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Mucosal boosting of H56:CAF01 immunization promotes lung-localized T cells and an accelerated pulmonary response to *Mycobacterium tuberculosis* infection without enhancing vaccine protection

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T cell-mediated protection against *Mycobacterium tuberculosis* (Mtb) is dependent upon the ability to localize within the site of pulmonary infection and directly interact with infected cells. In turn, vaccine strategies to improve rapid T cell targeting of Mtb-infected cells after pulmonary exposure are being actively pursued. Given parenterally, the subunit vaccine H56:CAF01 elicits polyfunctional CD4 T cells that localize to the lung parenchyma and confer durable protection. Here, we find that airway mucosal boosting of parenteral H56:CAF01 immunization greatly enhances the population of long-lived lung-resident T cells (Trm) and increases early vaccine T cell responses to pulmonary Mtb challenge in multiple mouse models. However, mucosal boosting does not alter the Th1/17 vaccine signature typical of H56:CAF01 and does not further improve durable control of pulmonary infection following aerosol Mtb-challenge. Additional mucosal boosting with H56:CAF01 further enhances the Trm response without further improving protection, while blocking the recruitment of non-Trm with FTY720-treatment failed to exposed Trm-mediated protection in mucosally boosting animals. These results demonstrate the limitations of maximizing lung-localized Trm in vaccine control of pulmonary Mtb infection, especially within an immunization protocol that is already optimized for the induction of mucosal-homing Th17 cells.

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INTRODUCTION

One quarter of the world's population is estimated to be infected with the bacterium *Mycobacterium tuberculosis* (Mtb), and tuberculosis (TB) disease remains the most common cause of death from a single infectious agent.[1,2] Given this setting, a novel, more effective anti-TB vaccine strategy is required to achieve the WHO's End TB targets of 90% reduction in TB incidence rate by 2035.[3]

T cell-mediated immunity is critical to control *M. tuberculosis* (Mtb) infection and TB disease. However, Mtb actively delays the adaptive T cell response after initial infection.[4] Thus, a cornerstone of TB vaccine development is to overcome this delay and recruit T cells for direct contact with infected lung phagocytes to control early bacterial growth.[5,6] Mtb-specific vaccine-induced memory Th17 responses have been associated with accelerated Mtb-specific CD4 T cell lung recruitment after Mtb-infection and improved protection in mice.[7] In addition to circulating central memory T cells (Tcm) and effector memory T cells (Tem), tissue-localized resident memory T cells (Trm) have also shown the capacity to increase the adaptive immune reaction to several pathogens and improve control of infection.[8–10] In this regard, developing vaccine delivery systems to increase lung-localized Mtb-specific T cells has been an emerging strategy, including mucosal delivery of vaccines.[9,11–14] While mucosal delivery of the only currently licensed TB vaccine, BCG, has shown varied results when given in the airways,[15–19] a recent study suggests that Trm induced by airway delivery of BCG can mediate protection against Mtb challenge.[20]

The anti-tuberculosis subunit vaccine H56 is a fusion protein of Mtb antigens Ag85B, ESAT-6, and Rv2660c.[21] When combined with the liposome-adjuvant CAF01 and given parenterally, H56:CAF01 elicits a polyfunctional Th1/Th17 CD4 T cell response and results in an early CD4 T cell response into Mtb-infected lung tissue and a significant reduction in CFU.[21–24] Additional studies using a CAF01-adjuvanted Group A Streptococcus antigen have shown that an immunization strategy comprising parenteral priming followed by intranasal mucosal boosting results in the development of lung-localized T cells with an enhanced recall response to antigen in the airways.[25] Here, we investigate utilizing this parenteral-priming mucosal-boosting strategy for the delivery of H56:CAF01. We hypothesized that this strategy will elicit an Mtb-specific lung-localized memory T cell population, further enhance an effective early anti-Mtb T cell response within the lung after initial Mtb exposure, and improve control of pulmonary Mtb infection. We found that subcutaneous immunization with H56:CAF01 followed by an intranasal boost, elicited a population of long-lived lung-resident Trm and an enhanced early lung T cell response to aerosol Mtb challenge. However, the T helper phenotype of the responding CD4 T cells was unaltered by airway

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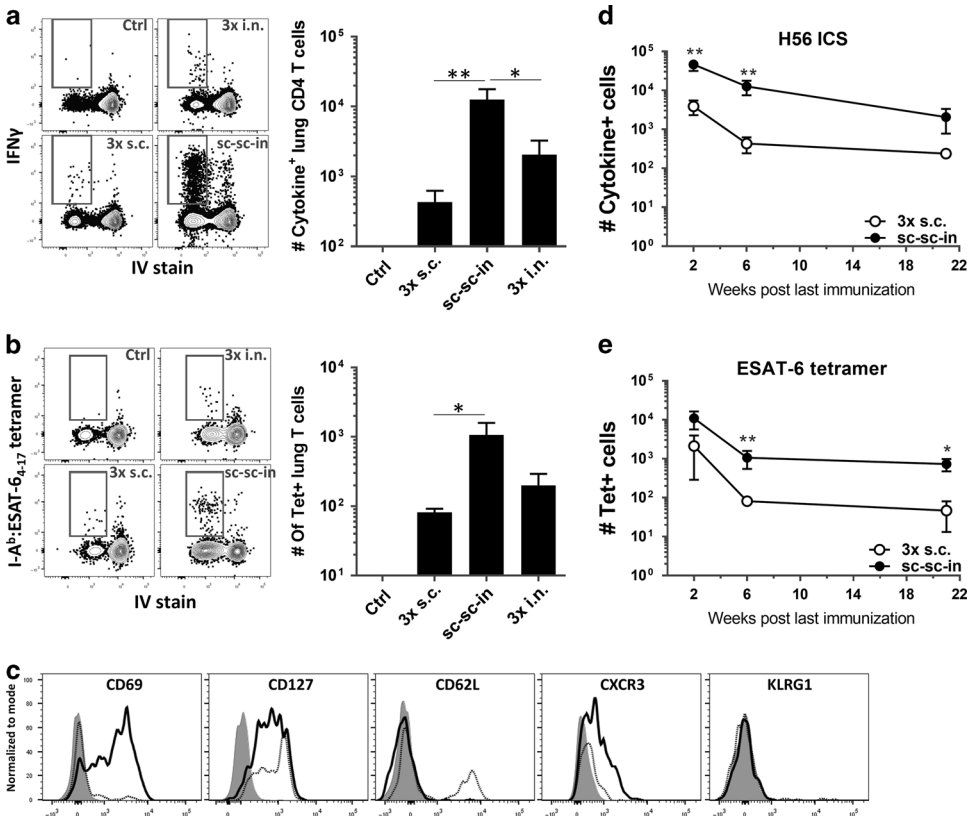


Fig. 1 Airway mucosal boosting of parenteral H56:CAF01 induces long-lived lung-localized Trm cells. CB6F1 mice were immunized 3 times at 2-week intervals via 3× s.c., 3× i.n., or 2× s.c. + 1× i.n. (sc–sc–in) routes. T cell populations in the lung parenchyma were assessed following in vivo intravenous staining to discriminate parenchyma tissue-localized and vasculature-localized circulating cells. **a**, **b** Sample FACS plots of individual mice (left) and compiled bar graphs ($n = 3$–5/group) of the number of **a** IV− lung CD4 T cells expressing IFNγ (left) or any of IFNγ/TNF-α/IL-2/IL-17 cytokines (right, bar graph) after ex vivo H56 restimulation (by ICS), and **b** IV− lung CD4 CD44^{hi} T cells positive for I-A^b:ESAT-6$_{4–17}$ tetramer staining, 6 weeks after the final immunization. **c** Surface marker expression of IV− "parenchyma" (solid line) and IV+ "circulating" (dotted line) tetramer+ CD4 lung T cells, and Tet+ IV− FMO staining controls (filled gray) concatenated from sc–sc–in H56:CAF01 mice from (**b**). **d**, **e** The number of vaccine-specific IV− lung CD4 T cells determined by **d** any cytokine+ after H56-restimulation and **e** I-A^b:ESAT-6$_{4–17}$ tetramer staining at 2, 6, and 21 weeks post final immunization. Bar/symbol, mean ± SEM. *$p < 0.05$, **$p < 0.01$ ANOVA with Tukey's posttest of log-transformed data

mucosal boosting and there was not a durable improvement in control of infection beyond H56:CAF01 given only subcutaneously.

