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

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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 $IL\text{-}17^+TNF^+IL\text{-}2^+$. 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 $IFN\text{-}\gamma^+TNF^+$ and to a lesser extent $IFN\text{-}\gamma^+TNF^+IL\text{-}2^+$. 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.

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 $CD4^+IFN\text{-}\gamma^+$ 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, $CD4^+IL\text{-}17^+$ T cells are particularly important in vaccine-mediated immunity [9,22] by promoting a more rapid recruitment of $CD4^+IFN\text{-}\gamma^+$ 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 $CD4^+IL\text{-}17^+$ T cells, including $CD4^+IL\text{-}17^+TNF^+IL\text{-}2^+$. 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 ± 0.23 $\log_{10}$ [25]. For bacterial load determination, mice were killed by CO_2 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 $Ag85B_{280\text{-}294}$ and $ESAT\text{-}6_{1\text{-}20}$-specific $CD4^+$ T cells was determined by ELISpot, as these peptides represent previously described $I\text{-}A^b$-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 Ionomycin 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: $1.8^{(\text{ct reference gene} - \text{ct target gene})} \times 100{,}000$.

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 ± 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

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