

BCG vaccination-induced long-lasting control of *Mycobacterium tuberculosis* correlates with the accumulation of a novel population of CD4⁺IL-17⁺TNF⁺IL-2⁺ T cells



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ARTICLE INFO

Article history:

Received 25 July 2014

Received in revised form 7 November 2014

Accepted 10 November 2014

Available online 20 November 2014

Keywords:

Tuberculosis

BCG vaccination

Multifunctional CD4⁺ T cells

Effector CD4⁺ T cells

Memory CD4⁺ T cells

ABSTRACT

Mycobacterium bovis Bacille Calmette-Guerin (BCG) is the only vaccine in use to prevent *Mycobacterium tuberculosis* (Mtb) infection. Here we analyzed the protective efficacy of BCG against Mtb challenges 21 or 120 days after vaccination. Only after 120 days post-vaccination were mice able to efficiently induce early Mtb growth arrest and maintain long-lasting control of Mtb. This protection correlated with the accumulation of CD4⁺ T cells expressing IL-17⁺TNF⁺IL-2⁺. In contrast, mice challenged with Mtb 21 days after BCG vaccination exhibited only a mild and transient protection, associated with the accumulation of CD4⁺ T cells that were mostly IFN- γ ⁺TNF⁺ and to a lesser extent IFN- γ ⁺TNF⁺IL-2⁺. These data suggest that the memory response generated by BCG vaccination is functionally distinct depending upon the temporal proximity to BCG vaccination. Understanding how these responses are generated and maintained is critical for the development of novel vaccination strategies against tuberculosis.

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1. Introduction

Tuberculosis (TB) remains a major public health problem. With approximately one-third of the world's population latently infected with *Mycobacterium tuberculosis* (Mtb), a staggering 8.7 million new cases and 1.4 million deaths are reported annually [1]. *Mycobacterium bovis* Bacille Calmette-Guerin (BCG) is the only vaccine currently available, but its efficacy against pulmonary TB is variable [2,3]. Nevertheless, BCG is still in use in multiple countries due to its efficacy in protecting against disseminated forms of TB in children [2]. For this reason, heterologous prime-boost regimens involving priming with BCG followed by an adjuvanted or vectored subunit boost are proposed as promising vaccine strategies against TB [4–6]. However, in a recent clinical trial, this approach proved insufficient in preventing Mtb infection or TB disease [7].

For a vaccine to induce protection against TB, antigen-specific T cells should be recruited rapidly to the lungs and activate the infected phagocytes to control Mtb [8,9]. In addition, the cells reaching the infection site should be able to survive within the phagocyte laden environment [10,11]. While CD4⁺IFN- γ ⁺ T cells are thought to be essential for Mtb control [12,13], the magnitude of the IFN- γ response does not provide a good correlate of protection against TB [14–16]. Moreover, recent data suggest that CD4⁺ T cells producing multiple cytokines, including IFN- γ , TNF, and IL-2 are associated with protection against Mtb infection [17–20] suggesting that, specific populations of CD4⁺ T cells that produce multiple cytokines play important roles in the control of TB.

In recent years, CD4⁺ T cells capable of producing IL-17 have been associated with protection against infection by several pathogens [21]. During TB, CD4⁺IL-17⁺ T cells are particularly important in vaccine-mediated immunity [9,22] by promoting a more rapid recruitment of CD4⁺IFN- γ ⁺ T cells to the lungs of Mtb infected mice, leading to an earlier control of Mtb proliferation [9]. The importance of multifunctional T cell populations [17–20] together with the differential efficacy of effector versus memory responses in the control of Mtb [23,24], led us to determine the

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phenotype and function of both the effector and memory responses induced by BCG vaccination in the control of Mtb infection. To this end, we challenged mice with Mtb 21 (effector) or 120 (memory) days after BCG vaccination to determine the protective efficacy of each regimen and to correlate protection with the phenotype of the CD4⁺ T cell response.

We show that, while both the effector and memory responses generated by BCG vaccination are equally effective at inducing early Mtb growth arrest, this does not correlate with the magnitude of the IFN- γ response. Indeed, both mice challenged with Mtb 21 and 120 days after BCG vaccination were equally able to induce Mtb growth arrest, however long-lasting protection was a feature of the 120 day but not the 21 day regimen. This long-lived protection is associated with the accumulation of multifunctional CD4⁺IL-17⁺ T cells, including CD4⁺IL-17⁺TNF⁺IL-2⁺. These data highlight the complexity of CD4⁺ T cell phenotypes that are generated by BCG vaccination and the importance of specific subsets in vaccine-mediated immunity. Understanding the mechanisms underlying the generation of these subsets will be critical in the development of novel vaccine strategies against TB.

