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Induction of mycobacterial protective immunity by sublingual BCG vaccination

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ABSTRACT

Tuberculosis (TB) remains a tremendous global health problem, with 1/4 of the world's population infected and causing > 1 million deaths annually. Intradermal Bacillus Calmette-Guérin (BCG) vaccine given during infancy protects against severe forms of acute disease but does not prevent chronic infection or development of pulmonary TB. TB vaccine mucosal targeting potentially could induce mucosal resident immune cells with increased protective capacity against pulmonary infection and disease. Sublingual (SL) administration of vaccines may be an optimal mucosal delivery platform based on the high absorptive capacity of this mucosal surface, the extensive lymphoid tissue, and published preclinical studies demonstrating efficacy of SL vaccination against other pathogens. To this end, we performed preliminary testing of sublingual TB vaccines. Vaccination of mice with SL BCG elicited potent mycobacteria-specific T cell responses which persisted 16 weeks post-immunization. The magnitudes of the T cell responses were similarly induced after sublingual, intranasal, and subcutaneous BCG vaccination. Interestingly, serum mycobacteria-specific antibody responses and systemic recovery of BCG post-vaccination were lower after SL BCG compared with systemic BCG immunization. However, more importantly, SL BCG vaccinated mice were significantly protected against an aerosolized virulent *M. tuberculosis* challenge ($P < 0.0001$ compared to unvaccinated mice). Furthermore, this protection was long-lived, persisting for 16 weeks with >1 log CFU reduction compared with naïve challenged mice in both lungs and spleens ($P < 0.0001$ and $P < 0.0028$, respectively). These exciting results provide strong support for further studies exploring the mechanisms of protective immunity induced by sublingual BCG vaccination.

1. Introduction

Tuberculosis (TB) is an airborne disease caused by infection with the organism *Mycobacterium tuberculosis* (Mtb), and is responsible for more deaths than any other infectious agent [1]. Nearly one in four individuals worldwide are infected with this mucosal pathogen [1]. After host inhalation of viable Mtb, the virulent mycobacteria invade pulmonary macrophages and replicate intracellularly. Most healthy, non-immunosuppressed individuals are able to survive acute infection; however, low-level mycobacteria persist resulting in establishment of a chronic, life-long infection. Five to ten percent of infected individuals will progress to active TB disease in which they develop chronic symptoms of fever, weight loss, cough, and sometimes hemoptysis and may develop severe lung pathology and/or disseminated infection which often have poor clinical outcomes. Long-term drug therapy combinations including isoniazid and rifamycins can be effective for treating latent TB infection and disease, provided the Mtb strains are drug-susceptible. However, multi-drug and extensive drug resistant (MDR and XDR, respectively) strains have emerged in which first and second line drugs are ineffective. New therapies are needed to efficiently treat and prevent these types of infections.

The only licensed vaccine available for TB is Bacillus Calmette-Guérin (BCG), developed more than 100 years ago and first used in humans in 1921. BCG was created by extensive passaging of *M. bovis* passaged over ~10 years in the laboratory, which resulted in significant attenuation. BCG vaccination has been delivered to billions of people over the past several decades, and in many TB-endemic areas of the world BCG is given to infants at birth.

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The most common routes of delivery include percutaneous, subcutaneous, and intradermal. All result in BCG replication in vivo, produce a local inflammatory cutaneous reaction leading to scaring, and induce TB-specific B and T cell responses. BCG has proven to be safe and effective in infancy, providing protection against the most severe forms of disease including disseminated and miliary TB [2–4]. Even though BCG provides significant benefit to infants and children, immunity in adolescence and adulthood wanes. In many studies, BCG vaccination during infancy has been shown to be ineffective in protecting adults against TB. Recent studies have demonstrated that BCG re-vaccination during adolescent years can reduce sustained Mtb infection [5]. Control and BCG vaccine recipients displayed similar Quanti-FERON TB (QFT) conversion rates; however, significantly higher frequencies of control vaccinees as compared to BCG-booster recipients displayed sustained QFT positivity without reversion. These results demonstrate the usefulness of BCG vaccination and re-vaccination and support further work in this area.

Because TB is acquired mucosally through inhalation of Mtb-contaminated aerosols and droplets, it stands to reason that mucosal immunity may provide enhanced protection against infection and disease. In mice, aerosolized and intranasal BCG administration result in local mycobacterial replication in alveolar macrophages, and induce lung-resident B and T cells providing protection against aerosolized challenge with virulent Mtb. However, these routes have not been studied yet in detail in humans although an early publication reported safety of aerosol BCG administration [6]. It is also possible to induce immune cells primed to rapidly migrate into the lungs by other vaccine routes due to shared pathways utilized by the common mucosal immune system (CMIS). For example, we have demonstrated in humans that oral BCG immunization can induce more potent anti-mycobacterial secretory IgA responses and higher numbers of lung-resident TB-specific T cells than observed after systemic BCG vaccination [7]. In these same studies, we have shown that circulating memory CD4+ T cells from oral and systemic BCG vaccine recipients display very different and unique molecular signatures, and differences in expression of the mucosal trafficking markers CXCR3 and $\alpha 4\beta 1$.

Another vaccination approach that may induce relevant mucosal immunity involves sublingual (SL) delivery. Sublingual immunotherapy (SLIT) for allergy treatment is safe and effective in humans, and has been used extensively for many years (reviewed in [8,9]). In preclinical studies, SL influenza vaccines have been shown to induce robust B and T cell immunity protective against respiratory challenges by multiple investigators [10–12]. Our current work is based on the hypothesis that SL BCG delivery in mice would induce T cell immunity protective against a biologically relevant aerosolized TB challenge.

