



# Rv2626c and Rv2032 activate TH1 response and downregulate regulatory T cells in peripheral blood mononuclear cells of tuberculosis patients

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## ABSTRACT

In the present study we have assessed T cell immuno-phenotypes in BCG vaccinated healthy individuals and patients with active pulmonary tuberculosis in response to two latency associated DosR Regulon Proteins Rv2626c and Rv2032. The proteins were shortlisted based on our previous bioinformatics analysis of the 48 DosR Regulon proteins. Both the proteins were seen to increase the percentage of CD4<sup>+</sup> and CD8<sup>+</sup> memory T cells in patients. Increase in expression of transcription factor T-Bet in response to the proteins suggested that the DosR proteins could be skewing the immune response toward the immune-protective TH1 type. This was confirmed with cell culture supernatant studies for release of TH1 and TH2 cytokines IFN- $\gamma$ , IL-2, TGF- $\beta$ , IL-4 and IL-10. A significant increase in frequency of CD4<sup>+</sup>/IFN- $\gamma$ <sup>+</sup> and CD8<sup>+</sup>/IFN- $\gamma$ <sup>+</sup> T cells in patients was observed in response to both our proteins. This was accompanied with a significant downregulation in regulatory T cell population. Based on our findings of increase in TH1 response and decrease in Treg cells responsible for suppressing the immunity, we project Rv2626c and Rv2032 as antigens capable of inducing a strong immune response against *Mycobacterium tuberculosis*.

## 1. Introduction

The rise of tuberculosis (TB) due to limited efficacy of Bacillus Calmette-Guérin (BCG) vaccine, HIV co infection and multi drug resistance demands serious and immediate action [1]. Earlier the vaccine strategy against TB was aimed at development of prophylactic vaccines. Animal studies have shown that although an active response is generated, sterilizing immunity is not achieved by prophylactic vaccines [2]. These vaccines are based on early stage antigens such as ESAT6 and CFP10 which are expressed in the first stage of infection [3]. As the infection advances, there is a change in expression of genes. The early stage antigens are dramatically downregulated later on whereas the late stage antigens such as those belonging to Dormancy survival regulon (DosR) are upregulated in the stationary phase of infection [4]. Therefore, early stage antigens in prophylactic vaccines are not recognized in late stage infection especially in case of latency. Since one

third of the global population is latently infected with TB, targeting this section of individuals becomes of utmost importance. Therefore, the focus, these days, is shifting towards developing a post-exposure booster vaccine involving early as well late stage antigens that can possibly cater to a large population of affected individuals at different stages of infection. These vaccines could serve to either completely eradicate the bacteria from the system or keep it in dormant/latent form preventing its reactivation [2]. DosR Regulon is a group of 48 late-stage expressing antigens. Investigation of latency associated antigens belonging to DosR Regulon of the bacterium as promising vaccine candidates have therefore emerged as the “new kid on the block” [5–7]. In our previous studies we have shortlisted promiscuous T cell antigens of DosR Regulon [6]. Two such antigens Rv2626c, a hypothetical protein and Rv2032, a nitroreductase, were analysed bio-informatically and were found to be highly immunogenic [8]. We have, therefore, studied the kind of T cell response produced by these two proteins in

**Abbreviations:** TB, Tuberculosis; BCG, Bacillus; Calmette, Guerin; Mtb, *Mycobacterium tuberculosis*; DosR, Dormancy survival regulon; Treg, regulatory T cells; IFN $\gamma$ , Interferon gamma; TNF- $\alpha$ , Tumor necrosis factor alpha; nTreg, naturally occurring regulatory T cells; iTreg, inducible regulatory T cells; FoxP3, forkhead- box P3; CTLA-4, Cytotoxic; Tlymphocyte, associated protein 4; GITR, glucocorticoid-induced TNFR family related gene; TGF- $\beta$ , transforming growth factor beta; PBMC, Peripheral Blood Mononuclear Cell; FCS, Fetal Calf Serum

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Peripheral Blood Mononuclear Cells (PBMCs) of BCG vaccinated healthy individuals and TB patients.

Clearance of tuberculosis infection involves a fine balance between protective and suppressive immune response [9]. However, the exact mechanism of the immune responses generated still needs to be understood. On encounter with the pathogen, first the innate immune response gets activated and tries to eliminate the infection [10–12]. When the innate arm is not able to generate adequate response, adaptive immunity comes into play [13]. CD4<sup>+</sup> T cells, part of adaptive immune system, have shown a prominent role in protection against Mtb [14]. On activation with antigen, naïve CD4<sup>+</sup> T cells are seen to show polarization into multiple categories depending on the cytokines produced by them. These are broadly known as TH1, TH2 and regulatory T (Treg) cells [15–17]. Studies have largely defined TH1 response to be immune-stimulatory and TH2 response to be immune-inhibitory in nature whereas, Tregs are seen to suppress efficient protective immune responses against the pathogen. In context of Mtb, expression of Treg cells is mediated via transforming growth factor beta (TGF- $\beta$ ) signaling. During the initial stage of Mtb infection, there is proliferation of Treg cells which causes a delay in the onset of adaptive immunity. Results from studies of patients with TB and experimental models have shown that Tregs are expanded and accumulated at the site of infection. These Tregs efficiently inhibit the arrival of effector T cells in the lungs, production of IFN- $\gamma$  and  $\gamma\delta$  T-cell responses to Mtb [18].

Here we propose examination of T cell portfolio activated by these antigens to unfold the balance and the relationship between T helper subsets TH1, TH2 and Treg in order to understand the vaccine candidature of these antigens in Indian population which is a high TB-burden country.