RESULTS

Mucosal boosting with H56:CAF01 induces long-lived lung-localized Trm

Similar to previous studies using parenteral H56:CAF01 vaccination,[21,22] CB6F1 mice were immunized subcutaneously (s.c.) three times at 2-week intervals. To compare the relative capacity of parenteral immunization to a parenteral-mucosal prime-boost strategy, the third immunization was given intranasally (i.n.) in a cohort of mice. After vaccination, lung tissue-localized CD4 T cells were assessed by flow cytometry, utilizing intravenous (IV) anti-CD45 antibody (Ab) staining to distinguish vasculature-localized (IV+) cells from lung parenchyma tissue-localized (IV−) leukocytes localized within lung interstitium/airways,[22,26] and vaccine-specificity by Th1/17 cytokine production via intracellular staining (ICS) following ex vivo H56 protein restimulation (Fig. 1a) or staining with I-A^b: ESAT$_{4-17}$ tetramer (Fig. 1b). Six weeks after vaccination, mice receiving 2× s.c. followed by 1× i.n. immunizations with H56:CAF01 (sc–sc–in; "prime-boost") resulted in a significantly higher number of lung parenchyma-localized vaccine-specific CD4 T cells than either parenteral-only (3× s.c.) or mucosal only (3× i.n.) immunized mice (Fig. 1a, b). These lung-localized CD4 T cells were phenotypically distinct from their circulating (IV+) counterparts, displaying a lack of central homing marker CD62L expression, increased amounts of principal Trm markers CD69 and CD127, as well as the chemokine receptor CXCR3, and reduced expression of KLRG1 (Fig. 1c).[20,27,28] Lung-localized vaccine-specific T cells are highly expanded 2 weeks post i.n. boost, followed by a contraction phase, after which the number of vaccine-induced lung-localized cells remained relatively stable from 6 weeks until at least 21 weeks post-vaccination (Fig. 1d, e). The Th1/17 cytokine profile of lung-localized cells was similar between the vaccinated groups, consisting of multifunctional Th1 and Th17 cells (Supplemental Figure 1). Additionally, as expected, H56:CAF01 did not prime a vaccine-specific CD8 T cell response[21,22,29] after s.c. or i.n. immunization (data not shown). Thus, mucosal boosting of s.c. H56:CAF01 induces long-lived lung tissue-resident CD4 T cells with Trm characteristics that are maintained in the absence of local antigen stimulus, suggesting they are bona fide resident memory T cells.

H56:CAF01 prime-pull enhances the magnitude of the early T cell response against aerosol Mtb infection

To determine if mucosal boosting improved the local lung T cell response to pulmonary Mtb infection, mice were rested 6 weeks after vaccination, to allow for the contraction of the adaptive response, and then given a low dose aerosol Mtb challenge.

As would be expected, 21 days after Mtb challenge, immunohistochemical analysis showed that H56:CAF01 vaccination

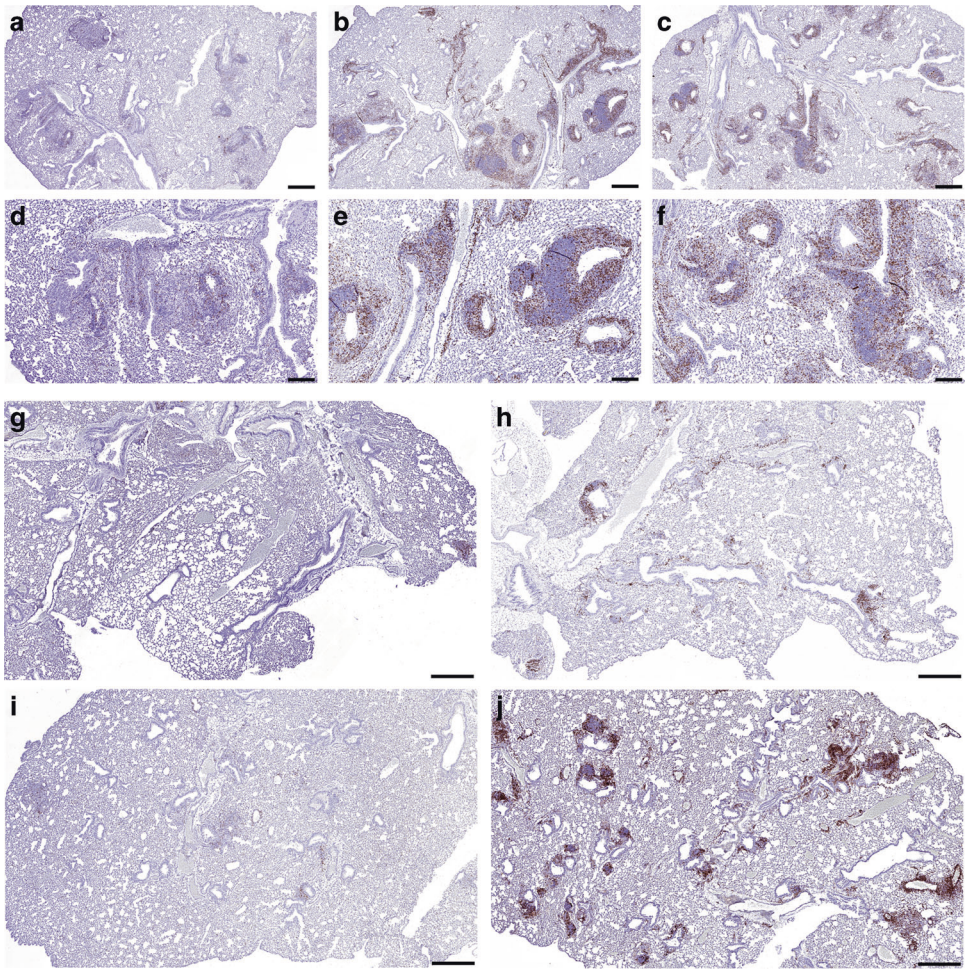


Fig. 2 Mucosal H56:CAF01 boosting enhances early CD4 T cell responses in the lung. Following low dose aerosol Mtb challenge, immunized mice were evaluated by immunohistochemistry (IHC) for CD4 lymphocyte responses with the lungs: **a–f** 21 days after Mtb challenge, lungs of 3× s.c. (**b**, **e**) and sc–sc–in (**c**, **f**) immunized mice contain larger numbers of brown staining CD4 within peri-airway lymphoid (darker blue) accumulations than do controls (**a**, **d**). Scale bar **a–c** = 500 μm, scale bar **d–f** = 200 μm. **g**, **h** 8 days and **i**, **j** 13 days post Mtb, peri-airway lymphoid accumulations containing CD4 lymphocytes (brown staining cells) are larger and more widely distributed in sc–sc–in (**h**, **j**) relative to 3× s.c. (**g**, **i**) immunized mice. Scale bar **g–j** = 500 μm

significantly increased pulmonary peri-airway $CD4^+$ lymphocyte numbers, compared to unvaccinated controls (Fig. 2a–f). Mice that received the vaccine by either protocol (3× s.c. or sc–sc–in) had a similar number, size, and distribution (peri-airway/perivascular) of lymphocyte foci, with indistinguishable $CD4^+$ cell infiltration at this time point (Fig. 2a–f). In contrast, comparison of lung tissue at earlier time points post Mtb infection revealed that mucosal H56:CAF01 boosting enhanced the number of distinguishable pulmonary peri-airway lymphocyte infiltrates containing $CD4^+$ cells as early as 8 days after Mtb challenge (Fig. 2g, h). By 13 days post challenge, the $CD4^+$ cellular response was further amplified in the mucosal H56:CAF01-immunized mice (Fig. 2i, j). CD4 cell staining in both H56:CAF01-immunized groups at all time points showed similar proportions of CD4 T cells localizing perivascularly, as is typically seen in response to aerosol Mtb challenge in resistant mouse strains.[30]

Overall, sc–sc–in H56:CAF01 mice had enhanced lymphocyte activity with greater CD4 T cell infiltrate around the airways, compared to 3× s.c. H56:CAF01 mice during the first 2 weeks after Mtb challenge. However, by 3 weeks post infection the 3× s.c. mice had "caught up" so that at this time point the overall lymphocytic, and in particular the CD4 lymphocyte, responses were indistinguishable between the vaccinated animals.

