Mucosal BCG Vaccination Induces Protective Lung-Resident Memory T Cell Populations against Tuberculosis


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ABSTRACT Mycobacterium bovis Bacille Calmette-Guérin (BCG) is the only licensed vaccine against tuberculosis (TB), yet its moderate efficacy against pulmonary TB calls for improved vaccination strategies. Mucosal BCG vaccination generates superior protection against TB in animal models; however, the mechanisms of protection remain elusive. Tissue-resident memory T (T_{RM}) cells have been implicated in protective immune responses against viral infections, but the role of T_{RM} cells following mycobacterial infection is unknown. Using a mouse model of TB, we compared protection and lung cellular infiltrates of parenteral and mucosal BCG vaccination. Adoptive transfer and gene expression analyses of lung airway cells were performed to determine the protective capacities and phenotypes of different memory T cell subsets. In comparison to subcutaneous vaccination, intratracheal and intranasal BCG vaccination generated T effector memory and T_{RM} cells in the lung, as defined by surface marker phenotype. Adoptive mucosal transfer of these airway-resident memory T cells into naïve mice mediated protection against TB. Whereas airway-resident memory CD4^{+} T cells displayed a mixture of effector and regulatory phenotype, airway-resident memory CD8^{+} T cells displayed prototypical T_{RM} features. Our data demonstrate a key role for mucosal vaccination-induced airway-resident T cells in the host defense against pulmonary TB. These results have direct implications for the design of refined vaccination strategies.

IMPORTANCE BCG remains the only licensed vaccine against TB. Parenterally administered BCG has variable efficacy against pulmonary TB, and thus, improved prevention strategies and a more refined understanding of correlates of vaccine protection are required. Induction of memory T cells has been shown to be essential for protective TB vaccines. Mimicking the natural infection route by mucosal vaccination has been known to generate superior protection against TB in animal models; however, the mechanisms of protection have remained elusive. Here we performed an in-depth analysis to dissect the immunological mechanisms associated with superior mucosal protection in the mouse model of TB. We found that mucosal, and not subcutaneous, BCG vaccination generates lung-resident memory T cell populations that confer protection against pulmonary TB. We establish a comprehensive phenotypic characterization of these populations, providing a framework for future vaccine development.

Since its introduction almost a century ago (1), live attenuated Mycobacterium bovis Bacille Calmette-Guérin (BCG) remains the only licensed vaccine against tuberculosis (TB) caused by the intracellular pathogen Mycobacterium tuberculosis. Although originally applied orally, today BCG is administered intradermally in early childhood and effectively prevents extrapulmonary TB, mainly disseminated miliary and meningeal forms in children (2). However, BCG fails to confer sufficient protection against the most common form of the disease, pulmonary TB. Thus, TB continues to cause significant global morbidity and mortality (3). The development and implementation of new and more efficient vaccines is mandatory if TB morbidity and mortality are to be reduced by 90% and 95%, respectively, to achieve the 2035 goal of the Stop TB Partnership (4, 5).

Induction of memory T cells has been shown to be essential for protective TB vaccines (6). In mice, protection against an M. tuberculosis challenge following subcutaneous (s.c.) BCG vaccination is dependent on Th helper type 1 (Th1) CD4^{+} T cell responses (7, 8). However, one of the shortcomings of s.c. BCG administration is the overall weak memory lymphocyte generation, which in addition lacks the mucosal-homing chemokine receptors that allow migration to the lung (9). Hence, mucosal vaccination has been suggested as a mimic of natural infection in order to improve local immunity at the site of infection (10–12). Comprehensive analyses of local immunity and correlates of protection in both the lung airways and the parenchyma are essential for the rational design of mucosal TB vaccination strategies using BCG (13, 14). Airway luminal T cells have been found to be critical for protec-
Confirming recent studies (19, 20), we found that multilungs were determined at various time points postinfection (p.i.) with M. tuberculosis dose of virulent tuberculosis infection. (A) B6 mice were BCG vaccinated either i.t. or s.c. with memory T cells, TRM cells, which permanently resides in nonlymphoid initial portals of infection (17). More recently, a third subset of blood and peripheral tissues, where they are thought to survey the mucosal administration of BCG.