## 2. Material and methods

### 2.1. Cloning, expression and purification of the DosR proteins

The genes encoding Rv2626c and Rv2032 proteins of DosR Regulon of Mtb were amplified from H37Rv genomic DNA. The PCR products were cloned into pGEM-T-Easy vector (Promega, USA) [19]. Genes were sub cloned into expression vector pET-28a(+)(Novagen, USA) in frame with a six N-terminal histidine tag using *Bam*HI and *Hind*III restriction enzymes [20]. The recombinant plasmids encoding these genes were transformed into *E. coli* BL21 (DE3) for protein over-expression in 1 l culture media (LB Broth). Bacterial cells were then harvested by centrifugation and suspended in 100 mM Tris-HCl (pH 8.0), 300 mM NaCl, and 1 mM phenyl methyl sulfonyl fluoride and lysed by sonication. Each recombinant protein was purified from sonication supernatant using Ni-NTA affinity chromatography and eluted with 250 mM imidazole under native conditions [21]. The purity of the prepared proteins was analyzed by SDS-PAGE and immune-blotting using anti-His antibodies (primary antibody: anti-His raised in mice (AbCam, USA), secondary antibody: peroxidase-conjugated goat anti-mouse IgG (Jackson, USA)) [22]. Purified proteins were dialyzed against PBS to remove imidazole. Purified proteins were passed through a column packed with polymyxin B-agarose beads after incubation for 1 h. at 4 °C to remove endotoxin contamination, if any. Evaluation of bacterial endotoxin was carried out using Limulus Amoebocyte Lysate assay kit (Pierce, USA). Protein concentration was estimated using BCA protein assay. SDS - PAGE and Western blot images for purified proteins are shown in supplementary figure S1.

### 2.2. Study subjects

Rv2626c and Rv2032 were used to stimulate PBMCs isolated from age and sex matched healthy subjects (n = 20) and TB patients (n = 20). The inclusion criteria were: (a) BCG vaccinated adult (18 years and above) men and women, (b) category I pulmonary TB patients. The exclusion criteria were: (a) HIV positive or suffering from

other viral or bacterial infections (b) patients with diabetes, cancer, autoimmune diseases or other conditions that may affect the immune system of the individual, (c) pregnant women, (d) children, (e) patients undergoing treatment, (f) patients suffering from multidrug-resistant TB. Samples were obtained from Rajan Babu Institute of Pulmonary Medicine and Tuberculosis Hospital, Mahatma Gandhi Marg, GTB Nager, Delhi. Informed consents were taken from all the subjects. The study was approved by the Institutional Human Ethical Committee (IHEC) of BR Ambedkar Centre for Biomedical Research, University of Delhi, Delhi and from Rajan Babu Institute of Pulmonary Medicine and Tuberculosis Hospital, Delhi according to the Declaration of Helsinki Principles.

### 2.3. Ex-vivo culture of PBMC

Peripheral blood (10 ml) was collected from all the subjects. Peripheral Blood Mononuclear Cells (PBMCs) were isolated by density gradient centrifugation on Ficoll-hypaque. Briefly, heparinized peripheral blood was layered over a histopaque cushion and centrifuged at 420xg for 30 min at 22 °C. Interface cells (PBMCs) were recovered, washed and resuspended in RPMI-1640 with 10% Fetal Calf Serum (FCS). Plated cells were stimulated with individual purified recombinant protein antigens at 1, 2, 5  $\mu$ g/ml for 24, 48 and 72 h. MTT assay (Promega, USA) was performed and viability of the cells was checked as per the manufacturer's instructions. By kinetic studies 48 h. time point for stimulation with 5 $\mu$ g/ml protein was found to be optimum.

### 2.4. Study of cell surface markers of T cells by Flow Cytometry

The un-stimulated and protein stimulated PBMC were cultured at  $1 \times 10^6$  cells/ml for 48 hs at 37 °C in 12 well culture plates supplemented with 10% FCS and antibiotics. Cells were harvested, washed in PBS supplemented with 0.5% BSA, and stained with fluorescently labeled antibodies. Antibodies used for phenotypic analysis were anti-CD4-FITC, anti-CD8-PE, anti-CD25-PE Cy7, anti- CD45RA-APC, anti-CD45RO-PE Cy7 (all antibodies were obtained from Biolegend, USA). Analysis of the flow cytometry data was performed using BD Accuri C6 software as well as *De novo* FCS express 5.0(USA) software [23].

### 2.5. Expression of transcription factors T-Bet and GATA3 by quantitative real time PCR

Expression of T-Bet and GATA3 was studied to assess the direction towards which the immune response is channelized: immunoprotective Th1 or immunosuppressive Th2. PBMCs from 10 BCG vaccinated healthy individuals, and 10 TB patients were plated. Cells were incubated at 37°C for 24 h. Stimulation was done with 5  $\mu$ g/mL of Rv2626c and Rv2032 each. After 24 h of stimulation RNA was isolated using RNA isolation kit (Qiagen, USA). DNase treatment was given using DNase kit (Sigma, USA) and cDNA was prepared using first strand cDNA synthesis kit (Sigma, USA) following manufacturer's instructions. Real time PCR was run in step-one real time PCR machine (ABI, USA). Primers were designed for T-Bet, GATA3 and housekeeping gene GAPDH. Primer sequences for the three genes are as follows:

T-Bet: FP 5'-CAAGCAGGGACGGCGGATGT-3'  
 RP 5'-TTGGACGCCCCCTTGTGTTT-3'  
 GATA3: FP 5'-CGGTCAGCACAGGCAGGGAGT-3'  
 RP 5'-GAGCCACAGGCATTGCAGACA-3'  
 GAPDH: FP 5'-AAGGGCATCTGGGCTACAC-3'  
 RP- 5'-GTCCACCACCCTGTGTGTAG-3'

Data was calculated using the  $2^{-\Delta\Delta CT}$  method and presented as fold induction. Fold change was normalized to GAPDH expression levels.

