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# Saponin TQL1055 adjuvant-containing vaccine confers protection upon *Mycobacterium tuberculosis* challenge in mice

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**ABSTRACT**

Tuberculosis (TB), caused by the intracellular pathogen *Mycobacterium tuberculosis* (*Mtb*), affects the lungs of infected individuals (pulmonary TB) but can also affect other sites (extrapulmonary TB). The only licensed vaccine *Mycobacterium bovis* bacillus *Calmette-Guerin* (BCG) protects infants and young children but exhibits variable efficacy in protecting against adult pulmonary TB. Poor compliance and prolonged treatment regimens associated with the use of chemotherapy has contributed to the development of multidrug-resistant (MDR) and extensively drug-resistant (XDR) *Mtb*. Thus, there is an urgent need for the design of more effective vaccines against TB. The development of safe and novel adjuvants for human use is critical. In this study, we demonstrate that saponin-based TQL1055 adjuvant when formulated with a TLR4 agonist (PHAD) and *Mtb* specific immunodominant antigens (ESAT-6 and Ag85B) and delivered intramuscularly in mice, the SA-TB vaccine induced potent lung immune responses. Additionally, the SA-TB vaccine conferred significant protection against *Mtb* infection, comparable with levels induced by BCG. These findings support the development of a SA-TB vaccine comprising TQL1055, as a novel, safe and effective TB vaccine for potential use in humans.

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## Introduction

Adjuvants included as components of vaccines have a major impact on vaccine efficacy via modulating and prolonging host immune responses. While vaccines are the most effective way to prevent and control infectious diseases, many pathogens that significantly impact human health remain without an effective vaccine. For example, *Mycobacterium tuberculosis* (*Mtb*) latently infects one-fourth of the world's population, causing pulmonary tuberculosis (PTB) in ~10.6 million people and resulting in ~1.6 million deaths each year.[1] The currently available TB vaccine, *Mycobacterium bovis* BCG (BCG), shows variable efficacy in protection against PTB.[2] In addition, multidrug resistant (MDR) *Mtb* strains have recently emerged. Thus, there is an urgent need for new TB vaccines.[3] TB vaccine development during the past decade has focused on targeting and production of the cytokine interferon-gamma (IFN-γ) from T helper 1 (Th1) cells to mediate early macrophage activation and bacterial killing.[4] A recombinant TB vaccine, MVA85A, was recently tested in human clinical trials. Despite inducing high levels of interferon gamma (IFN-γ) production from T cells,[5,6] this vaccine failed to protect against TB disease.[7,8] This is in contrast to the M72/AS01$_E$ TB vaccine, which also induced high levels of IFN-γ production from T cells, but prevented progression to pulmonary TB in adults.[9,10] We and others have shown that targeting the T helper type 17 (Th17) cells can enhance vaccine-induced immunity for TB.[11,12] In addition, B cells and antibodies were induced in the course of *Mtb* infection, and accumulate in the lung.[13] Antibodies from latently infected persons were found to be functionally protective when compared with patients with TB disease.[14] Additionally, vaccines that induce superior protection in rhesus macaques induced IgA, particularly at the mucosal surface.[15] In the absence of clear correlates of protection for TB vaccines, it is imperative that we explore new and effective approaches to target broad T and B cell responses for TB vaccine-induced immunity.

Shingrix (GSK) is a two dose, recombinant subunit vaccine that includes the adjuvant AS01B adjuvant (Liposomal QS-21 combined with the TLR4 agonist Monophosphoryl lipid, MPL). It provides significant (91–97%) efficacy in individuals ages 50 to 70$^+$ against shingles.[16] M72/AS01$_E$ vaccine comprises AS01E adjuvant (lower dose of adjuvant AS01B) along with a recombinant fusion protein, M72, derived from two *Mtb* antigens (*Mtb*32A and *Mtb*39A).[17] In general, saponins are excellent adjuvants, and QS-21 has been administered to millions of subjects and induces a balanced Th1/Th2 response.[18] However, some of the issues associated with use of AS01 is the tolerability of QS-21 with post-injection reactions, limited stability, and supply, expense and difficult procurement of QS-21, which is derived from a plant extract. The vaccine Mosquirix, which is a vaccine against malaria approved by the European Medicines Agency (EMA) also uses AS01 as an adjuvant.[19]

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TQL1055 is a new entity rationally designed and developed through extensive preclinical structure activity relationship (SAR) studies and is a simplified and stabilized version of natural saponin adjuvants with preclinical superiority for multiple vaccine antigens.[20,21] It does not rely on old-growth *Quillaja* and thus has a lower cost structure for manufacturing and can increase global saponin adjuvant output by many folds, compared to natural QS-21. Thus, TQL1055 is a saponin-based adjuvant that is preferable to use of AS01 as it is semi-synthetic, with increased and high-purity yield, increased stability, and increased cost-effectiveness.[20] The adjuvant used in this experiment included TQL1055 and the TLR4 agonist PHAD,[22,23] an MPLA mimic, rather than AS01.

In the current study, we tested whether SA-adjuvanted TB vaccine, specifically using TQL1055 and a TLR4 agonist will induce cellular and humoral immune responses and confer protection against *Mtb* infection. Our results show that intramuscular delivery of TQL1055 along with *Mtb* immunodominant antigens ESAT-6 and Ag85B (SA-TB vaccine) can confer *Mtb* control, to levels similar to BCG vaccination in mice but with reduced inflammation. Thus, our findings provide the basis for the development of a SA-TB vaccine as a novel, safe and effective TB vaccine for potential use in humans.

