM. Bacterial load was assessed in the lungs (d) and in the spleen (e) and presented as log₁₀ of the mean CFU ± SEM. Data are pooled from two independent experiments. The significance of differences between the groups was determined by ANOVA (*p < 0.05; **p < 0.01; ***p < 0.001).
was present in CysVac2/Advax-vaccinated lung samples compared to samples from unvaccinated animals. By contrast, percentages of IL-2, IL-17, or TNF cytokine-producing cells were markedly increased after i.t. vaccination with CysVac2/Advax compared to unvaccinated or CysVac2 vaccinated mice up to 8 weeks post-vaccination (Fig. 5b–d), however only IL-17 remained elevated post-challenge (Fig. 5b). Further analysis revealed that the major source of IL-17 originated from CD45 IV-CD4+ T cells that expressed RORγt (Fig. 5e). Furthermore, the Ag85Btet+ population was highly enriched in RORγt+ CD45 IV-IL-17+ CD4+ T cells (Fig. 5f). Overall, these data suggest that pulmonary vaccination with CysVac2/Advax promotes increased single and multifunctional cytokine-producing T cell populations both before and after aerosol challenge with M. tuberculosis, which is characterized by the development of a tissue-resident Th17-type response.

IL-17-mediated protection correlates with early recruitment of phagocytic cells and enhanced priming of pathogen-specific CD4+ T cells

Given the marked Th17 polarization of the CD4+ T cell response to pulmonary immunization with CysVac2/Advax, we next determined the impact of neutralizing IL-17 at the time of M. tuberculosis challenge (Fig. 6a). Treatment with anti-IL-17 mAb did not affect the capacity of CD4+ T cells to respond to infection, as the frequency of cytokine-producing CD4+ T cells was not altered between mice treated with anti-IL-17 or isotype control mAb (Fig. 6b). However, anti-IL-17 treatment had a detrimental effect on control of bacterial infection in the lung of CysVac2/Advax-immunized mice; bacteria numbers in anti-IL-17-treated mice were similar to unvaccinated mice, while immunized mice treated with isotype control mAb remained protected against infection (Fig. 6c).

We also studied the impact of IL-17 blocking on lung cell subsets after M. tuberculosis infection using flow cytometry phenotyping combined with an unsupervised visual implementation of t-distributed stochastic neighbor embedding (tSNE) analysis. The generated tSNE plot was calculated with 12 parameters and 10 clusters obtained using unsupervised analysis were subsequently assigned to a specific cell population (Fig. 6d), according to the expression level of each marker and previously described phenotypes (Supplementary Fig. 4). This analysis revealed that the percentage of neutrophils in the lung were elevated in vaccinated mice treated with control mAb, however, they returned to the level of unvaccinated animals after treatment with the anti-IL-17 mAb (Fig. 6e). A similar pattern was observed for monocytes and monocyte-derived-macrophages, however, no differences were observed for other phagocytic populations in the lung, such as alveolar macrophages (Fig. 6e).

We next investigated the effect of blocking IL-17 on the priming and proliferation of CD4+ T cells. To do this, we examined CD4+ T cells primed by the vaccination (Ag85Btet+) and compared to T cells responding specifically to M. tuberculosis (ESAT6-tet+). In the lung, we observed a greater proportion of Ag85B-tet+ cells in vaccinated mice compared to unvaccinated mice, however, anti-IL-17 treatment did not significantly alter the numbers of proliferating CD4+ T cells (Supplementary Fig. 5a–c) or the numbers of Ag85B-tet+ or ESAT6-tet+ cells (Supplementary Fig. 5d–f). However, in the mLN of vaccinated mice a distinct vaccine-induced population of proliferating CD4+ T cells expressing RORγt was distinguishable (Fig. 7a). IL-17 blocking resulted in reduced proliferation of total CD4+ T cells in the mLN of vaccinated mice.

