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Over-expression of superoxide dismutase obliterates the protective effect of BCG against tuberculosis by modulating innate and adaptive immune responses

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ABSTRACT

An efficient global control of tuberculosis requires development of alternative vaccination strategies that can enhance the efficacy of existing BCG vaccine. In this study, we evaluated the protective efficacy of a recombinant BCG (rBCG) vaccine over-expressing iron-cofactored superoxide dismutase (SOD-A), one of the prominent oxidative stress response proteins of *Mycobacterium tuberculosis*. Contrary to our expectations, over-expression of SOD-A resulted in the abrogation of BCG's ability to confer protection in guinea pig as well as in murine model. Analysis of immune responses revealed that over-expression of SOD-A by rBCG has pleiotropic effects on innate and adaptive immune responses. Macrophages infected *in vitro* with rBCG exhibited a marked reduction in apoptosis and microbicidal potential. In addition, rBCG vaccination of mice resulted in a reduced IFNγ and increased IL10 production when compared with the BCG vaccination. Further, we show that rBCG vaccination failed to generate an effective multi-functional CD4 T cell response. Altogether, our findings suggest that over-expression of SOD-A in BCG enhances the immuno-suppressive properties of BCG, characterized by skewing of immune responses towards Th2 type, an inefficient multi-functional T cell response and reduced apoptosis and microbicidal potential of macrophages leading to abolishment of BCG's protective efficacy.

1. Introduction

The ability to subvert the host immune system and withstand the phagocytic onslaught is critical for the intracellular survival of *Mycobacterium tuberculosis*. Reactive oxygen intermediates (ROI) represent one of the major components of the innate host defense [1,2] and production of superoxide dismutase (SOD) by *M. tuberculosis* provides protection against ROI-mediated killing by the activated macrophages [3]. *M. tuberculosis* produces two SOD proteins, (i) an iron-cofactored enzyme encoded by *sodA* (*Rv3846*) and (ii) a copper–zinc cofactored enzyme encoded by *sodC* (*Rv0432*) [4,5]. While, low levels of SOD-C are produced by almost all the mycobacterial species (saprophytic as well as pathogenic), copious amount of SOD-A is produced and secreted exclusively by the pathogenic mycobacterial species underlying its importance in the virulence [3]. Reduced production of SOD-A but not SOD-C results in a marked attenuation of *M. tuberculosis* virulence, which further reinforces the relative importance of SOD-A over SOD-C [5,6]. Moreover, SOD-A is up-regulated by *M. tuberculosis* during macrophage infection [7], is highly immunogenic and several of its B and T cell epitopes are recognized in PPD positive individuals as well as in tuberculosis (TB) and leprosy patients [8–10]. Thus, the immuno-dominant properties, extracellular abundance and the essential requirement of SOD-A for *in vivo* survival of *M. tuberculosis* make this antigen an attractive target for the development of new vaccines against TB.

M. bovis Bacille Calmette Guerin (BCG), provides a consistent protection against severe forms of childhood TB but it looses its potency gradually resulting in an inadequate protection against pulmonary TB in adults [11]. Thus, for an efficient control of TB, we require to develop new vaccination strategies to improve and sustain the protective efficacy of BCG. We have previously reported that a DNA vaccine expressing SOD-A induces a marked Th1 immune response in mice characterized by an elevated level of antigen specific IFNγ and a commensurate decline in IL10 level [12]. In addition, it imparts a notable protection against *M. tuberculosis* infection in guinea pigs, although, it could not surpass the

Abbreviations: BCG, Bacille Calmette Guerin; TB, tuberculosis; SOD, superoxide dismutase; rBCGsod, recombinant BCG over-expressing SOD; ROI, reactive oxygen intermediates; MFI, median fluorescence intensity; iMFI, integrated median fluorescence intensity; T_{Eff}, terminally differentiated T effector cells.

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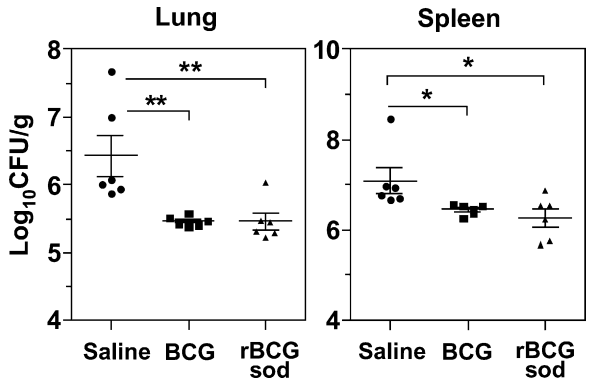

Fig. 1. Bacillary load in lung and spleen of guinea pigs following *M. tuberculosis* challenge. Lung and spleen CFU were determined at 10 weeks post-infection in guinea pigs ($n = 6$) and depicted by dot plot. Each point represents the $\log_{10}$ CFU value for an individual animal and the bar depicts mean (±SE) for each group. The lower limit of detection was 1.0 $\log_{10}$ CFU/g of tissue. $^{*}p < 0.05$; $^{**}p < 0.01$ (One-way ANOVA).

protective efficacy of BCG [12]. In the present study, we developed an rBCG strain over-expressing SOD-A and evaluated its protective efficacy against an aerosol infection of *M. tuberculosis* in guinea pig and murine model along with the analysis of cell-mediated immune responses.