## Materials and methods

### *Mice*

C57BL/6J (B6) mice (Jackson Laboratories, Bar Harbor, ME) were maintained under specific pathogen-free conditions at the Washington University in St. Louis. Six-week old female mice formed the various study groups. A single study with $n = 10$ individual mice per group for immunogenicity (Day 14 post-final vaccination dose) and challenge studies (Day 14 and 28 post *Mtb* challenge following vaccinations) was carried out as outlined under the NIH Subcontract HHSN272201700066C (Figure 1). All animal experiments were performed in accordance with the Institutional Animal Care and Use Committee at WashU following approved protocols.

## Vaccination and *Mtb* infection

The antigen proteins, ESAT-6 and Ag85B, were prepared in-house as described in our previous work.[24,25] The recombinant *Mtb* antigens containing a His-tag were expressed in *E. coli* BL21 (DE3) pLysS and purified using Ni+ NTA affinity chromatography. Chromatography was carried out in column format and after unbound proteins are washed away, and the target proteins were eluted using imidazole. The SA-TB vaccine was prepared by simple mixing of ESAT-6 (1 μg) and Ag85B (1 μg) antigens with increasing amounts of TQL1055 adjuvant 5, 15 and 30 μg (referred to as SA-TB5, SA-TB15 and SA-TB30) combined with 5 μg of the TLR4 agonist PHAD in a liposomal formulation. The adjuvant only group mice received 40 μg of TQL1055 (Adjuvance Technologies Inc. Lincoln, NE) combined with 5 μg PHAD (Avanti ® Polar Lipids, Alabaster, AL, USA) in a liposomal formulation. To ensure a consistent injection volume, which is essential for evaluating tolerability, we utilized the stock solution of TQL1055 for the no antigen control. By not diluting the stock solution with antigen, the concentration of TQL1055 in the control group remained at 40 μg, while the active groups received 30 μg. The bacterial stocks of BCG, *Mycobacterium bovis* Bacille Calmette Guerin (BCG Pasteur, Source: Trudeau Institute) and the strain *Mtb* H37Rv (BEI Resources, Manassas, VA) were cultured to mid-log phase in Proskauer Beck medium containing 0.05% Tween 80 and frozen as 1 mL aliquots at −80°C. Mice vaccinated with BCG received a single subcutaneous administration of $1 \times 10^6$ colony forming units (CFU) in 200ul, at the time of the third dose of SA-TB vaccinations in the other groups.

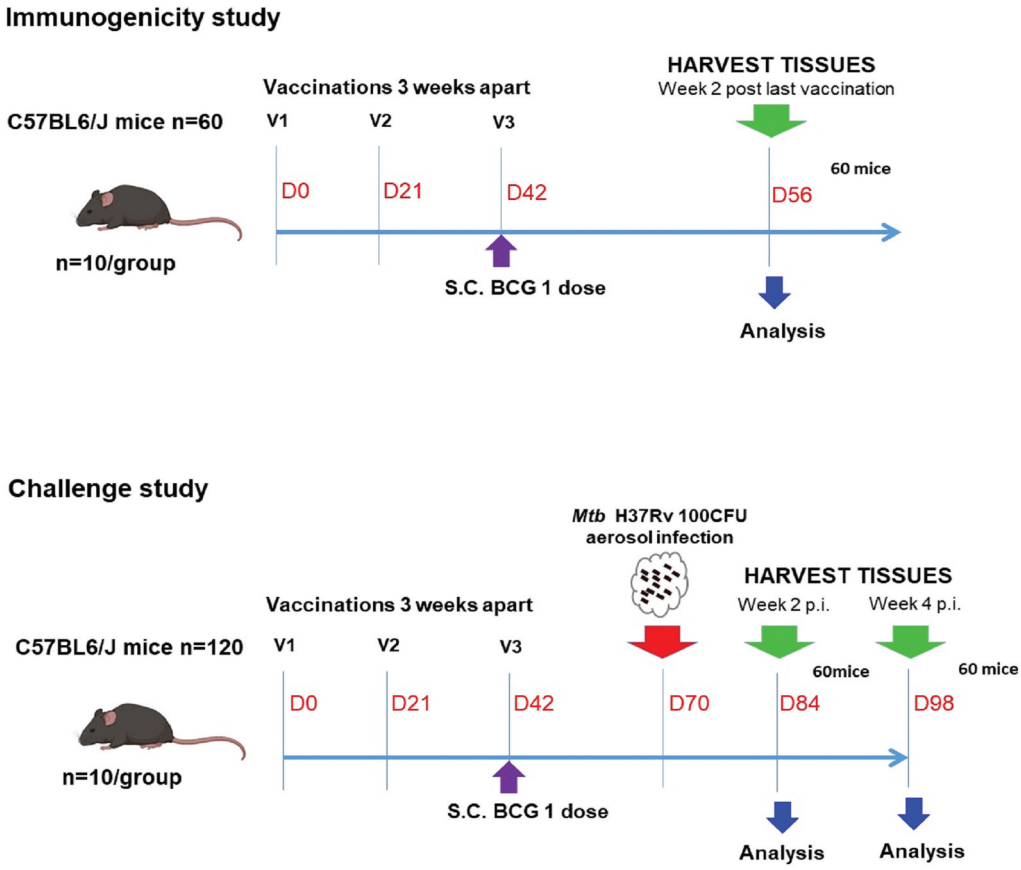
The SA-TB vaccine was delivered intramuscularly into the thigh muscle of the upper right leg of (50 μl per mouse) 6-week-old B6 mice, in three doses at three-week intervals, while mock-vaccinated mice received PBS as control. Some mice received adjuvant alone. Two weeks after the last booster, mice were subjected to immunogenicity assays mainly flow cytometry and serum immunoglobulin quantitation. Four weeks after the last booster vaccination, mice were challenged by aerosol with a low dose (100 CFU) of *Mtb* H37Rv (BEI Resources, Manassas, VA) using the Inhalation Exposure System (Glas-Col, Terre Haute, IN). 14 and 28 days after challenge, unvaccinated and vaccinated mice were sacrificed by $CO_2$ asphyxiation, and the lungs were aseptically excised and individually homogenized in physiological saline solution. Serial dilutions of lung homogenates were plated on 7H11 agar for CFU and counted after 3 weeks of incubation at 37°C as described before.[26]