## 2.6. Flow cytometry study of intracellular IFN- $\gamma$ or Foxp3

The un-stimulated and protein stimulated PBMC were cultured at  $1 \times 10^6$  cells/ml for 48 hs at 37 °C in 12 well culture plates supplemented with 10% FCS and antibiotics. After 20 h, Brefeldin A (10 $\mu$ g/ml, Biolegend, USA) was added. After 4 h, cells were harvested and stained for cell surface markers CD4 and CD8. Cells were washed with PBS and permeabilized for 20 min with permeabilization buffer (1X, Biolegend, USA). Cells were washed with PBS and stained with antihuman-IFN- $\gamma$ -APC or cells were stained for cell surface markers CD4 and CD25 and then intracellular anti-Foxp3-PE for 30 min, washed in PBS and acquired in BD Accuri C6 software as well as De Novo FCS express 5.0 software [23].

## 2.7. Cytokine analysis of cell culture supernatant by ELISA

The cytokines IFN- $\gamma$ , IL-2, TGF- $\beta$ , IL-4, IL-10 and IL-17 were assayed in cell culture supernatants after 48 hs stimulation by a solid phase sandwich ELISA using matched antibody pairs according to the manufacturer's instructions (eBioscience, USA). Range for detection of cytokines by the respective kit is as follows: IL-2 – 2–250 pg/mL, IL-4 – 2–200 pg/mL, TGF- $\beta$  – 8–1000 pg/mL, IFN- $\gamma$  – 4–500 pg/mL, IL-10 – 2–300 pg/mL, IL-17 – 4–500 pg/mL [24].

## 2.8. Statistical analysis

The results of BCG vaccinated healthy individuals and TB patients were presented as mean  $\pm$  SD. Wilcoxon matched-pairs signed-rank test was used for comparisons between basal levels of BCG vaccinated healthy individuals and TB patients as well as between unstimulated and protein - stimulated PBMC using Graph Pad Prism software version 5.02 (San Diego, CA, USA). Correlation between the two variables was analyzed using Spearman Correlation Coefficient.

## 3. Results

### 3.1. Cloning, expression and purification of the DosR proteins

Both the proteins were successfully cloned and expressed in pET-28a (+) expression vector. Rv2626c with molecular weight of 15.1 Kda and Rv2032 with molecular weight of 38.5 Kda were purified in native conditions from supernatant of sonicated bacterial culture. 1 l culture yielded 2 mg/ml and 2.5 mg/ml protein of Rv2626c and Rv2032 respectively. Proteins were passed through polymyxin B-agarose beads to remove endotoxin contamination followed by evaluation of bacterial endotoxin using Limulus Amoebocyte Lysate assay (Pierce, USA) which was < 0.5 EU/ml for both the proteins.

### 3.2. Recognition of T cells and upregulation of CD4, CD8 memory T cell response on stimulation with recombinant Rv2626c

PBMCs from healthy BCG vaccinated individuals (n = 20) and TB patients (n = 20) were stimulated with Rv2626c and Rv2032 to study the T cell response produced by them. Percentage of CD3<sup>+</sup> T cells co-expressing CD4/CD45RA, CD4/CD45RO, CD8/CD45RA and CD8/CD45RO was recorded by flow cytometry. Gating strategy is shown in supplementary figure S2.

A significant upregulation of CD4<sup>+</sup> and CD8<sup>+</sup> T cells depicting T cell activation was observed in TB patients in response to both the proteins. However, significant upregulation of memory-associated T cells (CD4<sup>+</sup>/CD45RO<sup>+</sup>, CD8<sup>+</sup>/CD45RO<sup>+</sup>) was observed only by Rv2626c in patients. No significant change was observed in CD8<sup>+</sup>/CD45RA<sup>+</sup> T cell population in any category of subjects (Fig. 1, supplementary Table T1).

Analysis of basal levels of T cell phenotypes was conducted between BCG vaccinated healthy individuals and TB patients. Percentage of

CD3<sup>+</sup> T cells expressing CD4/CD45RA, and CD8/CD45RA was significantly lower in patients as compared to healthy individuals (Supplementary table T1).

### 3.3. Increased expression of transcription factor T-Bet in response to Rv2626c and Rv2032

A 7.91  $\pm$  3.25-fold increase in response to Rv2626c and a 4.86  $\pm$  6.38-fold increase in response to Rv2032 was observed in expression of T-Bet in patients relative to the expression of GAPDH (Fig. 2). No significant change was observed in BCG vaccinated healthy individuals in response to the two proteins.

### 3.4. Polarization of immune response towards TH1 on stimulation with Rv2626c and Rv2032

Cell culture supernatants were analyzed for TH1 and TH2 cytokines IFN- $\gamma$ , IL-2, IL-10 and IL-4, TGF- $\beta$  and IL-17. On comparison between BCG vaccinated healthy individuals and patients, it was seen that the level of IFN- $\gamma$  was significantly lower in patients as compared to BCG vaccinated individuals. TGF- $\beta$  seems significantly higher in patients which is associated with expansion of Treg cells. Upon stimulation with the proteins, these IFN- $\gamma$  levels increased significantly in TB patients as compared to BCG vaccinated healthy individuals (Fig. 3). An increase in IL-2 and decrease in IL-10 (TH2 cytokine) levels was also observed but the changes were not found to be significant. No significant changes were observed in IL-17 levels too.