2. Methods

2.1. Mice and mycobacteria

Six to eight week-old female C57BL/6J mice (Jackson strain 000664) were used for these studies, and housed in microisolator cages (3–5 mice per cage) under pathogen-limiting conditions throughout. All studies were conducted in accordance with Saint Louis University Institutional Animal Care and Use Committee approvals. Connaught (Connaught Labs) and Danish (Statens Serum Institut) strains of BCG, and *M. tuberculosis* (Erdman strain; BEI Resources NR-15404) were grown in roller bottles at 37 °C in albumin-dextrose-catalase (ADC)-supplemented Middlebrook 7H9 media and stored in single-use aliquots at −80 °C until use. Mycobacteria were thawed and plated on oleic acid-albumin-dextrose-catalase (OADC)-supplemented 7H10 agar plates to determine concentrations (colony forming units; CFU) prior to use in vivo or in vitro. BCG viability post-incubation in PBS, 25% glycerin, or 50% glycerin was assessed by CFU plating (above) and by spiking samples into Mycobacterial Growth Indicator Tubes (MGIT) which were loaded into a MGIT320 instrument (BD). Here, the time to positivity (TTP) is inversely correlated with mycobacterial inoculation.

2.2. Vaccines

Aliquots of BCG were thawed, washed with PBS, and pelleted by centrifugation (3,700 × *g*, 20 min, 4 °C). Mycobacterial pellets were suspended in PBS or 50% glycerol in PBS to the required concentrations to generate doses as described in each figure legend (10^5–10^7 CFU/dose). For sublingual vaccinations, mice were first anesthetized with ketamine/xylazine (60 mg/kg and 5 mg/kg, respectively). Next, mice were immobilized, forceps used to open the mouth and displace the tongue, and 7 μl of diluted BCG placed on the floor of the mouth as previously shown [13]. Mice were allowed to rest in a horizontal state for 10 min prior to placement in the cages. Intranasal BCG vaccines suspended in PBS were administered to anesthetized mice in a volume of 40 μl split between the nares in 2 doses over 1–2 min using a P20 pipettor. Other groups of mice were administered BCG subcutaneously at the base of the tail in 100 μl PBS. Mice receiving intragastic (IG) BCG were fasted for four hours and IG treated with 0.5 ml of 1.5% sodium bicarbonate solution diluted in Hanks buffer 10 min prior to IG delivery of BCG (1×10^7 CFU in 100 μl PBS). Fifteen minutes after IG BCG administration, access to food and water access was returned.

2.3. Assessment of immunogenicity

Interferon-γ ELISPOT assays were utilized to study TB-specific T cell responses induced by SL BCG vaccination similar to studies reported previously [14,15]. Splenocytes were collected from groups of mice at various times post-BCG administration and passed over nylon mesh screens to obtain single cell suspensions. Red blood cells were removed using NH_4Cl lysis buffer and subsequent RPMI washing steps, and resuspended in assay media (10% FBS, 50U/ml penicillin, 50 μg/ml streptomycin, 2 mM L-Glutamine, 55 μM 2-mercaptoethanol, 1X non-essential amino acids, 1 mM sodium pyruvate, and 5 mM HEPES). In some experiments, bronchoalveolar lavage (BAL) samples were collected, pooled (in order to study functional T cell activity), and processed as described above. Cells were stimulated in anti-IFN-γ-coated nitrocellulose-bottom ELISPOT plates (BD Biosciences clone R46A2; and Millipore MAHAS4510) with various mycobacterial antigens [mycobacterial culture filtrate proteins (Mtb CF, BEI Resources, 10 μg/ml), or live Connaught strain BCG (MOI = 0.3–3)]. Media alone served as the negative control stimulation. After overnight incubation (37 °C, 5% CO_2), ELISPOT plates were developed as previously described using biotinylated anti-IFN-γ (BD Biosciences clone XMG1.2), streptavidin horseradish peroxidase (SA-HRP; Jackson ImmunoResearch), and AEC development solution. Plates were scanned and spots enumerated using a C.T.L. ImmunoSpot analyzer and software.

ELISA assays were conducted to assess mycobacteria-specific antibody responses. Briefly, Immulon-2 HB plates (Thermo Scientific) were coated overnight at 4 °C with 5 μg/ml Mtb whole lysate (MtbWL, BEI Resources) diluted in carbonate buffer (pH 9.6), washed with PBS + 0.05% Tween 20 (PBS-T), and blocked with 10% FBS in PBS for at least 2 h. Serum samples from non-vaccinated control and BCG vaccinated animals were serially diluted in 10% FBS/PBS and incubated at 4 °C overnight. Development of plates was accomplished with stepwise washing (PBS-T)

and addition of goat anti-murine IgG-HRP (Jackson Immuno Research), 5,5′-Tetramethylbenzidine (TMB substrate; Sigma), H_2SO_4, and data acquired with Tecan SLT Rainbow ELISA reader at 450/540 nm. Similar assays were performed to analyze vaccine-induced antibody responses to the major surface antigen of Mtb, lipoarabinomannan (LAM). Plates were coated with 4 μg/ml LAM diluted in 100% ethanol and allowed to dry at room temperature. Plates were washed and blocked overnight with 1% BSA in PBS-T. Serum samples were diluted (1:100) and added to plates, which were developed as described above.

2.4. Aerosol challenges

At times post-BCG vaccination indicated in the Figure Legends, groups of mice (N = 8–10 per group) were challenged with virulent *M. tuberculosis* via aerosol exposure. Mtb was thawed and 6 ml of 2×10^6 CFU/ml loaded into the nebulizer attached to the Glas-Col (Terre Haute, IN) Inhalation Exposure System (IES) based on previously described methods [16]. IES settings utilized for the aerosol challenges were: (1) 15-minute preheat, (2) 40-minute nebulization, (3) 40-minute cloud decay, (4) 15-minute UV decontamination, (5) 50 psi vacuum, and (6) 10 psi compressor. Two to five animals per group were euthanized immediately post-aerosol exposure to quantitate the delivery dosage. The above settings resulted in reproducible seeding of 50–100 CFU per animal.