Mucosal BCG vaccination confers improved protection against Mycobacterium-specific CD4+ and CD8+ T cells into the lung parenchyma. To identify possible mechanisms of improved protection following i.t. BCG vaccination, we performed a histological analysis of lung-infiltrating immune cells. Sixty days after mucosal vaccination (immediately prior to infection), unperfused lungs displayed greater cell infiltration and higher histological scores than those of naive and s.c. BCG-vaccinated mice (Fig. 2A and C, top). A large proportion of lung-infiltrating cells were CD3+ T cells, many of which were CD4+ (Fig. 2A and C, bottom). In contrast, 45 days after M. tuberculosis infection, there were no significant differences in the total number of T cells among the groups despite the lower histological scores of BCG-vaccinated animals (Fig. 2B and C).

To determine whether lung-infiltrating T cells were located in the lung parenchyma or the lung airways, we first removed the bronchoalveolar lavage fluid (BALF) and performed flow cytometry of the lung parenchyma tissue. Mucosal BCG vaccination induced higher numbers of CD4+ and CD8+ T cells in the lung parenchyma between days 22 and 45 following BCG vaccination (Fig. 2D, top). Intriguingly, this increase proved to be transient, as by day 60, the day of an M. tuberculosis challenge, there were no significant differences in the total lung parenchyma-infiltrating T cell numbers between the vaccination routes (Fig. 2D, top). At that time point, approximately 100 BCG CFU were detected in the lung (see Fig. S1A in the supplemental material). The majority of lung-parenchyma-infiltrating T cells displayed a memory phenotype, and a proportion stained positive for major histocompatibility complex (MHC) peptide tetramers derived from dominant mycobacterial antigens, namely, Ag85B-specific CD4+ (Ag85B: H-2I-Ab) and TB10.4-specific CD8+ (TB10.4:H-2Kb) T cell subpopulations (Fig. 2D, bottom; see Fig. S1B). However, apart from a small number of persisting TB10.4+–specific CD8+ T cells, the overall numbers of antigen-specific CD4+ and CD8+ T cells were comparable between the i.t. and s.c. BCG-vaccinated groups directly before an M. tuberculosis challenge (Fig. 2D, bottom). Furthermore, no significant differences in the numbers of lung alveolar macrophages (AMs) (CD11c+CD11b+Ly6G−/−F4/80+), dendritic cells (DCs) (CD11c+CD11b+F4/80+MHC class I+), or neutrophils (CD11b+Ly6G+) were observed over time between the two routes of vaccination, which suggests that changes in the myeloid compartment did not underlie increased protection (see Fig. S1C). Collectively, these results suggest that mucosal BCG vaccination drives a transient increase in Mycobacterium-specific CD4+ and CD8+ T cell populations in the lung parenchyma that recedes before a challenge.

I.t. BCG vaccination generates T cells seeding the lung airways. To further determine the contribution of airway-resident immune cells to improved vaccine-mediated protection, we collected BALF and performed a comprehensive analysis of airway-infiltrating cells in response to BCG vaccination and M. tuberculosis challenge. Analysis of the proportional changes in lumen-resident cell types revealed a cellular composition skewed toward resident lymphocytes following mucosal vaccination (Fig. 3A; see Fig. S2A). Although the total cell numbers in the BALF were comparable (see Fig. S2B), increased and decreased frequencies in airway cell populations were also reflected in the mucosal BCG vaccination confers better protection against M. tuberculosis infection than parenteral s.c. BCG vaccination for at least 100 days (Fig. 1B and C).