2. Materials and methods

2.1. Mice

Eight to 12-week-old female C57BL/6 mice were obtained from Charles River (Barcelona, Spain) and maintained at the ICVS animal facility. All animal experiments were performed according to the European Union Directive 86/609/EEC and were approved by the Portuguese national authority, Direção Geral de Veterinária.

2.2. Bacteria

Mtb H37Rv and *M. bovis* BCG Pasteur were originally obtained from the Trudeau Institute Mycobacterial Collection. Bacteria were grown in Proskauer Beck medium containing 0.05% Tween 80 to mid-log phase and frozen at -70 °C until use.

2.3. Vaccination, experimental infection and bacterial load determination

Mice were vaccinated with 1×10^6 BCG via the subcutaneous route. At 21 or 120 days after vaccination, mice were anesthetized with Ketamine/Medetomidine and infected via the intranasal route with 4×10^4 CFU of Mtb, resulting in a lung dose of $2.47 \pm 0.23 \log_{10}$ [25]. For bacterial load determination, mice were killed by CO₂ asphyxiation, the lungs were aseptically excised, individually homogenized, followed by plating serial dilution of the homogenate on nutrient 7H11 agar (BD Biosciences). CFUs were counted after 3 weeks of incubation at 37 °C. This procedure was followed to control for the presence of viable BCG in the lung at the time of Mtb challenge and no viable BCG bacteria were found in any of the experiments performed.

2.4. Quantification of antigen-specific responses by ELISPOT, and intracellular cytokine staining

Lungs and draining lymph nodes (LN) were aseptically removed and single cell suspensions prepared [25]. The quantification of Ag85B₂₈₀₋₂₉₄ and ESAT-6₁₋₂₀-specific CD4⁺ T cells was determined by ELISpot, as these peptides represent previously described I-A^b-restricted antigens to which there is an early focus of the T cell response [25,26]. Briefly, 1×10^5 cells were incubated in antibody-coated plates with irradiated splenocytes, 10 ng/ml of IL-2 and 1 µg/ml of cognate peptide. After 24 h of incubation, plates were processed for the detection of IFN- γ or IL-17 producing cells. Cells

cultured in the absence of antigen and cells from uninfected and unvaccinated mice were used as controls.

For intracellular cytokine staining, lung and LN cells were stimulated with 50 ng/ml of PMA plus 500 ng/ml of lonomycin in the presence of 10 µg/ml of Brefeldin A for 4 h at 37 °C. Cells were then fixed overnight at 4 °C before they were washed, permeabilized, using the Cytofix/Cytoperm Kit (BD Pharmingen), and stained for surface and intracellular antigens for 20 min at 4 °C. Antibodies for CD3 (145-2C11), CD4 (GK1.5), CCR6 (29-2L17), CCR7 (4B12), CD62L (Mel-14), CD11b (M1/70), Gr-1 (RB6-8C5), IFN- γ (XMG1.2), IL-17 (TC11-18H10.1), IL-2 (JES6-5H4) and TNF (MP6-XT22) were obtained from BD biosciences, eBioscience or Biolegend. Samples were acquired on a LSRII flow cytometer (BD Biosciences) and the data were analyzed using FlowJo software (TreeStar). The gating strategy can be found in Fig. S2A.

2.5. RT-PCR

Total lung RNA was extracted using TRIzol® Reagent (Invitrogen) and reverse transcribed using the SuperScript II (Invitrogen) and Oligo(dT) (Roche), according to the manufacturer's instructions. Target gene mRNA expression was quantified using SYBR green (Qiagen) and specific oligonucleotides [27]. A typical real-time RT-PCR protocol was performed under the following conditions: 15 min at 95 °C, followed by 40 cycles (95 °C denaturing for 15 s; 58 °C annealing for 20 s; 72 °C extension for 15 s), melting at 60 °C until 95 °C for 5 s, and finally cooling. The specificity of the SYBR green assays was confirmed by melting point analysis. Data were normalized to ubiquitin mRNA levels, using the following equation: $1.8^{(ct \text{ reference gene} - ct \text{ target gene})} \times 100,000$.