Consistent with previous reports, Mtb-specific lung T cells did not enter the lungs of unimmunized mice until 14–21 days post infection (Fig. 3a).[4,22] In contrast, pre-existing Mtb-specific lung T cells in 3× s.c. H56:CAF01-vaccinated animals had already increased in numbers by 8 days after challenge. Notably, at these earliest time points, sc–sc–in H56:CAF01-immunized mice displayed a further enhanced early lung tissue-localized T cell response, with a >20-fold increased number of Mtb(H56)-specific lung CD4 T cells, compared to 3× s.c.-immunized mice, 8 days after challenge (Fig. 3a). This relatively enhanced vaccine T cell response was transient, and lung-localized vaccine-specific T cell numbers were indistinguishable between vaccinated groups by 2–3 weeks after challenge (Fig. 3a).

Overall, intranasal mucosal boosting of parenteral H56:CAF01 significantly increased the recruitment/expansion of the lung-localized CD4 T cell response to pulmonary Mtb challenge.

H56:CAF01 prime-pull does not alter the cytokine profile of the lung CD4 T cells responding to aerosol Mtb infection

As mucosal immunization is often associated with an increase in Th17-skewed responses, we evaluated the cytokine profile of the responding cells in the lungs of H56:CAF01-immunized animals after Mtb challenge. We analyzed IV− lung CD4 T cell expression of Th1 and Th17 cytokines upon ex vivo H56 restimulation at 3

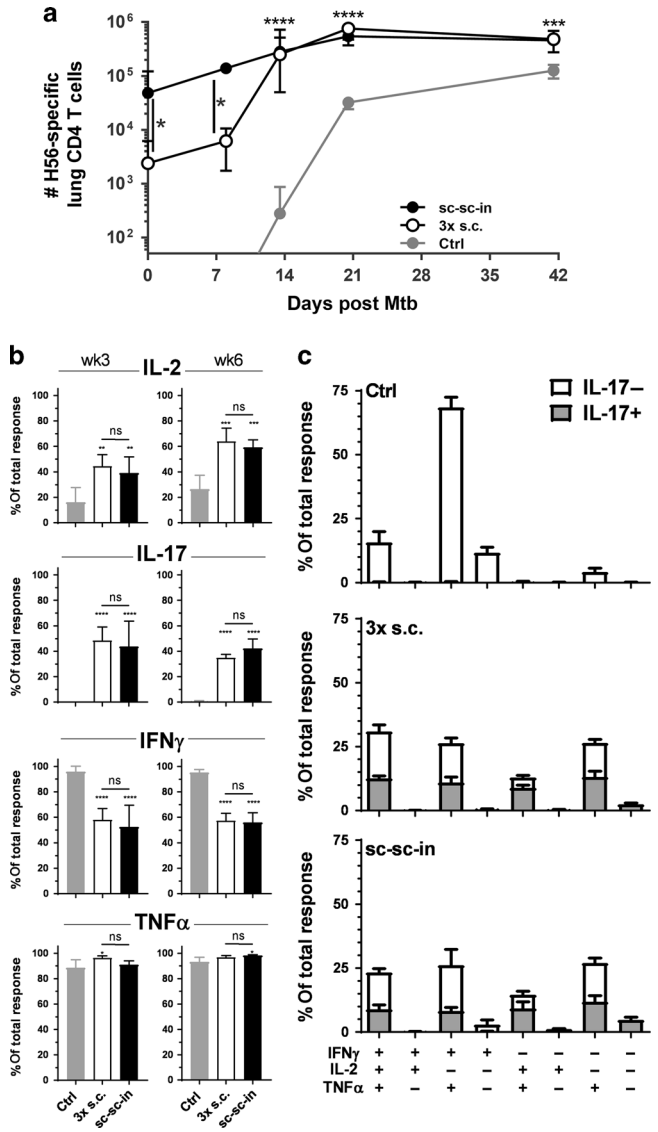
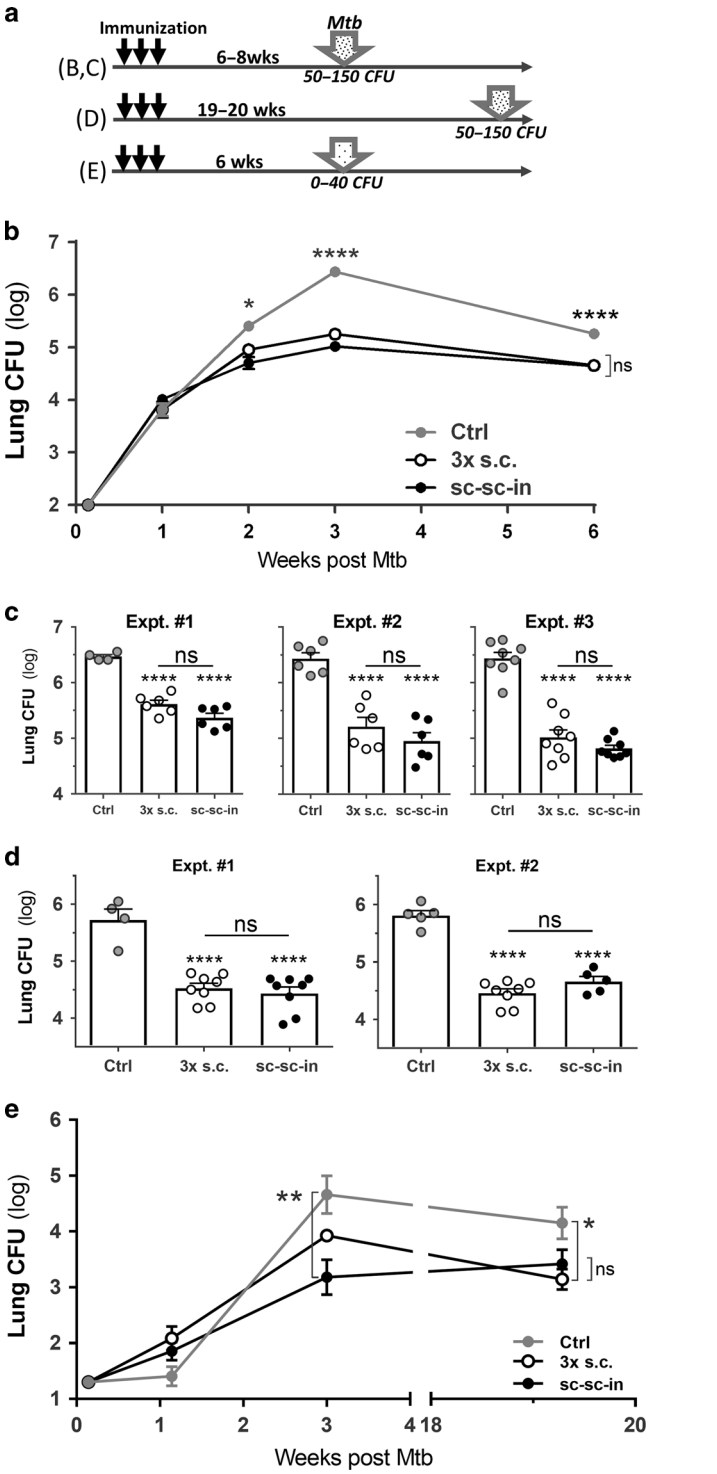


Fig. 3 Mucosal H56:CAF01 boosting enhances the magnitude, but not phenotype, of the early vaccine CD4 T cell response to pulmonary Mtb infection. CB6F1 mice were rested 6 weeks after vaccination and challenged with a low dose of aerosolized Mtb. **a** The number of H56-specific CD4 T cells in lung parenchyma (IV−) by ICS for expression of any of the cytokines IFNγ/TNF-α/IL-2/IL-17 over the course of infection. **b** The proportion of H56-specific cells from (**a**) expressing each individual cytokine is shown for 3 weeks (left) and 6 weeks (right) after Mtb challenge. **c** Combinatorial Boolean analysis of H56 lung CD4 T cells (from (**b**)) at 3 weeks post Mtb challenge are shown. Bar/symbol, mean ± SD. *$p < 0.05$, **$p < 0.01$ vs. Ctrl or as indicated via ANOVA with Tukey's posttest of log-transformed data (**a**) or raw percentage (**b**)

and 6 weeks post Mtb challenge. As expected, we found that the H56:CAF01 immunization altered the immune profile of responding lung CD4 T cells towards increased IL-2- and IL-17-expressing cells (Fig. 3b).[22,31] However, there was no difference in cytokine profile between 3× s.c. and sc–sc–in H56:CAF01-immunized mice, and further combinatorial Boolean analysis revealed a very similar pattern of enhanced polyfunctional CD4 T cells in both vaccinated groups (Fig. 3c). This was true at both the very early time point after challenge (day 8) when the number of lung H56-specific CD4 T cells was enhanced in the mucosal-boosted mice (data not shown), as well as at days 21 and 42 post infection, when the number was comparable between the H56:CAF01-immunized groups (Fig. 3). Therefore, airway mucosal boosting with H56:CAF01 did not skew the Th1/17 profile established by parenteral H56:CAF01 administration.