Fig. 3 Pulmonary vaccination with CysVac2/Advax induces antigen-specific serum antibodies and the formation of iBALT in the lungs of vaccinated mice. C57BL/6 mice (n = 3–4) were vaccinated by the i.t. route with CysVac2/Advax or CysVac2 (three times, 2 weeks apart). Before the first and second boost, as well as 2 and 4 weeks after the second boost, mice were bled from the lateral tail vein and serum was collected. Titers of IgG1 (a) and IgG2c (b) were calculated by ELISA over the course of the time points examined. IgA (c) was measured by ELISA in the serum of week 2 samples. Data are represented as the average of log10 titers ± SEM or average 450 nm absorbance ± SEM. Four weeks after the final vaccination, lung sections were stained with anti-mouse B220 (red) and anti-mouse CD3 (green) to visualize iBALT structures and imaged at ×20 magnification (d). Images are representative of three biological replicates/group; scale bars represent 50 microns. The significance of differences between the groups was determined by ANOVA (p < 0.05).
Fig. 4  Pulmonary vaccination with CysVac2/Advax induces Ag-specific persistent local resident CD4+ T cells. C57BL/6 mice (n = 3–4) were vaccinated by the i.t. route with CysVac2/Advax or CysVac2 (three times, 2 weeks apart). At weeks 2, 4, or 8 after the final immunization, lung cells were processed for Ag85B-I-A^+A^ tetramer staining. Representative dot plot from 8 weeks post-immunization of CD4^+^ T cells gated as outlined in Supplementary Fig. 1 (a). Number of Ag85B-I-A^+A^ tetramer-positive cells in the lung over time are shown in b. Also shown are the percentage of total CD4^+^ T cells expressing phenotypic makers markers associated with TRMs (CD45 IV^−^, CD11a^+^, CD69^+^, CD44^+^, PD-1^+^, KLRG-1^−^ (c)) and representative dot plots of TRMs markers on total lung CD4^+^ T cells (d) or in Ag85B-I-A^+A^ cells (e) at 8 weeks after last vaccination. Data are presented as mean ± SEM and are representative of two independent experiments. The significance of differences between the groups was determined by ANOVA (*p < 0.05; **p < 0.01; ***p < 0.001).
Fig. 5 Persistent CysVac2-specific IL-17 production by lung-resident CD4^+ T cells after pulmonary vaccination with CysVac2/Advax. C57BL/6 mice (n = 3–5) were vaccinated by the i.t. route with CysVac2/Advax or CysVac2 protein alone (three times, 2 weeks apart). Eight weeks after the last immunization mice were challenged with M. tuberculosis H37Rv by aerosol (~100 CFU). At 2, 4, or 8 weeks after last immunization (solid bars), and at 4 weeks after infection (striped bars), lung cells were restimulated ex vivo with CysVac2, and the production of IFN-γ (a), IL-17 (b), IL-2 (c), or TNF (d) by CD4^+ T cells determined by flow cytometry using the gating strategy described in Supplementary Fig. 1. Representative dot plots of co-expression of CD45 IV or RORγT with IL-17 by total CD4^+ T cells (e) or Ag85B:IA^b tetramer-positive cells (f) at 8 weeks after last vaccination. Data are represented as the percentage of cytokine-producing CD4^+ T cells ± SEM and are representative of two independent experiments. The significance of differences between the groups was determined by ANOVA (*p < 0.05; **p < 0.01; ***p < 0.001).