## Evaluation of inflammatory lesions in vaccinated mice by bright field microscopy

The right upper lung lobe was perfused with 10% buffered formalin and excised from mice for histological analysis of inflammation. The excised lobes were embedded in paraffin and sections (5 μm) of FFPE lung were cut using a microtome, stained with H&E and processed for light microscopy. Images were captured using the automated NanoZoomer digital whole-slide imaging system (Hamamatsu Photonics). Regions of inflammatory cell infiltration were delineated utilizing the NDP view2 software (Hamamatsu Photonics), and the percentage of inflammation was calculated by dividing the inflammatory area by the total area of individual lung lobes. All scoring was conducted in a blinded manner[27] utilizing $n =$ 10 mice per group.

## Flow cytometry staining

For flow cytometric analysis, staining was performed where cells were stimulated overnight with media (RPMI 1640 + 10% FCS) or ESAT-6 and Ag85B (10 μg/mL) at 37°C and subsequently incubated with Brefeldin A and Monensin (1 μg ml − 1; BioLegend, San Diego, CA, USA) for an additional 5 h at 37°C. Cells were surface stained with CD4, CD3, CD8 and CD44 together with Fc receptor block, followed by permeabilization with Cytofix/Cytoperm (BD Biosciences) and intracellular staining with IL-17 and IFN-γ. Cells were gated as singlets > lymphocytes > $CD4^+$ $CD3^+$ or $CD8^+CD3^+$ > $CD44^{hi}$ > $cytokine^+$. Cells single-stained with each fluorochrome were used as controls for the compensation matrix in the flow cytometry. Samples were acquired on a 4 Laser BD Fortessa flow cytometer, and the




| Group | Immunization | Presentation | Route | Immunogenicity Study | Challenge study |
|---|---|---|---|---|---|
| 1 | Unvaccinated- saline | NA | I.M. | N=10 | N=20 |
| 2 | BCG | 1X10$^6$ CFU/200 ul | S.C | N=10 | N=20 |
| 3 | TQL1055 adjuvant alone (40ug) | Reconstituted – 50ul | I.M. | N=10 | N=20 |
| 4 | ESAT6/Ag85B in TQL1055 (5ug) | Reconstituted – 50ul | I.M. | N=10 | N=20 |
| 5 | ESAT6/Ag85B in TQL1055 (15ug) | Reconstituted – 50ul | I.M. | N=10 | N=20 |
| 6 | ESAT6/Ag85B in TQL1055 (30ug) | Reconstituted – 50ul | I.M. | N=10 | N=20 |

**Figure 1.** Experimental scheme for immunogenicity and *Mtb* challenge studies : C57BL/6J mice ($n$ = 10 individual mice per group) for immunogenicity (day 14 post final vaccination dose) and challenge studies (day 14 and 28 post Mtb challenge following vaccinations) was carried out. Mice were vaccinated intramuscularly with the SA-TB vaccines [ESAT-6 1 µg, Ag85B 1ug with TQL1055 (5ug or 15 µg or 30 µg) in 50 µl], or adjuvant alone (TQL1055 40 µg), intramuscularly (in three doses at three week intervals). The positive controls were administered BCG vaccine subcutaneously once (1×10$^6$ CFU/200 µl). The unvaccinated group received 50 µl of PBS. The mice were then subjected to the immunogenicity or challenge study.

analysis was performed using FlowJo (TreeStar). The complete antibody panels, including fluorochrome, dilution factor, and representative gating strategies are represented in Supplementary Figure 1.

## Antibody quantification in serum using enzyme-linked immunosorbent assay

IgG1, IgG2b, IgG2c and IgA were measured in the serum samples from the immunized animals using the reagents indicated below from Southern Biotech. Briefly, the 96-well plates were coated with 2 µg/mL ESAT-6+Ag85B antigen overnight at 4°C. The next day, plates were washed and blocked with 1% BSA. After washing, plates were incubated with the sera samples (diluted and serially diluted up to 5 dilutions) followed by incubation at room temperature with the HRP-conjugated antibodies against IgG1 (Cat # 1070–05, SouthernBiotech, Birmingham, AL, USA), IgG2b (Cat # 1090–05, SouthernBiotech, Birmingham, AL, USA) and IgG2c (Cat # 1079–05), IgA (Cat # 1040–05, SouthernBiotech, Birmingham, AL, USA) and TMB substrate (VWR, Radnor, PA, USA). The dilution for all antibodies was 1:2000. The reaction was

stopped using 1N $H_2SO_4$, and the data were collected within 30 min using a plate reader (ELx405 BioTek, Winooski, VT, USA).