Mucosal boosting of H56:CAF01 does not enhance robust protection against aerosol Mtb

Consistent with previous studies, parenteral immunization with H56:CAF01 conferred significant protection against aerosol Mtb challenge with a reduction in lung bacterial burden of >1 log in the standard mouse challenge model (Fig. 4a, b; refs. [21,23,24,32]).

Fig. 4 Mucosal H56:CAF01 boosting does not provide improved stable protection against aerosol Mtb challenge. **a** Experimental design of efficacy studies in CB6F1 mice, with varied lengths of post-vaccination rest periods and dose of aerosol Mtb used for challenge that are represented in this figure (**b**–**e**). **b** Total lung CFU after low dose aerosol Mtb infection, compiled from three independent experiments with 4–8 mice/time point challenged with the same dose of Mtb. **c** Total lung CFU at 3 weeks after Mtb challenge from three independent experiments compiled in (**b**). **d** Total lung CFU from two independent experiments assessed 3 weeks after low dose Mtb challenge of 21–24 weeks rested immunized mice. **e** Total lung CFU assessed 3 weeks post ultra low dose aerosol Mtb challenge, representative of 2 experiments with similar results. Bar/line, mean ± SEM. *$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$ vs. Ctrl or as indicated via ANOVA with Tukey's posttest of log-transformed data. ns not significant. Dots on bars represent individual mice

However, contrary to our hypothesis, mucosal boosting of H56:CAF01 did not further reduce the lung bacterial burden after aerosol Mtb challenge (Fig. 4b). Though there was trend for a slight reduction (~0.25 log) in the lung CFU at 3 weeks post challenge in mucosally boosted mice, which was consistent across three independent experiments (Fig. 4c), this was transient and no improved protection was observed at later time points (Fig. 4b). Still, to better assess the impact of adaptive Trm-mediated improved protection, without potential confounding local innate immune changes in the lung environment, vaccinated mice were rested 21–24 weeks, when vaccine-specific lung Trm were still readily detectable (Fig. 1d, e), before low dose Mtb challenged (see Fig. 4a). In two independent experiments, both vaccinated groups controlled Mtb infection over non-vaccinated control (>1 log CFU reduction in the lung), confirming the long-term protective capacity of H56:CAF01 (Fig. 4d).[24] However, mucosal boosting of s.c. H56:CAF01 did not confer additional control of pulmonary Mtb, nor disseminated Mtb in spleen (Fig. 4d and Supplemental Figure 1).

To further assess infectious conditions in which mucosal boosting and an enhanced early lung tissue-localized T cell response might significantly improve control of Mtb-infection, we considered an extremely low dose challenge model, where as few as possible bacteria capable of causing a productive infection is utilized, and which may better mimic natural infection.[33] By reducing the infectious dose, we were able to deliver ~0–40 CFU into the lungs of infected mice (as assessed from whole lungs 1 day after infection), with productive infection of ~75% of exposed mice (the other 25% being stochastically uninfected). In this setting, we observed a similar finding as seen with a 50–150 CFU infecting dose; that mucosal boosting resulted in a transient reduction in lung CFU in the pre-peak phase of infection (3 weeks), but no improved protection during the chronic stage of infection (Fig. 4e).

Given the inability of mucosal boosting of H56:CAF01 to significantly improve protection over parenteral vaccination alone, we next wished to modify the immunization/infection settings to maximize the potential protective effect of pre-existing lung-localized anti-Mtb T cells. In one setting, we added a second i.n. H56:CAF01 boost to sc–sc–in mice (sc–sc–in→in) just 10 days before ultra-low dose Mtb challenge. As hypothesized, this second i.n. boost did indeed further increase the lung-localized T cell response by 9.4-fold over the sc–sc–in regiment at the time of infection (Fig. 5a). However, despite a now >2500-fold increase in H56-specific lung T cells at the time of challenge, sc–sc–in→ in H56:CAF01-immunized mice displayed no further reduction in lung CFU over 3x s.c.-immunized mice (Fig. 5b). Furthermore, vaccination did not inhibit productive Mtb infection, as the proportion of animals with detectable bacteria 3 weeks post ultra-low dose Mtb challenge in 3× s.c. (13/16, 81%), sc–sc–in (13/16, 81%), or sc–sc–in→in (15/16, 94%) H56:CAF01-immunized mice was not lower than that of control mice (6/8, 75%).

In another setting, we sought to isolate the protective capacity of the lung-localized Trm by inhibiting the recruitment of circulating Tcm/Tem following Mtb challenge. We treated control and immunized mice with FTY720 during the entire first 3 weeks of Mtb infection. FTY720 inhibits S1P1-mediated cell migration into efferent lymphatics resulting in inhibited recruitment of cells from secondary lymphoid organs into inflamed tissue. FTY720-treated mice were confirmed to have the expected lack of circulating naïve CD4 T cells throughout the experiment (Supplemental Figure 3). We hypothesized that FTY720 would inhibit the early lung T cell response in 3× s.c. H56:CAF01-immunized-mice, but not in mice that received a mucosal boost, in which the pre-existing lung Trm would compensate for deficient T cell recruitment and better control the lung infection. Day 8 after Mtb challenge, we confirmed that FTY720-treatment reduced the lung-localized (IV−) H56-specific T cell response in 3× s.c., but not in sc–sc–in, H56:CAF01-immunized mice (Fig. 5c). As expected, FTY720 treatment inhibited control of Mtb infection (Fig. 5d). Still, 3× s.c. H56:CAF01 was protective under FTY720 treatment, with ~1 log lung CFU reduction versus unvaccinated treated mice (Fig. 5d). However, FTY720 treatment did not reveal a specific protective role of Trm in mucosal-boosted H56:CAF01 sc–sc–in vaccinated mice, where lung bacterial burden was not different from 3× s.c. H56:CAF01-vaccinated mice (Fig. 5d).

Taken together, these data support that the enhanced pre-existing Trm population and resultant early lung-localized T cell response elicited by mucosal-boosted H56:CAF01-immunized mice does not readily contribute to improved control of pulmonary Mtb infection.

In our studies in which aerosol Mtb challenge was given 6 weeks (but not 20+ weeks) after immunization, we noted a trend of transient improved vaccine protective after mucosal boosting that was statistically significant in the ultra-low dose setting. Therefore we considered if innate differences in the lung environment after i.n. boosting could be contributing to increased or decreased Mtb control in our system, such as has been reported in other settings.[34] At 6 weeks post-vaccination, the number and activation state (assessed via CD86 and MHC II surface expression) of lung macrophages in H56:CAF01 3× s.c. and sc–sc–in immunized mice were not different from each other, or controls (Fig. 6a–c). Furthermore, after aerosol Mtb challenge, the activation stated of alveolar macrophages remained comparable between the 3× s.c. and sc–sc–in immunized mice (Fig. 6d, e). Moreover, the cytokine environment of the lungs in response to Mtb infection, including pro-inflammatory cytokines (e.g., IFNγ, IL-1β, IL-6, and IL-12) and Mtb-permissive cytokines (such as IL-5 and IL-10), and type I interferons were not different between 3× s.c. and sc–sc–in immunized mice (Fig. 6f). In contrast, mice that received a mucosal H56:CAF01 boost 10 days prior to challenge (sc–sc–in→in) had significantly increased number and activation state of lung macrophages prior to Mtb challenge (Fig. 6a–c). Early after Mtb challenge, sc–sc–in→ in immunized mice also showed increased activation of alveolar macrophages and increased IL-12p70 and IL-6 in the lung (Fig. 6d, f). However, this increased macrophage activation was notably not associated with improve protection (Fig. 5b). Overall, alteration of the innate immune environment by mucosal H56:CAF01 appeared to be nominal in our challenge studies comparing 3× s.c. and sc–sc–in immunizations, especially after a sufficient post-immunization resting period.

