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Recombinant BCG::Rv2645 elicits enhanced protective immunity compared to BCG *in vivo* with induced ISGylation-related genes and Th1 and Th17 responses

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ABSTRACT

There is a need to develop protective vaccines against tuberculosis (TB). Recently, we identified an immunodominant T-cell antigen, Rv2645, from the region of deletion 13 (RD13) of *M. tuberculosis* (*M. tb*) H37Rv, which is absent in Bacille Calmette-Guérin (BCG). Here, a recombinant BCG expressing Rv2645, namely, BCG::Rv2645, was constructed. Compared to BCG, we found that BCG::Rv2645 improved the antigen presentation capacity of dendritic cells (DCs) and elicited much stronger Th1 and Th17 responses, higher $CD44^{high}CD62^{low}$ effector memory $CD4^+$ T cells (T_{EM}), and fewer T regulated cells (Treg) and regulatory B10 in mice. Importantly, BCG::Rv2645 exhibited enhanced protective efficacy against virulent *M. tb* H37Rv challenge in both mice and rhesus monkeys, showing less severe pathology and reduced pathogens. Further, transcriptomic analysis and reverse transcription-quantitative real time PCR revealed that the mRNA levels of ISGylation (Isg)-related genes such as interferon-stimulated gene 15 (Isg15), and Th1- and Th17-related genes such as interferon-γ (IFN-γ) and interleukin-17A (IL-17A) were significantly up-regulated in splenocytes and macrophages after stimulation with Rv2645. This study shows that BCG::Rv2645 is a promising TB vaccine candidate with enhanced protective immunity. The enhanced Th1/Th17 immune responses and up-regulation of ISGylation-related genes induced by Rv2645 may be major factors contributing to the protective immunity of BCG::Rv2645.

1. Introduction

Tuberculosis (TB) remains a leading human infectious disease and a major public health problem in low-income countries. Currently, Bacilli Calmette-Guérin (BCG), an attenuated strain of *Mycobacterium bovis*, is the only tuberculosis vaccine available for human use and has been given to more than 3 billion people since 1921 [1]. However, BCG only decreases childhood tuberculosis (TB), and its protective efficacy against adult pulmonary disease in endemic areas is insufficient, ranging from 0 to 80% [2]. These data indicate that the BCG vaccine lacks the immunogenicity required to generate protective immunity in adult populations. Therefore, the development of more efficacious TB vaccines that can confer potent protection *in vivo* remains a major challenge to TB vaccinologists.

Comparative genomics studies identified 129 coding sequences absent from BCG and/or *Mycobacterium bovis* but present in virulent *M. tb* H37Rv, the principal agent of human tuberculosis [3]. These genes are called region of deletion (RD) genes. The functional consequences of most of these RD genes have not been studied. Some of these coding sequences encode potential antigens that could improve immunogenicity if reintroduced into BCG, generating recombinant BCG (rBCG) [4,5]. Many of the most promising candidates for vaccine development are secreted RD proteins [6], such as ESAT-6, CFP10 and Ag85B [5,7–11]. However, we believe that other dominant antigens, with potential capacity to induce protective immune responses, lurk in other RD regions.

The gene product of the open reading frame Rv2645 of *M.tb* H37Rv was annotated as a conserved hypothetical protein in the database, and its function has not been reported [12]. Recently, we discovered that Rv2645, an early secreted 15 kD protein from

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the RD13 region, evoked the highest level of antigen- specific IFN-γ-producing $CD4^+$ T cells among the 40 tested RD proteins, including the well-known ESAT6 and CFP10 [12]; Epitope mapping revealed that $Rv2645_{30–44}$ and $Rv2645_{136–143}$ may be the dominant T-cell and B-cell epitopes, respectively, of Rv2645 [12]. Here, we further evaluated the value of Rv2645 as a candidate antigen introduced into BCG and assessed the immunogenicity and protective ability of recombinant BCG::Rv2645 against *M. tb* H37Rv in mice and monkeys.

2. Materials and methods

2.1. Strains, plasmids and animal protocol

M. tb H37Rv [strain ATCC 25618] and *M. bovis* BCG [strain ATCC 35734] were maintained on 7H9 middle brook liquid medium and harvested while in log-phase growth. Bacilli were washed in phosphate buffered saline (PBS) with 0.05% Tween 80 and triturated uniformly before use. The plasmid pMV361 (kindly provided by Dr. Jiaoyu Deng, Wuhan Institute of Virology, Chinese Academy of Sciences) was used for construction of shuttle expression plasmid in mycobacterium. The 7H11 middle brook solid medium was used for screening single colonies.

BALB/c mice (6–8 weeks of age) and rhesus monkeys (Chinese origin, 3–6 years old) obtained from Animal Laboratory Center, Wuhan University were used in this study. All bacterial cultures and animal tests were carried out in the ABSL-3 Laboratory of the Wuhan University School of Medicine. The animal experimental protocol was approved by the Institutional Animal Care and Use Committee of Wuhan University. The animal bacterial challenge protocols were performed in compliance with all guidelines and were approved by Institutional Animal Care and Use Committee of Wuhan University.

2.2. Construction of the shuttle prokaryotic expression plasmid encoding Rv2645

The entire Rv2645 gene sequence from *M. tb* H37Rv chromosomal DNA was amplified by PCR and subcloned into the shuttle expression vector pMV361. Primer sequences were 5′-ATTGAATT CATGACCACCACGC-3′ (forward) and 5′-AATAAGCTTCCGCCGGTGT TCGC-3′ (reverse). The underlined oligonucleotides represent *Eco*RI and *Hin*dIII sites, respectively, to facilitate cloning. The amplified products were purified with the Axgen Nucleic Acid Purification kit (Axygen, China), digested with *Eco*RI and *Hin*dIII and ligated into pMV361 that were digested by *Eco*RI and *Hin*dIII. The resulting plasmid pMV361-Rv2645 was confirmed by restriction enzyme digestion and DNA sequencing.