2.6. Histological and morphometric analysis

The caudal lobe of each lung was inflated with neutral buffered formalin and processed for hematoxylin and eosin stain. Sections were screened and scored in a blinded manner by a qualified pathologist [25].

Immunofluorescence was performed on formalin-fixed tissue sections as previously described [28]. Briefly, antigens were unmasked and blocked with donkey serum and FcBlock, and endogenous biotin was neutralized. Sections were probed with purified goat anti-iNOS (M-19) followed by a secondary donkey anti-goat antibody (Invitrogen). SlowFade Gold antifade with DAPI (Invitrogen) was used to detect nuclei. Images were obtained with an Olympus BX61 microscope and were recorded with a digital camera (DP70).

2.7. Statistical analysis

Data points represent means \pm SEM. Two-way ANOVA with Bonferroni's posttest was used to compare groups using Graphpad Prism Software. Means were considered significant for $p \leq 0.05$.

3. Results

3.1. The efficacy of BCG-mediated control of Mtb is dependent on the length of time between vaccination and Mtb challenge

To address the efficiency of the effector versus the memory response generated by BCG vaccination, we challenged mice with Mtb 21 (effector) or 120 (memory) days after vaccination and determined their ability to induce early growth arrest and maintain long-lasting control of Mtb. Mice challenged 120 days after BCG vaccination manifested improved and early control of Mtb, when compared to mice challenged 21 days after BCG vaccination (Fig. 1A). Moreover, the latter group showed a transient protection

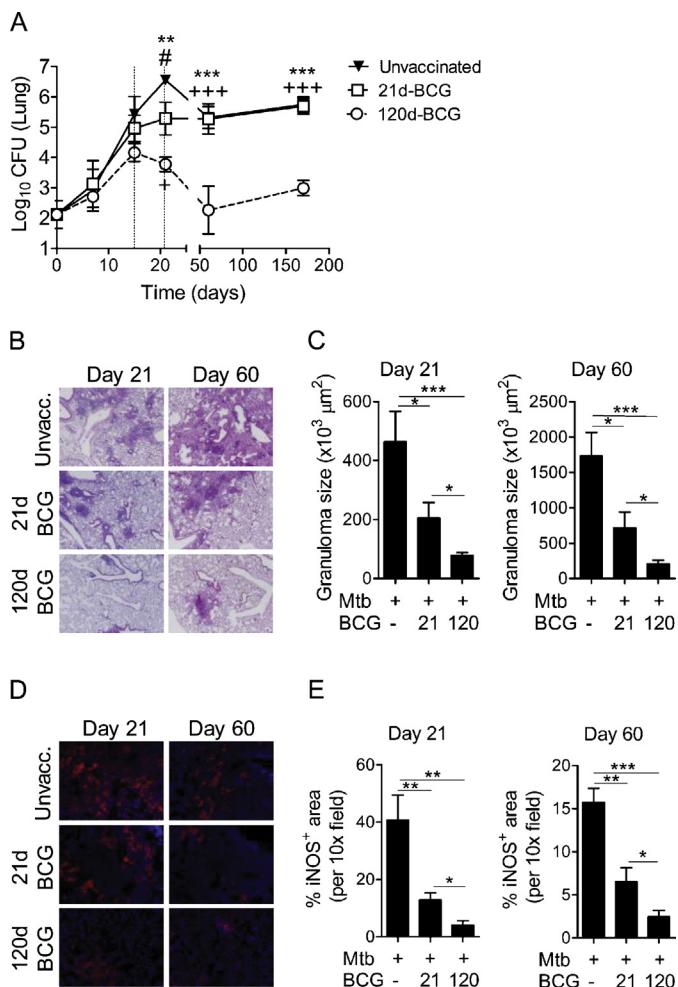


Fig. 1. Growth arrest and long-lasting control of Mtb depends on the time between BCG vaccination and Mtb challenge. (A) Lung Mtb burdens at days 7, 15, 21, 60 and 180 days in mice that were left unvaccinated (black triangles) or vaccinated with BCG 21 (white squares) or 120 days (white circles) before challenge with Mtb ($n=4-8$). * $p<0.05$; ** $p<0.01$; *** $p<0.001$ determined by two-way ANOVA; #, represents statistical differences between unvaccinated and 21d-BCG vaccinated mice; +, represents statistical differences between 120d-BCG and 21d-BCG vaccinated mice. (B) Microscopic lesions of Mtb-infected mice, and (C) morphometric analysis of the inflammatory lung lesions ($n=4-8$). * $p<0.05$; *** $p<0.001$ determined by two-way ANOVA. (D) iNOS (red) and nuclei (blue) staining and (E) percentage of iNOS occupied lung area ($n=4-8$). * $p<0.05$; ** $p<0.01$; *** $p<0.001$ determined by two-way ANOVA. Results are from one representative out of four independent experiments (For interpretation of the color information in this figure legend, the reader is referred to the web version of the article.).