**RESULTS**

Mucosal BCG vaccination confers superior protection against M. tuberculosis infection. To investigate the role of lung-resident T cells in immune protection against TB following BCG vaccination, we compared local (mucosal) BCG vaccination via the intratracheal (i.t.) route to parenteral vaccination by s.c. administration of BCG. Sixty days after vaccination, mice were challenged aerogenically with M. tuberculosis and the bacterial loads in their lungs were determined at various time points postinfection (p.i.) (Fig. 1A). Confirming recent studies (19, 20), we found that mucosal BCG vaccination confers improved protection against M. tuberculosis infection than parenteral s.c. BCG vaccination for at least 100 days (Fig. 1B and C).
FIG 2  I.t. BCG vaccination causes transient influx of T cells into the lung parenchyma. Histological staining of lung sections from control and i.t. or s.c. BCG-vaccinated mice 60 days after BCG immunization (A) and on day 45 after M. tuberculosis infection (B). Lung sections were stained with H&E (top) and IF (bottom) for CD3 (red), CD3 (white), and CD4 (blue). (C) Histological scores (top) and numbers of CD4+ T cells per 10 high-power fields (hpf) (bottom) at designated time points after BCG vaccination (gray) and an M. tuberculosis challenge (black). Scale bar, 100 μm. Flow cytometric quantification of lung parenchyma (D) TCRβ+ CD4+ and CD8+ T cells (top) and antigen-specific Ag85B:H-2I-Ab CD4+ and TB10.4:H-2Kd CD8+ T cells (bottom) at designated time points after BCG vaccination (gray) and an M. tuberculosis challenge (black). Results are presented as mean values ± the standard error of the mean from two pooled independent experiments (n = 8 to 10 mice per group). The statistical significance of differences between s.c. and i.t. BCG immunizations is shown. ****, P ≤ 0.0001; ***, P ≤ 0.001; **, P ≤ 0.01; *, P ≤ 0.05. (C, D) Analysis of variance with Tukey’s posttest for significance.

total cell numbers (see Fig. S2C). In contrast to the kinetics of local parenchymal T cell populations, we identified increased frequencies and numbers of airway luminal T cells after i.t. BCG vaccination that persisted until the challenge (Fig. 3B). Influx of T cells into the lung airways was detected at later experimental time points than parenchymal infiltration and started around day 24 after vaccination. Most strikingly, i.t. BCG vaccination led to a profound change in the composition of lung-residing immune cells that was characterized by a numerical and proportional increase in T cells (Fig. 3B; see Fig. S2A), many of which were specific for mycobacterial antigens by tetramer staining (Fig. 3C; see Fig. S3). Additionally, CXCR3, a chemokine receptor required for migration of T cells into the lung airways and parenchyma (22), was highly expressed on antigen-specific T cells after i.t. BCG vaccination (Fig. 3D), indicating recent targeted migration to the lung airways.

T\text{EM} and T\text{RM} cells infiltrate the lung airways after i.t. BCG vaccination. Because of the striking increase in the number of luminal T cells following i.t. vaccination, we interrogated whether airway-infiltrating T cells following i.t. BCG administration phenotypically resembled T\text{EM} (CD44hi CD62Llo CD103hi) and T\text{RM} (CD44hi CD62Llo CD103hi CD69lo) cells. Particularly the T\text{RM} population has been shown to confer protection against viral and bacterial pulmonary infections (23, 24). We found that, indeed, i.t. BCG vaccination recruited significantly higher frequencies and absolute numbers of CD4+ and CD8+ T\text{RM} and T\text{EM} cells to the airways than s.c. BCG vaccination (Fig. 4A and B). Similarly, characterization of parenchymal T cells revealed higher numbers of CD4+ and CD8+ T\text{RM} and T\text{EM} cells in i.t. BCG-vaccinated mice (Fig. 4C). Collectively, our results demonstrate that i.t. BCG vaccination induces CD4+ and CD8+ T\text{RM} and T\text{EM} cell recruitment to the lung airway spaces and the lung parenchyma.