2.3. Construction of recombinant BCG::Rv2645

The shuttle expression plasmid pMV361-Rv2645 was electrotransformed into the BCG strain by Gene Pulser XcellTM (Bio-Rad, USA) in the 7H11 medium with kanamycin selection. Then, the recombinant vaccine strains were generated, confirmed by PCR with Rv2645 primers mentioned above, and Western blotted with anti-Rv2645 and Southern blot as described below.

2.4. Western blotting and Southern blotting for confirming the recombinant vaccine strain

A positive monoclonal colony was selected from 7H11 medium with kanamycin and amplified in 7H9 liquid medium. Then, the harvested bacteria were used for Western blotting and Southern blotting. For Western blotting, the harvested bacteria cell lysates underwent SDS-PAGE and were then probed with Rv2645 rabbit polyclonal antibodies prepared in our lab [12] as primary antibody diluted 1:10000 with PBS containing 0.05% (v/v) Tween-20 (PBST), followed by horseradish peroxidase (HRP) conjugated goat anti-rabbit IgG as a second antibody with 1:4000 dilution. For Southern blotting, the BCG::Rv2645 genome was extracted by the Bacterial Genome Extraction Kit (TianGen, China); then, 10 μg genomic DNA was digested by *Sau*III enzyme, electrophoresed in agarose gel, and transferred onto polyvinylidene fluoride (PVDF) membrane. The reaction was performed with biotin-labeling at the 5′ end of the Rv2645 probe: 5′-CCGGCGATTCACTACACG GAACCGCCCGTGTTGGGG-3′ that was synthesized by Invitrogen company (Shanghai, China) and then blotted with HRP-rabbit anti-biotin antibody (Abcam, UK) diluted with 1:2000 in PBST. All color development following the addition of the substrate Immobilon Western (Millipore, Shanghai, China).

2.5. Flow cytometry (FCM) analysis

Splenocytes and lymph node cells from the immunized mice were isolated 30 days after immunization using the BDTM IMag Mouse $CD4^+$ T and $CD8^+$ T lymphocyte enrichment set-DM (BD, Biosciences Pharmingen, USA) via negative selection. The purified $CD3^+CD4^+$ or $CD3^+CD8^+$ T cells were cultured in 6-well plates (5×10^6 cell per well). Then, cells were stimulated with Tuberculin purified protein derivative (PPD) (1 μg/mL) or Rv2645 protein (1 μg/mL) at 37 °C in 5% CO2 for 24 h. Brefeldin A (BFA, 1 μg/mL), an inhibitor of intracellular protein transport, was added for 12 h to block cytokine release at 37 °C in 5% CO2 atmosphere. The cells were then fixed in 4% paraformaldehyde in PBS at room temperature (RT) for 15 min, permeabilized and stained with PE-anti-mouse IFN-γ, FITC-anti-mouse IL-17A, PerCP/Cy5.5-anti-mouse IL-2, PE-anti-mouse TNF-α, PE-anti-mouse GrB, or PE-anti-mouse perforin (eBiosence, USA). The memory $CD4^+$T cells were stained by PE-anti-mouse CD44 and PerCP/Cy5.5-anti-mouse CD62L. Treg were gated by PE-anti-mouse $FoxP3^+$ and PE-cy5-anti-mouse $CD25^+$ from purified $CD3^+CD4^+$T cells, while B10 cells were gated by APC-anti-mouse $CD19^+$ FITC-anti-mouse IL-10^+.

Dendritic cells (DC) from peripheral lymph nodes were stained with FITC-anti-mouse CD11c, PE-anti-mouse IL-10, PE-anti-mouse IL-12p40, PE-CD83, APC-CD86, PE-CD80, PE-CD40 and PerCP/Cy5.5-MHC-II (BioLegend, USA).

For analysis of lymphocyte proliferation, splenocytes from each immunized mouse were detected by measuring Ki67 expression using a FITC-Ki67 antibody (16A8, BioLegend) by FCM.

All samples were processed on an Accuri C6 flow cytometer, and results were analyzed using the Accuri C6 Flow software (BD Bioscience, Franklin Lakes, NJ).

2.6. Serum antibody measurement by ELISA

Briefly, 96-well microtiter plates were coated with PPD and Rv2645 proteins at 10 μg/ml (100 μl/well) in coating buffer (0.1 M carbonate/bicarbonate, pH 9.6) and incubated at 4 °C overnight. After washing four times with PBS containing 0.05% (v/v) Tween-20 (PBST), the plates were blocked with 200 μl/well blocking buffer (2% bovine serum albumin (BSA) in PBST) at 37 °C for 1 h. After washing, 200-fold diluted mouse eyeball blood was added and incubated at 37 °C for 1 h. The plates were thoroughly washed and then incubated with HRP-conjugated goat anti-mouse IgG1 and IgG2a antibodies at 37 °C for 30 min, followed by the addition of 100 μl/well TMB substrate. The reaction was stopped by the addition of 50 μl of 2 M H_2SO_4. The optical densities were then measured at 450 nm within 10 min with a PerkinElmer 2030 multilabel reader. All samples were tested in duplicate wells in each

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