while those vaccinated 120 days prior to challenge maintained a long-lasting control of Mtb (Fig. 1A). Consistent with these data, mice that were challenged with Mtb 120 days after vaccination had a significant reduction of cell infiltrates (Fig. 1B), granuloma size (Fig. 1C) and iNOS foci (Fig. 1D and E) in the lung, when compared to mice challenged with Mtb 21 days after vaccination. These data support the hypothesis that the effector response induced after BCG vaccination, despite being able to induce early Mtb growth arrest, is less effective than the memory response at maintaining long-lasting control of this pathogen.

3.2. BCG-mediated control of Mtb correlates with the IL-17 response and is independent of the magnitude of the IFN- γ response in the lung

Based on the differences in protection afforded by BCG, shown above, we asked whether these responses were associated with

the presence of particular antigen-specific T cell populations at the time of challenge.

To answer this, we first determined the frequencies of effector memory (T_{EM}) and central memory (T_{CM}) CD4 $^{+}$ T cells within the lungs of the vaccinated and unvaccinated mice just prior to challenge [29]. We found a predominance of T_{EM} cells in the lungs of mice that were vaccinated 21 days before Mtb challenge, whereas T_{CM} cells were more prevalent in mice vaccinated for 120 days (Fig. 2A). Next, we characterized the Ag-specific T cell response, by comparing the number and frequency of cytokine producing Ag85-specific T cells. Mice that were vaccinated for 21 days before the Mtb challenge had a higher number (Fig. 2B) and frequency (Fig. S1A) of IFN- γ -producing Ag85-specific T cells than mice vaccinated 120 days before the Mtb challenge. In contrast, there was an increased number (Fig. 2D) and frequency (Fig. S1B) of IL-17-producing T cells in the mice vaccinated 120 days prior to challenge. That this ex vivo response mimicked the in vivo situation is illustrated by the decreased expression of IFN- γ (Fig. 2C) and increased expression of IL-17 mRNA (Fig. 2E) in the whole lung tissue of the mice vaccinated 120 days before challenge. We also characterized the CD4 $^{+}$ T cells within the lungs of mice prior to challenge and found that there was a significantly higher frequency of CD4 $^{+}$ T cells expressing CCR6, a chemokine receptor important for the migration of Th17 cells [30] in the lungs of the mice vaccinated for 120 days when compared to mice vaccinated for 21 days (Fig. S1C). Consistent with the enhanced IL-17 response there was also an enhanced granulocyte (CD11b $^{+}$ GR1 $^{+}$) accumulation in the lungs of mice vaccinated 120 days before the Mtb infection (Fig. S1D). Together these data support the hypothesis that the nature of the T cells within the lung prior to Mtb challenge between the 21 day and 120 day vaccinated mice were quite different.

In order to determine if these different populations of antigen-specific T cells mounted a similar response to infection we compared the kinetics of antigen-specific cytokine responses following Mtb challenge. In unvaccinated mice, the Mtb infection resulted in the accumulation of Ag85-specific IFN- γ and IL-17-producing T cells peaking at day 21 post-infection, stabilizing thereafter (Fig. 2F). A similar profile was observed for IFN- γ and IL-17 mRNA levels, although the initial increased transcription of genes encoding these cytokines was followed by a decrease after day 21 (Fig. 2G). As described above, there were higher numbers of both Ag85-specific-IFN- γ - and IL-17-producing T cells in the lungs of vaccinated mice prior to Mtb infection (Fig. 2B, D and F). Upon Mtb challenge, the number of IFN- γ and IL-17-producing antigen-specific T cells did not change dramatically and were maintained at levels above completely naive mice throughout the infection (Fig. 2F). Thereafter, mice vaccinated for 120 days had lower numbers of Ag85-specific T cells (Fig. 2F). The same trend was observed for IFN- γ and IL-17-producing ESAT-6-specific T cells (Fig. S1E).