## Statistical analysis

Statistical analyses of all data were performed using GraphPad Prism (La Jolla, CA, USA). One-way ANOVA with Tukey's correction test was done for comparisons with the six groups. For correlation analysis Pearson's correlation coefficient was used

## Results

### *SA-TB vaccine induces robust T cell and antibody responses in mice*

The development of safe and novel adjuvants for vaccines for human use is a significant bottleneck in vaccine development for TB. Thus, we wanted to test the potential use of a novel semi-synthetic saponin-based adjuvant TQL1055 combined with the TLR4 agonist PHAD in a liposomal formulation, in the form of a TB vaccine. Therefore, we first carried out immunogenicity studies (Figure 1) and determined whether vaccination of TQL1055/PHAD along with *Mtb* immunodominant antigens could drive robust T cell responses and antigen-specific antibody responses. C57BL/6 mice were vaccinated and boosted twice with SA-TQL1055 adjuvant formulated with two immunodominant *Mtb* antigens ESAT-6 and Ag85B whole proteins (SA-TB vaccine), with the vaccine formulation containing increasing amounts of TQL1055 from 5, 15 and 30 µg (referred to as SA-TB5, SA-TB15 and SA-TB30) and fixed 5 µg dose of the TLR4 agonist PHAD. Unvaccinated controls were included, as well as a group of mice that received BCG were also included as positive controls. Additionally, one group was vaccinated with the highest dose of adjuvant TQL1055/PHAD alone, without any *Mtb* antigens. Two weeks after the last booster vaccination, we determined antigen-specific IFN-γ and IL-17-producing $CD4^+$ T cell responses in lungs and spleens of vaccinated mice. Our results show that when compared to unvaccinated mice, B6 mice vaccinated with BCG induced activated $CD4^+CD44^{hi}$ cells in the lung. Importantly, delivery of adjuvant alone did not induce lung activated $CD4^+CD44^{hi}$ cells, while mice vaccinated with SA-TB vaccine induced activated lung $CD4^+CD44^{hi}$ responses, with SA-TB15 inducing the most potent activation and accumulation of lung $CD4^+CD44^{hi}$ cells (Figure 2a). Indeed, as reported before, BCG vaccination induced both IL-17 and IFN-γ-producing $CD4^+$ T cell responses in the lung.[26] Vaccination with SA-TB, especially SA-TB15 and SA-TB30 showed increased induction of *Mtb*-specific IFN-γ-producing T cells, and coincident induction of IL-17-producing cells T cells. Figure 2a. Vaccination with TQL1055/PHAD alone did not induce neither activated $CD4^+CD44^{hi}$ nor rigorous *Mtb*-specific IFN-γ-producing T cells. With respect to $CD8^+$ T cells, only SA-TB15 vaccination induced activated $CD8^+CD44^{hi}$ T cells, and was effective in inducing IFN-γ-producing $CD8^+$ T cells. Increase in IL-17-producing $CD8^+$ T cells were detected in SA-TB5 and SA-TB30 vaccinated groups, over levels detected in unvaccinated mice (Figure 2b). Together these results show that SA-TB15 followed by SA-TB30 induced the activation and accumulation of prominent lung *Mtb*-specific $CD4^+$ and to a lesser extent $CD8^+$ activation, as well as differentiation into cytokine-producing cells.

To determine activation of T cell responses following vaccination, we studied $CD4^+$ and $CD8^+$ T cell responses in the spleens of unvaccinated and vaccinated groups of mice. Surprisingly, except for the group of mice receiving adjuvant alone, none of the other vaccinated groups showed increase in $CD4^+$ or $CD8^+$ $CD44^{hi}$ cells. However, similar to the lung responses, SA-TB15 was the most efficient at inducing IFN-γ-producing $CD4^+$ T cell responses, while no significant increase in IL-17-producing $CD4^+$ T cells were detected in any of the groups except with the BCG group (Figure 2c–d). Additionally, with respect to cytokine-producing $CD8^+$ T cells, BCG was effective in priming both IL-17-producing and IFN-γ-producing $CD8^+$ *Mtb*-specific T cells, while only SA-TB15 induced both IL-17-producing and IFN-γ-producing $CD8^+$ T cells (Figure 2c–d). Thus, our results show that both in the lung and spleen, SA-TB vaccination induced potent $CD4^+$ cytokine-producing T cell responses, with a preference for IFN-γ production rather than IL-17 production.

To determine humoral immune responses, we measured the levels of antigen-specific antibodies in the serum following vaccination and observed that mice that were vaccinated SA-TB had high magnitude of antibody titers for all isotypes (IgG1, IgG2b, IgG2c and IgA) measured by end point dilution (Figure 3a–e). No major differences were observed between the high antibody levels induced by any of the three doses of TQL1055 used, while BCG vaccination did not induce high levels of serum antibodies. BCG lacks ESAT-6 due to the deletion of the genomic region RD1,[28] and this factor may be attributed to the reduced antibody responses in serum derived from BCG vaccinated mice even though BCG expresses Ag85B, while still providing BCG-induced protective immunity.[29] We also determined antigen-specific IgA antibody titers in the serum of immunized animals and found only a small increase in SA-TB vaccinated mice, when compared with controls, likely a limitation of using serum rather than bronchoalveolar lavage fluid, which was not collected in this study. Importantly, adjuvant alone vaccination did not induce high levels of antigen-specific antibody responses similar to levels induced in unvaccinated mice. We also observed that immunization with the most effective vaccine SA-TB15 showed balanced Th1/Th2 responses (Table 1) Overall, our data show that SA-TB vaccines induced elevated antibody responses following vaccination.