2. Materials and methods

2.1. Bacterial strains and preparation of antigens for immunization

rBCGsod was generated by engineering a *Mycobacteria* – *Escherichia coli* shuttle vector pSD5.pro [13,14] to over-express *sod*A (*Rv3846*) of *M. tuberculosis* under transcriptional control of promoter of the *hsp65* gene of *M. leprae* as depicted in Supplementary Fig. 1. BCG (Danish strain, BCG laboratories, Chennai, India), rBCGsod and *M. tuberculosis* (H37Rv strain, ATCC no. 25618, AIIMS, New Delhi, India) were grown to mid-log phase in Middlebrook 7H9 media and stocks were prepared as described earlier [15].

2.2. Experimental animals

Specific pathogen free 200–300 g female outbred guinea pigs (Dunkin Hartley strain) were procured from Disease Free Small Animal House Facility, Haryana Agricultural University, Hissar, India. Specific pathogen free 20–30 g inbred mice (Balb/c strain) were procured from National Centre for Laboratory Animal Sciences, National Institute of Nutrition, Hyderabad, India. The animals were maintained in a BSLIII facility and routinely cared for according to the guidelines of CPCSEA (Committee for the Purpose of Control and Supervision on Experiments on Animals), India. All the animal experiments were reviewed and approved by the Institutional Animal Ethics Committee.

2.3. Immunization of guinea pigs and evaluation of protective efficacy

For protective efficacy study, two guinea pig experiments were carried out (Supplementary Fig. 2a) and in each experiment animals ($n = 6$–7) were immunized with the following regimens: (i) 5×10^5 CFU of BCG (in 100 μl of saline) by intradermal route, (ii) 5×10^5 CFU of rBCGsod (in 100 μl of saline) by intradermal route and (iii) 100 μl of saline by intradermal route (control group). In Exp-I, guinea pigs were challenged at 6 weeks after immunization with 250–500 bacilli of *M. tuberculosis via* respiratory route in an aerosol chamber (Glasscol Inc.) and were euthanized at 10 weeks post-infection. In Exp-II, guinea pigs were challenged at 12 weeks post-immunization and were euthanized at 16 weeks post-infection by intraperitoneal injection of thiopentone sodium (100 mg/kg body weight) (Neon Laboratories Ltd.). The methodologies employed in this study for immunization, infection and evaluation of protective efficacy are based on the methods (high dose infection model) developed by EU TB vaccine cluster for screening of TB vaccine regimens in guinea pig model [16].

After dissecting the animals, lungs, liver and spleen were scored for gross pathological changes as described previously [15]. Lung and spleen bacillary load were measured along with the evaluation of histopathological changes as described previously [15]. The detection limit in case of both lung and spleen CFU was 1.0 $\log_{10}$ CFU/g.

2.4. Immunization of mice and evaluation of immune responses and protective efficacy

For this, BALB/c mice ($n = 4$–6) were immunized with either of the following: 5×10^5 CFU of BCG or rBCGsod or 100 μl of saline by subcutaneous route (Supplementary Fig. 2b and c). Immune responses were evaluated by assessment of (i) cytokine responses and (ii) multi-functional T cell responses. For evaluation of protective efficacy, mice were immunized and infected following the same protocol as used for guinea pigs. At 4 weeks post-infection, bacillary load in lungs and spleen was determined. The protection study was carried out once with 4 mice in each group.

2.5. Assessment of cytokine responses

In the first experiment (Supplementary Fig. 2b), at 4, 8 and 12 weeks post-immunization, antigen specific cytokine (IFNγ and IL10) responses were measured as described previously [17]. Briefly, splenocytes were stimulated *in vitro* in a 96-well cell culture plate (5×10^5 cells/well) with purified recombinant SOD (20 μg/ml) in RPMI. After 90 h, supernatants were collected for estimation of IFNγ and IL10 by using cytokine specific kits (BD Biosciences).

2.6. Assessment of multi-functional T cell responses

In the second experiment (Supplementary Fig. 2c), at 12 weeks post-immunization multi-functional T cell responses were assessed by intra-cellular cytokine staining of CD4 and CD8 T cells for IFNγ, TNFα, and IL2. Briefly, splenocytes were cultured in RPMI-GlutaMAX™ (containing 10% heat inactivated FBS and 1X antibiotic–antimycotic) (Invitrogen) in the presence of PPD (20 μg/ml) (Statens Serum Institut) or purified SOD (20 μg/ml) for 2 h at 37 °C. Thereafter, GolgiStop was added and cells were incubated for an additional 4 h before intracellular cytokine staining. Following stimulation, T cells were purified by using BD IMag™ Mouse T Lymphocyte Enrichment Kit and stained for cell surface markers, CD4 (FITC) or CD8 (FITC) along with intracellular cytokines, IFNγ (RPE), TNFα (PerCPCy5.5) and IL2 (APC) by using BD Cytofix/Cytoperm kit. Cells (50,000–100,000) were acquired by using a FACS Calibur flow-cytometer (by using Cell Quest Pro software) and analysed by using FlowJo software (Tree Star). Cell frequency, median fluorescence intensity (MFI) and integrated MFI (iMFI = % frequency × MFI) for different cytokines were calculated. For stimulation and FACS analysis, cells pooled from all 4 mice from a group were used and the data were normalized to respective media control. Multifunctional T cells derived from splenocytes stimulated with PPD and SOD antigens have been referred to as PPD and SOD specific T cells, respectively, here onwards. Cells that produce three cytokines (IFNγ, TNFα and IL2) have been referred to as