These data demonstrate that the presence of both IFN- γ - and IL-17-producing T cells in vaccinated mice at the time of challenge is associated with early growth arrest and long-lasting control of Mtb.

3.3. Accumulation of a novel population of CD4 $^{+}$ IL-17 $^{+}$ TNF $^{+}$ IL-2 $^{+}$ cells correlates with long-lasting control of Mtb

While IFN- γ is of critical importance in the control of Mtb [12,13], the expression of this cytokine is not, by itself, a good correlate of protection [14–16]. We initially compared the frequency of IFN- γ producing CD4 $^{+}$ T cells (grouped by dashed line in Fig. 3A) and found that mice vaccinated only 21 days prior to challenge had a higher frequency of these cells than the mice vaccinated 120 days prior to challenge (Fig. 3A). We then compared the frequency of CD4 $^{+}$ T cell co-expressing IFN- γ , TNF and IL-2, which have been associated with protection mediated by BCG [17–20]. We found

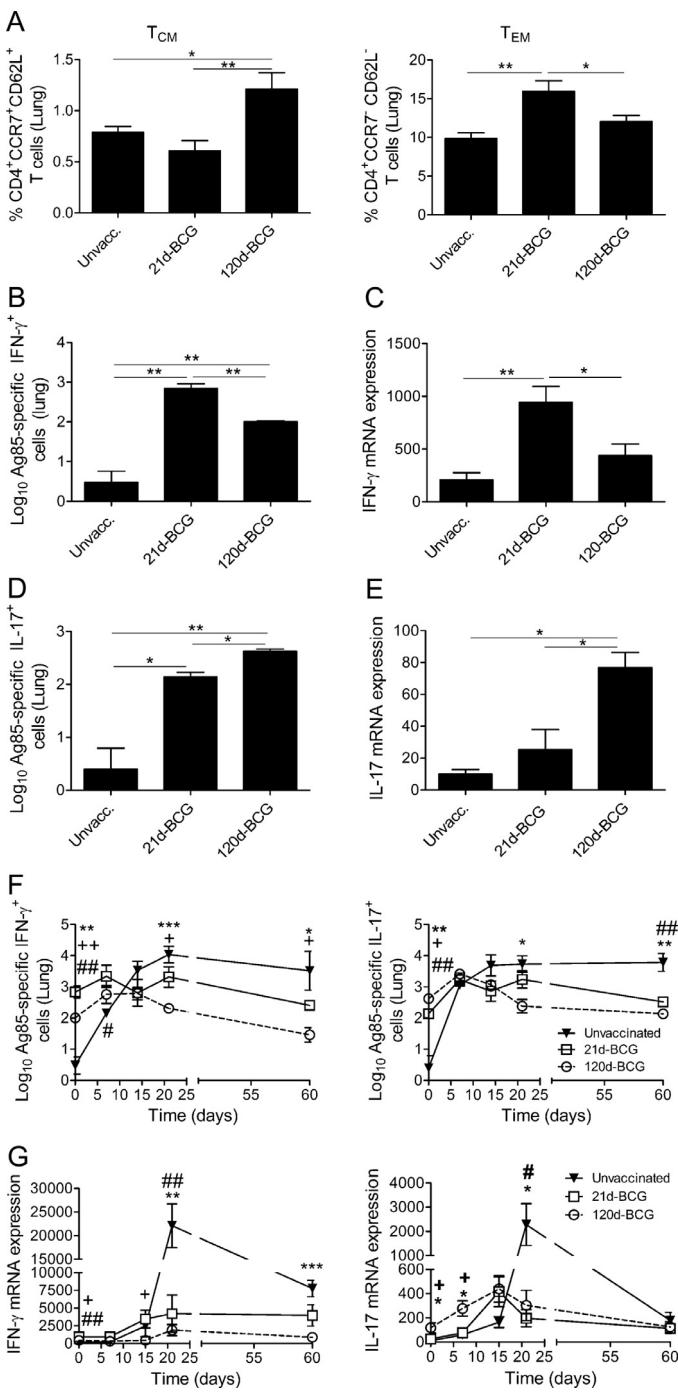


Fig. 2. The time between BCG vaccination and Mtb challenge impacts the phenotype and functionality of the CD4⁺ T cell response. Mice were left unvaccinated (Unvacc.) or vaccinated with BCG for 21 days (21d-BCG) or 120 days (120d-BCG) prior to Mtb challenge. Cells were generated from the lungs, and the expression of CCR7 and CD62L was determined by flow cytometry in CD3⁺CD4⁺ T cells (A) and the production of IFN-γ (B) or IL-17 (D) was determined by ELISpot, after antigen stimulation. Messenger RNA was also isolated from the lungs of the same groups of mice and the level of expression of IFN-γ (C) or IL-17 (E) determined by RT-PCR. ($n=6$), * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ determined by two-way ANOVA. Unvaccinated (black triangles) and mice vaccinated for 21 days (white squares) or 120 days (white circles) were challenged with Mtb and the number of CD4T cells capable of producing IFN-γ (F, left panel) or IL-17 (F, right panel) was determined by ELISpot. The expression of mRNA for IFN-γ (G, left panel) and IL-17 (G, right panel) was also determined over time, post challenge ($n=6$). *+, #, +, **+, ##, +, **+, ##, +, *** $p < 0.05$; **+, ##, +, **+, ##, +, ** $p < 0.01$; *** $p < 0.001$, determined by two-way ANOVA; *, represents statistical differences between Mtb and 120d-BCG; #, represents statistical differences between 21d-BCG and 120d-BCG; +, represents statistical differences between 21d-BCG and 120d-BCG. Results are from one representative out of four independent experiments.

