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Persistent BCG bacilli perpetuate CD4 T effector memory and optimal protection against tuberculosis

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ABSTRACT

Tuberculosis (TB) remains one of the most important infectious diseases of man and animals, and the only available vaccine (BCG) requires urgent replacement or improvement. To facilitate this, the protective mechanisms induced by BCG require further understanding. As a live attenuated vaccine, persistence of BCG bacilli in the host may be a crucial mechanism.

We have investigated the long term persistence of BCG following vaccination and the influence on the induced immune response and protection, using an established murine model. We sought to establish whether previously identified BCG-specific CD4 T_{EM} cells represent genuine long-lived memory cells of a relatively high frequency, or are a consequence of continual priming by chronically persistent BCG vaccine bacilli.

By clearing persistent bacilli, we have compared immune responses (spleen and lung CD4: cytokine producing T effector/T_{EM}; TCR-specific) and BCG-induced protection, in the presence and absence of these persisting vaccine bacilli. Viable BCG bacilli persisted for at least 16 months post-vaccination, associated with specific CD4 T effector/T_{EM} and tetramer-specific responses. Clearing these bacilli abrogated all BCG-specific CD4 T cells whilst only reducing protection by 1 $\log_{10}$.

BCG may induce two additive mechanisms of immunity: (i) dependant on the presence of viable bacilli and T_{EM}; and (ii) independent of these factors.

These data have crucial implications on the rational generation of replacement TB vaccines, and the interpretation of BCG induced immunity in animal models.

1. Introduction

Tuberculosis (TB) caused by infection with *Mycobacterium tuberculosis* (*M. tb*) or *Mycobacterium bovis* (*M. bovis*) remains one of the most important infectious diseases of man and animals, respectively; inflicting a huge cost in both health, welfare and financial terms [1].

At present the only vaccine against TB is *M. bovis* bacille Calmette–Guérin (BCG), which demonstrates variable efficacy in humans and cattle [2,3]. In particular, BCG appears effective in childhood, but not in adolescents and adults [4]. Despite this performance, BCG remains the most widely used human vaccine, and due to its partial efficacy and proven safety record, is unlikely to be withdrawn and remains the benchmark to improve upon.

It is clear that optimal protection against TB requires CD4 T cells, as well as the effector cytokines IFN-γ and TNF-α (reviewed in [5]). However, as other studies demonstrate; CD4 T cell derived IFN-γ is not an exclusive component of vaccine-mediated immunity [6] and identification of other critical components of protection remains elusive.

To compound our incomplete knowledge, the study of BCG induced immune memory has also proven difficult. The chronic nature of TB infection, lack of sterilising immunity, and transient protective window, all contribute to complicate the characterisation of vaccine-specific T cell memory.

Memory T cells exist in a number of subsets. Classically, these are understood to be partitioned into effector memory (T_{EM}) and central memory (T_{CM}) categories [7]; but recent evidence argues for a complex functional diversity within these two broad categories *in vivo* (reviewed in [8]).

There has been an intensive effort to characterise T cell memory induced by BCG immunization in both animal models [9–14] and humans [15–17]. Given its variable efficacy, it is of critical importance to understand the mechanisms underlying its protective capacity, if improved vaccines or vaccination strategies are to be progressed.

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The majority of these studies report BCG to induce a predominant CD4 T_{EM} response, defined by $CD62L^{lo}$ expression, often associated with cytokine multifunctionality [9,16,18]; but few identify BCG-specific $CD62L^{hi}$ or $CCR7^{hi}$ CD4 T_{CM} responses [19–22]. We recently reported CD4 T_{EM} cells to persist 18 months following BCG immunization [9], and consistently, observe no defined contraction of immune responses following immunization.

Given the potential of BCG to persist in the immuno-competent host [23–27], combined with the absence of immune contraction; we hypothesised whether these CD4 T_{EM} cells represent: (a) genuine long-lived high frequency memory cells, or alternately; (b) result from continual priming by persistent BCG bacilli.

Therefore, we sought to investigate the persistence of live BCG long after immunization and the influence of this on immune responses and protection against *M. bovis* challenge, in a mouse model [28].

We report here that live BCG vaccine persisted for the 16 month period of study and that clearance of these bacilli by antibiotic treatment resulted in abrogation of the BCG-specific CD4 T cell population; but protective immunity was only reduced by ~50%. Thus, we propose the existence of two separate additive mechanisms of protection induced by BCG; one dependent on, and one independent of persistent BCG and associated T_{EM} population. These data may have crucial implications on the rational generation of replacement or adjunct TB vaccines, and the interpretation of BCG induced immunity in animal models.

2. Materials and methods

2.1. Ethics

All animal work was carried out in accordance with the UK Animal (Scientific Procedures) Act 1986; under appropriate licences. The study protocol was approved by the AHVLA Animal Use Ethics Committee (UK PCD number 70/6905).

2.2. Animals

Female BALB/c mice were obtained from SPF facilities at Charles River UK Ltd and used at 8 weeks of age. All animals were housed in appropriate BSL3 containment facilities at AHVLA.

2.3. Mycobacteria

The vaccination strain was the human vaccine *M. bovis* BCG Danish 1331, prepared as per manufacturer's instructions (SSI, Denmark).

Mycobacterium bovis isolate AF2122/97 was used for all challenge experiments as previously described [9].

2.4. Mycobacterial antigens

A pool of 7 recombinant mycobacterial proteins (Rv1886c, Rv0251, Rv0287, Rv0288, Rv3019c, Rv3763, Rv3804c), were used for all stimulations as previously described [9]. All proteins were extensively dialyzed and re-suspended in physiological buffer (HBSS) before use.

2.5. Immunization, mycobacterial enumeration and challenge

Mice were immunized with a single intradermal injection (50 μl) containing 2×10^5 CFU of BCG, or sham control (HBSS) in the base of the tail. For enumeration of viable BCG, the spleen, lung, liver and the pooled LNs draining the site of immunization [29] (inguinal, iliac and axillary) were aseptically removed, homogenized and plated in their entirety onto modified Middlebrook 7H11 agar (Difco™) plates [30]. CFU were enumerated twelve weeks after incubation at 37 °C. Limit of detection (LOD) was 2 CFU. A sample of colonies at 16 months was verified as BCG by molecular typing [31].

Additionally, thirty weeks following immunization, mice were challenged intranasally with ~600 CFU of *M. bovis* as previously described [28]. Bacterial loads in spleen and lungs were enumerated four weeks after challenge as previously described [28].