### 3.5. Rv2626c and Rv2032 increase frequency of CD4<sup>+</sup>/IFN- $\gamma$ <sup>+</sup> and CD8<sup>+</sup>/IFN- $\gamma$ <sup>+</sup> T cells

PBMCs were analysed for co-expression of CD4<sup>+</sup>/IFN- $\gamma$ <sup>+</sup> and CD8<sup>+</sup>/IFN- $\gamma$ <sup>+</sup> by flow cytometry in CD3<sup>+</sup> T cells of BCG vaccinated healthy individuals and TB patients. Significant upregulation was observed in CD4<sup>+</sup>/IFN- $\gamma$ <sup>+</sup> and CD8<sup>+</sup>/IFN- $\gamma$ <sup>+</sup> T cells in response to both Rv2626c and Rv2032 in TB patients when compared with unstimulated cells (Fig. 4, supplementary table T2). However, no change was observed after 48 hs of stimulation. Gating strategy is shown in supplementary figure S2.

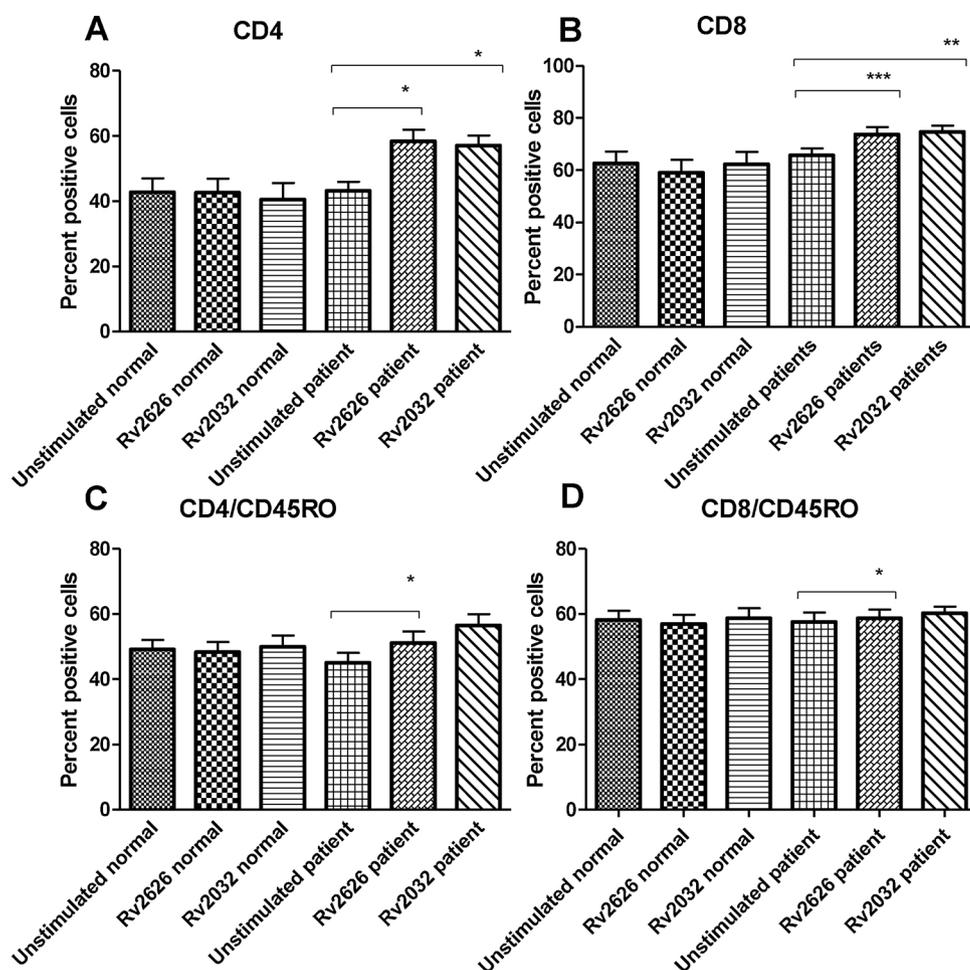
### 3.6. DosR Regulon proteins down regulate the immune suppressive Treg cells (CD4<sup>+</sup>/CD25<sup>+</sup>/FoxP3<sup>+</sup>)

Population of regulatory T cells was much higher in patients as compared to BCG vaccinated healthy individuals. This Treg cell population was seen to decrease significantly on stimulation with Rv2626c and Rv2032 (Fig. 5A), Supplementary Table T1. Gating strategy is shown in supplementary figure S2.

Ratio of Treg cells and T effector cells (CD4<sup>+</sup>/CD25<sup>+</sup>) was plotted for BCG vaccinated healthy individuals and patients. The ratio was seen to be higher in patients than in BCG vaccinated healthy individuals. Both DosR proteins were seen to lower the ratio significantly in patients (Fig. 5B).

## 4. Discussion

The alarming situation posed by global TB threat, more so in high TB burden countries has triggered a wave of studies in the lookout for new potential vaccine candidates [25]. A large proportion of population is latently infected with Mtb and has the highest risk of developing and transmitting the disease. Mtb, "the smart pathogen" persists among humans without its presence being felt for a prolonged duration. It is believed that there is an alteration in the repertoire of antigens that may be recognized by T cells in later stages of infection in which the bacteria are dormant [4]. Therefore, the widely accepted vaccine development strategy involves incorporation of both early as well as late

