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Brief report

Interferon-gamma production by mononuclear cells in Bacille Calmette-Guérin-revaccinated healthy volunteers predicted long-term antimycobacterial responses in a randomized controlled trial

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ABSTRACT

The Bacille Calmette-Guérin (BCG) vaccine is the only vaccine currently available for tuberculosis, and it demonstrates variable efficacy against the disease. The assessment of new vaccine strategies is hindered by the small annual probability that an infected individual will develop tuberculosis, and the lack of simple and reliable surrogate markers of protection. The frequency of cytokine-producing T cells as well as the production of IFN-γ have been disputed as surrogate markers of protection. We evaluated the evolution of these immune parameters in a population from a high burden city where BCG revaccination has been shown to result in mild protection. We found that individuals whose in vitro IFN-γ responses to mycobacterial antigens had increased by more than 3.3-fold were more likely to maintain higher responses after 1 year and to show increased expansion of IFN-γ-producing T lymphocytes than those with lower or null increase of IFN-γ.

1. Introduction

Tuberculosis (TB) remains a major public health problem worldwide, despite over 80% worldwide coverage achieved by the Bacille Calmette-Guérin (BCG) vaccine. BCG efficacy is highly variable among populations and is often low against pulmonary TB, which limits its effectiveness at reducing TB transmission. Biomarkers are urgently needed to evaluate new vaccine strategies [1]. In spite of being widely assessed the IFN-γ production to mycobacterial antigen has been questioned as a valid biomarker [2].

In the BCG-protected population of the United Kingdom [3] IFN-γ production to *Mycobacterium tuberculosis* protein purified derivative (PPD) in whole blood cultures increased up to 11-fold [4] 1 year after BCG vaccination, while in Malawi where the vaccine has no efficacy BCG vaccination demonstrated up to 2-fold increase in the levels of this cytokine [5]. Similar high IFN-γ responses among children from the UK were also associated with the expansion of IFN-γ-producing T cells [6]. In the animal model, the total IFN-γ response has demonstrated better association with the rate of protection than the response of antigen-specific $CD4^+$ or $CD8^+$ T cells [7].

In Salvador, Bahia, Brazil BCG revaccination at school age demonstrated moderate additional protection against TB, relative to the priming vaccination [8]. Additionally, the IFN-γ response to mycobacterial antigens was increased at 2 months after BCG revaccination only in a subset of the revaccinated children [9]. Therefore, we evaluated whether the fold increase in the in vitro IFN-γ production to whole mycobacterial antigen post-BCG revaccination could be associated with the frequency of IFN-γ^+/$CD4^+$ and $CD8^+$ T cells as well as predict the response to the same stimulus at 1 year post-revaccination for potential use as a parameter to guide the selection of newly proposed immunization strategies against TB.

Abbreviations: BCG, Bacille of Calmette-Guérin; CI, confidence interval; HIV, human immunodeficiency virus; IFN-γ, interferon-gamma; IL, interleukin; Mtb, M. tuberculosis H37Rv culture lysate; PBMC, peripheral blood mononuclear cells; PHA, phytohemagglutinin; PPD, protein purified derivative; roc, Receiver operating characteristic; TB, tuberculosis; TST, tuberculin skin test; TNF, tumor necrosis factor.