that both groups of vaccinated mice had an increased percentage of lung IFN-γ⁺TNF⁺IL-2⁺ CD4⁺ T cells at the time of Mtb challenge, relative to unvaccinated mice (Fig. 3A and B) and that mice vaccinated 21 days before the Mtb challenge had the highest percentage of this population (Fig. 3A and B, blue bar and pie section). Moreover, the percentage of these cells remained high until day 12 post-infection, with mice vaccinated for 21 days consistently showing the highest frequency (Fig. 3B).

We next analyzed the characteristics of the CD4⁺IL-17⁺T cells (represented by solid line in Fig. 3A). Since we did not detect CD4⁺IFN-γ⁺IL-17⁺ cells (not depicted), we focused on the IFN-γ⁻ population. The IL-17⁺ population was strikingly higher in the 120 day vaccinated mice relative to the 21 day vaccinated and unvaccinated mice both prior to infection (Fig. 3A) and for the first 21 days post challenge (Fig. 3C). Among these CD4⁺IL-17⁺ cells, we found that the triple producing IL-17⁺TNF⁺IL-2⁺ were the population that differed the most between both groups of vaccinated mice (Fig. 3A, shown by purple bar and purple pie slice and Fig. 3D). The differences in the lung populations were also observed in the draining lymph nodes (Fig. S2B-D).

Overall, these data suggest that a novel subset of multifunctional CD4⁺IL-17⁺TNF⁺IL-2⁺ T cells present at the time of challenge and during the early part of the response are associated with the ability of BCG-induced memory populations to provide long-lasting control of Mtb. The nature of the protective function of these cells will be dissected in the future namely by the determination of the role of IL17 itself or of any other molecule expressed by this subset of T cells.

4. Discussion

The Th1/Th2 paradigm proposed by Mosman and Coffman [31], followed by the identification of other Th cell phenotypes [32], brought new understanding of protective immune responses to infection. However, it is now well established that each T cell phenotype incorporates a myriad of sub-populations that likely have different roles during infection [17,33–36]. Specifically for TB, the temporal correlation of Mtb growth arrest with the accumulation of CD4⁺IFN-γ⁺ T cells suggests a critical role for this population in the control of the infection [8]. However, previous observations that IFN-γ is not a good correlate of protection [14–16], achieving an early IFN-γ response is not enough to control Mtb infection [37], and the data presented herein suggest that the CD4⁺ T cell populations that mediate immunity to TB are likely not IFN-γ-single producing Th1 cells, but subpopulations of this phenotype together with other cells whose effector function is not mediated by IFN-γ [38,39].