Intranasal boosting enhances early lung T cell response with limited impact on protection in susceptible mouse strains

The CB6F1 mouse strain is relatively resistant to aerosol Mtb infection, with a prolonged period of asymptomatic chronic/latent tuberculosis. In contrast, other inbred strains display increased

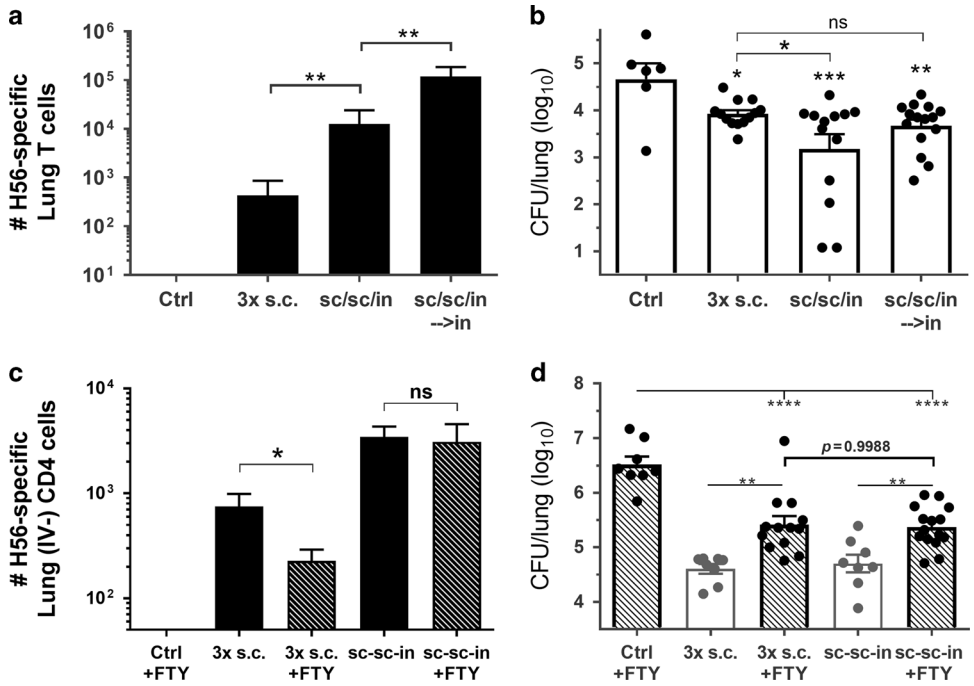


Fig. 5 Lung-localized Trm elicited by H56:CAF01 airway boosting do not improve control of pulmonary Mtb infection. **a**, **b** CB6F1 mice were vaccinated 3–4 times with H56:CAF01, as indicated, and assessed for **a** total number of H56-specific lung-localized (IV−) CD4 T cells 6 weeks after the third vaccination ($n = 5$ mice/group), and **b** total lung CFU 3 weeks after low dose Mtb aerosol challenge. **c**, **d** In a separate single experiment, CB6F1 mice were vaccinated with H56:CAF01 as indicated, rested 6 weeks, and challenged with low dose aerosol Mtb. One day prior to challenge some mice began and continued to receive FTY720 (FTY). **c** The total number of lung-localized H56-specific CD4 T cells was assessed 8 days following Mtb challenge in mice treated/non-treated with FTY720 ($n = 5$ mice/group). **d** Total lung CFU 3 weeks post challenge of control and vaccinated mice receiving FTY720-treatment. Bar, mean ± SEM, dots represent individual mice. *$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$ vs. Ctrl or as indicated via ANOVA with Tukey's posttest of log-transformed data. ns not significant

susceptibility to aerosol Mtb challenge. Therefore, we investigated if an intranasal boosting regiment would significantly increase or otherwise alter the lung-localized T cell response and control of Mtb infection in strains with genetic susceptibility to infection.

129S2 mice are highly-susceptible to Mtb infection due to a negative effect of increased type I IFN on lung phagocyte recruitment following aerosol challenge.[35] Similar to results in CB6F1 mice, subcutaneous H56:CAF01 vaccination improved control of low dose aerosol Mtb infection in 129S2 mice, while mucosal H56:CAF01 boosting did not further improve pulmonary Mtb control (Fig. 7a). Mucosal H56:CAF01 boosting did increase the early (day 8) lung-localized vaccine T cell response to Mtb challenge (Fig. 7b). However, as in CB6F1 mice, there was no effect on the phenotype of the responding CD4 T cells. Lung-localized H56-specific CD4 T cells in the Mtb-infected lungs of H56:CAF01-vaccinated mice had the same Th1/17 cytokine profiles, with enhanced IL-2 and IL-17 expression (Fig. 7c) and relative T-bet/RORγT expression profiles (Fig. 7d) regardless of route of vaccination.

C3H is another mouse strain highly-susceptible to Mtb infection.[36] Dissemination of Mtb from the lungs is greatly delayed in these mice, and this is associated with a delayed initiation of adaptive cellular immune response (relative to B6 mice).[37] Similar to 129 Sv and CB6F1 mice, subcutaneous H56:CAF01 vaccination of C3H mice provided significant protection against low dose aerosol Mtb challenged C3H mice, compared to unvaccinated controls (Fig. 7e). Mucosal boosting with H56:CAF01 also greatly increased (>40-fold) the early lung-localized vaccine T cell response to pulmonary Mtb challenge (Fig. 7f) over 3× s.c. H56:CAF01-vaccinated mice, but did not provide additional protection (Fig. 7e). Notably, as in the other two mouse strains, mucosal boosting also did not alter Th1/17 profile of the responding lung-localized vaccine CD4 T cells (Fig. 7g).

Overall, the results in the susceptible mouse strains were very similar to those of the resistant strain; despite an increased lung T cell response, no change in vaccine-specific T cell profile nor addition protection was conferred by mucosal boosting of s.c. H56:CAF01 immunization.

DISCUSSION

We have confirmed and expanded previous findings that parenteral vaccination with H56:CAF01 induces a combined Th1/Th17 population of memory T cells that accelerates the T cell response to aerosol Mtb challenge and improves control of infection.[21,22,24] We further report that boosting H56:CAF01 parenteral immunization with an intranasal boost results in the induction of a population long-lived lung-localize Trm and enhances the early vaccine T cell response within lymphocytic foci in the lungs following aerosol Mtb infection. However, we were surprised to find that this pre-existing lung T cell population and the resultant robust increase in the early lung T cell response did not improve protection against aerosol Mtb challenge. These data suggest practical limitations on the ability of vaccine-elicited T cells to control aerosol Mtb infection.

There is growing recognition that Trm, non-circulating T cells that reside within tissue parenchyma after infection, can provide secondary immune protection in various settings.[28] Parabiosis studies remain the gold-standard in confirming true tissue-resident status of a given T cell. However, phenotypic characterization of Trm has also begun to identify biological markers, and, when performed in combination with in vivo intravenous staining,[26] the identification of tissue-localized Trm via flow cytometry has greatly advanced. We report that the lung-localized CD4 T cells enhanced after airway mucosal boosting of parenteral H56:CAF01 display many characteristics of true Trm.