2.6. Antibiotic clearance of BCG bacilli (BCG abbreviated)

Drinking water containing antibiotics (100 μg/ml ethambutol, 200 μg/ml isoniazid and 100 μg/ml rifampicin) (all Sigma, UK), was provided *ad libitum*, replenished twice weekly for the period of treatment. Placebo comprised $D.H_2O$ containing the same volume of solvent (DMSO) used to prepare antibiotics.

2.7. Cell isolations and stimulations

On euthanasia, spleen, lung and LNs (inguinal, iliac, axillary, brachial, cervical and popliteal) were aseptically removed and spleen and interstitial lung cells prepared as previously described [9]. LN cells were prepared as spleen cells. Following washing (300 g/8 min), all cells were re-suspended at 5×10^6 ml^{-1} for assays. Cells were cultured with the specific protein cocktail as described, each antigen at final concentration of 2 μg/ml for all assays.

2.8. ELISPOT

Cells were incubated with antigen and the frequency of antigen-specific IFN-γ secretors detected by ELISPOT (Mabtech, Sweden), as previously described [9].

2.9. Flow cytometry

For intracellular staining (ICS), cells isolated from spleen or lungs were stimulated with antigen pool and anti-CD28 (BD Biosciences) as previously described [9]. They were surface stained with CD4–APC-H7, CD19-PE-CF594, CD11b-PE-CF594 (all BD Bioscience), CD44–eFluor 450, CD62L – PE or – PerCP-Cy5.5, CD27–PE and LIVE/DEAD® Fixable Yellow Dead Cell Stain ('YeViD', Invitrogen). Subsequently, cells were washed, fixed and permeabilised and stained for ICS with IFN-γ–APC (BD Bioscience), IL-2–PE-Cy7 and TNF-α–FITC as previously described [9].

For MHC class II-peptide tetramer staining, RBC were removed (spleen samples only) using RBC lysis buffer (eBioscience, USA). Cells were stained (45 min/37 °C/5% CO_2) in culture media with Rv0288 (TB10.4) peptide: MHCII I-$A(^d)$ (SSTHEANTMAMMARDT) tetramer-complex, labeled with APC; or I-$A(^d)$ negative control (PVSKMRMATPLLMQA) tetramer–APC (both provided by NIH MHC Tetramer Core Facility, USA). Following washing, they were stained (15 min/4 °C) in staining buffer with CD4–APC-H7, CD44–FITC, CD62L–PerCP-Cy5.5, and YeViD, washed and fixed with Cytofix.

All antibody conjugates were purchased from eBioscience except where stated. Data were acquired using a Beckman Coulter CyAn ADP (retrofitted with a 562/40 bandpass filter in the second channel of the violet laser) and analyzed on Summit v4.3 (Beckman Coulter, USA) or Flowjo v7.6.5 (Tree Star, USA) software. All analyses were gated on a minimum of 100,000 live lymphocytes.

2.10. Statistical analyses

All data were analyzed with GraphPad Prism 5 software (GraphPad, USA) using un-paired student's two-sided *t*-test (2 treatment groups) or one- or two-way ANOVA with Bonferroni post-test (3

treatment groups). Mycobacterial counts were $\log_{10}$ transformed before comparison. A Two-tailed correlation analysis was used to obtain coefficient of determination (r^2) from the Pearson correlation coefficient (r). Differences with a p value <0.05 were considered significant and denoted with *, <0.01 with ** and <0.001 with ***.

3. Results

3.1. BCG-bacilli and -specific IFN-γ responses persist for at least 16 months in vaccinated mice and demonstrate a defined correlation

To establish the long-term persistence of viable BCG bacilli, groups of mice were immunized at week 0 with a standard dose (2×10^5 CFU) of the licensed human vaccine BCG Danish 1331. At sequential monthly time-points, the BCG burden of individual mice was determined in pooled draining lymph nodes (d.LNs), spleen and lungs; plating the entire organs/tissues to maximise detection. Fig. 1A demonstrates that viable BCG bacilli were cultured from the d.LNs throughout the experimental duration of 16 months. The burden was highest and most consistent at 6 weeks post immunization (p.i.) at 3.0 $\log_{10}$ CFU (±0.5), decreasing to 2.4 $\log_{10}$ CFU (±0.5) at 16 months p.i.

BCG were cultured from the majority of spleen samples, although with large replicate variability. CFU counts increased from 1.7 $\log_{10}$ CFU (±1.7) at 6 weeks p.i. to 2.3 $\log_{10}$ CFU (±2.3) at 17 weeks p.i., decreasing to 0.0 $\log_{10}$ CFU (±2.0) by 16 months p.i. Culture of BCG from the lungs was sporadic and only possible in 1 or 2 replicates at each time point up to 22 weeks p.i., after which it was undetected.

Given the established importance of IFN-γ producing CD4 T cells in protection against TB, the frequency of BCG-specific IFN-γ secretors in the spleen was evaluated by *ex vivo* ELISPOT using defined protein cocktail at defined time-points following BCG immunization. Fig. 1B shows that whilst IFN-γ secreting cell frequency was maximal at 6 weeks p.i. (1197 SFU/million cells) and declined thereafter; substantial frequencies of IFN-γ secreting cells (478 SFU/million cells) were present 16 months p.i., as previously described [9].

Regression analyses between the mean spleen IFN-γ ELISPOT frequency and the mean bacterial burden in d.LNs showed a statistically significant correlation, demonstrating a clear link between antigen load (from the most reliable tissue indicator) and IFN-γ responses circulating through the spleen (Fig. 1C).

