

# Evaluation and Identification of *in vitro* Cellular Immune Response to Culture Filtrate Antigens of *M. tuberculosis* Culture. Implications for Vaccine Design

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## Abstract

In the current study, fractions of culture filtrate proteins isolated at different time periods from *M. tuberculosis* sputum culture were evaluated for T cell activity (ADA, IFN- $\gamma$ , TNF- $\alpha$ , & IL-12) using *in vitro* PBMC model. These isolated fractions were later on partially characterized by antibody detection assay using panel of six *M. tuberculosis* H37Rv antigens (Ag85B, ESAT-6, CFP-10, GroES, 45 KD, and Hsp 16). Our results suggested us that PBMCs induced with culture filtrate proteins particularly those secreted towards later phase of growth curve of *M. tuberculosis* culture have good potential T cell activity as compared to BCG vaccine. On partial characterization we found levels of all secretory antigens increased towards later phase fractions (fraction C). Moreover on further evaluating individual purified *M. tuberculosis* H37Rv antigens for T cell activity, we found that PBMCs induced with these antigens induces good T-cell response but not as consistent as fraction C. Thus to conclude, Culture Filtrate Proteins of *M. tuberculosis* sputum culture are important T-cell targets, and thus potential of such culture filtrate proteins may be further explored in near future for development of effective vaccination strategies for improving efficacy of currently available TB vaccine.

**Keywords:** Culture Filtrate Proteins (CFP); *M. tuberculosis*; PBMC model; BCG vaccine

## Background

Tuberculosis (TB) caused by the intracellular bacterium *Mycobacterium tuberculosis* (MTB) remains a major worldwide health problem responsible for approximately three million deaths annually [1]. The level of protection conferred by only available TB vaccine, Bacillus Calmette Guerin (BCG) is very variable and differs according to the form of pulmonary TB [2]. For more than 80 years, no new TB vaccine has successfully been developed [3]. With TB eradication on the horizon, new vaccines with better protection rates than BCG or improvement in current immunization program is urgently needed. Vaccine candidates currently in clinical trials include improved recombinant BCG vaccines, virus-based recombinant vaccines, and subunit vaccines comprised of dominant secreted antigens [4].

Secreted proteins, regularly described as culture filtrate proteins (CFP's), are the main targets of the T-cell response in TB [5]. In recent years, research has been focused on antigens released by live MTB in culture medium; pools of such extracellular antigens have been tested in several laboratories as subunit vaccines with substantial levels of protection in animal models [6]. The demonstration that non-living vaccines based on secreted proteins could effectively protect against subsequent MTB infection in animal models, has led to the initiation of extensive antigen discovery programs which aimed to identify crucial antigenic molecules in culture filtrates [2]. Recent data demonstrates that some antigens are expressed in culture filtrates of MTB but absent in *Mycobacterium bovis* BCG and most environmental mycobacterial species investigated [7]. These findings have thus increased our interest in these molecules both as potential vaccine candidate and a novel specific diagnostic reagent.

Despite its widespread use, BCG has failed to reduce global burden of TB, hence newer vaccination strategies or focus on new molecules alternate to BCG are needed [8]. In our study, culture filtrates of MTB bacilli isolated from sputum samples were used to evaluate T-cell

activity. Purpose of this was to identify and evaluate T-cell profile of different antigens that may be present in MTB sputum culture. In our earlier studies, we showed effectiveness of heterologous prime boost regimes by boosting with Ag85B molecule [Husain et al.2011, unpublished data], Ag 85 B is major secretory protein present in culture filtrate; likewise large pools of secretory antigens are present in culture filtrate, these antigens play an important role in MTB growth and metabolism, thus our present study focused in exploring vaccine potential of these secretory antigens.

A number of research groups have identified vaccine potential of CFP's. The ESAT-6 antigen purified from strongly stimulatory, low-molecular-mass fraction of culture filtrate has attracted considerable interest in recent years, as it is recognized early during infection in several species, including mice [9]. In addition, secreted antigens such as the Ag85A or 85B antigens, and Mtb72F have proven to be promising candidates for BCG-boosting vaccines in mice, guinea pigs, and nonhuman primates [5]. Another study done by Lindblad et al. showed that immunization with culture filtrate antigens in the presence of different adjuvant provided protection in mice challenged with MTB, and protection was mediated by gamma interferon (IFN)-producing CD4 cells [10].

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In order to reduce the immense burden of TB in developing countries, improved vaccination strategies are urgently needed. Secretary antigens of MTB represent an important target area for vaccine research. By exploring vaccine potential of these secretary antigens, we can make amendments in area of vaccine research. The objective of our present study was to identify and evaluate vaccine potential of secretary antigens isolated at different time periods from MTB sputum culture using *in vitro* PBMC model. In this study, we have also tried to partially characterize the CFP's of MTB sputum culture using an antibody detection assay protocol in order to identify the phase wise antigenic population secreted in MTB sputum culture and also to evaluate T cell activity of individual purified antigens so as to get an overview of ability of individual purified antigens to stimulate T-cell response.