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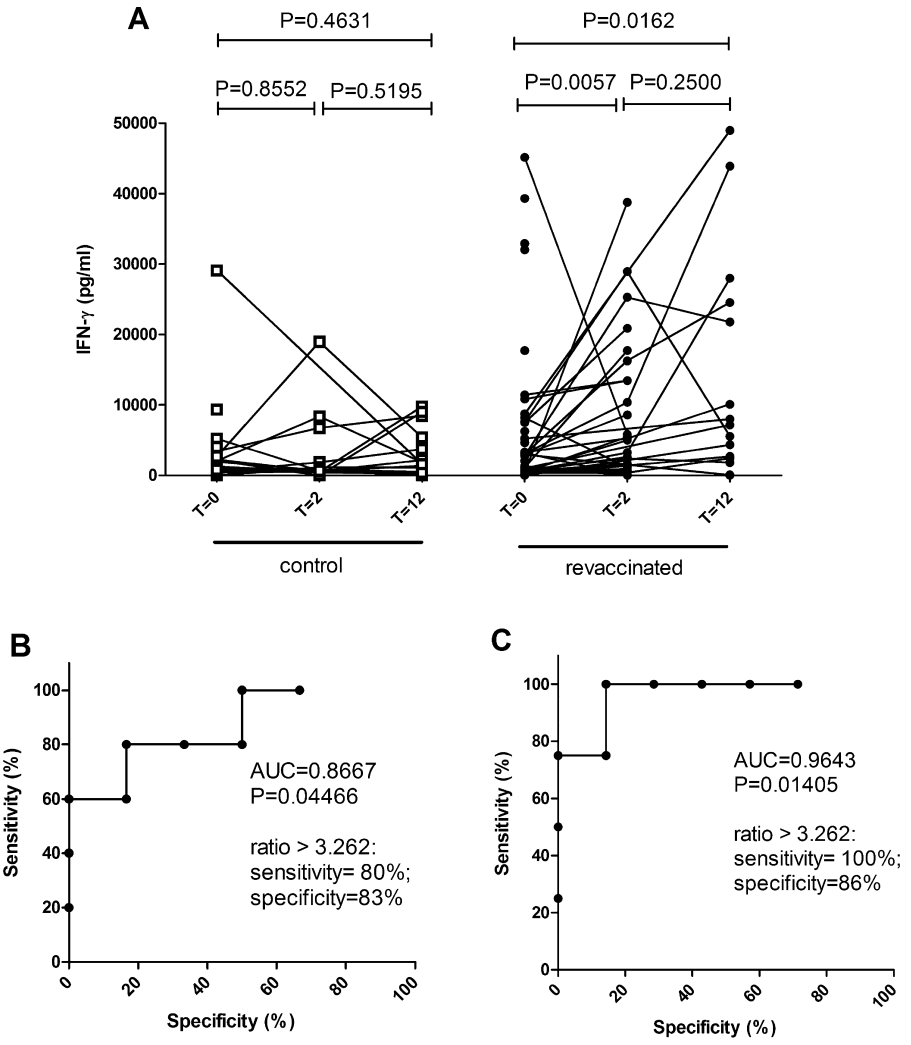

Fig. 1. A, IFN-γ production in whole blood cultures of volunteers randomly assigned to control (29) and BCG-revaccinated (46) groups. The numbers of individuals present at follow-up evaluations were: at $T=2$, 28 revaccinated and 14 control subjects; at $T=12$, 16 revaccinated and 14 controls (Supplementary Fig. 2). B and C, roc curves for the prediction of IFN-γ production at 12 months post-revaccination: B, model representing the prediction of IFN-γ responses above the median value obtained for the revaccinated group; C, model representing the prediction of IFN-γ responses above the mean value plus twice the standard deviation observed in the cultures of control individuals.