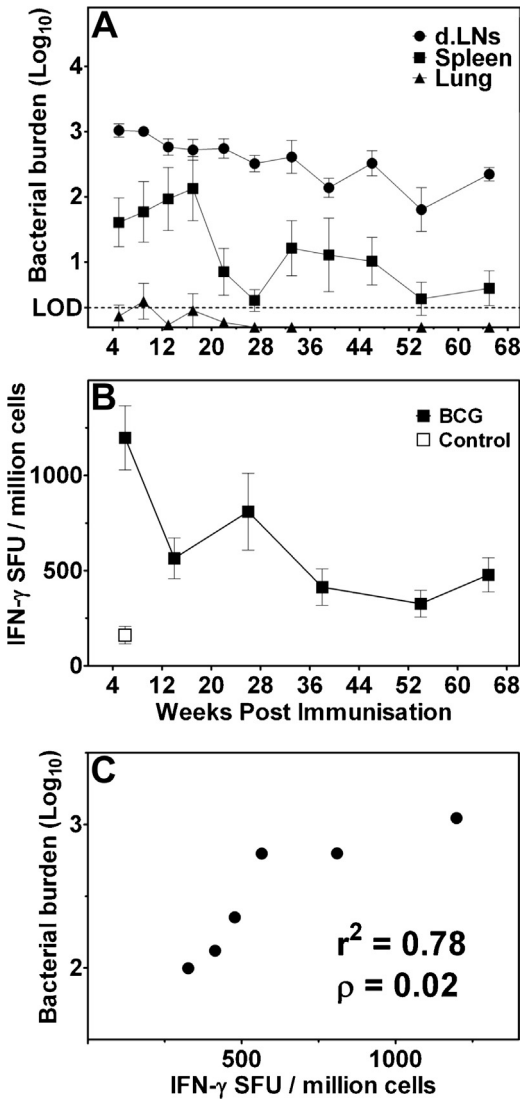

Fig. 1. *BCG-bacilli and -specific IFN-γ response persist for at least 16 months in the vaccinated host and demonstrate a defined correlation.* (A) Sequential mycobacterial burden in d.LNs, spleen and lung of mice after immunization with BCG. Symbols represent mean (±S.E.) CFU (LOD = 2 CFU, n = 7–8). (B) The frequency of BCG antigen-specific IFN-γ producing spleen derived cells measured by ELISPOT following immunization. Placebo control shown at week 6. Symbols represent mean (±S.E.) SFU adjusted per 10^6 cells, (n = 4–8). (C) There was a significant correlation between the mean frequency of IFN-γ producing spleen cells and the mean BCG bacterial burden in the d.LNs. r^2 = correlation analysis coefficient of determination.

3.2. Antibiotic treatment abrogates BCG persistence and specific IFN-γ effector response

To establish the minimum treatment regimen to clear persistent bacilli after BCG immunization, groups of mice were immunized with BCG for 6 weeks (previously shown to induce protection) [9,28]. They were then given a 0, 1, 2 or 3 month course of antibiotic treatment (subsequently termed BCG abbreviated) of ethambutol, isoniazid and rifampicin *via* drinking water. Mice were returned to normal water for a further two weeks following the cessation of treatment, to flush any residual *in vivo* antibiotics inhibiting bacterial culture.

At the end of each treatment regimen, bacterial burden in the individual organs/tissues was determined as described previously; with the inclusion of the liver as an additional potential reservoir of bacilli. Fig. 2A shows that 1 month of treatment was sufficient to clear residual bacilli from the spleen; but a further 2 months of treatment were required to consistently clear persistent BCG from the d.LNs in all animals. The pre-treatment burdens observed in both the spleen and d.LNs were equivalent to previous experiments (Fig. 2A *cf.* Fig. 1A). BCG in lungs and liver were undetectable in this experiment.

As further experiments were critically dependant on consistent efficacy of treatment, a further experiment included vaccinated mice given an additional 3 months rest after cessation of 3 months treatment. In contrast to immunised, untreated mice (which had a burden of 2.7 $\log_{10}$ CFU (±0.6) in the d.LNs ~7.5 months p.i.), no viable BCG were detected in the treatment group (Fig. 2B) confirming the efficacy of antimicrobial treatment.

To evaluate the effect of persistent BCG bacilli on specific IFN-γ responses, groups of mice were immunized with BCG or placebo control for 6 weeks, prior to treatment with antibiotics or placebo for 3 months. To ensure that: (a) analyses were not influenced by short-lived effector T cell responses; and (b) BCG bacilli were effectively cleared, animals were rested for 3 months after treatment.

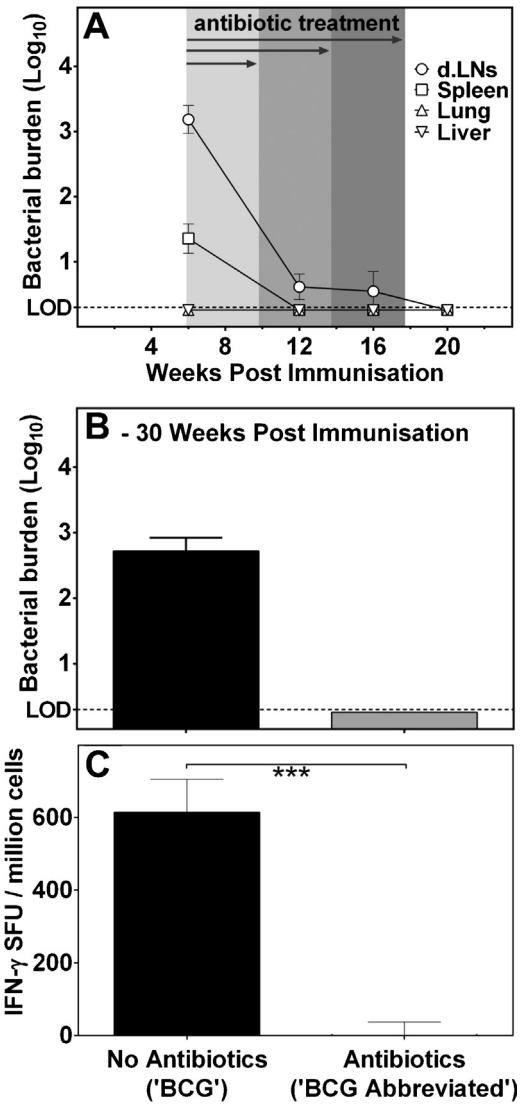

Fig. 2. *Antibiotic treatment abrogates BCG persistence and the specific IFN-γ response.* Mice were immunized with BCG for 6 weeks followed by a 1, 2 or 3 month course of antibiotic treatment, as indicated. Mice were rested for 2 weeks after cessation of treatment. (A) mycobacterial burden in whole organ homogenates. (B) In a second experiment, mycobacterial burden was assessed from the d.LNs 3 months following cessation of 3 month antibiotic treatment course. Symbols and bars represent mean (±S.E.) CFU, (LOD = 2 CFU, $n = 7–8$). **(C)** IFN-γ production by ELISPOT of spleen cells after a 3 month treatment regimen. Symbols represent mean (±S.E.) SFU adjusted per 10^6 cells with placebo responses deducted. *** $p < 0.001$, students *t*-test. Data are representative of two independent experiments ($n = 6–8$).