Phenotypic characterization of airway-infiltrating T cells generated by i.t. BCG vaccination. T\text{RM} cells vary in phenotype and function, depending on the tissue they reside in (25–27). The phenotype of T\text{RM} cells in lung airways following mucosal BCG vaccination has not been characterized. Hence, we performed

![Image](https://mbio.asm.org/)

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transcriptional gene expression profiling of sorted BALF CD4$^+$ and CD8$^+$ T$_{EM}$ and T$_{RM}$ cell subpopulations induced by i.t. BCG vaccination with a Fluidigm Dynamic Array. The purity of the different sorted cell populations was routinely assessed at 86 to 99% (see Fig. S4). Increased transcription levels of typical markers associated with tissue residency of CD4$^+$ and CD8$^+$ TRM such as Itgae (CD103) and Itga1 (VLA-1) were confirmed (Fig. 5A and B). CD4$^+$ TRM cells displayed a regulatory profile, with high Foxp3 and Il10 mRNA expression (Fig. 5A and B). Additionally, CD4$^+$ TRM cells expressed T-bet, as well as Foxp3, at the protein level (Fig. 5C). Importantly, each marker was expressed by distinct sub-populations, suggesting a heterogeneous population comprising effector and regulatory T cells (28). Therefore, we concluded that CD4$^+$ TRM cells, defined here as CD4$^+$ CD103$^+$ CD69$^+$ cells, comprise a mixture of regulatory and effector T cells rather than solely belonging to the T$_{RM}$ subset. On the other hand, CD8$^+$ T$_{RM}$ cells expressed significantly higher levels of gamma interferon (IFN-$\gamma$) (Ifng), tumor necrosis factor alpha (TNF-$\alpha$) (Tnfa), and Cxcr6 (Fig. 5B) (29) and statistically insignificantly higher levels of perforin (Prf1) and granzyme B (Gzmb) than their CD8$^+$ TEM counterparts (Fig. 5A).

To further characterize the phenotypes of T$_{EM}$ and T$_{RM}$ cells infiltrating the airways after i.t. BCG vaccination, we also assessed interleukin-2 (IL-2) receptor alpha chain (CD25), IFN-$\gamma$, and CXCR3 protein expression levels. I.t. BCG vaccination generated CD25- and CXCR3-expressing, IFN-$\gamma$-producing CD8$^+$ T$_{RM}$ cells, as well as CXCR3$^+$-expressing, IFN-$\gamma$-producing CD4$^+$ airway-resident T cell subpopulations (Fig. 5D). Collectively,
these data indicate that i.t. BCG vaccination induced airway-resident T cells with a heightened ability to migrate to the lung and produce the key protective proinflammatory cytokine IFN-γ. Although CD4+ T cells could be categorized as TEM and TRM on the basis of surface markers, transcriptional profiling revealed more heterogeneous populations.

**Mucosal transfer of airway-resident T cell populations confers protection against TB.** To determine the subset(s) of airway-infiltrating T cells critical for improved protection after mucosal vaccination, we adaptively transferred sorted airway T cell subpopulations directly into the tracheas of naive C57BL/6 (B6) mice 1 day prior to an aerogenic *M. tuberculosis* challenge (Fig. 6A; see Fig. S4 in the supplemental material). All of the transferred subsets provided some degree of protection 28 days after the *M. tuberculosis* challenge (Fig. 6B). Intriguingly, transfer of as few as 3,500 sorted CD8+ T RM cells into naive mice conferred the most profound protection against a *M. tuberculosis* challenge, on a per-cell basis (Fig. 6B). Transfer of CD8+ T RM cells was associated with significantly lower AM numbers, higher numbers of CD4+ T cells, and increased numbers of B cells in the lung 28 days after the *M. tuberculosis* challenge (Fig. 6C). We also performed airway CD4+ and CD8+ T cell depletion (Fig. S5; see Fig. S6). These experiments yielded an opposite effect compared to the transfer of different T cell subsets (Fig. 6C). Whereas the mucosal CD4+ T
cell depletion efficiency was around 90%, the CD8+ T cell deple-
tion efficiency was only around 50% (data not shown). Because of the low efficiency of CD8+ T cell depletion, we could not draw any
definitive conclusions. Therefore, despite its great additive value
to the overall conclusion, we were not able to specifically delete
T RM cell populations from the airway.

Intriguingly, when the bacterial load in the whole lung was
determined without previously performing lavage, the transfer of
all airway T cell subsets reduced bacterial loads at equal levels and
the improved protective effect of CD8+ T RM cells was lost
(Fig. 6D). These results suggest that i.t. BCG vaccination induces
(i) multiple subpopulations of local T RM cells that contribute to
protection against M. tuberculosis and (ii) compartmentalized
protective effects in lung airways but not in lung parenchyma.