2. Materials and methods

2.1. Recruitment and study design

This was a prospective randomized controlled trial of BCG revaccination among tuberculin skin test (TST) negative and human immunodeficiency virus (HIV) negative university students from Salvador, Bahia, Brazil aged 18 years or older that had been vaccinated with BCG in early childhood and presented distinguishable prime vaccination scars, identified in a TST survey [10]. Informed consent was obtained from all participants. Subjects donated blood at baseline (T0), 2 months (T2) and 12 months (T12) after the first intervention (Supplementary Fig. 1). Revaccination was performed intradermally using a 26G × 1/2″ needle and 1 ml syringe in the deltoid region of the right arm at least 2 cm apart from the previous BCG scar with one dose of 0.1 ml *M. bovis* BCG Moreau-RJ vaccine suspension (Fundacao Ataulpho de Paiva, reg. MS 101660001/007-6, Lot nr. 02P). Hemogram and hematocrit values were measured using a hemocytometer (Coulter Electronics, Hialeah, FL, USA). Serology against HIV-1 and HIV-2 was performed using a rapid test (Determine™ HIV-1/2, Abbott Laboratories, Tokyo, Japan). A follow up clinical evaluation was performed by one clinician and one dermatologist. The Ethical Committee of the Centro de Pesquisas Gonçalo Moniz approved this study (CEP-CPqGM/FIOCRUZ, 19/2002) that complied with the ethical principles contained in the Helsinki Declaration and the Brazilian National Health Council Resolution 196/96 Guidelines.

2.2. Cultures

Whole blood cultures were performed as described in [9] from vacuum-collected blood, with or without 10 μg/ml of *M. tuberculosis* H37Rv culture lysate (Mtb, Colorado State University, USA). Plates were incubated at 37 °C with 5% CO_2 and 98% humidity for 72 h and the culture supernatants were then recovered and stored at −20 °C until analysis.

Peripheral blood mononuclear cells (PBMCs) were obtained from the T0 and T2 samples using a Ficoll-Histopaque gradient (Sigma–Aldrich, MO, USA). Cells (2×10^5/well) were plated with no stimuli or with phytohemagglutinin 5 μg/ml (PHA; Sigma–Aldrich, MO, USA), 10 μg/ml Mtb, or live *M. bovis* BCG Moreau-RJ strain at 10 mycobacteria per monocyte (10:1 MOI). Live monocytes were estimated from percent propidium iodide negative (1 μg/ml,