The frequency of BCG-specific IFN-γ secreting cells in the spleen was then evaluated by *ex vivo* ELISPOT stimulated with the defined protein cocktail.

Fig. 2C shows that the significant IFN-γ response induced by BCG immunization (613 SFU/million cells) was completely abrogated in BCG abbreviated animals ($p < 0.001$). These data clearly demonstrate that, the persisting IFN-γ responses observed in BCG immunized animals were due to persistent BCG bacilli, rather than long-term memory.

3.3. The presence of BCG-specific multifunctional CD4 T_{EM} cells is dependent upon the presence of live BCG bacilli

To further investigate whether this ablation of the IFN-γ responses (ELISPOT) in BCG abbreviated mice was specific to CD4 T cells and of what memory phenotype, the CD4 T cell responses specific to BCG in spleen and lung were assessed by intracellular cytokine staining (ICS) after stimulation with defined protein cocktail (Fig. 3). Fig. 3A shows BCG immunization induces significant populations of multifunctional CD4 T cells (IFN-γ^+/IL-2^+/TNF-α^+, IFN-γ^+/TNF-α^+ and IL-2^+/TNF-α^+), in both spleen and lung-derived cells, with frequencies considerably higher in the lungs as reported previously [9]. ICS performed on d.LN samples of BCG immunized mice in previous experiments were unable to detect significant populations of cytokine producing cells (data not shown), and so were not performed here. Consistently, no BCG-specific CD8 responses were detectable in any of these, or previously reported experiments [9].

Further, these data demonstrate the clearance of persistent BCG bacilli significantly ablates ($p < 0.001$) the presence of all cytokine producing CD4 T cells in both the spleen and lungs (Fig. 3A). Consistent with previous data [9] these multifunctional CD4 T cells consist entirely of $CD44^{hi}$ $CD62L^{lo}$ cells indicative of a T_{EM} phenotype (spleen—99.3%; lung—99.6% of total cytokine$^+$ cells) as shown in Figs. 3B (representative plots of spleen and lung CD4 T cells) and S1 (gating strategy).

We considered that the absence of a measurable T_{CM} ($CD62L^{hi}$) response may be due to the effector cell focus of the assays thus used. We therefore used a class II MHC – TB10.4 (73–88 a.a.) peptide-tetramer complex to detect the total CD4 T cell population specific to this immunodominant antigen in spleens of vaccinated or BCG abbreviated mice, (Figs. 3C and D). As shown in Fig. 3C, 0.23% of total spleen CD4 T cells were $CD62L^{lo}$ Tet^+; reduced to 0.03% $CD62L^{lo}$ Tet^+ following BCG abbreviation (Figs 3C and D). There were no vaccine-specific $CD62L^{hi}$ Tet^+ CD4 T cells in the spleen (Fig. 3C) or LNs (data not shown). Tetramer analysis of lung cells was not performed due to insufficient yields.

These data demonstrate both systemic and mucosal CD4 T cell responses to BCG vaccination are dependent on the persistence of live bacilli, and that these responses are dominated by multifunctional CD4 T_{EM} cells, with no detectable CD4 T_{CM} cells.

3.4. Protection is optimal in the presence of, but not dependant on, persistent BCG bacilli

To determine the effect of these persistent viable vaccine bacilli upon BCG-induced protection; equivalent groups of mice were subjected to this antibiotic treatment regimen, prior to intranasal challenge with *M. bovis* for 4 weeks. As described in Fig. 4, both BCG and BCG abbreviated immunized mice exhibited significant protection compared to placebo controls in both the spleen (Fig. 4A): BCG—protection 1.6 $\log_{10}$ ($p < 0.001$); BCG-abbreviated—0.8 ($p < 0.001$), and the lungs (Fig. 4B): BCG—protection 1.7 $\log_{10}$ ($p < 0.001$); BCG-abbreviated—0.7 ($p < 0.01$). Protection in BCG-abbreviated mice, however, was significantly less compared to untreated BCG vaccinates (spleen 52% reduction *cf.* untreated, $p < 0.01$; lungs 40% *cf.* untreated, $p < 0.001$).

These data demonstrate that whilst BCG induced protection is optimal when persistent bacilli are present; significant protection is maintained after clearance of these bacilli.

4. Discussion

As BCG remains the benchmark to improve upon, it is critical to understand the mechanisms underlying its protective efficacy if improved vaccines or vaccination strategies for TB are to progress. Primary to this aim must be further investigation on the establishment and maintenance of BCG-induced memory.

We report that intradermal immunization with a relatively low dose of BCG (2×10^5 CFU) results in a persistent 'infection', with viable vaccine bacilli present in the secondary lymphoid organs