2.5. Quantitation of mycobacteria post-challenge

At times indicated in the specific Figure Legends post-Mtb aerosol challenge, groups of animals were euthanized prior to aseptic removal of lungs and spleens. In efficacy studies, mice and resulting tissue samples were numbered and results evaluated in a blinded fashion. Tissues were rinsed in PBS, then homogenized in 1 ml of ADC-supplemented 7H9 Middlebrook media using a Tissue-Tearor (BioSpec). Ten-fold serial dilutions of homogenized tissues were plated on OADC-supplemented 7H10 Middlebrook agar plates with 5 μg/ml Thiophen-2-carboxylic acid hydrazide (TCH) to distinguish BCG from Mtb. After 4 weeks of incubation at 37 °C, colonies were enumerated. Similar methods were conducted to evaluate BCG dissemination and bacterial loads post-BCG immunization.

2.6. Statistical analyses

Mann-Whitney U-tests and student t-tests were performed using Graphpad Prism v5.

2.7. Acknowledgments

The following reagents were obtained through BEI Resources, NIAID, NIH: *Mycobacterium tuberculosis*, Erdman strain (NR-15404), *Mycobacterium tuberculosis* strain H37Rv Culture Filtrate Proteins (NR-14825), Whole Cell Lysate (NR-14822), and purified lipoarabinomannan (LAM; NR-14848).

3. Results

3.1. Potent T cell reactivity induced by SL-BCG

Systemic and mucosal administration of BCG vaccine has been shown by multiple investigators to generate robust TB-specific T cell immunity in animal models [17,18]. Sublingual vaccines for influenza, RSV, and HPV have also been demonstrated to induce potent pathogen-reactive T cell immunity [10,12,19,20]. In humans, SL immunotherapy (SLIT) is widely utilized for allergy desensitization, and has been shown to be safe and effective (reviewed in [8,9]). Glycerin is a commonly used carrier for these therapies, because it is generally inert, well-tolerated, and increases the viscosity of the SLIT dosages. We first investigated whether BCG viability would be adversely affected by suspension in glycerol/PBS solutions. We incubated equivalent amounts of BCG diluted in PBS, 25% glycerol/PBS, and 50% glycerol/PBS for 2 h at room temperature, then assessed BCG viability by CFU plating and MGIT assays. As shown in Fig. 1A, BCG viability was not compromised by glycerol exposure. We next performed pilot immunogenicity experiments in which mice were vaccinated SL with BCG suspended in PBS or 50% glycerol. Control groups included naïve animals and mice vaccinated SC with BCG. T cell reactivity to TB antigens was assessed 6 weeks later by IFN-γ ELISPOT assay. As demonstrated in Fig. 1B, BCG delivered via SL route induced robust T cell reactivity to mycobacterial antigens similar in magnitude to those induced by SC vaccination. Suspension of BCG vaccine in glycerol/PBS was beneficial, resulting in >2-fold increased frequencies of mycobacterial-specific IFN-γ-producing T cells as compared to responses from mice vaccinated with the same dosages of BCG administered in PBS alone. Interestingly, similar T cell responses were observed in mice receiving 1×10^5 – 1×10^7 BCG delivered SL.

We also investigated TB-specific antibody responses in these same groups of mice vaccinated with BCG via SL and SC routes. As expected, systemic delivery of BCG resulted in potent antibody responses specific for both MtbWL and LAM (Fig. 1C&D). However, only very low-level anti-mycobacterial antibodies were detected in serum samples from SL BCG vaccinated mice, regardless of dosage or carrier.

3.2. Evaluation of BCG strains utilized for SL vaccination

We next investigated whether different strains of BCG may influence SL vaccination-induced immunogenicity. Groups of B6 mice were vaccinated with 1×10^6 – 1×10^7 CFU of Connaught or Danish BCG (C-BCG and D-BCG, respectively) delivered via SL route. As shown in Fig. 2A, mice receiving SL C-BCG and D-BCG developed robust and similar T cell reactivity to mycobacterial antigens. Similar to results displayed in Fig. 1C&D, SL BCG immunized animals failed to mount an effective antibody response to TB antigens (Fig. 2B). To determine whether BCG colonizes pulmonary and systemic tissues, we evaluated lung and spleen mycobacterial burdens in mice vaccinated 7 weeks prior with SL and IN BCG. BCG vaccination results in long-term BCG persistence (≥7 weeks) and dissemination to systemic organs after IN delivery (Fig. 2C). However, very low if any BCG was detected in lungs and spleens of mice after SL vaccination with BCG. These results were confirmed in additional qualitative growth experiments in which 2 of 3 mice immunized via the SL route failed to colonize lung, cervical lymph nodes, or spleens. BCG was detected in all 3 tissues after SC BCG vaccination (N = 2, data not shown).