## Vaccination with SA-TB confers protection in a mouse model of *Mtb* infection.

Mice were vaccinated three times with three-week intervals with SA-TB5, SA-TB15 and SA-TB30, or with adjuvant alone (Figure 1). Additionally, we included mice that received BCG vaccination alone. Mice receiving PBS were included as unvaccinated controls. All groups of vaccinated and unvaccinated mice were rested for 4 weeks, and then challenged with a low

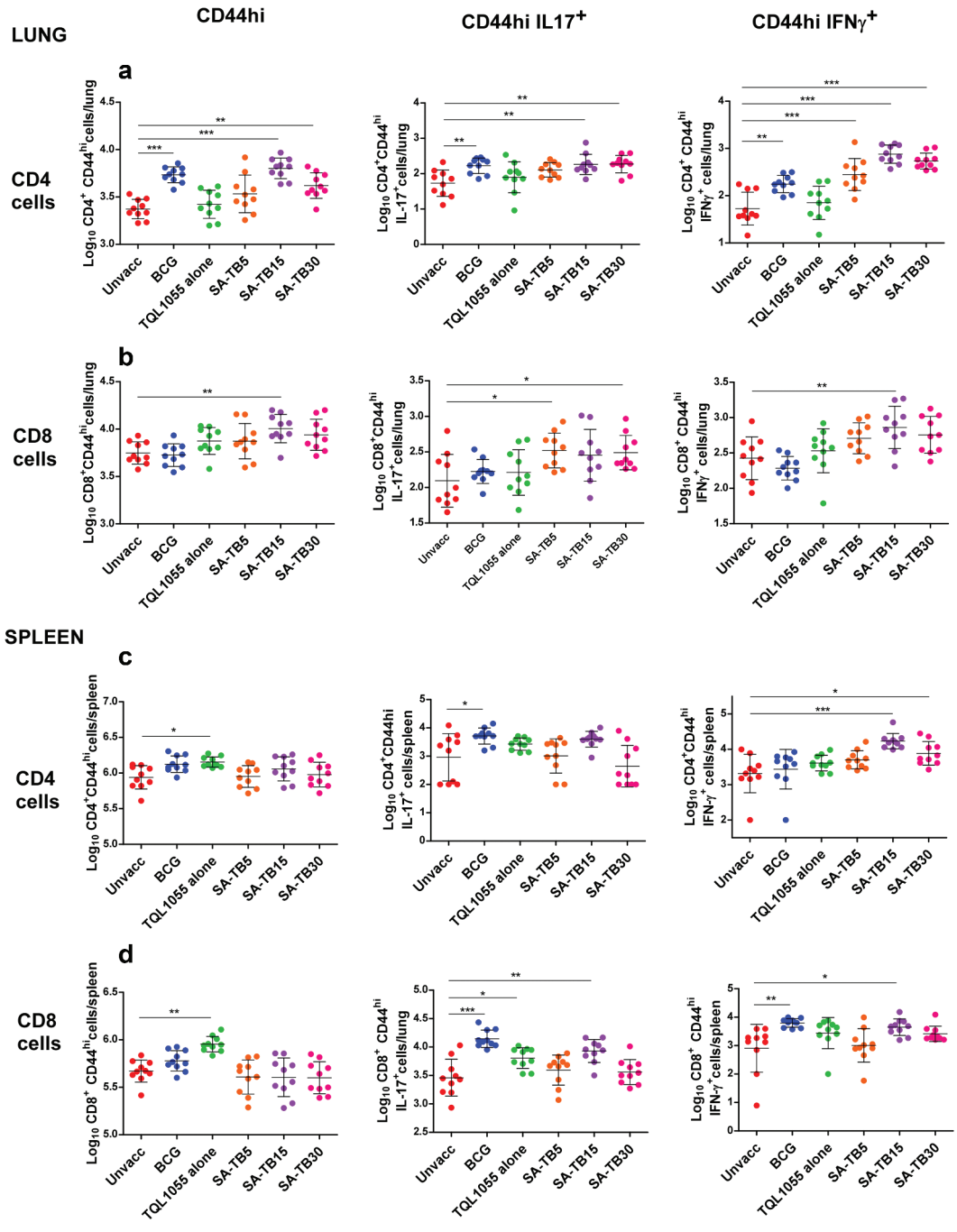



**Figure 2.** SA-TB vaccine induces robust T cell and antibody responses in mice. B6 mice ($n = 10$ per group) were vaccinated 3 times I.M. at 3-week intervals with adjuvants + *Mtb* antigens (ESAT-6+Ag85B), or BCG vaccinated or received only adjuvant, and or left unvaccinated. Using flow cytometry lung *mtb*-specific responses were determined in single cell lung (a) or spleen (b) suspensions from vaccinated mice 14 dpi (days post vaccination) and unvaccinated mice. The cells were restimulated with *Mtb*antigens ESAT-6 and Ag85B. The number of lung $CD4^+$ and $CD8^+$ $CD44^{hi}$ producing cells were quantitated by flow cytometry. Data for cell responses was calculated by subtracting the values for respective unstimulated controls from the values for corresponding antigen-stimulated samples. Statistical significance was verified by one-way Anova with Tukey's correction; $n = 10$ biological replicates. All data are mean ± s.d. *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; ****, $p \leq 0.0001$.

dose of aerosolized *Mtb* H37Rv. As expected, BCG vaccination resulted in significant protective efficacy upon *Mtb* challenge at 14 dpi (Figure 4a) with *Mtb* CFU further reduced significantly at 28 dpi compared to unvaccinated mice (Figure 4b). One mouse out of the ten died following challenge with *Mtb* in the BCG group. SA-TB vaccination, together with adjuvant alone vaccination resulted in early *Mtb* control at levels similar to BCG, suggesting that both innate and adaptive immune responses may be activated in SA-TB vaccinated groups and conferring early protection (Figure 4a). However, at 28 dpi, all the three SA-TB immunized groups showed significant *Mtb* control, while adjuvant alone immunized group showed *Mtb* CFU levels similar to unvaccinated controls, suggesting that long-term sustained protection was dependent on adaptive immune responses. Importantly, within the dose range of SA-TB, SA-TB15 and SA-TB30 conferred the best and least variable *Mtb* control, coinciding with the rigorous cellular responses induced by these doses. These results suggest that