Oral and i.n. vaccinations mimic i.t. BCG vaccination. Finally,
although the i.t. BCG administration employed in our model is a low-invasion intervention, it is unlikely to be broadly
applicable as a human vaccination strategy. Clinically more feasi-
ble intranasal (i.n.) and oral BCG vaccinations strikingly induced
infiltration of T cells into the lung parenchyma and airways very
similar to that induced by i.t. BCG vaccination, which was re-
spected in overall increased numbers of TEM and TRM cells
compared to the appropriate internal control as indicated. (A)
Fold changes in the expression of selected genes of sorted BALF T cell populations. (B) Fold changes in the expression of selected genes of sorted BALF T cell populations. Triplicates of 100 BALF CD4+ and CD8+ TEM cells from i.t. BCG-vaccinated mice were sorted. Quantitative PCR was run with the data collection software (36 cycles) from Fluidigm. mRNA concentrations of all sorted T cell populations were normalized to β-actin (NM_007393.4) expression. The color code indicates fold changes (2^ΔΔCt) in transcripts relative to the appropriate internal control as indicated. (B) Representative histograms of selected surface activation markers and IFN-γ expression by CD4+ and CD8+ TEM and TRM cells. Results are presented as pooled individual data points ± the standard error of the mean (B), representative fluorescence-activated cell sorter plots (C), or histograms (D) from two pooled independent experiments (n = 6 to 8 mice per group). The statistical significance of differences from the T RM cell subset (B) is shown. ****, P ≤ 0.0001; ***, P ≤ 0.001; **, P ≤ 0.01; *, P ≤ 0.05. (B) Analysis of variance with Tukey’s posttest for significance.

FIG 5 Phenotypic characterization of lung-infiltrating T cells generated by i.t. BCG vaccination (A, B). B6 mice were BCG vaccinated i.t., and BALF T cell subsets were sorted 60 days later by fluorescence-activated cell sorting gated as described in the legend to Fig. 4A. Sorted naive (TCRβ+ CD44hi CD62Llo) BALF T cells or splenic TEM cells (TCRβ+ CD44hi CD62Llo) from i.t. BCG-vaccinated mice 60 days after immunization were also used as controls. (A) Heat map showing gene expression from sorted BALF T cell populations. Triplicates of 100 BALF CD4+ and CD8+ TEM cells from i.t. BCG-vaccinated mice were sorted. Quantitative PCR was run with the data collection software (36 cycles) from Fluidigm. mRNA concentrations of all sorted T cell populations were normalized to β-actin (NM_007393.4) expression. The color code indicates fold changes (2^ΔΔCt) in transcripts relative to the appropriate internal control as indicated. (B) Fold changes in the expression of selected genes of sorted BALF CD4+ and CD8+ TEM and TRM cells from i.t. BCG-vaccinated mice compared to the appropriate internal control. Quantitative PCR was run with the data collection software (36 cycles) from Fluidigm as described for panel A. (C, D) BALF immune cell phenotype measured by flow cytometry 60 days after i.t. BCG vaccination. (C) Representative flow cytometry of intracellular Foxp3 and T-bet expression by sorted BALF CD4+ TEM and TRM cells. (D) Representative histograms of selected surface activation markers and IFN-γ expression by CD4+ and CD8+ TEM and TRM cells.
We describe an in-depth in vivo approach to dissection of the immunological mechanisms associated with improved protection of mucosal BCG vaccination against pulmonary TB. We conclude that lung-resident CD4+ and CD8+ T cells, comprising CD8+ TRM cells, are a main component underlying the enhanced efficacy of mucosal BCG administration. Airway-resident CD4+ T cells comprised a mixture of T-bet+ effector and Foxp3+ expressing regulatory T cells. In contrast, airway-resident CD8+ T cells displayed prototypical CD69 surface phenotype associated with tissue sequestration (32, 33).