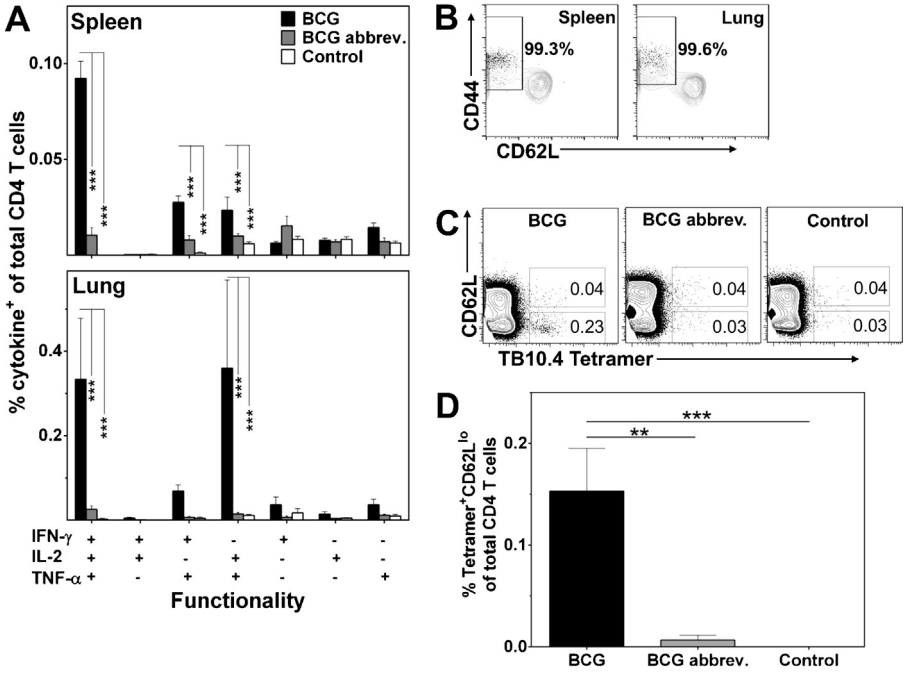

Fig. 3. *BCG abbreviation abrogates multifunctional CD4 T cell response.* Mice were immunized with BCG or placebo control 6 weeks prior to receiving a 3 month course of antibiotic chemotherapy to abbreviate bacilli persistence. After a further 3 months, lymphocytes from the spleen or lung were isolated, antigen stimulated and stained by intracellular staining (ICS). Alternately unstimulated cells were stained with specific MHC class II-TB10.4 peptide complex tetramer, together with cell surface markers. (A) Analysis of the 7 potential combinations of cytokine producers, from the three experimental groups. Bars represent mean (±SE) % frequency of cells of indicated T cell phenotype as a % of total $CD4^+$ cells. *** $p < 0.001$, ANOVA with Bonferroni post-test ($n = 6–8$). Data are representative of two independent experiments. (B) Representative flow cytometry plots (spleen/lung cells), from BCG immunized mice, gated on live $CD4^+$ lymphocytes, identifying the antigen-specific cytokine$^+$ (any combination of: IFN-γ^+, IL-2^+, TNF-α^+) cells analyzed for CD44 and CD62L expression. Cytokine$^+$ events overlay contours displaying the expression of CD44 and CD62L by all CD4 T cells, numbers represent $CD44^{hi}$ $CD62^{lo}$ cytokine$^+$ cells as a % of all cytokine$^+$ cells. Data are representative of 6 separate experiments. (C) Representative flow cytometry plots (spleen cells), gated on live $CD4^+$ lymphocytes, identifying class II MHC—TB10.4 (73–88 a.a.) peptide-tetramer$^+$ cells analyzed for $CD62L^{lo/hi}$ expression, from the three experimental groups. Numbers represent events within the indicated gates as a % of total $CD4^+$ cells. Data are representative of 6 separate experiments. (D) Analysis of spleen tetramer$^+$ $CD62L^{lo}$ cells. Bars represent mean $CD62L^{lo}$ tetramer$^+$ cells as a % of total $CD4^+$ cells with control tetramer staining values subtracted (±S.E.). ** $p < 0.01$, *** $p < 0.001$, ANOVA with Bonferroni post-test ($n = 7$).

(SLO) for up to 66 weeks. Further, we demonstrate a direct relationship between the BCG burden and the frequency of IFN-γ responses. We establish that clearance of these bacilli requires sustained antibiotic treatment, and abrogates the cytokine producing vaccine-specific CD4 T cells derived from the spleen and the lungs. Strikingly, although substantially decreased, significant pulmonary and systemic protection was still present following clearance of bacilli. Together these data suggest BCG may induce two mechanisms of immunity: (i) dependant on the presence of viable bacilli and associated T_{EM}; and (ii) a further mechanism, independent of persisting bacilli and T_{EM}. The exact details of the latter mechanism are yet to be elucidated, and are the subject of current investigation.

The question of BCG persistence has been noted in previous studies in mice [24,25,27,32–35], other animal models [23,26] and humans [36,37]. In a similar study using C57BL/6 mice and *M. tb* challenge [27], spleen protection was reduced by 75%, but in contrast lung immunity was unaffected. This disparity with our study could be due to: mouse strain, challenge organism, incomplete BCG bacilli clearance, or the shorter duration between chemotherapy and challenge.

To date, however, no relationship between BCG persistence and the predominance of CD4 T_{EM} responses has been reported [9,16,18,38]. Our data indicate a clear link between BCG antigen load and T cell responses, which as demonstrated here and previously, are multifunctional (IFN-γ^+/IL-2^+/TNF-α^+, IFN-γ^+/TNF-α^+ and IL-2^+/TNF-α^+) $CD62L^{lo}$CD4 T cells which we consider T_{EM} [9]. We also demonstrate that antigen-specific IFN-γ could used as a direct surrogate of viable bacilli (with the caveat of appropriate antigen stimulation).

We cannot rule out that our antibiotic regimen did not completely eliminate the persistent BCG without performing subsequent immunosuppression [39], which was beyond the scope of our study. However, our data clearly demonstrate reproducible elimination to a point that no BCG baciili and antigen-specific cells could be detected after 3 months of 'rest'. Therefore, we consider this sufficient BCG clearance for the objectives of this study.

We define these IFN-γ^+/IL-2^+/TNF-α^+ triple- or bi-functional cells as CD4 T_{EM} based on $CD62L^{lo}$ $CCR7^-$ expression [9]. As CD62L can be cleaved by metalloproteases, we previously conducted studies using the inhibitor TAPI-2 [40] to demonstrate that identification of stimulated-responder cells as $CD62L^{lo}$ was not due to non-specific mechanisms of CD62L down-regulation (data not shown). We have also confirmed this by sorting $CD62L^{lo/hi}$ cells prior to functional assay (Kaveh & Hogarth, unpublished data).

Following BCG clearance, this absence of CD4 T_{EM} cells, and indeed any detectable antigen-specific IFN-γ responses (ELISPOT) or cells expressing a T cell receptor (TCR) specific to an immunodominant antigen; indicates that all detectable responses result from continual priming by BCG bacilli, as has been reported during chronic *Salmonella* infection [41], and that they are relatively short-lived. Whilst determination of specific CD4 T_{EM} cell longevity was beyond the scope of this study; they were absent at four months following last detection of viable bacilli, indicating a lifespan of <four months; conforming to the hypothesis that T_{EM} cells are responsible for intermediate, rather than long-term memory [42]. Interestingly, our previous data show lung T_{EM} responses were sustained at 12 months following BCG immunization. [9], at which point we show here there are no detectable bacilli in the lung, indicating traffic from a site of active priming such as the SLO; according