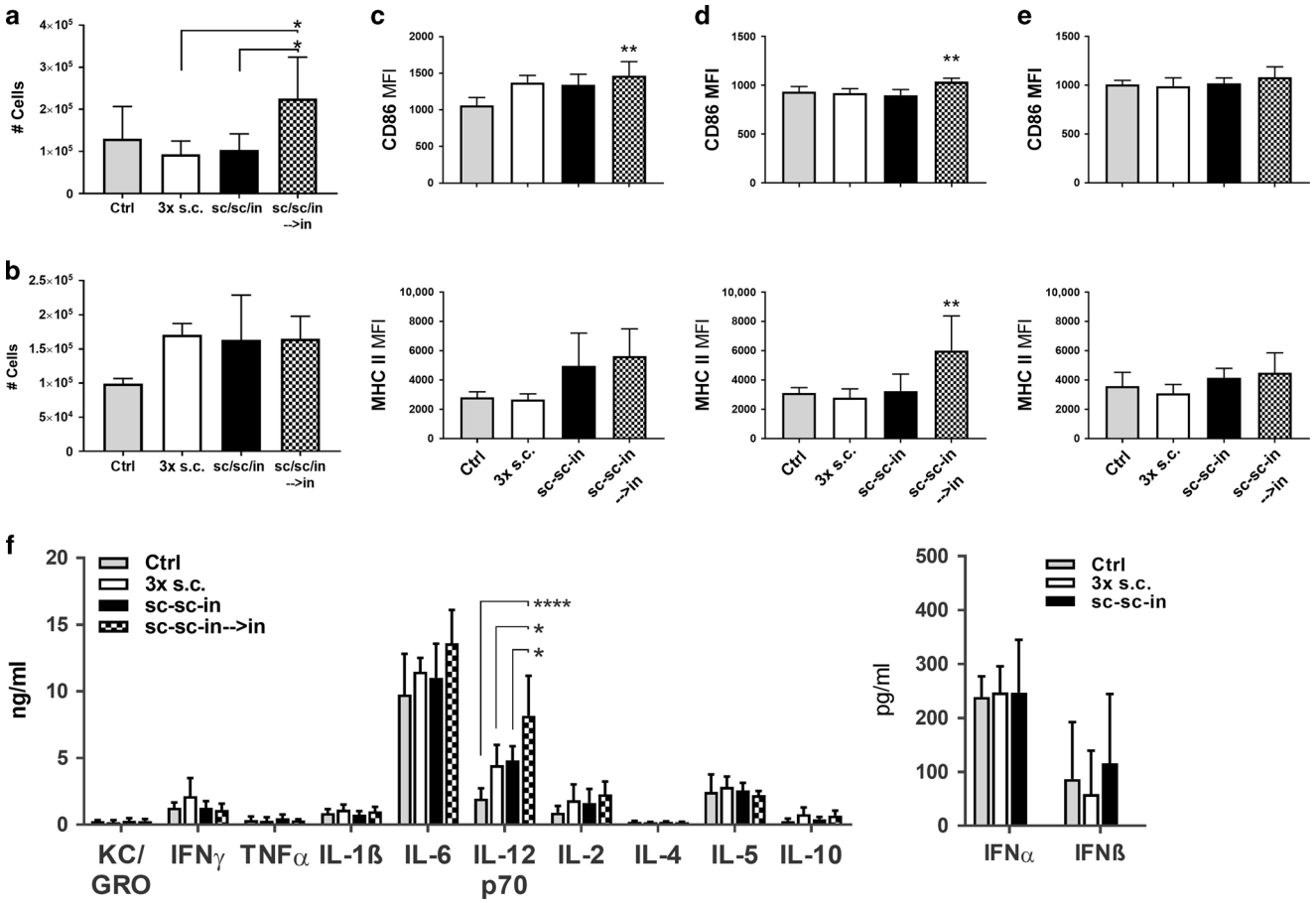


Fig. 6 Innate inflammatory environment in the lungs of H56:CAF01-immunized mice. The total number of **a** lung interstitial tissue macrophages (IV$^-$, CD11b$^+$, CD11c$^-$, Ly6G$^-$) and **b** alveolar macrophages (CD11c^{hi}, CD11b^{mid}, CD103$^-$, autofluorescent) were enumerated by flow cytometry 6 weeks after the third immunization, and **c** the relative surface expression of CD86 and MHC-II on alveolar macrophages assessed and expressed as geometric mean fluorescent intensity. **d**, **e** CD86 and MHC-II surface expression on alveolar macrophages **d** 8 days and **e** 21 days after ultra-low dose aerosol Mtb infection. **f** Lung cytokine levels 8 days post Mtb challenge. Ctrl, [gray bars]; H56:CAF01 (via 3× s.c. [open bars] or sc–sc–in routes [solid bars]); sc–sc–in immunized mice given a second i.n. boost 10 days prior to challenge (sc–sc–in–in, [checkered bars]). Bars, mean ± SD. *$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$ vs. Ctrl or as indicated, determined by one-way ANOVA with Tukey's posttest. Data are representative of two independent experiments with similar results

IV-staining shows that these T cells are located within the lung tissue and display the canonical Trm phenotype of CD62L− CD69 +, which functionally associates with tissue retention, as well as displaying a CD127+ CXCR3+ KLRG1 phenotype which has also been associated with Trm (Fig. 1).[20,27,28] Moreover, while quantitative histology shows that insufficient liberation from tissue results in a woeful under-counting by flow cytometry, we were able to readily detect these cells after vaccination. Most importantly, the H56-specific T cells were detected in the lung tissue as late as 21 weeks after immunization in mice, functionally confirming their long-lived lung-localized memory Trm phenotype.

Given that mucosal boosting enhanced lung Trm and the very early lung-localized T cell response to aerosol Mtb challenge, it was somewhat surprising that this did not translate into improved protection beyond s.c.-only H56:CAF01 immunization. Indeed, a recent study showed that overcoming the delayed accumulation of vaccine-elicited CD4 T cells into the lungs, by intratracheal delivery of activated Mtb antigen-loaded dendritic cells, greatly improved Mtb control.[38] One possible explanation for the lack of improved control is that while the enhanced Trm accelerated the vaccine response kinetics, the early T cells were unable to target the appropriate cells necessary to control infection. Mtb is known to actively prevent antigen presentation and activation of infected cells, which could have real implications for what a T cell vaccine can effectively achieve in vivo.[39,40] This leads to a model in which infected cells are severely limited in their ability to generate an effective cognate interaction with vaccine-elicited T cells at the site of infection within the first weeks of Mtb infection[41] and suggests limitations of a vaccination approach that depends upon tissue-localized T cells as a first line of defense against infected cells at the site of pulmonary infection. Our combined flow cytometry and histological data confirms that while mucosal H56:CAF01 boosting further enhanced the CD4 T cell response in the lungs within the first 2 weeks post infection, this did not enhance Mtb control. During these first 2 weeks, CD4 T cells in mucosally boosted mice were localized into peri-airway/perivascular foci with a similar distribution to that seen in all H56:CAF01-vaccinated mice at later time points. This suggests that the cells were anatomically situated to confer protection, and were therefore otherwise deficient in their capacity to do so. Notably, even further expansion of this early Trm-mediated response by >10-fold using an addition i.n. boost prior to challenge was also ineffective towards improving protection over 3× s.c. H56:CAF01-immunized mice (Fig. 5). Furthermore, isolating the Trm-mediate protection by blocking Tcm/Tem recruitment via FTY720 treatment demonstrated that the expanded Trm population in i.n.-boosted mice did not improve protection against Mtb challenge (Fig. 5). Together,