TRM cells have been implicated in protection following mycobacterial lung infection, extending its relevance to lung-resident memory T cell responses. VLA-1, a1B1-integrin, is an adhesion molecule known to be highly expressed by respiratory virus-specific memory CD8+ T cells in the airways, retaining them in the lung through attachment to the extracellular matrix (35). Our study is the first to ascribe protective relevance to intraluminal T cells following mucosal BCG vaccination, which includes CD8+ TRM cells in the lung airways in the context of TB.

CD8+ TRM cells have been implicated in protection following viral infections (36), but their beneficial role following bacterial infection is just being appreciated (37). Recent work has highlighted the potential of CD8+ TRM to activate bystander NK and B cells via IFN-γ, TNF-α, and IL-2, in addition to their well-known cytolytic role (24). Mucosal i.t. transfer of airway T cell populations into na"ive mice identified a crucial role for CD8+ TRM cells in the airways, retaining them in the lung through attachment to the extracellular matrix (35). Intriguingly, when BALF was collected from infected mice prior to CFU enumeration, transferred CD8+ TRM not only displayed superior protection against M. tuberculosis challenge but also reduced the number of AMs and increased the local CD4+ T and B cell numbers. In contrast, when CFU counts in the complete lung (BALF plus lung tissue) were determined, the protective capacity of transferred CD8+ TRM cells was lower. Thus, it is tempting to speculate that cytolytic CD8+ TRM cells limit the entry of M. tuberculosis into lung tissue by killing infected AMs in the lung airways, constraining the cellular reservoir for M. tuberculosis. It is also possible that CD8+ TRM cells contribute to protective immunity attained by i.t. BCG vaccination in the lung (i) by means of killing infected AMs and (ii) by recruiting CD4+ T cells to the site

CD8+ T cells, comprising mixed CD4+ T cell populations and CD8+ TRM cells.

**DISCUSSION**

We describe an in-depth in vivo approach to dissection of the immunological mechanisms associated with improved protection of mucosal BCG vaccination against pulmonary TB. We conclude that lung-resident CD4+ and CD8+ T cells, comprising CD8+ TRM cells, are a main component underlying the enhanced efficacy of mucosal BCG administration. Airway-resident CD4+ T cells comprised a mixture of T-bet+ effector and Foxp3+ expressing regulatory T cells. In contrast, airway-resident CD8+ T cells displayed prototypical CD69 surface phenotype associated with tissue sequestration (32, 33).

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of *M. tuberculosis* infection. CD8+ T
\textit{RM} cells’ killing abilities, as well as their compartmentalized protective role, should be addressed in future studies.

Comprehensive transcriptional and flow cytometric analysis of airway CD4+ memory T cells identified a heterogeneous population comprising Foxp3+- or T-bet+-expressing T cell subsets. Further studies are needed to analyze the functional properties of CD103−CD69+CD4+ memory T cells that have been described by others as most concordant to the CD4+ T \textit{RM} Population (38). In addition, enhanced IL-10 transcripts suggest diverse roles for lung CD4+ T cells besides the classical Th1 responses previously considered correlates of protection. Although it was beyond the scope of this study to dissect the underlying protective mechanism, CD4+ Foxp3+ T-cell-derived IL-10 emerges as a strong candidate for ameliorating immunopathology (39) and at the same time has been shown to promote the maturation of CD8+ T cells (40). The exact role of airway-resident CD4+ T-cell-derived IL-10 and its functional impact on local anti-*M. tuberculosis* immunity should be elucidated in future studies.

Further studies are required to dissect the mechanisms of protection induced by the transfer of total BALF. A minute number of influenza virus-specific CD8+ T cells in the airways was recently shown to be sufficient to transfer protection against a subsequent influenza virus infection (36). Therefore, it is possible that even fewer than the 2,500 lung-resident T cells induced by BCG vaccination that were transferred here could mediate protection after mucosal transfer. Although it is beyond the scope of this study, identifying the minimal number of T cells required to transfer protection will be valuable additional information.