(Fig. 7b) including the vaccine-primed RORγT^+ cells (Fig. 7c). When the frequency of the vaccine-primed CD4^+ T cells (Ag85B-tet^+) was compared to those primed only by M. tuberculosis infection (ESAT6-tet^+) (Fig. 7d), Ag85B-tet^+ cells were significantly higher in vaccinated mice compared to the unvaccinated group, however, anti-IL-17 treatment did not affect the numbers of Ag85B-tet^+ cells (Fig. 7e). By contrast, the numbers of ESAT6-tet^+ positive CD4^+ T cells were reduced after IL-17 blocking in the mLNs (Fig. 7f). Overall, neutralizing IL-17 reduced phagocytic cells in the lung of immunized animals and the numbers of pathogen-specific CD4^+ T cells in the draining LNs, which correlated with a loss of vaccine-mediated protection.
Protection afforded by pulmonary CysVac2/Advax against aerosol *M. tuberculosis* is dependent on IL-17 and correlates with lung phagocytic cell recruitment. C57BL/6 mice (*n* = 5–6) were vaccinated by the i.t. route with CysVac2/Advax (three times, 2 weeks apart), and at 8 weeks after the last immunization mice were challenged with *M. tuberculosis* H37Rv by aerosol (~100 CFU). One day before aerosol, mice were treated i.p. with anti-IL-17 or an isotype control mAb (twice weekly for 3 weeks) (a). Cells from the lungs of infected mice were restimulated ex vivo with CysVac2 and cytokines secretion (IFN-γ, IL-2, IL-17, TNF) determined by flow cytometry using the gating strategy described in Supplementary Fig. 1 (b). Bacterial load was assessed in the lungs and is presented as Log_{10} of the mean CFU ± SEM (c). Representative tSNE dimensions 1 and 2 plots of the total live cells in the lung (d). Bar graphs show the average of the percentage ± SEM of identified lung cell subsets (e). Data are pooled from two independent experiments. The significance of differences between the groups was determined by ANOVA (*p* < 0.05; **p** < 0.01; ***p** < 0.001).
adjuvants resulting in protective immune responses. When γ the CysVac2/Advax vaccine (Fig.5), an important post-challenge that did not correlate with the protective effect of Indeed, in our data IFN- α administered to the respiratory mucosa, highly in inflammation and pathology. We found that i.t. delivery of CysVac2/Advax was significantly more protective than i.m. vaccination against challenge with M. tuberculosis (Fig. 1). Strikingly, i.t. vaccination, despite being more protective, did not induce appreciable numbers of multifunctional CD4+ T cells (IFN-γ IL-2 TNFα; Fig. 1) and even more surprisingly removal of CpG from the formulation did not reduce vaccine protection, despite a loss of multifunctional CD4+ T cell generation (Fig. 2). Induction of multifunctional T cell responses has been used as a key criterion for vaccine progression to human trials, however recent evidence in both mice and humans indicates that the generation of IFN-γ secreting T cells does not necessarily correlate with protection. Indeed, in our data IFN-γ was the only cytokine analyzed pre- and post-challenge that did not correlate with the protective effect of the CysVac2/Advax vaccine (Fig. 5), an important finding for the selection of vaccines for progression to human trials.

We observed that pulmonary vaccination with CysVac2 vaccination induced a lung-resident CD4+ population that expressed markers of TRM-like cells (Fig. 4). We have previously shown that a recombinant influenza vaccine conferred protection against M. tuberculosis in the absence of circulating memory T cells, suggesting an important role for M. tuberculosis-specific TRM. In the current study, detailed phenotypic analysis of CysVac2/Advax-induced, antigen-specific CD4+ TRM-like cells showed that in addition to well-characterized markers of tissue-resident memory cells (CD69+ CD44hi CD62Llow CD45 IV+), this population displayed high levels of CD11a with minimal expression of CD103, which low level of CD103 expression contrasts with the increased expression of CD103 on CD8+ TRM, where this integrin is thought to be essential in the retention of these cells within tissue. We also noted the distinct formation of iBalt structures in the lung after CysVac2/Advax-delivery (Fig. 3); iBalt is thought to provide a survival niche for TRM and thus may participate in the protective response afforded by CysVac2/Advax vaccination. Antigen-specific TRM cells induced following CysVac2/Advax vaccination also displayed a dominant PD-1+ KLRG-1+ phenotype (Fig. 4). During M. tuberculosis infection, PD-1 expression indicates an earlier stage of CD4+ T cell differentiation that is associated with a higher proliferative capacity, while KLRG-1+ cells are more terminally differentiated and produce greater levels of cytokines. IL-2-secreting KLRG-1+ T cells induced by subunit booster vaccination are associated with protection against chronic M. tuberculosis, owing to the maintenance of their proliferative capacity and circulating KLRG-1+ CD4+ T cells induced by subcutaneous H56/CAF01 vaccination display the ability to home to the lungs. Our findings confirmed an association between vaccine-induced, PD-1+ KLRG-1+ CD4+ T cells that reside within the lung parenchyma and protection against M. tuberculosis. It is possible these cells possess greater effector functions and proliferative capacity than their KLRG-1− counterparts and are therefore capable of enhanced protection against M. tuberculosis infection.