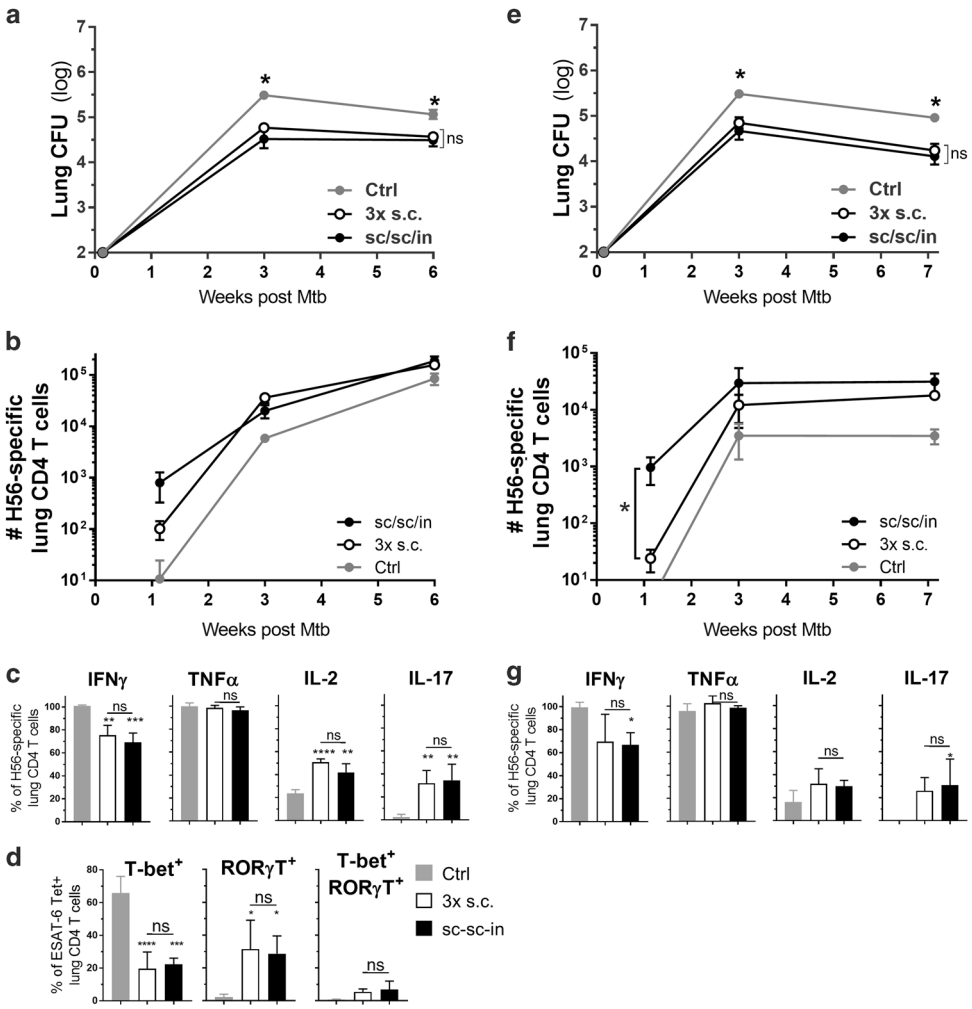


Fig. 7 Mucosal H56:CAF01 boost elicits early lung-localized T cell responses without additional protection in susceptible mouse strains. 129S2 (**a**–**d**) and C3H (**e**–**g**) mice were rested 6 weeks after vaccination and aerosol challenged with low-dose Mtb. Post challenge, lungs were assessed for (**a**, **e**) total CFU, and (**b**, **f**) the total number of H56-specific CD4 T cells in lung parenchyma (IV-) by ICS for expression of any of the cytokines IFNγ/TNF-α/IL- 2/IL-17. (**c**, **g**) The proportion of H56-specific cells from (**b**, **f**, respectively) expressing each an individual cytokine is shown for 3 weeks after Mtb challenge. (**d**) The proportion of I-Ab:ESAT-64-17 tetramer+ lung IVCD4 T cells expressing the indicated transcription factor. Symbol, mean±SEM of $n = 8$ (**a**, **e**) $n = 4$ (**b**, **f**) mice. Bar, mean±SD of $n = 4$ mice. *$p < 0.05$, **$p < 0.01$ vs. Ctrl or as indicated, via ANOVA with Tukey's post-test of log-transformed data (**a**, **b**, **e**, **f**) or raw percentages (**c**, **d**, **g**). ns not significant

these data support the interpretation that the Trm-mediated response itself cannot provide additional Mtb control in this setting. Here, it is worth noting that, 3× s.c. H56:CAF01 itself did induce a small, but detectable, long-lasting lung-localized T cell population in immunized mice with Trm markers (Fig. 1, data not shown and ref. [22]) and that likely conferred protection in the presence of FTY720. It is possible that this small population alone conferred some tissue-localized protection, which further expansion of this population by mucosal boosting did not enhance.

Airway vaccination with other anti-Mtb vaccines has shown promise in other settings. Lung-resident $CD4^+$ memory T cells induced after pulmonary immunization with an anti-Mtb recombinant influenza virus vaccine are associated with protection in mice.[42] Intranasal immunization with BCG further improves Mtb control in mice in several, but not all, studies,[15–19] and clinical development of aerosolized BCG delivery is ongoing.[43] While a mechanistic interpretation of such improved protection after pulmonary BCG infection must be carefully considered due to non-specific effects, one study suggests that the protection is CD8 Trm-dependent.[20] Intranasal delivery of recombinant adenovirus Mtb-vaccine is more protective than parenteral immunization, although here the natural respiratory mucosa tropism of the adenoviral vector leading to increased magnitude of vaccine take is likely a factor.[44,45] In contrast, intranasal boosting of recombinant adenovirus with a recombinant vesicular stomatitis virus (VSV) resulted in reduce T cell-mediated control of Mtb via a type I interferon-mediated inhibition of the lung macrophage inflammatory response, including reduced activation in vivo after Mtb infection.[34] Notably, in our studies, the activation status of the alveolar macrophages in response to aerosol Mtb challenge was not diminished by i.n. administration of H56:CAF01 (Fig. 6), indicating that a mucosal vaccine-induced reduction of macrophage capacity was not the reason that the enhanced early T cell response in sc–sc–in H56:CAF01-immunized mice was unable to further improve control of Mtb. To the contrary, mice that received a second i.n. administration of H56:CAF01 (sc–sc–in→ in) displayed increased activation of alveolar macrophages and increased lung IL-12 after Mtb challenge, suggesting that (initially) after H56:CAF01 i.n. boosting the innate lung environment is pro-inflammatory. However, by 6 weeks post-vaccination, the basal lung environment and subsequent macrophage response to aerosol Mtb challenge was not different between control, 3× s.c.

and sc–sc–in H56:CAF01-immunized mice, demonstrating that our experimental design using an adjuvanted subunit vaccine and sufficiently long resting periods mitigated such transient effects of mucosal immunization.

Recently, i.n. immunization with a recombinant Mtb antigen fusion protein combined with a cyclic dinucleotide adjuvant was demonstrated to be more protective than subcutaneous immunization.[46] Notably, this enhanced protection was associated with Th17-skewing of the immune response after i.n. immunization and the resultant combined Th1 + Th17 vaccine profile.[46] Likewise, i.n. immunization with another protein subunit vaccine in a Th1-skewing adjuvant, ID93:GLA-SE, also resulted in a dramatically Th17-skewed T cell response.[47] Similarly, i.n. BCG induces a Th17 immune response that confers protection in the DBA/2 mouse model of TB where s.c. BCG, which does not induce Th17 immunity, is not protective.[48] Moreover, increasing the Th17 response to parenteral BCG immunization, via blocking IL-10 during vaccination, or i.n. BCG by adjuvanting with cholera toxin further improves control of Mtb infection.[19,49] Indeed, in combination with anti-Mtb Th1 cells, vaccine-elicited Th17 cells accelerate vaccine immunity and are protective against Mtb infection.[7] Taken together, these observations suggest that in many cases, the improved protection after airway mucosal immunization is provided not by enhancing a vaccine-elicited Trm population, but by skewing the vaccine-induced Th1 response into a Th1 + Th17 response. When given parenterally, H56:CAF01 already elicits a Th1 + Th17 anti-Mtb response, which is not altered by mucosal boosting (Figs. 3 and 6). This pre-existing vaccine-induced Th1 + Th17 profile of s.c. H56:CAF01 may well explain why, in contrast to BCG and other anti-Mtb subunit vaccines, mucosal boosting of parenteral s.c. H56:CAF01 did not enhance protection.

Improved efficacy of a vaccine-elicited Th1 response by addition of a Th17 response is likely pathogen specific. Indeed, in a set of studies comparing adjuvant impact on vaccine immunogenicity and efficacy, H56, was most protective against Mtb infection when it was combined with the Th1 + Th17-skewing CAF01 adjuvant.[23] In contrast, the efficacies of both an anti-chlamydia and an anti-influenza vaccine did not correlate with adjuvant-dependent Th17 responses.[23] In Mtb infection, Th17 cells have been associated with acceleration of the initial response to Mtb challenge and organization of cells within a protective granuloma structure to provide long-term control.[7,50,51]

Over the past two decades, various T cell populations with different targets and functionalities have been identified as being protective against Mtb. Finding the optimal combination of complementary T cells to be induced by a vaccine against Mtb is still a mystery. Certainly, even the idea that there is one such combination is likely an oversimplification of the task at hand. Early T cell responses elicited by vaccination against Mtb are protective, but have inherent limits in the course of Mtb infection. Aerosol delivery of vaccines to maximize Trm responses is intriguing, but the impact on increased and true vaccine-memory protection may be vaccine dependent. The ability for airway route of vaccination to show improvement over parenteral vaccination may depend on the specific vaccine used and the population to be vaccinated for protection (e.g., genetic susceptibility, Mtb exposure-level of the environment). Different vaccine candidates may be "lacking" different T cell subsets for their optimization. Here, we show that in the case of parenteral H56:CAF01, expanding the lung-localized population of Trm does not complement the existing vaccine-elicited protective CD4 T cells.