Some remaining questions need to be addressed in future studies to determine the role of live antigen in the lung following mucosal BCG vaccination. Although the cellular analyses of the lung revealed similar results with s.c. and i.t. BCG-vaccinated mice, the presence of a low-grade ongoing infection in the lungs hampers the use of CD44 as a memory marker, as CD44 is also a marker of effector cells during ongoing infection. The crossover of CD69 as both an early activation marker and a resident memory marker requires further transfer experiments with T cell subpopulations to address their long-term viability and recall responses in the absence of antigen in order to validate them as "true" memory populations. Because there are no singular defining markers for T \textit{RM} cells yet, particularly for the CD4+ subset, in this study, we chose to perform mRNA phenotyping of CD4 and CD8 T cells infiltrating the airways postvaccination, which revealed heterogeneous expression of transcription factors and effector molecules and confirmed their ability to mount a recall response to an infectious challenge. Furthermore, it will also be important to determine the contribution of non-*M. tuberculosis*-specific memory T cells (e.g., influenza virus-specific T cells) in mediating protection after adoptive transfer. Although unspecified mechanisms for protection cannot be ruled out entirely, the fact that not all *M. tuberculosis*-specific T cell subsets protected equally well after adoptive transfer suggests that non-*M. tuberculosis*-specific effects (41) contribute little to protection against TB following vaccination.
Nevertheless, only transfer of *M. tuberculosis*-unrelated memory T cell subsets from the lung will definitively address the role of noncognate effects.

Taken together, our results highlight the value of better understanding the mechanisms underlying mucosal vaccination against TB. Our findings emphasize that mucosal vaccination offers an option for improving protective efficacy against TB either by BCG or by second-generation vaccine candidates. We recommend that optimization of mucosal vaccine administration should complement the design of novel vaccine candidates that either substitute for or boost BCG immunization.

**MATERIALS AND METHODS**

**Animals and bacteria.** B6 mice were maintained under specific-pathogen-free conditions. All experiments were conducted in accordance with the requirements of and approval by the State Office for Health and Social Services. *M. tuberculosis* strain H37Rv (ATCC no. 27294) and BCG SSI 13311 (ATCC no. 35733) were grown by previously described protocols (42). Prior to vaccination, vaccine stock vials were thawed and cells were harvested and resuspended in phosphate-buffered saline (PBS). For CFU enumeration, serial dilutions were performed and plated onto Middlebrook 7H11 agar. Plates were incubated at 37°C for 3 to 4 weeks prior to counting.

**Immunizations and infection.** B6 mice were immunized with 5 × 10⁵ (i.t. and i.n.), 1 × 10⁶ (s.c.), or 1 × 10⁷ (oral) CFU (12, 31, 43). For i.t. immunization, anesthetized mice (1:1:8 xylazine-ketamine–PBS) were intraperitoneally inoculated with the oophoropharynx with 50 μl of bacteria (44). To determine protective efficacy, mice were challenged via the aerosol route with 200 CFU of *M. tuberculosis* H37Rv 60 days postvaccination by using a Glas-Col inhalation exposure system.

**Histology and IF assay.** Unperfused lungs from BCG-vaccinated or *M. tuberculosis*-infected animals were fixed for 24 h in 4% (wt/vol) paraformaldehyde and then dehydrated and embedded in paraffin for histological analysis. Two-micrometer sections were deparaffinized and stained with hematoxylin and eosin (H&E). For immunofluorescence (IF) assay, heat-induced antigen retrieval in citrate buffer (10 mM citric acid, pH 6.0) was performed prior to incubation with anti-CD31 (clone S231; Dianova), anti-CD3 (clone M-20; Santa Cruz), anti-CD4 (clone 145-2C11; BD Pharmingen), or anti-CD4 (clone 4SM5; eBioscience) antibodies.

**Cell isolation.** Intra-airway luminal cells were removed from the lung by bronchial lavage as described previously (45). Supernatant was frozen at −80°C until protein analysis, and the remaining cells were analyzed by flow cytometry. Lungs were perfused with PBS through the left ventricle and cut into small pieces, and single-cell suspensions were prepared by mechanical dissociation through a 70-μm nylon mesh (46).