The type of T cell that should be induced by vaccination to prevent Mtb infection and TB disease remains largely unknown [40,41]. Previous published data suggest that heterologous prime-boost strategies that potentiate IFN-γ responses may not be enough [7]. Our data support this hypothesis and show that the effector and memory responses induced by BCG vaccination are functionally distinct, with the latter being more efficient at maintaining long-lasting control of Mtb. Indeed, the effective long-term memory response is characterized mainly by CD4⁺IL-17⁺ multifunctional T cells, whereas the effector response is skewed to CD4⁺IFN-γ⁺ multifunctional populations. These data are important as they provide a better understanding on the importance of different populations of CD4⁺ T cells in the control of primary Mtb infection and vaccine-mediated immunity. It has recently been suggested that one of the short comings of BCG is the poor induction of CD4⁺ central memory populations [42] specifically, CD4⁺TNF⁺IL-2⁺ and CD4⁺IFN-γ⁺TNF⁺IL-2⁺ multifunctional memory cells [17]. While our data support these observations, we were able to see distinct

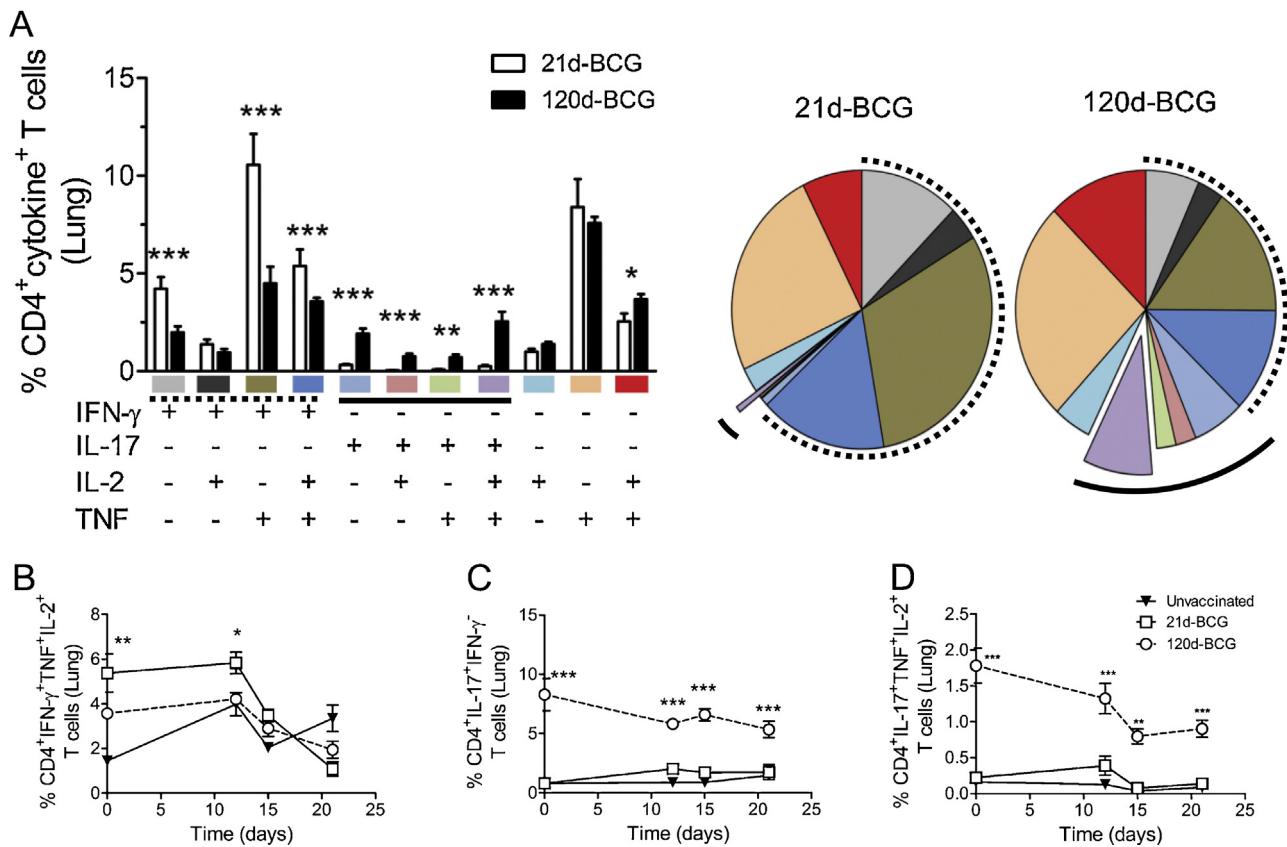


Fig. 3. Accumulation of IL-17⁺IL-2⁺TNF⁺ producing T cells induced by BCG vaccination correspond to long-lasting control of Mtb. Mice were left unvaccinated (black triangles in B-D) or vaccinated with BCG 21 days (21d-BCG in A, white squares in B-D) or 120 days (120d-BCG in A, white circles in B-D) prior to Mtb challenge ($n=8$). (A) Percentage of lung CD4⁺ T cells expressing different combinations of cytokines prior to Mtb challenge (left panel). Pie graphs represent the percentage of lung CD4⁺ T cells expressing specific combinations of cytokines with the colours from the left panel indicating the cytokines being expressed within each slice. CD4⁺ T cells expressing IFN- γ prior to challenge are grouped with a dashed line while those producing IL-17 are grouped with a solid line. (B-D) The cytokine producing phenotype of T cells within the lung was also followed over the first three weeks post challenge by flow cytometry ($n=4$). * $p<0.05$; ** $p<0.01$; *** $p<0.001$ determined by two-way ANOVA. Results are from one representative out of two independent experiments.

multifunctional CD4⁺IL-17⁺ populations after long-term BCG vaccination. We hypothesize that this population supplies the protective CD4⁺ T cell lung response as a result of becoming terminally differentiated [42]. The superior function of the IL-17 multifunctional memory populations is likely associated with their improved ability to survive and mediate effector function within the lung inflammatory environment. Indeed, it has been shown that human Th17 memory cells are relatively resistant to apoptosis and retain plasticity, converting into other Th cells [43]. Moreover, we have recently shown that different CD4⁺ T cell populations display a differential ability to survive and perform effector function in the infection site [10]. Future experiments are planned to address this issue. CD4⁺IL-17⁺ cells have been recently shown to induce vaccine-mediated immunity in a CXCL13-dependent, IFN- γ -independent way, by promoting the recruitment and correct location of pro-inflammatory cytokine secreting CXCR5 cells within the lung tertiary lymphoid structures [44,45]. IL-17 is also an important