Lung-resident CD4+ TRM was found to be an important source of IL-17 induced after Advax:CysVac2 i.t. delivery, and this was not observed after i.m. delivery of the vaccine. This is in agreement with the complexity of the immunological environment in the lung potentially contributing to suboptimal pathogen responses. This may be particularly detrimental in the case of M. tuberculosis infection, where priming and recruitment of effector T lymphocytes to the lungs is delayed, allowing unchecked growth of the organism. For this reason, mucosal vaccination has been of interest in the field of TB vaccines, with pulmonary delivery of BCG, live recombinant viruses, or protein/adjuvants resulting in protective immune responses. When administered to the respiratory mucosa, highly inflammatory adjuvanted vaccines may induce protective immunity, but this is often accompanied by excessive inflammation, mucus accumulation, and eosinophilia. Thus, there is a need for adjuvants that can induce protective lung immunity in the absence of deleterious inflammation and pathology. We found that i.t. delivery of CysVac2/Advax was significantly more protective than i.m. vaccination against challenge with M. tuberculosis. The respiratory tract is the preferred port of entry of M. tuberculosis infection, where priming and recruitment of effector T cells in the mLN (Fig. 4). We have previously shown that a
Bacterial strains

*M. tuberculosis* H37Rv (BEI Resources, USA) and BCG Pasteur were grown at 37 °C in Middlebrook 7H9 medium (Becton Dickinson, BD) supplemented with 0.5% glycerol, 0.02% Tylopxol, and 10% albumin-dextrose-catalase (ADC), or on solid Middlebrook 7H11 medium (BD) supplemented with oleic acid–ADC.

Mouse immunization, treatments, and infection

CysVac2 fusion protein (Ag85B-CysD) was recombinantly expressed in *E. coli*, purified from inclusions bodies by ChinaPeptides (Shanghai, China), and refolded in Tris-buffer. Advax (delta-inulin, 50 mg/ml and AdvaxCpG (delta-inulin plus CpG, 50 mg/ml and 500 μg/ml, respectively) were provided by Vaxine Pty Ltd (Adelaide, Australia). Mice were anesthetized by intraperitoneal (i.p.) injection of Ketamine/Xylazine (80/100 mg/kg mouse) and then vaccinated with 1 mg of Advax or AdvaxCpG and 3 μg of CysVac2 in a final volume of 50 μl PBS via i.m. route, using an insulin syringe (BD), or via the i.t. route, using PennCentury Microsprayer Aerosoliser (PennCentury, PA, USA). Three μg of CysVac2 alone or sterile PBS were administered as controls where appropriate. Mice were immunized subcutaneously with 5 × 10^5 BCG for protection experiments.

For neutralization of IL-17, mice were injected i.p. with 250 μg of anti-IL-17A (clone TC11-18H10.1, Biolegend) or isotype control (clone RTK2071, Biolegend) one day prior to *M. tuberculosis* infection and then every three days for three weeks.

For *M. tuberculosis* challenge experiments, 6 or 8 weeks after the last vaccination mice were infected with *M. tuberculosis* H37Rv via the aerosol route using a Middlebrook airborne infection apparatus (Glas-Col, IN, USA) with an infectious dose of ~100 viable bacilli. Three or 4 weeks later, the lungs and spleen were harvested, homogenized, and plated after serial dilution on supplemented Middlebrook 7H11 agar plates. Colonies forming units (CFU) were determined 3 weeks later and expressed as log_{10} CFU.

For intravascular staining of leucocytes, 3 min before euthanasia, mice were i.v. injected with 200 μl biotin-conjugated anti-CD45 mAb in PBS (15 μg/ml, Biolegend, clone 104) into the lateral tail vein. The detection of biotin was performed with APC-Cy7-conjugated streptavidin (BioLegend).

Cell isolation, peptide stimulations, and flow cytometry

Mice were bled from the lateral tail vein and the PBMCs were isolated from whole blood by stratifying uncoupled whole blood on Histopaque1083 (Sigma-Aldrich). Single-cell suspensions were prepared from the lung by digesting the tissue with Collagenase IV and DNase (Sigma-Aldrich) for 45 min and dissociated using a gentleMACS dissociator (Miltenyi Biotec), the cell recovered were incubated in ACK lysis buffer to remove red blood cells. PE-conjugated Ag85B 240fl–254:I-Ab tetramer and APC-conjugated ESAT6, 26–36:A4 tetramer were provided by the NIH Tetramer Core Facility. For staining, cells were incubated with tetrathers at a 1:200 dilution for 1 h at 37 °C. Cells were stained using the marker-specific fluorochrome-labeled mAbs indicated in Supplementary Table 1. The gating strategy for the identification of specific cell populations is shown in Supplementary Fig. 1. To assess antigen-specific cytokine induction by T cells, PBMCs or single-cell suspensions from the lung were stimulated for 4 h with CysVac2 (5 μg/ml) and then supplemented with brefeldin A (10 μg/ml) for a further 10–12 h. Cells were surface stained with Fixable Blue Dead Cell Stain (Life Technologies) and the marker-specific fluorochrome-labeled antibodies indicated in Supplementary Table. Cells were then fixed and permeabilized using the BD Cytofix/CytopermTM kit according to the manufacturer’s protocol. When required, intracellular staining was performed using mAbs against the specific cytokines (Supplementary Table 1). Samples were acquired on a BD LSR-Fortessa (BD) and analyzed using FlowJoTM analysis software (TreeStar, USA). A Boolean combination of gates was used to calculate the frequency of single-, double- and triple-positive CD4+ T cell subsets. tSNE was run using default FlowJo parameters (iterations = 1000, perplexity = 30). Samples were randomly downsampled to 2000 events per sample and analysis was run on equal numbers of events per sample using the FlowJo tSNE plugin.