**Flow cytometry, intracellular cytokine staining, and tetramer staining.** Identification of innate cell populations was performed with antibodies against CD11b (M1/70), CD11c (HL3), Ly6G (1A8), Siglec-F (E50-2440), F4/80 (BM8), and MHC class II (M5/114.15.2). Surface identification of T cells was performed with antibodies against T-cell receptor β (TCRβ) (H57-597), CD8 (GK1.5), CD4 (GK1.5), CD44 (IM7), CD62L (ME-14), CD103 (M290), and CD69 (H1.2F3). For memory phenotyping, CCR6 (CXCRC3-173) and CD25 (PC61.5) antibodies were included. DCs were characterized as CD11c⁺ MHC-II⁺, AMs were characterized as CD11c⁺ Siglec-E⁻ CD11b-low/− autophagy-positive cells, neutrophils were characterized as Ly6G⁺ CD11b⁺, eosinophils were characterized as CD11c⁺low− Siglec-F⁺ NK cells were characterized as TCRβ⁻ NK1.1⁺, and CD4⁺ and CD8⁺ T cells were characterized as TCRβ⁺ CD4⁺ or TCRβ⁺ CD8⁺. Intracellular staining for transcription factors Foxp3 (FJK-16s) and T-bet (4B10) was performed with the Foxp3 staining buffer kit (eBioscience). To determine IFN-γ (AS.53) cytokine levels, intracellular staining was performed with the BD Cytofix/Cytoperm Fixation/permeabilization kit according to manufacturer’s instructions.

Ag65BH-2L-Aβ (280 to 294; FQDAtYNAAGHHNAVF) tetramers were provided by the National Institutes of Health tetramer facility, and TB10.4:H-2K¹ (4 to 11: IMYNYPAM) tetramers were prepared in house. Tetramer staining was performed at room temperature for 1 h prior to additional surface staining. Analysis was performed on an LSR II or Canto II (Becton, Dickinson) flow cytometer. Data were analyzed with FlowJo (TreeStar).

**Mucosal T cell transfer.** CD4⁺ and CD8⁺ TEm and TEM cells were sorted from BALF collected from i.t. BCG-vaccinated mice 60 days postvaccination. B6 recipient mice were anesthetized and received 50 μl of a cell suspension in PBS containing sorted T cell populations i.t. The specific cell numbers transferred are indicated in the figures.

**Mucosal T cell depletion.** B6 mice were BCG vaccinated i.t., and 2 days prior to a challenge, CD4 and CD8 T cell subsets were mucosally depleted through i.t. administration of anti-CD4 (GK1.5), anti-CD8 (53-6.7), or anti-control IgG (Ctrl). Two days following mucosal depletion, depleted and untreated mice were aerosol infected with *M. tuberculosis* and lung CFU counts were determined 28 days later.

**Gene expression analysis.** Gene expression was analyzed simultaneously with the 48.48 Dynamic Array Integrated Fluidic Circuits from Fluidigm as previously described (47). Preamplification of genes by reverse transcription and cDNA synthesis (18 cycles) was performed with the Cells Direct one-step qPCR kit (Life Technologies, Inc.) and TaqMan gene expression assay mix (Applied Biosystems). Triplicates of 100 BALT or lung parenchyma cells were sorted, and mRNA amounts were normalized to β-actin (NM_007393.4) expression. Data represent fold changes (2⁻ΔΔCt) in transcripts relative to the appropriate internal control.

**Statistical analyses.** Statistical analyses were performed with GraphPad Prism software (San Diego, CA). For in vivo experiments, data from two independent experiments were pooled. P values of <0.05 were considered statistically significant.

**SUPPLEMENTAL MATERIAL**

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.01686-16/-/DCSupplemental.

Figure S1, EPS file, 1 MB.
Figure S2, EPS file, 3.1 MB.
Figure S3, EPS file, 1 MB.
Figure S4, EPS file, 1.3 MB.
Figure S5, EPS file, 0.9 MB.
Figure S6, EPS file, 1.1 MB.
Figure S7, EPS file, 0.6 MB.

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