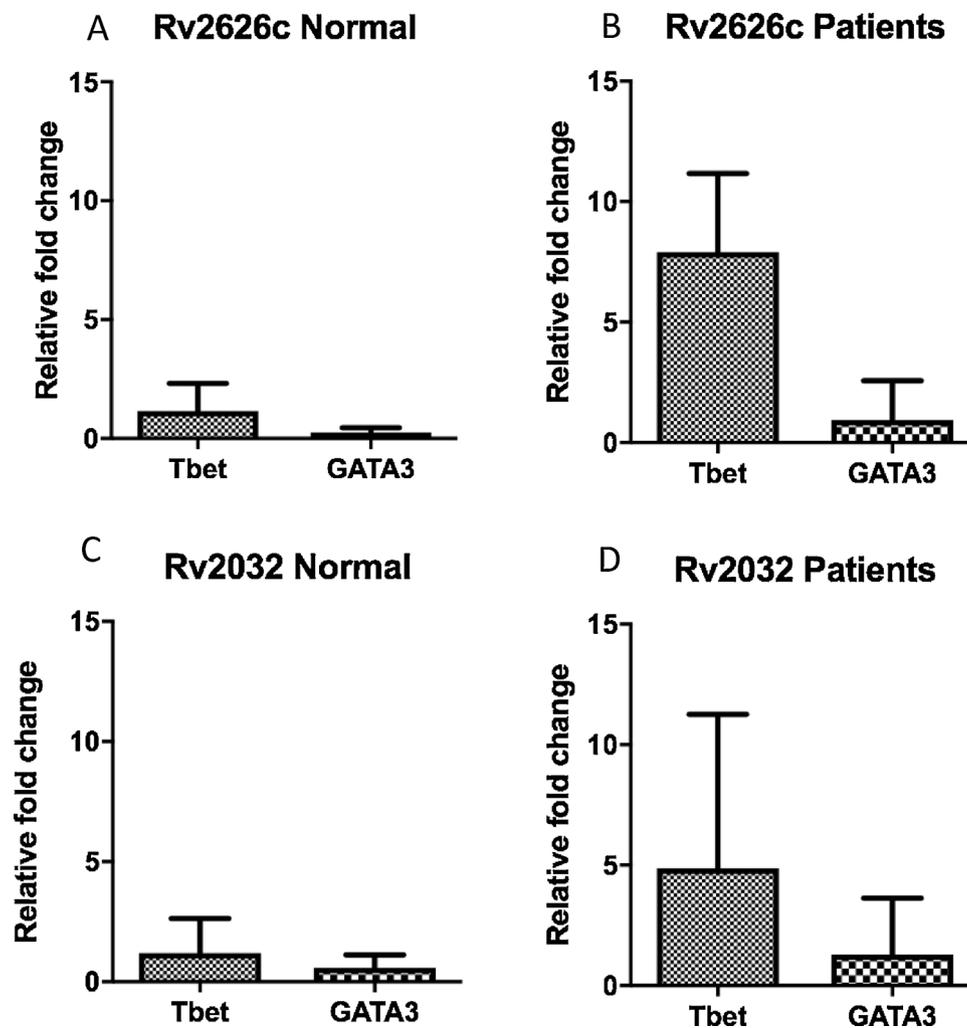


**Fig. 1.** CD4/CD8 T cell in response to Rv2626c and Rv2032 in BCG vaccinated healthy individuals and TB patients. Cell surface expression of (A) CD4, (B) CD8, (C) CD4/CD45RO, (D) CD8/CD45RO were determined as percentage of cells by Flow Cytometry BCG vaccinated healthy individuals (N = 20) and TB patients (N = 20). When compared with unstimulated PBMCs (gated CD3<sup>+</sup> T cells), expression of CD4 and CD8, was significantly upregulated in response to Rv2626c and Rv2032, while CD4/CD45RO was upregulated only in response to Rv2626c in TB patients. In both the category of subjects statistical analysis was done on CD3<sup>+</sup> T cells between unstimulated and protein-stimulated samples with the non-parametric Wilcoxon matched-pairs signed-rank test. Results are represented as mean  $\pm$  SD. \*P < 0.05, \*\*P < 0.01 and \*\*\*P < 0.001.

stage antigens of Mtb to combat the bacteria in different stages of infection. A group of 48 latency associated genes of DosR Regulon which become upregulated in Mtb exposed individuals emerge as a promising pool of T cell antigens [26]. In our literature and bioinformatics analysis we identified sixteen DosR Regulon proteins as potential T cells-stimulating antigens [6]. We further pursued two of these proteins namely, Rv2626c and Rv2032 and studied the kind of immune response generated by them in human PBMCs. The immunogenic potential of the two proteins was compared in healthy BCG vaccinated individuals and BCG vaccinated TB patients. Our MTT assay demonstrated that there was a considerable proliferation in CD4<sup>+</sup> and CD8<sup>+</sup> T cells in TB patients in response to the proteins which could be one of the most important reasons of an increase in immunity. When naïve T cells are stimulated by antigens, they undergo proliferation and differentiation into effector T cells [27] which was evidenced by an increase in the expression of CD4<sup>+</sup>/CD45RA<sup>+</sup> T cells in patients in response to the proteins. This upregulation ascertained that the T cells were activated to acquire effector functions in response to both the proteins. Further in the course of T cell activation, T cells lose the expression of CD45RA and start expressing CD45RO isoform, which is a marker of memory T cells [28–30]. Memory response is the most crucial factor in development of vaccines. The currently available BCG vaccine elicits CD4 and CD8 mediated T cell response but the effect is not long lasting. Therefore, various studies aim at boosting BCG with post exposure vaccines that can generate an effective memory response. In studies performed by Marin et al., it is reported that frequency of memory T cells is reduced in patients with active TB [31]. In our study also we have also observed a low frequency of memory T cells in patients. But, interestingly on stimulation with Rv2626c and Rv2032, we observed a significant increase in memory T cells (CD4<sup>+</sup>/CD45RO<sup>+</sup> and CD8<sup>+</sup>/

CD45RO<sup>+</sup>).