3.3. SL BCG induces recall splenic and BAL T cell responses after repeat pulmonary BCG exposure

To determine whether SL BCG primes T cells of mucosal relevance, we first vaccinated groups of mice with BCG via SL or IN routes. Nine weeks after vaccination, mice received an intranasal BCG challenge. Three days later, we studied systemic (spleen) and pulmonary (bronchoalveolar lavage) samples in mycobacterial-specific IFN-γ ELISPOT assays. SL and IN BCG vaccinated mice developed similar systemic TB-specific T cell responses that were nearly equivalent after re-exposure via pulmonary challenge (Fig. 3A). Interestingly, we identified marked increases in numbers of TB-specific T cells primed by SL BCG which reside in

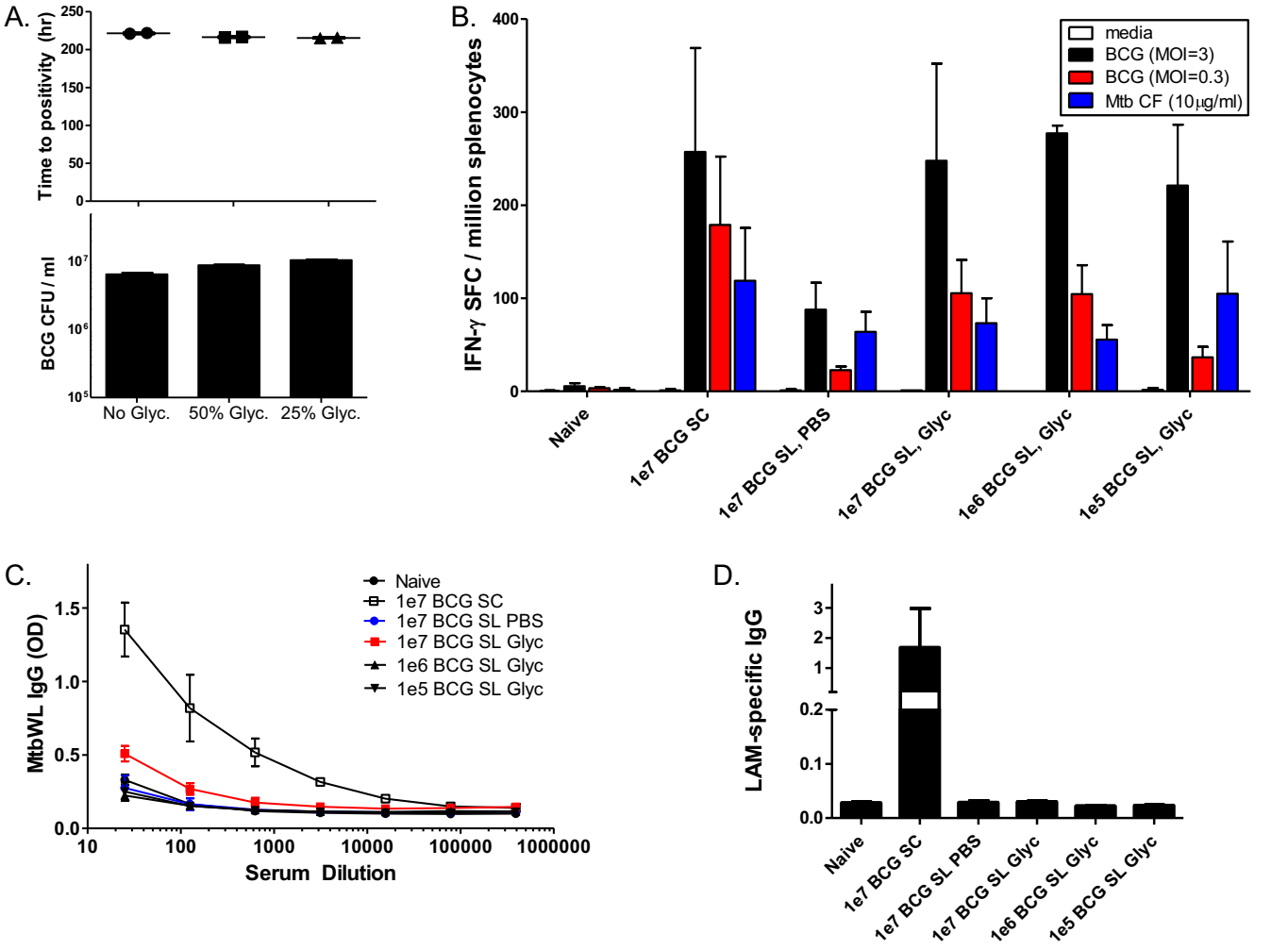

Fig. 1. Sublingual BCG vaccination induces robust mycobacteria-specific T cell, but not B cell, immunity. (A) BCG was diluted in PBS, 50% glycerin in PBS, or in 25% glycerin in PBS and incubated for 2 h prior to BCG quantification using standard CFU plating as well as MGIT Bactec (time to positivity) methods. (B) Connaught strain BCG was diluted in PBS or 50% glycerin and delivered to groups of B6 mice via SL or SC routes. After 6 weeks, mycobacteria-specific T cell immunogenicity was assessed using total splenocytes in IFN-γ ELISPOT assays after stimulation with Mtb culture filtrate (CF) or BCG (0.3–3.0 MOI). (C&D) Sera were collected 6 weeks post-vaccination and studied in Mtb lysate (C; MtbWL) and lipoarabinomannan (D; LAM)-specific IgG ELISAs. For the above experiments, 2 pools per group of splenocytes and sera were prepared from 2 to 3 animals each.