## Materials and Methods

### Bacterial strains and culturing

MTB bacilli isolated from sputum samples were grown in Middle brook 7H9 liquid medium along with OADC enrichment and antibiotic supplements in BAC/T culture bottles (Biomerieux, france) and incubated at 37°C in *BacT/Alert system* ( Biomerieux,france) for 30 days.

### Collection and isolation culture filtrate antigens

Fractions were collected at different time points (3, 6, 10, 14, 21 & 28 days) from growth phases of MTB culture. For isolation of culture filtrate antigens, fractions were centrifuged (12,000 rpm for 15 min) and supernatants collected were stored at 4°C until use. At the time of experiments, culture filtrate antigens isolated from MTB culture were pooled to obtained following fractions:

Fraction A (3-6 day)

Fraction B (10-14 day)

Fraction C (21-28 days)

**Ethical committee approval:** Blood collection was done from healthy individuals, seronegative for PPD, HIV, and HBV for PBMC isolation. The protocols for this study were approved by ethical committee of Central India Institute of Medical sciences and in accordance with the NIH guidelines (NIH publication No. 80-23; revised 1978).

### Peripheral blood mononuclear cells (PBMC) isolation and culturing

For separation of PBMCs, 5 ml venous blood was obtained from a healthy BCG vaccinated, PPD negative individuals in sterile EDTA vacutainer tubes. PBMC cells were extracted from whole blood using a density gradient Ficoll Histopaque method. After isolation; PBMCs were cultured keeping the concentration at  $2 \times 10^5$  cells/well. PBMCs were stimulated with purified protein derivative (PPD) (25 µg/ml) (Span diagnostics). For evaluation of T-cell activity of different culture filtrate antigens from MTB sputum culture, study was divided into following phases: In phase I cultured PBMCs were induced with 100 µl of different phase fractions of culture filtrate protein (fraction A, B, C) from MTB sputum culture and also separately by BCG vaccine. BCG vaccine was taken as a positive control. Cells without induction were taken as control. Cells were incubated at 37°C in CO<sub>2</sub> incubator. Cell supernatants were collected at different time points (0, 24, 48, 72 hrs) for estimation of T-cell markers, Adenosine Deaminase (ADA),

IFN-γ, IL-12, TNF-α. In phase II, cell supernatants of phase I were characterized by antibody assay against panel of MTB antigens using ELISA protocol. In Phase III, purified antigens were used for assessment of T-cell activity. Procedure used was similar as used in Phase I except in place of culture fraction, purified antigens were used for induction.

### Evaluation of T-cell Activity

**Adenosine Deaminase (ADA):** ADA activity in the supernatant (Phase I &III) was determined at 37°C according to the method of Guisti and Galanti based on the Berthlot reaction, which is the formation of colored indophenol complex from ammonia liberated from adenosine and quantified spectrophotometrically (U.V. Visible spectrophotometer, Systronic-Model). One unit of ADA is defined as the amount of enzyme required to release one mmol of ammonia/min from adenosine in standard assay conditions. Results were expressed as units per liter per minute (U/L/min).

### Cytokines estimation

Cytokines (IFN-γ, IL-12, TNF-α) in Phase I & III were measured by an Enzyme Linked Immunosorbant Assay (ELISA) according to the manufacturer's instructions (Bender Med System, Austria). In brief, anti (IFN-γ, IL-12, TNF-α) monoclonal coating antibody was adsorbed onto microwells. After two hours of incubation at room temperature, the wells were washed and blocked with 0.5% BSA in Phosphate Bufferd Saline (PBS). After one hour of incubation at room temperature, cell supernatant followed by biotin-conjugated anti-cytokine antibody (IFN-γ, IL-12, TNF-α) was added to the coated wells. After another two hours of incubation, streptavidin–HRP (horseradish peroxidase) was added to the wells. After one hour of incubation, streptavidin–HRP was removed by washing and substrate solution reactive with HRP was added to the wells. A colored product was formed in proportion to the amount of cytokine present in the sample. The reaction was terminated by the addition of 4 N sulphuric acid and the absorbance was measured at 450 nm.

Characterization of culture filtrate antigens in cell culture supernatants using Antibody detection assay using panel of six MTB antigens by ELISA Protocol.