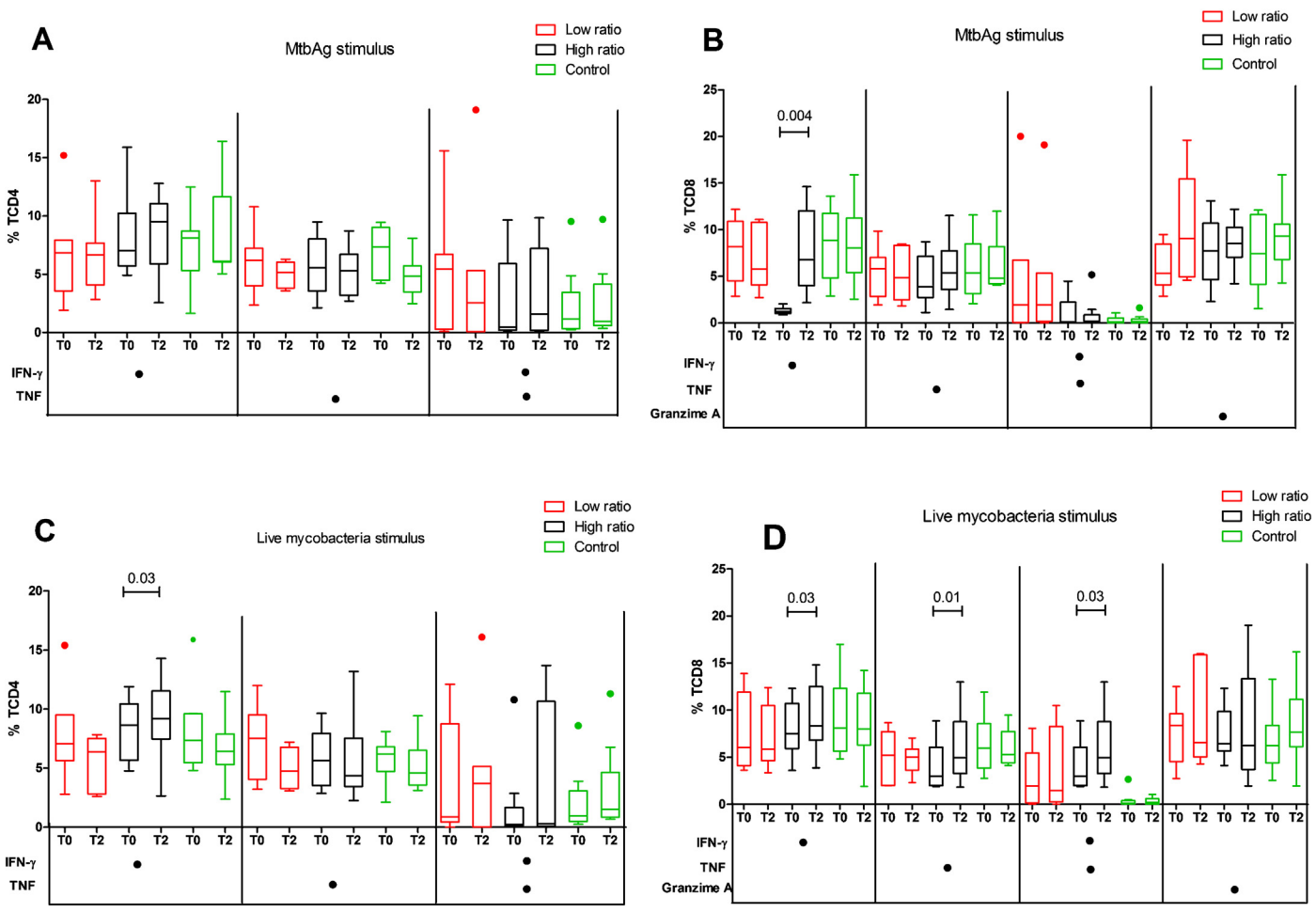

Fig. 2. Expansion of cytokine-producing CD4⁺ (A, C) and cytokine/granzyme A-producing CD8⁺ (B, D) T cells in PBMC cultures stimulated with Mtb (A, B) or live BCG (C, D). Samples with less than 80% viable cells were discarded. The cultures from sixteen revaccinated (7 low ratio and 9 high ratio) and 9 control individuals with PBMC from both $T=0$ and $T=2$ were analyzed.

Sigma-Aldrich, MO, USA) CD14 positive (Becton-Dickinson Biosciences, San Jose, CA) of total PBMC counts.

The bacterial inocula were prepared as described [11]. Live BCG-stimulated cultures were incubated for 2 h in antibiotic-free medium, washed and suspended in medium with antibiotics and maintained for 6 or 48 h. The cytokines were measured in culture supernatants (Supplementary Table 2) using multiplexed bead-based immunoassays (BD Cytometric Bead Array™, Becton-Dickinson Biosciences, San Jose, CA). The T0 and T2 supernatants were evaluated simultaneously, whereas the T12 supernatants were assessed 1 year later. IL-17 was not assessed at T12. Cytokine and granzime A-producing T cells were quantitated by flow cytometry using fluorescent antibodies (Becton-Dickinson Biosciences, San Jose, CA) as described [12].

2.3. Statistical analyses

Volunteers were randomly assigned to the revaccinated or control groups using Microsoft Excel (Microsoft Co., Redmond, WA). Frequencies were compared using Fisher's exact test or the chi-squared test. The cytokine levels and ratios were compared using the Mann-Whitney U test or the Wilcoxon signed rank test. All statistical tests were two tailed, and the differences were considered significant at $P \leq 0.05$. Databanks were mounted in EpiData Entry (EpiData Association, Denmark), and the data were processed and analyzed using EpiData Analysis and Prism (GraphPad Inc., San Diego, CA).