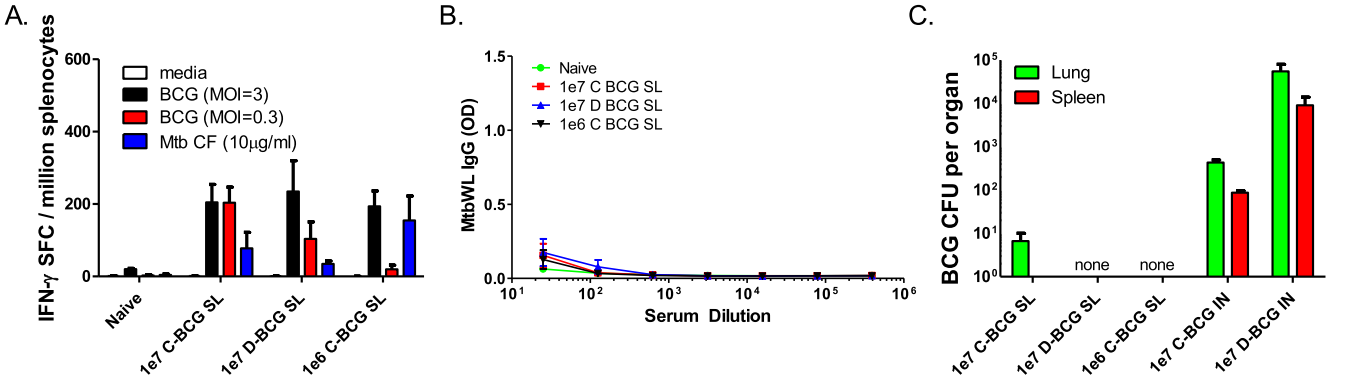

Fig. 2. Connaught and Danish BCG induce similar T cell immunity in the absence of BCG dissemination. (A) Connaught (C-BCG) and Danish BCG (D-BCG) were diluted in 50% glycerin in PBS and administered SL to B6 mice. Six weeks later, mycobacteria-specific T cell immunogenicity was assessed using IFN-γ ELISPOT assays. Total splenocytes were incubated in medium alone, or stimulated with BCG (0.3–3.0 MOI), or Mtb culture filtrate (CF; N = 3 mice per group). (B) Antibody responses were evaluated 6 weeks post-immunization using Mtb lysate-specific (MtbWL) IgG ELISAs using serum samples from N = 4–5 mice per group. (C) BCG recovery was determined in the lungs and spleens 7 weeks after SL and IN BCG administration in 3 mice per group.

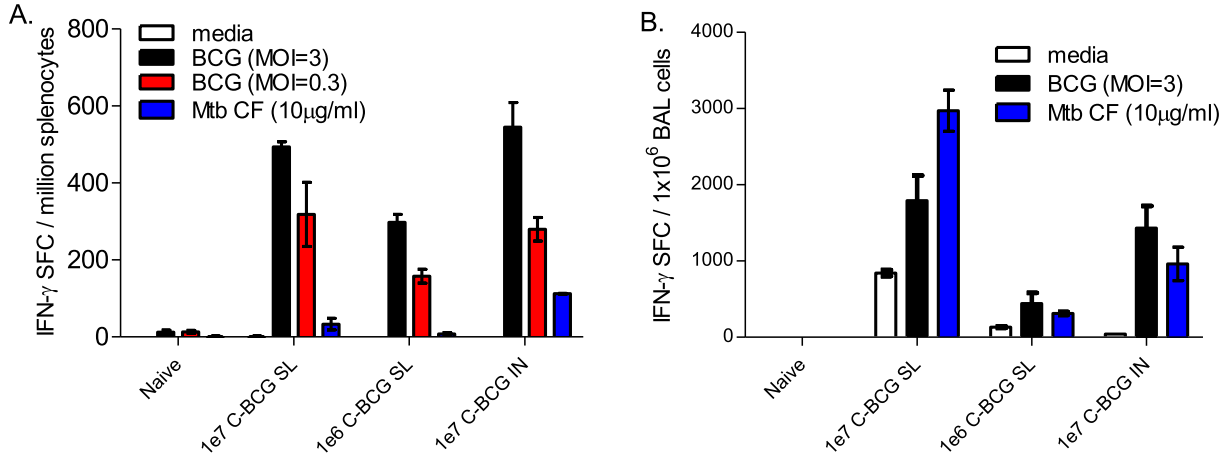

Fig. 3. Sublingual BCG vaccination induces both TB-specific mucosal and systemic T cell immunity. Wild type B6 mice were vaccinated with $1 \times 10^{6\text{-}7}$ CFU of Connaught (C) BCG via sublingual or intranasal route. Mice were challenged 9 weeks later with 1×10^{7} D-BCG IN. After 3 days, splenocytes (A) or bronchoalveolar lavage cells (B) were pooled from N = 4 mice/group and stimulated in overnight IFN-γ ELISPOT assays with nothing, live BCG, or Mtb culture filtrate (CF).

or rapidly infiltrate the lungs after pulmonary pathogen exposure (Fig. 3B).

3.4. SL BCG immunization induces immunity protective against Mtb challenge

As shown above, mice vaccinated with BCG via the SL route mount robust TB-specific T cell responses but fail to produce measurable amounts of TB-specific serum IgG. We have previously demonstrated that oral BCG vaccination in humans induces robust pulmonary TB-specific T cell responses. Therefore, we sought to determine whether SL and/or intragastric (IG) BCG vaccination induce immunity protective against a biologically relevant Mtb challenge. All routes of BCG vaccination (SC, SL, and IG) induced systemic T cell immunity (Fig. 4A). Next, groups of vaccinated and control mice were exposed to aerosolized Mtb, resulting in pulmonary seeding of ~155 CFU per mouse. Four weeks post-Mtb challenge, mice were euthanized and mycobacterial burdens evaluated in systemic (spleen) and pulmonary (lung) organs. As shown in Fig. 4B and C, SC, SL, and IG BCG vaccination resulted in significantly decreased numbers of Mtb recovered in both the spleens and lungs as compared to unvaccinated mice (all $P \leq 0.0005$). Although not found to be statistically significant, the fewest numbers of disseminated organisms were recovered from mice vaccinated with SC BCG as compared to mice vaccinated with SL or IG BCG (Fig. 4B).

3.5. Long-term protection induced by SL BCG vaccination

Because systemic BCG vaccination in infants provides short-term protection but fails to induce long-term protection, we next sought to determine whether SL BCG vaccination provides long-term protection in mice. As described above, mice were vaccinated with BCG via SC or SL route, and T cell immunity determined 16 weeks later. Both routes induced long-term mycobacteria-specific T cell responses (Fig. 5A). Additional groups of mice were challenged with Mtb at 16 weeks post-vaccination via aerosol exposure, and four weeks later mycobacteria were enumerated in spleen and lung homogenates.