The milieu and balance between TH1 and TH2 cytokines which is mediated by T-Bet and GATA3 transcription factors respectively, govern the control and eradication of mycobacteria. Therefore, expression levels of T-Bet and GATA3 were monitored to ascertain the direction towards which the immune response moves - either immunoprotective TH1 or immunoregulatory TH2. PBMC stimulation with Rv2626c and Rv2032 led to increased expression of T-Bet in patients. This result was further substantiated by TH1 and TH2 cytokine analysis by ELISA which showed release of significant amount of TH1 cytokine IFN- $\gamma$  in patients in response to the proteins. The protective role of IFN- $\gamma$  producing T cells is well established. Gene knockout studies have clearly demonstrated that lack of IFN- $\gamma$  leads to a higher risk and lower clearance of infection [32]. A remarkable increase in TH1 cytokines (IFN- $\gamma$  and IL-2) and down regulation of TH2 cytokines (IL-4 and IL-10) showed polarization of immune response towards a protective TH1 type in response to Rv2626c and Rv2032. Recent studies have shown that although IFN- $\gamma$  may be produced by various cells, it is the IFN- $\gamma$  producing CD4<sup>+</sup> T cells that play a prominent role in optimal protection. These CD4<sup>+</sup> T cells also supplement IFN- $\gamma$  production from CD8<sup>+</sup> T cells and therefore helps in bringing about immune response from both CD4 as well as CD8 [33]. In corroboration of these findings, we observed a significant increase in IFN- $\gamma$  release by PBMCs of patients on stimulation with the two DosR proteins which might have been the cause of a significant increase in the percentage of CD4<sup>+</sup> and CD8<sup>+</sup> T cells. This fact is further highlighted by the significant increase in both CD4<sup>+</sup>/IFN- $\gamma$ <sup>+</sup> and CD8<sup>+</sup>/IFN- $\gamma$ <sup>+</sup> T cells in response to the two proteins in the present study. Our findings support the fact that not just CD4<sup>+</sup> T cells but CD8<sup>+</sup> T cells are also significant contributors to immunity against TB [34] as we observed a significant increase in CD8<sup>+</sup>/



**Fig. 2.** Increase in T-Bet expression in *ex-vivo* PBMC culture of BCG vaccinated healthy individuals and TB patients on stimulation with Rv2626c and Rv2032. Expression levels of T-Bet and GATA-3 (A) in BCG vaccinated healthy individuals (n = 10) in response to Rv2626c. (B) in TB patients (n = 10) in response to Rv2626c. (C) in BCG vaccinated healthy individuals (n = 10) in response to Rv2032. (D) in TB patients (n = 10) in response to Rv2032. T-Bet expression was increased in patients in response to both Rv2626c and Rv2032.

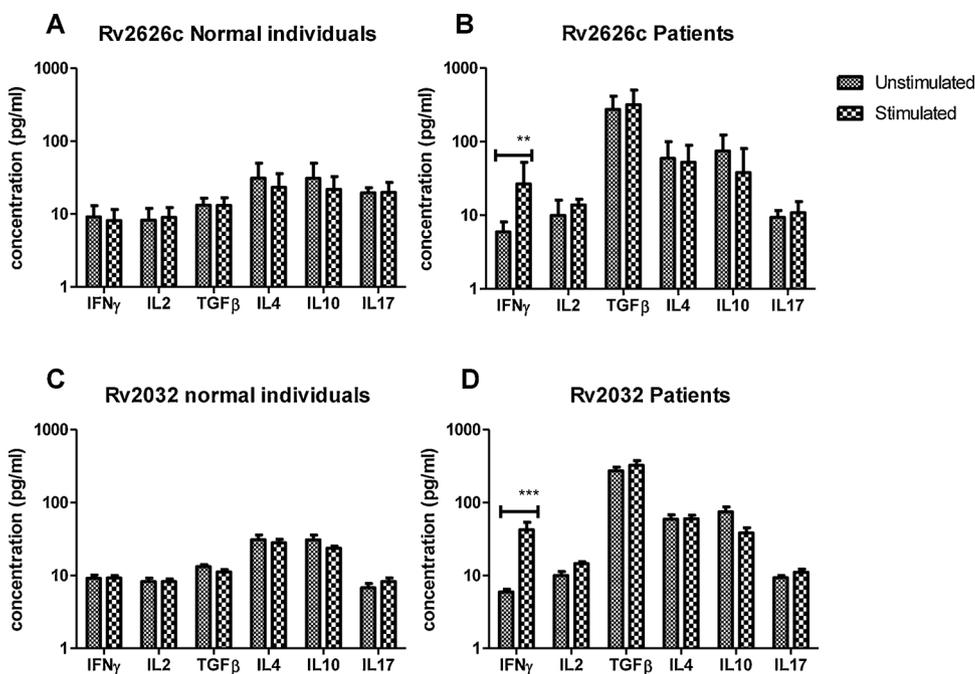
CD45RO<sup>+</sup> T cells as well as CD8<sup>+</sup>/IFN- $\gamma$ <sup>+</sup> T cells in response to the DosR proteins. As IFN- $\gamma$  is the key player in control of Mtb and its production by PBMCs shows a direct relationship with the clinical manifestations of tuberculosis [35], our findings strongly suggest that Rv2626c and Rv2032 can play a protective role against TB infection.