3. Results and discussion

3.1. Modulation of cytokine responses to Mtb upon revaccination with BCG

Only revaccinated individuals demonstrated increased IFN-γ and IL-10 production (Fig. 1A and Supplementary Table 2) in Mtb-stimulated cultures at T2 compared to T0, and only IFN-γ levels differed between the revaccinated and control subjects at T2 ($P=0.0362$). The TNF levels were increased in both the control and revaccinated subjects (Supplementary Table 2), which may reflect a nonspecific response to the TST performed prior to volunteers' enrollment. IFN-γ production at T12 was also higher for revaccinated individuals compared to the T0 levels (Fig. 1A), although these levels were not different from those observed in control volunteer cultures ($P=0.0845$). The other evaluated cytokines were similar between the evaluation periods and between the control and revaccinated groups (Supplementary Table 2).

3.2. Prediction of the IFN-γ response at 12 months post-BCG revaccination

In Salvador, we previously found that the IFN-γ responses in Mtb-stimulated cultures from BCG-revaccinated adolescents were

increased up to 8-fold [9]. To determine whether the T2/T0 IFN-γ ratio in early whole blood cultures stimulated with Mtb could predict the IFN-γ response at 12 months post-BCG revaccination we analyzed two endpoints: (i) IFN-γ production above the median level at T12 (Fig. 1B) and (ii) IFN-γ production above the mean value plus twice the standard deviation found in cultures of control individuals at T12 (Fig. 1C). There was a significant association with both endpoints, with high sensitivity and specificity for the cut-off value corresponding to a ratio of 3.262. This was not observed when considering the absolute values of IFN-γ production obtained post-BCG revaccination, which is in agreement with the current view that the magnitude of the IFN-γ response is unlikely to reflect vaccine efficacy [2]. These data further suggest that there is a dichotomy in the response of the individuals to BCG revaccination, by which only part of the revaccinated subjects show the development of a long lasting Th1 response. It must be stressed that the number of individuals tested and the period of follow-up were insufficient to investigate any direct association between the ratio of IFN-γ production and protection against the development of TB disease.

3.3. *In vitro expansion of T cell subsets from volunteers stratified according to the IFN-γ ratio*

We used the ratio of 3.262 that was found to predict the long term IFN-γ response as a cut-off to assign the volunteers to "low-ratio" or "high-ratio" groups (Supplementary Fig. 2). We compared the expansion of cytokine- or granzyme A-producing T cells in PBMC cultures at T0 and T2 between the control, high-ratio and low-ratio individuals (Fig. 2). T cell expansion (IFN-γ^+, TNF^+ or granzyme A^+ or IFN-γ/TNF^+ $CD4^+$ or $CD8^+$ lymphocytes) following Mtb or live BCG stimulation relative to unstimulated cultures was not different between the control, low-ratio, and high-ratio individuals at T0 or T2. However, there was greater expansion of $CD4^+$IFN-γ^+ cells upon stimulation with live BCG at T2 compared to T0 among high-ratio volunteers (Fig. 2C) that was not observed among the controls or low-ratio individuals. Similarly, greater $CD8^+$ lymphocytes expansion (both single-positive and IFN-γ^+/TNF^+) was observed at T2 only in the high-ratio group (Fig. 2B and D). No differences in $CD8^+$ granzyme A^+ cells expansion were observed following Mtb or live BCG stimulation between pre- and post-BCG revaccination cultures. These findings are in agreement with those of Kagina et al. [13], who failed to demonstrate any difference in BCG-induced T cell frequencies when comparing children who developed TB with vaccine-protected children.

The possibility that the ratio of the IFN-γ response within a short time pre- and post-vaccination may reflect vaccine efficacy may signal novel approaches to improve the analysis of ongoing vaccine trials. IFN-γ release assays are relatively inexpensive, highly reproducible, and easily applicable to large-scale field studies and continue to be developed and applied for the diagnosis of TB and clinical outcome prediction [14]. The development of similar tests to validate vaccine candidates would be relatively easy and could have a significant impact on accelerating vaccine research and development toward obtaining a better TB vaccine. Further epidemiologic studies are needed to establish a possible direct link between the IFN-γ response and protection against TB development in diverse human populations.

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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.2013.04.079.

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