Both SC and SL routes protected against disseminated infection ($P < 0.003$ compared to non-vaccinated animals; Fig. 5B). Systemic BCG vaccination resulted in more potent protection in systemic tissue (spleen) as compared to SL BCG vaccination ($P = 0.0123$). However, SL and SC BCG vaccination provided similar levels of protection in the lungs ($P < 0.0001$ compared to non-vaccinated animals; Fig. 5C).

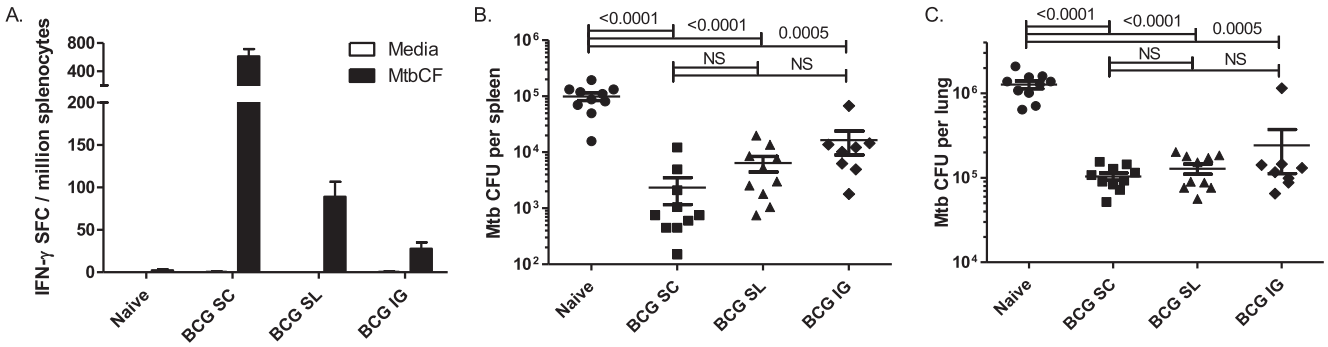

Fig. 4. SL and IG BCG vaccinations provide protection against mycobacterial challenge. Groups of female B6 mice were vaccinated with 1×10^{7} Connaught strain BCG via SC, SL, or IG route. Six weeks later, mycobacterial-specific T cell immunity was measured in 5 representative mice per group via IFN-γ ELISPOT assay upon stimulation with Mtb culture filtrate proteins (MtbCF; A). Additional mice were challenged with *M. tuberculosis* via aerosol exposure 6 weeks post-vaccination. Four weeks later mycobacterial burdens were evaluated in spleen (B) and lung (C) homogenates. Each dot represents average CFU/organ from a single mouse. Statistical comparisons were performed using 2-tailed unpaired t tests.

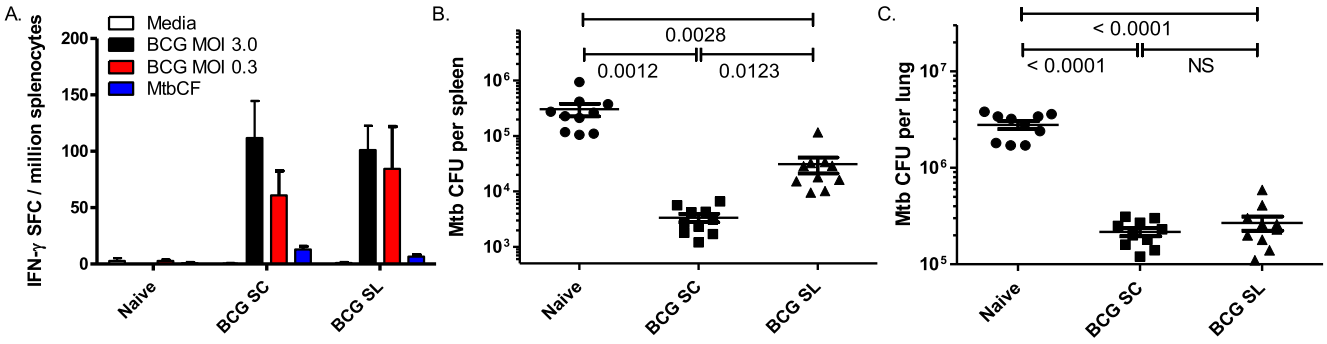

Fig. 5. SL vaccination provides long-term protection against aerosolized Mtb challenge. Female B6 mice were vaccinated via SC or SL route with 1×10^7 Connaught strain BCG. After 16 weeks, 3 representative mice per group were euthanized for study of mycobacteria-specific T cell immunity. Splenocytes were stimulated in overnight IFN-γ ELISPOT assays with Mtb culture filtrate (MtbCF) and live BCG (A). Additional mice were challenged with *M. tuberculosis* via aerosol exposure 16 weeks post-vaccination. Four weeks later mycobacterial burdens were evaluated in spleen (B) and lung (C) homogenates. Each dot represents calculated CFU/organ from a single mouse. Statistical comparisons were performed using 2-tailed unpaired t tests.

4. Discussion

The protection induced by BCG vaccination is only partial, thus improved TB vaccines and vaccine strategies are needed. Our group and others are pursuing vaccination regimens designed to induce more robust and effective immunity. We have found that oral BCG vaccination in humans induces more potent lung T cell immunity than intradermal BCG vaccination [7]. Other groups have demonstrated induction of protective immunity in preclinical animal models using pulmonary (intranasal and aerosol) BCG vaccination routes [17,21,22]. Intravenous vaccination induces very potent protective immunity in non-human primates [23]. The safety of the above regimens (intranasal, aerosol, and intravenous) has not been established, although BCG administration via each of these routes results in mycobacterial replication in the lungs. Our studies utilizing sublingual delivery of BCG have demonstrated prolonged efficacy against Mtb challenge (Fig. 5), even in the near absence of BCG vaccine dissemination and replication (Fig. 2). Further work is required to fully evaluate the safety and efficacies of the various vaccination routes.