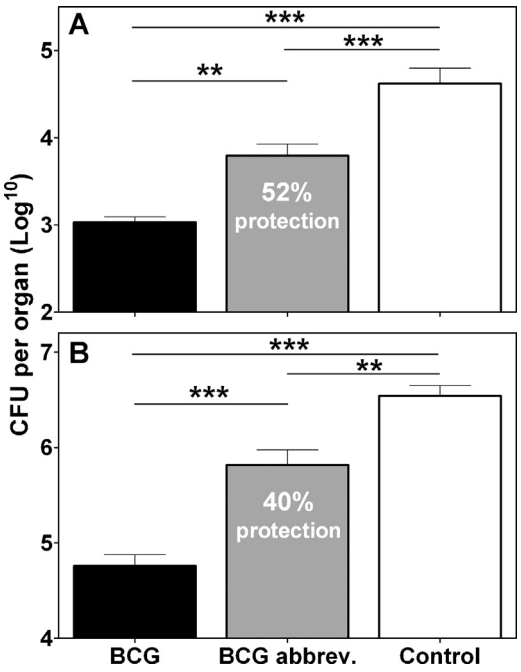

Fig. 4. *Degree of protection is dependent on the presence of viable BCG.* Mice were immunized with BCG or placebo control 6 weeks prior to receiving a 3 month course of antibiotic chemotherapy to abbreviate bacilli persistence. After a further 3 months, mice were challenged with *M. bovis* for 4 weeks and the: (A) spleens and (B) lungs assayed for *M. bovis* bacterial burden. Bars represent mean CFU (±S.E.). ** $p < 0.01$, *** $p < 0.001$, ANOVA with Bonferroni post-test ($n = 8$). The indicated % protection is calculated from that observed in BCG immunized (non-abbreviated) when compared to placebo control mice.

with reports that responses to mycobacteria are initiated in the LN [43,44].

Despite their short-lived nature, CD4 T_{EM} cells appear to make a significant contribution to protective immunity, as the reduction in bacterial burden was reduced by up to 60% in their absence. CD4 T_{EM} have been reported as important mediators of protection in *M. tuberculosis* [45] malaria [46] and Leishmania [38], among other infections. We acknowledge, however, that a direct protective, rather than associative role of these cells remains to be shown; but at present, the lack of technologies to allow the sorting of live T cells based on cytokine production, preclude the T_{EM} adoptive transfer experiments required to definitively demonstrate such a role.

It is intriguing to speculate whether at least a proportion of the protection afforded by BCG during childhood is due to persisting bacilli and associated T_{EM}. There is evidence that BCG may persist for many years in humans [37,47–52] and together with the observed waning of immune responses to BCG through childhood [36]; this may represent gradual clearance of bacilli and associated T cells.

Long-term memory, however, is considered dependent on the generation of T_{CM} responses. At present, few reports directly identify an antigen-specific CD4 T_{CM} cells induced in mice by BCG alone [19,22]; some describe T_{CM}-like cells after clonal expansion induced by prime-boost vaccination, challenge or reinfection [14,21,53]. In humans, T_{CM} may only appear after contraction of the BCG-specific T_{EM} response [20]. This situation is confounded by our incomplete understanding of T_{CM} cell phenotypes. Conflicting evidence is often published, and there is clearly substantial plasticity between memory T cell phenotypes (reviewed in [42,54]). Unequivocal identification of these cells is also complicated by the weak expression of characterisitic cells markers (*e.g.* CCR7) and their often mutual expression by the naïve T cell population.

ICS by flow cytometry is often used, but has a distinct effector bias relying immediate cytokine production, and so is unlikely optimal for T_{CM} detection [55,56]. To circumvent this, we performed class II-peptide tetramer staining, but were unable to detect any $CD4^{+}CD62L^{hi}$ antigen-spepcific T_{CM} cells. Perhaps the paucity of reported T_{CM} responses to vaccination is due to rarity of these cells, estimated at 0.025–0.0025% of total CD4 T cells [57]. The background responses of most assays in naïve mice (<0.05% CD4 T cells) may obscure such populations [57]. Indeed, recent studies have had to employ enrichment of tetramer^{+} cells [58], to allow detection of rare T_{CM} cells in BCG vaccinates [19,22]. Other *in vitro* expansion approaches, such as cultured ELISPOT [59] may also help to resolve this population.

Therefore, we cannot rule out the existence of undetected BCG-specific T_{CM}. The existence of potential T_{CM} cells has been demonstrated in adoptive-transfer experiments, where cells with a potential T_{CM} phenotype ($CD62L^{hi}/CD45RB^{hi}$, but unknown for CCR7) conferred modest protection [12,60].

In the absence of a robust T_{CM} response, other potential mechanisms of protection in BCG abbreviated mice may include alternate T cell subsets secreting cytokines not examined in this study (*e.g.* T_H17 [13]), or undetected CD8 T cells, B cells or 'innate' cell activation and imprinting [61].

Current models for assessing TB vaccines compare performance against the BCG 'gold standard', which likely include persistent bacilli and thus active T_{EM} responses. This may account for the inability to improve upon BCG often reported [62]. A model where protection is assessed against only long-term memory, such as the abbreviation method used here, or other strategies to remove constant priming; may allow an enhanced 'window of protection' and subsequent identification of vaccines with potential for improved performance.

This report has implications for the interpretation of immunity in pre-clinical models, with predominant responses dependent on antigen persistence. Therefore, studies which include such persistent BCG, not only demand a vaccine candidate to outperform the 'gold standard' in the face of constantly primed $T_{Effector}$ and T_{EM} responses; but also confound interpretation of the immunological analyses, with the dominant responses induced by live BCG undoubtedly obscuring the immune responses responsible for long-term memory-mediated protection. This underscores the importance of understanding the mechanisms of T cell memory.

Author contributions

Conceived and designed the experiments: **PJH DAK**. Performed the experiments: **DAK CGP**. Analyzed the data: **DAK PJH**. Wrote the paper: **DAK PJH**.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2014.10.041.