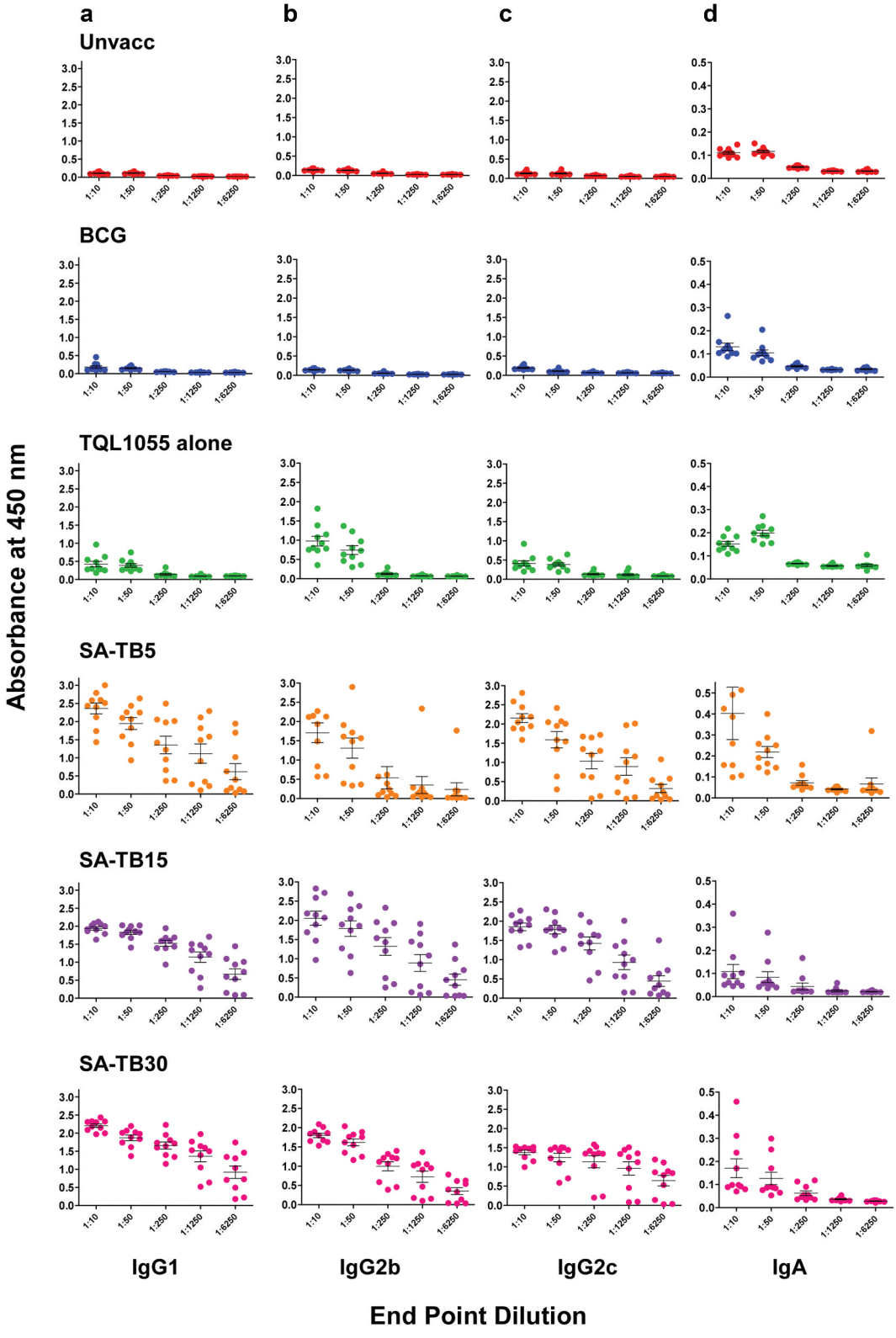



**Figure 3.** SA-TB vaccine induces antigen-specific IgG responses in serum. B6 mice were vaccinated 3 times I.M. at 3-week intervals with adjuvants + *Mtb* antigens (ESAT-6+Ag85B), or BCG vaccinated or received only adjuvant, and unvaccinated mice were also included. 14 days post last vaccination, serum was collected from the mice and immunoglobulin specificity against mtb antigens ESAT-6 and Ag85B in peripheral blood serum (diluted 1:15) was determined by ELISA using antibodies against IgG1(**a**), IgG2b(**b**), IgG2c(**c**) and IgA(**d**).

SA-TB mucosal vaccine is protective upon *Mtb* challenge and provides protection, similar to that provided by BCG.

Although prevention of infection and *Mtb* control are readouts of vaccine efficacy, alleviation of TB disease is another outcome for an effective vaccine response. Thus, we measured lung inflammation in the different groups of vaccinated *Mtb*-infected mice. We found that while BCG vaccinated *Mtb*-infected mice demonstrated increased

**Table 1.** Balanced Th1/Th2 responses represented by ratios of serum IgG2c/IgG1 levels in SA-TB15 vaccinated mice.

| Mice # | IgG2c/IgG1 |
|---|---|
| 1 | 1.187793427 |
| 2 | 1.157894737 |
| 3 | 0.965533981 |
| 4 | 0.74034695 |
| 5 | 1.08046683 |
| 6 | 0.959446093 |
| 7 | 1.002101944 |
| 8 | 1.029485257 |
| 9 | 0.847930703 |
| 10 | 0.641713748 |

inflammation at the time of necropsy (28 dpi) as previously reported,[24,25] vaccination with SA-TB in all three groups or with TQL1055 alone did not induce significant inflammation (Figure 4c–d). There is a significant linear correlation between the accumulation of $CD4^+CD44^{hi}$ T cells and $CD8^+CD44^{hi}$ T cells and increased *Mtb* control, as well as correlation between the accumulation of cytokine producing $CD4^+$ and $CD8^+$ T cells and increased *Mtb* control (Supplementary Figure.2a-f). Correlation analysis also revealed that reduction of bacterial burden correlated with decreased disease severity in the SA-TB vaccinated mice in comparison to unvaccinated controls (Supplementary Figure 2 g). These results suggest that vaccination using the SA-TB, induced *Mtb* control and limited disease inflammation in *Mtb*-infected mice.