As known from literature, regulatory T cells formerly known as suppressor T cells, suppress or downregulate induction and proliferation of effector T cells [36]. Since Treg cells are known to compromise protective immunity and are associated with pathogen persistence, it is believed that a good vaccine candidate should suppress the Treg cell population [37,38]. It is also reported that Treg cells limit vaccine immunogenicity [39]. They are seen to dampen the protective efficacy of TH1 cytokines [40] and their depletion is associated with improved CD4<sup>+</sup>T cell response [41]; therefore, the down regulation of Treg cells could play a promising role in the discovery of new vaccine candidates against TB. This strategy of targeting Treg cells along with stimulating a protective response in the form of IFN- $\gamma$  release was also used in MVA85A vaccine [42]. The vaccine showed promising results in animal studies therefore, a lookout for more such antigens becomes all the more important. Also since there is no gold standard assay for detection of latent infection, identification of such antigens may prove to be a major breakthrough in classifying individuals into a high risk category, especially the latently infected individuals in a high TB-burden country. In corroboration of these facts, we observed that the frequency of Treg

cells was high in TB patients which on stimulation with our proteins, decreased significantly. Treg depletion has previously been related to increase in IFN- $\gamma$  synthesis [43]. Our study is in agreement with this fact as we have observed a negative correlation between Tregs and IFN- $\gamma$ .

Differentiation of CD4<sup>+</sup>/CD25<sup>+</sup> T cells into Treg cells is critically dependent on the cytokine milieu of IL-10 and TGF- $\beta$  [44,45]. In turn, Treg cells also secrete TGF- $\beta$  and IL-10 which further program CD4<sup>+</sup> T cells to form Treg cells that mediate suppression of immune response [46]. Previous studies and our own observations indicate that there is a negative correlation between IFN- $\gamma$  and Treg cells and a positive correlation of immunosuppressive cytokines IL-10 and TGF- $\beta$  with Treg cells. Thus, controlled levels of TGF- $\beta$  and IL-10 in response to our proteins might be the reason of accentuated down-regulation of Tregs in TB patients. It can be inferred from this data that these proteins are able to down-modulate the immune suppressive Treg cells and therefore become very important candidates for post-exposure vaccine development studies. Also, the ratio of Treg and effector T cells (CD4<sup>+</sup>/CD25<sup>+</sup>) was also seen to decrease thereby depicting the skewing of immune response towards TH1 type. The tilt towards T effector cells could be due to a possible conversion of Treg cells into T effector cells which has also been reported in previous studies [47].

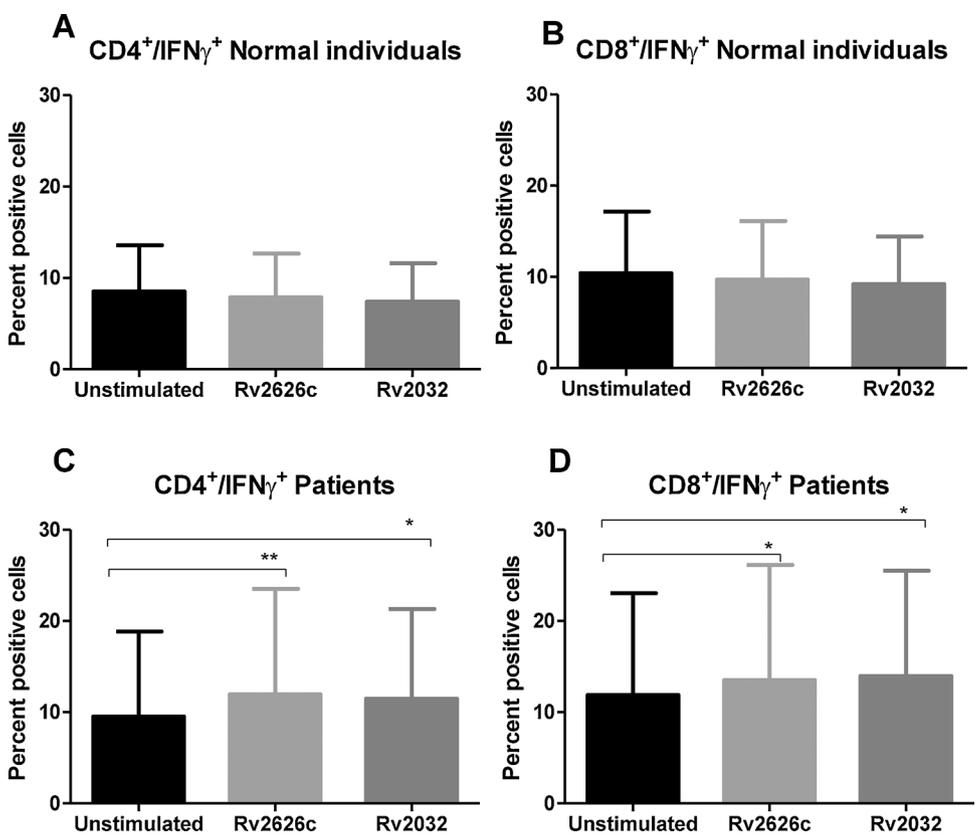
Studies have determined that DosR Regulon antigens are recognized in latently infected individuals in different geographical regions such as