References

[1] Ashford DA, Whitney E, Raghunathan P, Cosivi O. Epidemiology of selected mycobacteria that infect humans and other animals. Rev Sci Tech 2001;20:325–37.
[2] Bloom BH, Fine PEM. The BCG experience: implications for future vaccines against tuberculosis. In: Bloom BH, editor. Tuberculosis: Pathogenesis, Protection and Control. Washinton, DC: ASM Press; 1994.
[3] Hogarth PJ, Hewinson RG, Vordermeier HM. Development of vaccines against bovine tuberculosis. J Pharm Pharmacol 2006;58:749–57.
[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] Cooper AM. Cell-mediated immune responses in tuberculosis. Annu Rev Immunol 2009;27:393–422.
[6] Gallegos AM, van Heijst JW, Samstein M, Su X, Pamer EG, et al. A gamma interferon independent mechanism of CD4 T cell mediated control of *M. tuberculosis* infection in vivo. PLoS Pathog 2011;7:e1002052.
[7] Sallusto F, Lanzavecchia A. Heterogeneity of CD4+ memory T cells: functional modules for tailored immunity. Eur J Immunol 2009;39:2076–82.
[8] Jameson SC, Masopust D. Diversity in T cell memory: an embarrassment of riches. Immunity 2009;31:859–71.
[9] Kaveh DA, Bachy VS, Hewinson RG, Hogarth PJ. Systemic BCG immunization induces persistent lung mucosal multifunctional CD4 $T_{(EM)}$ cells which expand following virulent mycobacterial challenge. PLoS One 2011;6:e21566.
[10] Horvath CN, Shaler CR, Jeyanathan M, Zganiacz A, Xing Z. Mechanisms of delayed anti-tuberculosis protection in the lung of parenteral BCG-vaccinated hosts: a critical role of airway luminal T cells. Mucosal Immunol 2012;5:420–31.
[11] Connor LM, Harvie MC, Rich FJ, Quinn KM, Brinkmann V, et al. A key role for lung-resident memory lymphocytes in protective immune responses after BCG vaccination. Eur J Immunol 2010;40:2482–92.
[12] Kipnis A, Irwin S, Izzo AA, Basaraba RJ, Orme IM. Memory T lymphocytes generated by *Mycobacterium bovis* BCG vaccination reside within a CD4 $CD44^{lo}CD62$ $Ligand^{hi}$ population. Infect Immun 2005;73:7759–64.
[13] Wozniak TM, Saunders BM, Ryan AA, Britton WJ. *Mycobacterium bovis* BCG-specific Th17 cells confer partial protection against *Mycobacterium tuberculosis* infection in the absence of gamma interferon. Infect Immun 2010;78:4187–94.
[14] Henao-Tamayo MI, Ordway DJ, Irwin SM, Shang S, Shanley C, et al. Phenotypic definition of effector and memory T-lymphocyte subsets in mice chronically infected with *Mycobacterium tuberculosis*. Clin Vaccine Immunol 2010;17:618–25.
[15] Kagina BM, Abel B, Scriba TJ, Hughes EJ, Keyser A, et al. Specific T cell frequency and cytokine expression profile do not correlate with protection against tuberculosis after bacillus Calmette–Guérin vaccination of newborns. Am J Respir Crit Care Med 2010;182:1073–9.
[16] Soares AP, Scriba TJ, Joseph S, Harbacheuski R, Murray RA, et al. Bacillus Calmette–Guérin vaccination of human newborns induces T cells with complex cytokine and phenotypic profiles. J Immunol 2008;180:3569–77.
[17] Tena-Coki NG, Scriba TJ, Peteni N, Eley B, Wilkinson RJ, et al. CD4 and CD8 T-cell responses to mycobacterial antigens in African children. Am J Respir Crit Care Med 2010;182:120–9.
[18] Lindenstrom T, Agger EM, Korsholm KS, Darrah PA, Aagaard C, et al. Tuberculosis subunit vaccination provides long-term protective immunity characterized by multifunctional CD4 memory T cells. J Immunol 2009;182:8047–55.
[19] Ancelet LR, Aldwell FE, Rich FJ, Kirman JR. Oral vaccination with lipid-formulated BCG induces a long-lived, multifunctional $CD4^+$ T cell memory immune response. PLoS One 2012;7:e45888.
[20] Soares AP, Kwong Chung CK, Choice T, Hughes EJ, Jacobs G, et al. Longitudinal changes in $CD4^+$ T-cell memory responses induced by BCG vaccination of newborns. J Infect Dis 2013;207:1084–94.
[21] Lindenstrom T, Knudsen NP, Agger EM, Andersen P. Control of chronic mycobacterium tuberculosis infection by CD4 KLRG1-IL-2-secreting central memory cells. J Immunol 2013;190:6311–9.
[22] Vogelzang A, Perdomo C, Zedler U, Kuhlmann S, Hurwitz R, et al. Central memory $CD4^+$ T cells are responsible for the recombinant bacillus Calmette–Guérin ΔureC::hly vaccine's superior protection against tuberculosis. J Infect Dis 2014 [epub ahead of print].
[23] Lurie MB. The Fate of BCG and associated changes in the organs of rabbits. J Exp Med 1934;60:163–78.
[24] Lugosi L. Multiple comparison of dried BCG vaccines: stability at 37 degrees C and persistence of strains in the mouse spleen. Vaccine 1984;2:149–56.
[25] Orme IM, Collins FM. Aerogenic vaccination of mice with *Mycobacterium bovis* BCG. Tubercle 1986;67:133–40.
[26] Palmer MV, Thacker TC, Waters WR, Robbe-Austerman S, Lebepe-Mazur SM, et al. Persistence of *Mycobacterium bovis* Bacillus Calmette–Guérin in white-tailed deer (*Odocoileus virginianus*) after oral or parenteral vaccination. Zoonoses Public Health 2010;57:e206–12.
[27] Olsen AW, Brandt L, Agger EM, van Pinxteren LA, Andersen P. The influence of remaining live BCG organisms in vaccinated mice on the maintenance of immunity to tuberculosis. Scand J Immunol 2004;60:273–7.
[28] Logan KE, Gavier-Widen D, Hewinson RG, Hogarth PJ. Development of a *Mycobacterium bovis* intranasal challenge model in mice. Tuberculosis (Edinb) 2008;88:437–43.
[29] Harrell MI, Iritani BM, Ruddell A. Lymph node mapping in the mouse. J Immunol Methods 2008;332:170–4.
[30] Gallagher J, Horwill DM. A selective oleic acid albumin agar medium for the cultivation of *Mycobacterium bovis*. J Hyg (Lond) 1977;79:155–60.
[31] Kamerbeek J, Schouls L, Kolk A, van Agterveld M, van Soolingen D, et al. Simultaneous detection and strain differentiation of *Mycobacterium tuberculosis* for diagnosis and epidemiology. J Clin Microbiol 1997;35:907–14.
[32] Aldwell FE, Cross ML, Fitzpatrick CE, Lambeth MR, de Lisle GW, et al. Oral delivery of lipid-encapsulated *Mycobacterium bovis* BCG extends survival of the bacillus in vivo and induces a long-term protective immune response against tuberculosis. Vaccine 2006;24:2071–8.
[33] Minassian AM, Ronan EO, Poyntz H, Hill AV, McShane H. Preclinical development of an in vivo BCG challenge model for testing candidate TB vaccine efficacy. PLoS One 2011;6:e19840.
[34] Roch F, Bach MA. Strain differences in mouse cellular responses to *Mycobacterium lepraemurium* and BCG subcutaneous infections. I. Analysis of cell surface phenotype in local granulomas. Clin Exp Immunol 1990;80:332–8.
[35] Leversen NA, Sviland L, Wiker HG, Mustafa T. Long-term persistence of BCG pasteur in lungs of C57BL/6 mice following intranasal infection. Scand J Immunol 2012;75:489–99.
[36] Weir RE, Gorak-Stolinska P, Floyd S, Lalor MK, Stenson S, et al. Persistence of the immune response induced by BCG vaccination. BMC Infect Dis 2008;8:9.
[37] Whittaker JA, Bentley DP, Melville-Jones GR, Slater AJ. Granuloma formation in patients receiving BCG immunotherapy. J Clin Pathol 1976;29:693–7.
[38] Darrah PA, Patel DT, De Luca PM, Lindsay RW, Davey DF, et al. Multifunctional TH1 cells define a correlate of vaccine-mediated protection against *Leishmania major*. Nat Med 2007;13:843–50.
[39] McCune RM, Feldmann FM, Lambert HP, McDermott W. Microbial persistence. I. The capacity of tubercle bacilli to survive sterilization in mouse tissues. J Exp Med 1966;123:445–68.
[40] Jabbari A, Harty JT. Simultaneous assessment of antigen-stimulated cytokine production and memory subset composition of memory CD8 T cells. J Immunol Methods 2006;313:161–8.
[41] Nelson RW, McLachlan JB, Kurtz JR, Jenkins MK. $CD4^+$ T cell persistence and function after infection are maintained by low-level peptide:MHC class II presentation. J Immunol 2013;190:2828–34.
[42] Masopust D, Schenkel JM. The integration of T cell migration, differentiation and function. Nat Rev Immunol 2013;13:309–20.
[43] Chackerian AA, Alt JM, Perera TV, Dascher CC, Behar SM. Dissemination of *Mycobacterium tuberculosis* is influenced by host factors and precedes the initiation of T-cell immunity. Infect Immun 2002;70:4501–9.
[44] Wolf AJ, Desvignes L, Linas B, Banaiee N, Tamura T, et al. Initiation of the adaptive immune response to *Mycobacterium tuberculosis* depends on antigen production in the local lymph node, not the lungs. J Exp Med 2008;205:105–15.
[45] Kapina MA, Shepelkova GS, Mischenko VV, Sayles P, Bogacheva P, et al. $CD27^{low}$ CD4 T lymphocytes that accumulate in the mouse lungs during mycobacterial infection differentiate from $CD27^{high}$ precursors in situ, produce IFN-gamma, and protect the host against tuberculosis infection. J Immunol 2007;178:976–85.
[46] Stephens R, Langhorne J. Effector memory Th1 CD4 T cells are maintained in a mouse model of chronic malaria. PLoS Pathog 2010;6:e1001208.
[47] Bouton J, Mainwaring D, Smithells RW. BCG dissemination in congenital hypogammaglobulinaemia. Br Med J 1963;1:1512–5.
[48] Trevenen CL, Pagtakhan RD. Disseminated tuberculoid lesions in infants following BCG vaccination. Can Med Assoc J 1982;127:502–4.
[49] Tajima Y, Takagi R, Nakajima T, Kominato Y. An infant with asymptomatic hepatic granuloma probably caused by bacillus Calmette–Guérin (BCG) vaccination found incidentally at autopsy: a case report. Cases J 2008;1:337.
[50] Barouni AS, Augusto C, Queiroz MV, Lopes MT, Zanini MS, et al. BCG lymphadenopathy detected in a BCG-vaccinated infant. Braz J Med Biol Res 2004;37:697–700.
[51] Armbruster C, Junker W, Vetter N, Jaksch G. Disseminated bacille Calmette–Guérin infection in an AIDS patient 30 years after BCG vaccination. J Infect Dis 1990;162:1216.
[52] Reynes J, Perez C, Lamaury I, Janbon F, Bertrand A. Bacille Calmette–Guérin adenitis 30 years after immunization in a patient with AIDS. J Infect Dis 1989;160:727.
[53] Henao-Tamayo M, Obregon-Henao A, Ordway DJ, Shang S, Duncan CG, et al. A mouse model of tuberculosis reinfection. Tuberculosis (Edinb) 2012;92:211–7.
[54] Mueller SN, Gebhardt T, Carbone FR, Heath WR. Memory T cell subsets, migration patterns, and tissue residence. Annu Rev Immunol 2013;31:137–61.
[55] Zaph C, Uzonna J, Beverley SM, Scott P. Central memory T cells mediate long-term immunity to *Leishmania major* in the absence of persistent parasites. Nat Med 2004;10:1104–10.