Sublingual immunotherapy (SLIT) has been used for many years for treatment of severe allergies [8,9]. Because of the strong safety record of SLIT and its effectiveness, the SL route for vaccine delivery has garnered increased attention over the past several years. For example, many influenza vaccine formulations (eg- live-attenuated virus, hemagglutinin subunit) have been tested in mice and were demonstrated to be immunogenic and protective against live viral challenges [10–12]. More recent studies have shown that a SL delivered subunit TB vaccine containing Ag85B and ESAT-6 adjuvanted with glycolipid alpha-galactosylceramide (αGalCer) induced immunity protective against aerosolized Mtb challenge [24]. Only a handful of sublingual vaccines have progressed to human clinical trials. A human papilloma virus vaccine failed to induce strong HPV-specific serum IgG or neutralizing antibodies [25], however, the authors noted that further optimization of the vaccine formulation (eg-adjuvants) should be conducted. In contrast, a SL polyvalent bacterial vaccine (Bactek) for treatment of recurrent respiratory tract infections was shown to induce immune stimulation of CD4+ T cells [26], and a sublingual bacterial vaccine (MV 140-Uromune) was recently shown to reduce the incidence of recurrent urinary tract infections in women [27]. Because of the superior safety profile of SL as compared to injection delivery of antigens, further exploration of these types of vaccines are warranted.

Despite inducing robust T cell immunity and protection against a biologically relevant aerosol Mtb challenge, SL BCG failed to induce measurable mycobacterial-specific antibody responses. One possible explanation for this could be the lack of disseminated infection observed after SL BCG administration, leading to a lower overall antigen dose as compared with the SC vaccination route. This leads us to speculate that enhancing the uptake or presentation of antigens delivered via SL route might increase B and T cell immunogenicity. For example, various mucosal adjuvants could be used to enhance SL BCG effectiveness. Exploration of novel strategies (gels, etc.) to allow greater antigen uptake after SL vaccination may also be warranted. Though we have clearly shown that SL and IG delivery of BCG induce robust T cell immunity and protection against aerosolized Mtb challenge, the exact mechanism of protection is unknown. Further studies should be undertaken to evaluate the polyfunctionality of SL BCG-induced T cells (expression of multiple cytokines, etc.), and to assess the extent to which T cells induced by various BCG routes (SL, intranasal, subcutaneous) can inhibit intracellular mycobacterial growth. It would also be important to characterize the T cell memory phenotype of pulmonary T cells induced by SL BCG vaccination to inform future iterative vaccine designs.

The results above demonstrate that SL BCG vaccination induces potent mycobacteria-specific T cells and durable protection against Mtb challenge. In humans, intradermal BCG is often given during the first day of life in order to provide protection against severe disease for the most vulnerable populations. However, vaccine-induced immunity wanes over many years, and thus not much protection is afforded to adolescents and adults. Vaccines designed to boost immunity in these older populations are needed. As discussed above, early results from a large clinical trial demonstrated that adolescents receiving a systemic BCG booster vaccination were significantly protected against sustained infection [5]. Our group has shown that combined ID and PO BCG vaccination in humans induces superior lung T cell immunity compared to ID BCG vaccination alone[7]. Thus, combinations of mucosal and systemic BCG vaccinations might induce the best combination of mucosal and systemic immune responses and provide optimal protective efficacy. Preclinical studies in mice and non-human primates are needed to evaluate the boosting effects of BCG vaccination given via different routes (ID, PO and SL, both alone and in combination).

5. Conclusion

In summary, we have shown that SL BCG is immunogenic in mice, inducing mycobacteria-specific peripheral T cells of similar magnitude as IN and SC BCG. Notably absent in SL BCG vaccinated animals are mycobacterial-specific serum IgG responses and BCG

dissemination, however, SL BCG primes T cells that reside within and/or migrate to the lungs following a pulmonary mycobacterial challenge. Most importantly, SL BCG vaccination provided long-term protective immunity against an aerosolized Mtb challenge, warranting further exploration of this safe and effective vaccination route.

Declaration of Competing Interest

Daniel Hoft, Azra Blazevic, Emma Killoran, and Daniel Hoft report no competing interests. Mary Morris is employed by Allergy Associates of La Crosse and is a strong proponent of sublingual immunotherapy.