Detection of anti-CysVac2 antibodies

Microtiter plates were incubated overnight with 5 μg/ml CysVac2 antigen at 4 °C, blocked with 3% BSA, and serially diluted serum samples were incubated for 1 h at RT. Plates were washed and biotinylated polyclonal goat anti-mouse IgG2c (1:10,000, Abcam, cat# ab97253), rat anti-mouse IgG1 (1:500, clone RMG1-1, Biolegend, cat# 406603) or rat anti-mouse IgA

METHODS

Ethics statement

Female C57BL/6 (6–8 weeks of age) were purchased from Australian BioResources (NSW, Australia), and housed at the Centenary Institute animal facility (Sydney, Australia) in specific pathogen-free conditions. All mouse work was performed according to ethical guidelines as set out by the Sydney Local Health District (SLHD) Animal Ethics and Welfare Committee, which adhere to the Australian Code for the Care and Use of Animals for Scientific Purposes (2013) as set out by the National Health and Medical Research Council of Australia. All experiments within this manuscript were approved under protocol number 2017/011 by the SLHD Animal Ethics and Welfare Committee.
Immunofluorescence staining
Following perfusion, the superior right lung lobe from euthanized mice was excised and inflated with 4% paraformaldehyde (ThermoScientific) in PBS using a needle and syringe. Following fixation, lobes were transferred to 20% sucrose solution and then snap frozen at −80°C in Optimal Cutting Temperature compound (VWR chemicals). Lobes were sectioned using a Cyrotome E (ThermoScientific) to 12 µm thickness and slide allowed to dry at RT overnight before storage at −80°C. Prior to staining, slides were washed in PBS and non-specific antibody binding blocked with 3% Normal Goat Serum (Sigma) with 0.1% Triton-X 100 (Sigma) in PBS. Slides were probed with AF488-conjugated anti-mouse-CD3 at 1:100 dilution (Clone 17A2, Biologend, cat# 100210) and AF594-conjugated anti-mouse-B200 at 1:200 dilution (Clone RA3-6B2, Biolegend, cat# 103254) to visualize T and B cells, and NucBlue fixed cell stain ReadyProbes reagent (Life Technologies) to stain nuclei. Following staining, samples were mounted with Prolong Gold Antifade mountant (Thermo Fisher) and imaged using a BX51 microscope (Olympus, Japan) microscope.

Statistical analysis
Statistical analysis was performed using GraphPad Prism 6 software (GraphPad Software, USA). The significance of differences between experimental groups was evaluated by one-way analysis of variance (ANOVA), with a pairwise comparison of multi-grouped data sets achieved using the Tukey post-hoc test. Differences are considered as statistically different when \( p \leq 0.01 \).

Reporting summary
Further information on research design is available in the Nature Research Reporting Summary linked to this article.

DATA AVAILABILITY
The authors confirm that all relevant data are included in the paper and its Supplementary Information.

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AUTHOR CONTRIBUTIONS

C.C., N.P., W.J.B., and J.A.T. conceived and designed the study. C.C., A.A., K.F., G.N., E.S., and N.D.B. performed the experiments. All authors processed and analyzed the data. C.C. and J.A.T. wrote the first draft of the manuscript, all authors reviewed and approved the final manuscript version.

COMPETING INTERESTS

N.P. is the research director for Vaxine P/L. E.L.S. was an employee of Vaxine P/L. The remaining authors declare that they have no competing interests.

ADDITIONAL INFORMATION

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