## Discussion

TB is a significant infectious cause of global mortality and morbidity largely due to lack of an efficacious modern human vaccine, as BCG vaccination remains the only licensed vaccine against TB. Thus, there is an emergent need for the preclinical development of safe and novel adjuvants for TB vaccine use. In this study, we test and demonstrate that TQL1055 saponin-based adjuvant delivered along with the TLR4 agonist PHAD and *Mtb*-specific immunodominant antigens (SA-TB vaccine) induced increased lung T cell and antibody immune responses. Additionally, the SA-TB vaccine conferred significant protection against *Mtb* infection compared to the unvaccinated and adjuvant-only controls, and level of protection was comparable to that induced by BCG. These findings strongly support the development of a SA-TB vaccine with TQL1055 as a novel, safe and effective, TB vaccine for potential use in humans in the future.

A candidate subunit vaccine, $M72/AS01_E$, which contains two immunodominant antigens along with AS01E, has recently been demonstrated to induce IFN-γ production in protecting latently infected young adults against progression to disease.[9] Whether $M72/AS01_E$'s potential protection involves $CD4^+$ T cell IFN-γ production or other immune mechanisms that mediate protection is not fully clear. In general, saponin-based adjuvants induce balanced Th1/Th2 immune responses.[30] Our study shows that SA-TB vaccine containing TQL1055 and PHAD induced enhanced $CD4^+$ and $CD8^+$ IFN-γ production and to a lesser extent, IL-17-producing T cell responses, following vaccination both in the lung and spleen. In our prior work, we have demonstrated that the localization of cytokine-producing T cell responses in the lung provides a platform for accelerated immune responses and superior protection against *Mtb* infection.[31,32] Therefore, the enhanced accumulation of antigen-specific $CD4^+$ and $CD8^+$ cytokine-producing T cells in the lungs, likely contribute to the rigorous *Mtb* control observed in SA-TB vaccinated *Mtb*-challenged mice. Indeed, even vaccination with the adjuvant alone induced early protection in the lung at 14 dpi (days post infection), suggesting that the adjuvant may be activating innate pathways and should be explored for use as an innate training agent in future studies.[33] Whether B cells and/or antibodies contribute to protection against TB, and to vaccination-induced protection mediated by BCG, remains unclear. Despite lacking ESAT-6, the Ag85B protein of BCG is required for the early interaction of the vaccine with host cells, but deletion of the antigen does not affect BCG-induced protective immunity against *Mtb* aerosol challenge. Therefore, BCG contains other antigens other than Ag85B that are sufficient for protection against *Mtb* infection in the murine model.[29] The $M72/AS01_E$ vaccine candidate has also been shown to be an inducer of antibodies.[34] Our data show that SA-TB vaccines at all doses used are rigorous in inducing antibody responses across broad isotypes, and may be contributing to enhance protection against *Mtb* infection. Together our results demonstrate that our novel SA-TB containing TQL1055/PHAD adjuvants induced both T cells responses and antibody responses, and possibly long-term innate training.

The mechanism by which AS01E, which contains QS-21 and MPL in liposomal formulation, induces immune responses is thought to be via activation of the NLRP3 inflammasome, release of caspase-1 dependent cytokines, IL-1β and IL-18, and induction of IFN-γ responses.[35] However, a major bottleneck for use of QS-21 in many vaccine platforms including Shingrix, Mosquirix and $M72/AS01_E$ is the supply chain issues associated with sourcing QS-21. QS-21 adjuvant is extracted from older *Quillaja* trees native to Chile, and traditionally extracted in small amounts from the bark of mature trees, a process that is not sustainable for long-term use in vaccines. Given the current limitations with sourcing and processing of QS-21, the market demands for this saponin-based adjuvant far outweigh the availability of this adjuvant. Therefore, it is imperative that novel saponin-based adjuvants that are synthetic or semi-synthetic such as TQL1055 and activate similar immune pathways to QS-21 be tested for use in vaccine formulations to alleviate these bottlenecks. Our results show that SA-TB vaccine containing TQL1055 saponin combined with the TLR4 agonist PHAD-based adjuvant functions similar to $M72/AS01_E$ in the induction of IFN-γ responses, but also confers significant protection against *Mtb*, at levels comparable to the gold standard BCG vaccination in mice. These results suggest that TQL1055/PHAD is a safe, immunogenic and potential novel adjuvant for use in TB vaccines and functions well when used with TQL1055 doses between 15 and 30 μg.