METHODS

Mice

Six-to-eight-week-old female C3H/HeNHsd and CB6F1 (BALB/c × C57BL/6) (Envigo, Netherlands), and 129S2PasCrl (Charles River, Germany) mice were housed at Statens Serum Institut and rested 1 week before any procedures. All handling and procedures were performed in compliance with the European Community Directive 86/609 for the care and use of laboratory animals.

Immunizations

Mice were immunized three times at 2-week intervals. Subcutaneous (s.c.) immunizations comprised 200 µl of CAF01 (250 µg DDA/50 µg TDB) alone ("Ctrl") or mixed with 5 µg H56 protein, produced as previously described.[23] Intranasal (i.n.) immunizations comprised 5 µg H56 in CAF01 (0.25–1.0× of s.c. dose) split over 15 µl per nostril.

Mtb infection and lung CFU enumeration

Following immunization, mice were rested 6–20 weeks prior to aerosol challenge with Mtb. Virulent *M. tuberculosis* Erdman (TMC 107, ATCC) grown to log-phase in Sauton medium (BD Pharmingen "BD", San Diego, CA, USA) stored at −80 °C. was suspended in PBS Tween 20 (0.05%) and aerosolized for inhalation using a Biaera exposure system controlled via AeroMP software (Biaera, Hagerstown, MD, USA). Average low dose infection delivered 50–150 CFU/animal and as confirmed by CFU plating of whole lung homogenates 1 day after infection. For ultra-low dose infections, 1/5 dose was delivered resulting in productive infection of ~75% of animals with 0–40 lung CFU at 1 day post infection.

To enumerate lung bacteria, left lung lobes from individual mice were homogenized using Miltenyi AutoMACS dissociator and M-tubes (Miltenyi, Germany), serially diluted in PBS, grown for 2 weeks at 37 °C, and CFU enumerated as previously described.[21] For ultra low dose infections, mice without a detectable productive infection (i.e., no CFU detectable, limit of detection = 6 CFU/lung) were excluded from analyses of changes in bacterial burden.

Intravenous staining

Anti-CD45.2 FITC (BD), 2.5 µg/mouse was given intravenously via tail vein 3–6 min prior to organ harvest to distinguished parenchymal-localized (IV−) and vasculature-associated (IV+) cells.

Tissue preparation and cytokine analysis

Lungs were aseptically removed from euthanized mice into 4 °C 2.5 ml RPMI media, mildly dissociated via AutoMACS (C-tubes, Miltenyi) and supernatants harvested for cytokine analyses, before digested for 30–60 min with collagenase IV (Sigma-Aldrich) and forced through a 70 µm filter and washed twice before direct staining for MHC II tetramers and surface markers or stimulated for ICS analysis. Lung and spleens for CFU were frozen at −80 °C until further use.

Total lung supernatants were assayed for cytokines (IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p70, IFNγ, TNFα, KC/GRO) via a 10-plex murine V-plex assay (MesoScaleDiagnostic, Rockville, MD, USA) and for murine type I interferons using individual ELISA kits for IFNα (eBioscience, San Diego, CA, USA) and IFNβ (PBL Assay Science, Piscataway, NJ, USA).

Flow cytometry

Intracellular staining (ICS) for cytokines. 1–2 × 10^6 lung MNCs were stimulated in vitro in V-bottom 96-well plates at 37 °C in media containing anti-CD49d (1 µg/ml) and anti-CD28 (1 µg/ml) without antigen or in the presence of H56 protein at 5 µg/ml for 1 h, plus 6 h in the presence of 10 µg/ml brefeldin A (Sigma-Aldrich), after which cells were maintained at 4 °C for 2–8 h before staining. Cells were surface-stained using anti-CD4-allophycocyanin (clone RM4-5; BD Biosciences; BD) before fixation and permeabilization using Cytoperm/Cytofix kit (BD) as per manufacturer's instructions, and subsequently stained for intracellular cytokines with anti–IFN-γ PE-Cy7 (XMG1.2; BD), anti–TNF-α-PE (MP6-XT22; BD), IL-2-

allophycocyanin-Cy7(JES6-5H4; BD), and IL-17A-PerCP-Cy5.5 (I7B7; eBiosciences, San Diego, CA, USA). Antigen-specific cell numbers determined by Nucleocounter (ChemTec, Deerfield Beach, FL, USA) enumeration in combination with ICS data or by direct event count enumeration of known sample volumes run by flow cytometry. Non-specific background cytokine positive events from paired non-H56 stimulated were subtracted for each combinatorial Boolean gate.

Tetramer and surface staining. I-A^b:ESAT-6$_{4-17}$-APC or PE, and I-A^b:hCLIP negative control, were provided by the NIH tetramer facility (Atlanta, GA, USA). When applicable, cells were stained with tetramers for 30 min at 37 °C before surface staining at 4 °C with anti-CD4-BV786 (GK1.5; BD), CD44 Alexaflour700 (IM7; Biolegend, San Diego, CA, USA) or BV711 (BD), CD127- PE/CF594 (SB/199; BD), KLRG1- BV711 (2F1; BD), CXCR3-PerCP/Cy5.5 (CXCR3-173; eBioscience), and CD69-PE/Cy7 (H1,2F3, BD), CD62L-APC/Cy7 (MEL-4; eBioscience), CD11b-PE/Cy7 (M1/70;BD), CD11c-BV421 (HL3;BD), CD86-BV510 (GL1;BD), I-A/I-E-BV605 (MHC II, M5/114.15.2;BD), Ly6G-PE (1A8;BD), CD103-BV786 (BD). Dead cells were excluded by the fixable viability e506 or e780 (eBioscience). For transcription factor staining, the FoxP3/transcription factor buffer set (eBioscience) with anti-T-bet-APC (4B10; BD) and anti-RORγT-PE/CF594 (Q31-378; BD) was used.

Data was collected using a LSR Fortessa (BD) and data analyzed Flow cytometry analyses including file concatenation and Boolean analysis was performed with FlowJo software v.10 (Tree Star, Ashland, OR, USA).

FTY720 protocol

Mice were given daily oral administration of 70 µg FTY720 (Sigma-Aldrich) (200 µl, 350 µg/ml) beginning 1 day prior to Mtb challenge and continuing until organ harvest post infection.

Immunohistochemical staining

Lung tissue was fixed in 4% buffered paraformaldehyde formalin (VWR, Leuven, Belgium) and stored at 4 °C until further processing at BioSiteHisto (Helsinki, Finland). Briefly, fixed tissue was embedded in paraffin and sectioned prior hematoxylin and eosin staining or anti-CD4 antibody staining with immunoperoxidase detection and hematoxylin counterstaining. Stained sections were analyzed by an independent and blinded pathologist.

Statistical analysis

Prism software (v.7.04, GraphPad, San Diego, CA) was used to perform all statistical analyses as described in figure legends. CFU data and total cytokine positive cells numbers were log-transformed to normalize variance before analysis.

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AUTHOR CONTRIBUTIONS

J.S.W., E.M.A., R.M., and P.A. developed the concept and designed the experiments. D. C. contributed expertise to the immunization strategy and formulations. J.S.W. performed the experiments and analyzed the results. J.P.C. analyzed the histology data in a blinded manner. J.S.W. wrote the manuscript with critical input and revision for intellectual content from P.A. All authors approved of the final version.

ADDITIONAL INFORMATION

The online version of this article (https://doi.org/10.1038/s41385-019-0145-5) contains supplementary material, which is available to authorized users.

Competing interests: P.A. is a co-inventor on patents relating to cationic liposomes as vaccine adjuvants and to the TB fusion protein Ag85B-ESAT-6-Rv2660c (H56). All rights have been assigned to the Statens Serum Institut. The other authors declare no competing interests.

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