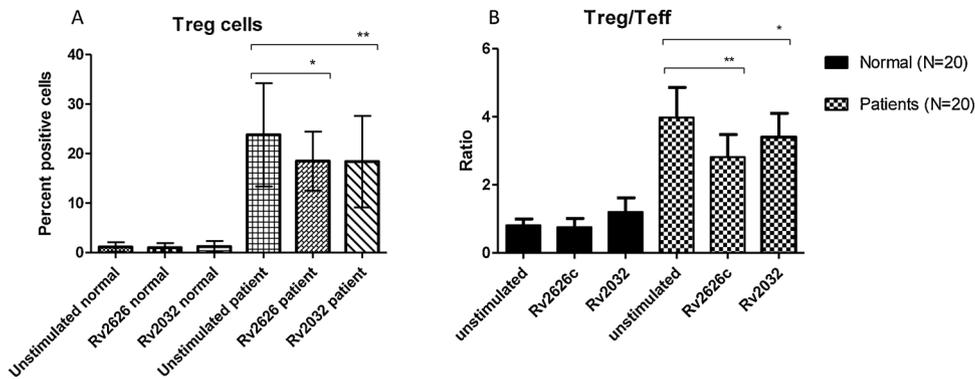
**Fig. 3.** Cytokine profile in cell culture supernatant of PBMCs of (A) in BCG vaccinated healthy individuals in response to Rv2626c. (B) in TB patients in response to Rv2626c. (C) in BCG vaccinated healthy individuals in response to Rv2032. (D) in TB patients in response to Rv2032. Cell culture supernatant was collected after 48 h. stimulation with the proteins. Sandwich ELISA was done for IFN- $\gamma$ , IL-2, IL-10, IL-4, TGF- $\beta$  and IL-17. Rv2626c and Rv2032 showed significant upregulation in IFN- $\gamma$  and a non-significant downregulation in IL-10 depicting a protective response in TB patients. Data of PBMC is representative of twenty independent donors in each category. In each category of subjects statistical analysis was done on CD3<sup>+</sup> T cells between unstimulated and protein-stimulated samples with the non-parametric Wilcoxon matched-pairs signed-rank test. Results are represented as mean  $\pm$  SD. \*P < 0.05, \*\*P < 0.01 and \*\*\*P < 0.001.

Netherlands, Italy, Germany, Japan, Ethiopia, Gambia and Uganda [48]. We analyzed the immunological profile of CD3<sup>+</sup> T cell in north Indian population for the first time and observed that TB patients strongly recognized the two DosR Regulon proteins. We have shown that the two latency associated proteins Rv2626c and Rv2032 produced strong *in-silico* evidences of protective and immunogenic response. In our studies on human PBMCs, these proteins were seen to trigger a four-way beneficial response against TB: (a) by increasing the frequency of memory-associated T cells (CD4<sup>+</sup>/CD45RO<sup>+</sup> and CD8<sup>+</sup>/CD45RO<sup>+</sup>),

(b) by increasing CD4<sup>+</sup>/CD8<sup>+</sup> mediated IFN- $\gamma$  release which is known to be associated with protection, (c) by augmentation of expression of transcription factor T-Bet which is the master regulator of Th1 cytokines followed by upregulation of Th1 cytokines IFN- $\gamma$  and IL-2 and down regulation of Th2 cytokines IL-10 and TGF- $\beta$  and most importantly (d) by down modulation of Treg cells which are associated with immune-suppression. It is known that 41 out of the 48 DosR Regulon genes in BCG show 97% similarity with those of Mtb [49]. As reported in previous studies, despite being highly similar, BCG does not



**Fig. 4.** Intracellular estimation of (A) CD4<sup>+</sup>/IFN  $\gamma$ <sup>+</sup> T cells of BCG vaccinated healthy individuals, (B) CD8<sup>+</sup>/IFN  $\gamma$ <sup>+</sup> T cells of BCG vaccinated healthy individuals, (C) CD4<sup>+</sup>/IFN  $\gamma$ <sup>+</sup> T cells of TB patients and (D) CD8<sup>+</sup>/IFN  $\gamma$ <sup>+</sup> T cells of TB patients. PBMCs (gated CD3<sup>+</sup> T cells) were stimulated for 48 hs. Flow cytometry was done for CD4<sup>+</sup>/IFN $\gamma$ <sup>+</sup> and CD8<sup>+</sup>/IFN $\gamma$ <sup>+</sup> T cells. In each category of subjects (n = 20 each), statistical analysis was done on CD3<sup>+</sup> T cells between unstimulated and protein-stimulated gated CD3<sup>+</sup> T cells samples with the non-parametric Wilcoxon matched-pairs signed-rank test. Results are represented as mean  $\pm$  SD. \*P < 0.05, \*\*P < 0.01 and \*\*\*P < 0.001.



**Fig. 5.** (A): Expression of Treg cells on stimulation with Rv2626 and Rv2032. Co-expression of CD4<sup>+</sup>/CD25<sup>+</sup>/FoxP3<sup>+</sup> (Treg cells) shows no significant change in expression of Treg cells in PBMCs (gated CD3<sup>+</sup> T cells) of BCG vaccinated healthy individuals (N = 20) in response to Rv2626c and Rv2032 when compared with unstimulated PBMCs. Treg cell population was significantly downregulated in TB patients (N = 20) in response to Rv2626c and Rv2032 when compared with unstimulated PBMCs. (B) Ratio of Treg and T effector cells in BCG vaccinated healthy and TB patients on stimulation with Rv2626c and Rv2032. Ratio was significantly lowered in TB patients in re-

sponse to both the proteins.

In each category of subjects statistical analysis was done on CD3<sup>+</sup> T cells between unstimulated and protein-stimulated samples with the non-parametric Wilcoxon matched-pairs signed-rank test. Results are represented as mean  $\pm$  SD. \*P < 0.05, \*\*P < 0.01 and \*\*\*P < 0.001.

recognize DosR Regulon antigens which is evidenced in our study also as BCG vaccinated healthy individuals failed to induce any significant response against Rv2626c and Rv2032 [49–52]. A plausible explanation which is also widely accepted is that BCG which is administered through the vaccine does not enter the state of latency so incorporation of DosR Regulon proteins in BCG vaccine could provide a better protection as also seen in studies conducted on mice with rBCG expressing DosR Regulon antigen [49–52]. The other fourteen potential vaccine candidates identified by us may also be tested similarly for their T cell-based immunogenicity [6].

### Conflict of interest

The authors have no competing interests to declare.

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### Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.cimid.2018.11.016>.

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