inflammatory mediator for the recruitment of neutrophils during TB [21,46]. While the role of neutrophils during TB is still controversial, they can have protective roles including, but not limited to, the rapid clearing of Mtb released by dying macrophages in the granuloma [46,47]. Therefore, one can speculate that a vaccination approach that promotes a sustained, but controlled, recruitment of neutrophils in the context of an IFN- γ rich environment within the lung is likely more advantageous than an approach that promotes the recruitment of macrophages alone, that serve as host cells for Mtb.

It is not only important that we define what comprises protective immunity but also how to maintain these responses with minimal pathological consequences to the host [48]. The data presented herein suggest that the protective memory response to TB must undergo a process of maturation capable of significantly reducing bacterial burdens without pathological consequences to the host. It is likely that the strain, dose, route and time post-BCG vaccination as well as the genetic background of the host impact the generation and the kinetics of these populations. This would explain the differences observed in the generation of memory populations and protection afforded by BCG [42]. Moreover, while previous exposure to mycobacteria has been one of the factors associated with the variable protection afforded by BCG [3,49], our data suggest that exposure to Mtb soon after vaccination may also contribute to the reduced efficacy of this vaccine. Likely this exposure further promotes the expansion of the effector response, hampering the development of the memory response.

Overall, our data show that the memory response generated by BCG vaccination can be very effective at mediating Mtb growth arrest and maintaining long-lasting control of bacterial burdens. Specifically, the accumulation of CD4⁺IL-17⁺TNF⁺IL-2⁺ T cells appears to be critical for these functions. This population is functionally distinct from the effector response and requires time to develop. It will be important in the future to determine whether the kinetics of this response is specific for BCG or a more general phenomenon induced by other live or subunit vaccines, currently in clinical trials. Understanding the mechanisms underlying the

development and expansion of this population will have important implications for the development of novel vaccination strategies against TB and in heterologous prime-boost strategies wherein BCG is used as priming agent.

Acknowledgements

We thank the ICVS animal facility personnel for excellent animal husbandry. This work was supported by Fundação para a Ciência e Tecnologia, Portugal and cofunded by Programa Operacional Regional do Norte (ON.2—O Novo Norte), Quadro de Referência Estratégico Nacional (QREN), through the Fundo Europeu de Desenvolvimento Regional (FEDER) project grants PTDC/SAU-MII/101977/2008, PTDC/BIA-BCM/102776/2008, and from Projeto Estratégico – LA 26 – 2013–2014 (PEst-C/SAU/LA0026/2013). AMC was funded by the National Institute of Allergy and Infectious Diseases at the National Institutes of Health grant AI46530. A.C. received a personal FCT grant SFRH/BPD/3306/2007 and M.S. is an FCT Investigator fellow.

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.2014.11.013>.

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