A hallmark of pulmonary TB in both humans and experimental animals is the associated inflammation that is induced

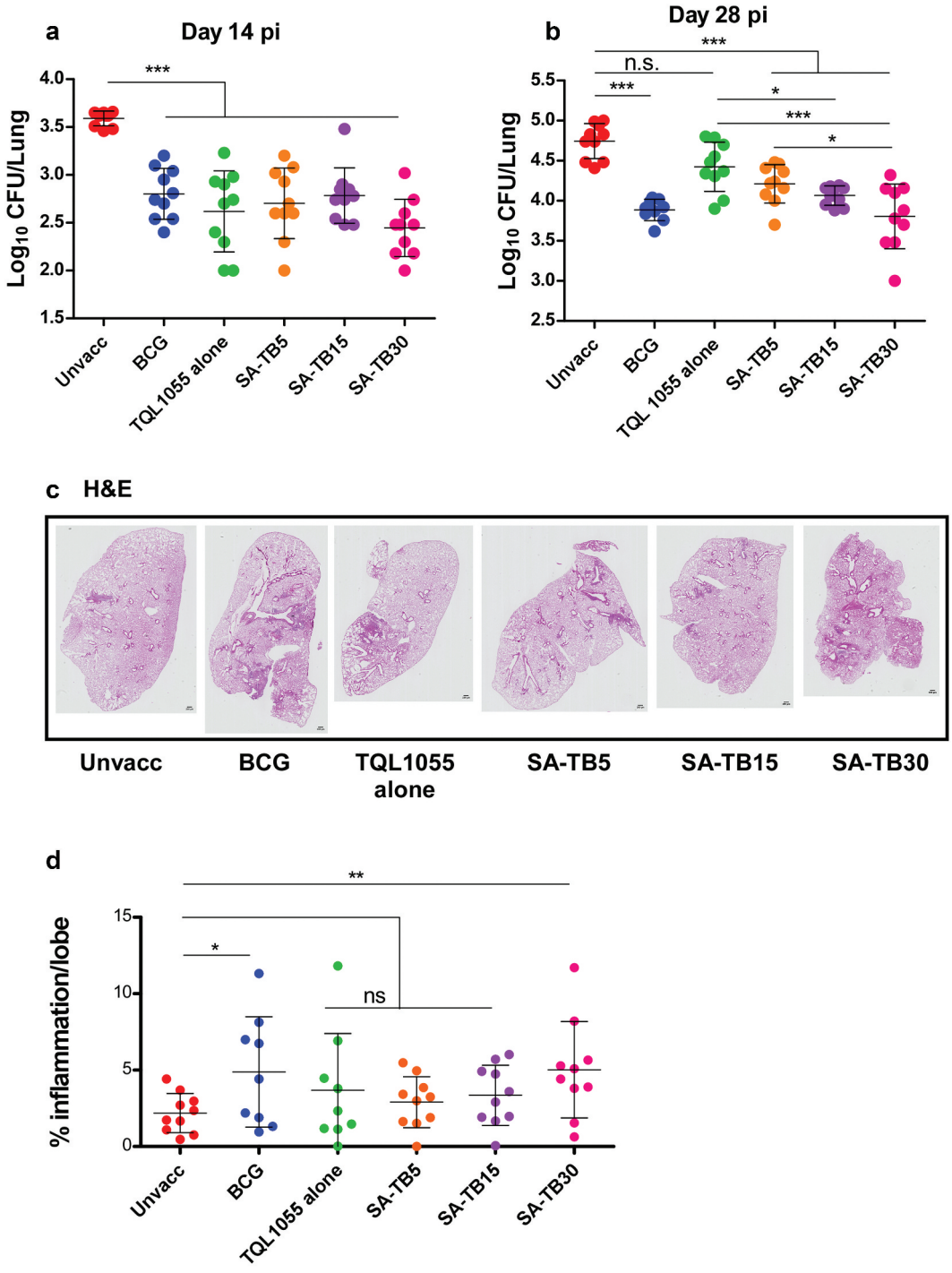



**Figure 4.** Vaccination with SA-TB confers protection in a mouse model of Mtb. B6 mice were vaccinated 3 times I.M. at 3-week intervals with adjuvants + *Mtb* antigens (ESAT-6+Ag85B), or BCG vaccinated or received only adjuvant, and unvaccinated mice were also included as controls. The mice were, rested for 4 weeks and then challenged with *Mtb* H37Rv (100 CFU). *Mtb* CFU was determined in lung homogenates by plating 14 days post infection (a) and 28 days post infection (b). FFPE lung sections were subjected to H&E staining (c). Lung inflammation was quantitated on formalin-fixed, paraffin-embedded lung sections from mice on H&E-stained FFPE lung sections on 28 dpi (d). Statistical significance was verified by one-way Anova with Tukey's correction, $n = 10$ biological replicates. All data are mean ± s.D. *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; ****, $p \leq 0.0001$.

in the form of granulomas in the lung. Our results show that while use of BCG as a vaccine is effective in controlling *Mtb* infection, it mediates significant inflammation in the lung.[24] In contrast, the SA-TB vaccine while mediating similarly effective *Mtb* control, particularly the lower dose of SA-TB5 did not induce lung inflammation. Therefore, the use of SA-TB vaccine may be effective in not only providing protection against *Mtb* infection in host but may also aid in alleviating inflammation in the lungs, and perhaps even limiting TB reactivation in latently infected individuals. Additionally, in future studies it can also be tested if further titration of the TQL1055 between SA-TB15 and SA-TB30, or use of SA-TB15 as a booster to BCG vaccine will act cooperatively to further enhance protection against *Mtb* challenge. In addition, while in this study we used ESAT-6 and Ag85B as immunodominant antigens, in future studies SA-TB vaccines can be formulated

and tested with M72 antigen or other potential antigen candidates to study effects on immunogenicity and *Mtb* control. Further validation of the SA-TB vaccine in a non-human primate animal model will be crucial to test the efficacy of the SA-TB vaccine candidate for use in humans for protection against *Mtb* infection and disease.

In summary, we demonstrate that the SA-TB vaccine induces enhanced IFN-γ responses in $CD4^+$ and $CD8^+$ T cells, as well as robust antibody responses to significantly protect against the *Mtb* in mice. While conferring *Mtb* protection, the SA-TB vaccine offers the added advantage of reducing inflammation. These findings support and enable the development of a SA-TB vaccine as a novel, safe and effective, Th1-inducing vaccine for use in humans, and a substitute for QS-21.

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