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References

[1] WHO. Tuberculosis fact sheet; 2018.
[2] Colditz GA, Berkey CS, Mosteller F, Brewer TF, Wilson ME, Burdick E, et al. The efficacy of Bacillus Calmette-Guérin vaccination of newborns and infants in the prevention of tuberculosis: Meta-analyses of the published literature. Peds 1995;96:29–35.
[3] Colditz GA, Brewer TF, Berkey CS, Wilson ME, Burdick E, Fineberg HV, et al. Efficacy of BCG vaccine in the prevention of Tuberculosis: Meta-analysis of the published literature. JAMA 1994;271:698–702.
[4] Trunz BB, Fine P, Dye C. Effect of BCG vaccination on childhood tuberculous meningitis and miliary tuberculosis worldwide: a meta-analysis and assessment of cost-effectiveness. Lancet 2006;367:1173–80.
[5] Nemes E, Geldenhuys H, Rozot V, Rutkowski KT, Ratangee F, Bilek N, et al. Prevention of M. tuberculosis infection with H4:IC31 vaccine or BCG revaccination. N Engl J Med 2018;379:138–49.
[6] Rosenthal SR, McEnery JT, Raisys N. Aerogenic BCG vaccination against tuberculosis in animal and human subjects. J Asthma Res 1968;5:309–23.
[7] Hoft DF, Xia M, Zhang GL, Blazevic A, Tennant J, Kaplan C, et al. PO and ID BCG vaccination in humans induce distinct mucosal and systemic immune responses and CD4(+) T cell transcriptomal molecular signatures. Mucosal Immunol 2018;11:486–95.
[8] Tosca MA, Licari A, Olcese R, Marseglia G, Sacco O, Ciprandi G. Immunotherapy and asthma in children. Front Pediatr. 2018;6:231.
[9] Blanco C, Bazire R, Argiz L, Hernandez-Pena J. Sublingual allergen immunotherapy for respiratory allergy: a systematic review. Drugs Context 2018;7. 212552.
[10] Song JH, Nguyen HH, Cuburu N, Horimoto T, Ko SY, Park SH, et al. Sublingual vaccination with influenza virus protects mice against lethal viral infection. Proc Natl Acad Sci U S A 2008;105:1644–9.
[11] Park HJ, Ferko B, Byun YH, Song JH, Han GY, Roethl E, et al. Sublingual immunization with a live attenuated influenza a virus lacking the nonstructural protein 1 induces broad protective immunity in mice. PLoS ONE 2012;7. e39921.
[12] Gallorini S, Taccone M, Bonci A, Nardelli F, Casini D, Bonificio A, et al. Sublingual immunization with a subunit influenza vaccine elicits comparable systemic immune response as intramuscular immunization, but also induces local IgA and TH17 responses. Vaccine 2014;32:2382–8.
[13] Munoz-Wolf N, Rial A, Saavedra JM. Chabalgoity JA. Sublingual immunotherapy as an alternative to induce protection against acute respiratory infections. Journal of Visualized Experiments: JoVE 2014.
[14] Blazevic A, Eickhoff CS, Stanley J, Buller MR, Schriewer J, Kettelson EM, et al. Investigations of TB vaccine-induced mucosal protection in mice. Microbes and Infection/Institut Pasteur 2014;16:73–9.
[15] Eickhoff CS, Van Aartsen D, Terry FE, Meymandi SK, Traina MM, Hernandez S, et al. An immunoinformatic approach for identification of Trypanosoma cruzi HLA-A2-restricted CD8(+) T cell epitopes. Human Vaccines Immunotherapeutics 2015;11:2322–8.
[16] Orme IM, Roberts AD. Animal models of mycobacteria infection. Current protocols in immunology/edited by John E Coligan [et al]. 2001;Chapter 19: Unit 19 5.
[17] Derrick SC, Kolibab K, Yang A, Morris SL. Intranasal administration of Mycobacterium bovis BCG induces superior protection against aerosol infection with Mycobacterium tuberculosis in mice. Clin Vaccine Immunol 2014;21:1443–51.
[18] Chen L, Wang J, Zganiacz A, Xing Z. Single intranasal mucosal *Mycobacterium bovis* BCG vaccination confers improved protection compared to subcutaneous vaccination against pulmonary tuberculosis. Infect Immun 2004;72:238–46.
[19] Lee HJ, Cho H, Kim MG, Heo YK, Cho Y, Gwon YD, et al. Sublingual immunization of trivalent human papillomavirus DNA vaccine in baculovirus nanovector for protection against vaginal challenge. PLoS ONE 2015;10. e0119408.
[20] Fu YH, Jiao YY, He JS, Giang GY, Zhang W, Yan YF, et al. Sublingual administration of a helper-dependent adenoviral vector expressing the codon-optimized soluble fusion glycoprotein of human respiratory syncytial virus elicits protective immunity in mice. Antiviral Res 2014;105:72–9.
[21] Barclay WR, Busey WM, Dalgard DW, Good RC, Janicki BW, Kasik JE, et al. Protection of monkeys against airborne tuberculosis by aerosol vaccination with bacillus Calmette-Guerin. Am Rev Respir Dis 1973;107:351–8.
[22] Garcia-Contreras L, Wong YL, Muttil P, Padilla D, Sadoff J, Derousse J, et al. Immunization by a bacterial aerosol. Proc Natl Acad Sci U S A 2008;105:4656–60.
[23] Sharpe S, White A, Sarfas C, Sibley L, Gleeson F, McIntyre A, et al. Alternative BCG delivery strategies improve protection against Mycobacterium tuberculosis in non-human primates: Protection associated with mycobacterial antigen-specific CD4 effector memory T-cell populations. Tuberculosis (Edinb) 2016;101:174–90.
[24] Khan A, Singh S, Galvan G, Jagannath C, Sastry KJ. Prophylactic sublingual immunization with mycobacterium tuberculosis subunit vaccine incorporating the natural killer T Cell agonist alpha-galactosylceramide enhances protective immunity to limit pulmonary and extra-pulmonary bacterial burden in mice. Vaccines (Basel) 2017. 5.
[25] Huo Z, Bissett SL, Giemza R, Beddows S, Oeser C, Lewis DJ. Systemic and mucosal immune responses to sublingual or intramuscular human papilloma virus antigens in healthy female volunteers. PLoS ONE 2012;7:e33736.
[26] Alecsandru D, Valor L, Sanchez-Ramon S, Gil J, Carbone J, Navarro J, et al. Sublingual therapeutic immunization with a polyvalent bacterial preparation in patients with recurrent respiratory infections: immunomodulatory effect on antigen-specific memory CD4+ T cells and impact on clinical outcome. Clin Exp Immunol 2011;164:100–7.
[27] Lorenzo-Gomez MF, Padilla-Fernandez B, Garcia-Cenador MB, Virseda-Rodriguez AJ, Martin-Garcia I, Sanchez-Escudero A, et al. Comparison of sublingual therapeutic vaccine with antibiotics for the prophylaxis of recurrent urinary tract infections. Front Cell Infect Microbiol 2015;5:50.