[56] Pakpour N, Zaph C, Scott P. The central memory CD4+ T cell population generated during *Leishmania major* infection requires IL-12 to produce IFN-gamma. J Immunol 2008;180:8299–305.
[57] Taylor JJ, Jenkins MK. CD4+ memory T cell survival. Curr Opin Immunol 2011;23:319–23.
[58] Moon JJ, Chu HH, Hataye J, Pagan AJ, Pepper M, et al. Tracking epitope-specific T cells. Nat Protoc 2009;4:565–81.
[59] Godkin AJ, Thomas HC, Openshaw PJ. Evolution of epitope-specific memory CD4+ T cells after clearance of hepatitis C virus. J Immunol 2002;169: 2210–4.
[60] Andersen P, Smedegaard B. CD4(+) T-cell subsets that mediate immunological memory to *Mycobacterium tuberculosis* infection in mice. Infect Immun 2000;68:621–9.
[61] Jeyanathan M, Damjanovic D, Shaler CR, Lai R, Wortzman M, et al. Differentially imprinted innate immunity by mucosal boost vaccination determines anti-tuberculosis immune protective outcomes, independent of T-cell immunity. Mucosal Immunol 2012;6(3):612–25.
[62] McShane H, Jacobs WR, Fine PE, Reed SG, McMurray DN, et al. BCG: myths, realities, and the need for alternative vaccine strategies. Tuberculosis (Edinb) 2012;92:283–8.