### Procedure

Characterization of Antigens in cell culture supernatants (Phase II) were done using antibody detection assay using panel of six MTB H<sub>37</sub>R<sub>v</sub> antigens (Ag 85B, 45kDa, GroES, Hsp 16, CFP-10 and ESAT-6) by ELISA protocol. Briefly 100 µl of panel of antigens (Ag 85B, 45kDa, GroES, Hsp 16, CFP-10 and ESAT-6) were coated to the eight separate micro titer wells. After overnight incubation, plates were blocked with 0.5% BSA in phosphate buffered saline (PBS-T) for two hrs. After blocking, wells were washed with PBS-T thrice and were kept overnight at 4°C till further analysis. At the day of experiment, 100 µl of cell culture supernatants (1:400 dilutions in PBS-T) were added and incubated for 45 min at 37°C. The wells were washed, followed by addition of the secondary antibody (goat anti human IgG–HRP 1:10,000) and were incubated for 45 min at 37°C. For color development, 100 µl of TMB/H<sub>2</sub>O<sub>2</sub> substrate solution was added to the wells and incubated at room temperature for about 10 min. The reaction was stopped using 100 µl of 2.5N H<sub>2</sub>SO<sub>4</sub> and absorbance of each well was read at 450 nm.

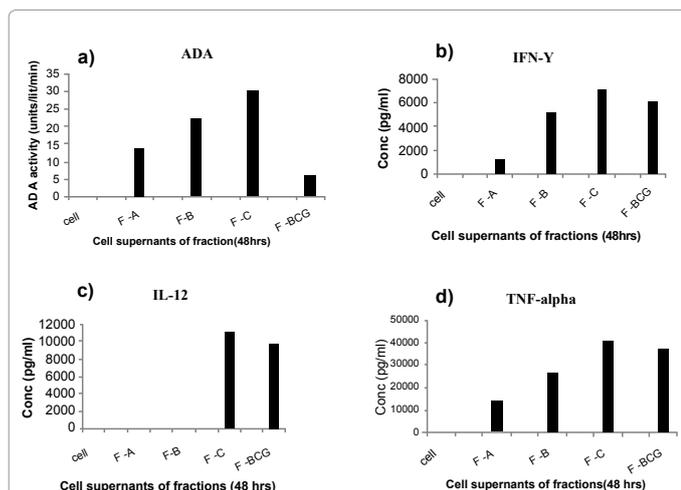
### Statistical Analysis

Data are expressed as mean ± standard deviation (SD). Comparison of t-test was used for obtaining statistical significance. P value < 0.05 was considered statistically significant.

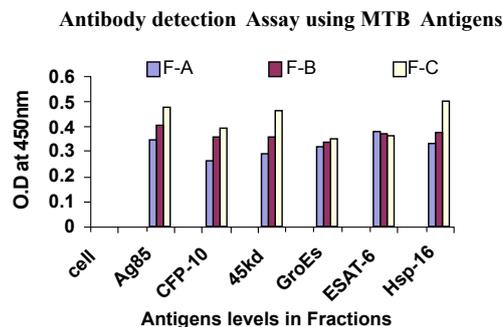
## Results

Our current study focused on testing vaccine potential of CFPs isolated from different phases of MTB sputum culture using *in vitro* PBMC model. To achieve our objective, we divided our study in three different phases. In Phase I, collected PBMCs were isolated and induced with different fractions of MTB CFPs and BCG vaccine. BCG vaccine was used as a positive control in order to compare T-cell activity of MTB CFPs with that of BCG. Cell supernatants were collected at different time points to study markers of T-cell activity. Figure 1a shows T cell activity-ADA, in 48 hrs cell supernatants of PBMC's induced with culture filtrate fractions A, B, C and BCG vaccine. Cell culture supernatants induced with different culture filtrate fractions showed good ADA activity, which was statistically significant as compared BCG vaccine. Among fractions, PBMCs induced with Fractions C showed better ADA activity, which was found to be highly significant ( $p=0.0001$ ) as compared to BCG. These results showed that the culture filtrate fraction, especially fraction C may have ability to stimulate T-cell better than BCG vaccine which showed poor ADA levels. Similarly after cytokine analysis we found PBMCs induced with fraction C induced good IFN- $\gamma$  response as compared to BCG vaccine ( $p=0.005$ ) (Figure 1b). Cell supernatant of Fractions C also showed good IL-12 levels ( $p=0.007$ ) as compared to BCG; however no IL-12 activity was reported in fraction A and B (Figure 1c). Cell supernatants of all culture filtrate fractions showed TNF- $\alpha$  response, with increasing activity from fractions A to C (Figure 1d). Fraction C showed TNF- $\alpha$  response better than BCG vaccine ( $p=0.005$ ) and fraction A and B. Thus phase I results showed us that among culture fractions, PBMCs induced with fraction C showed better T cell response.

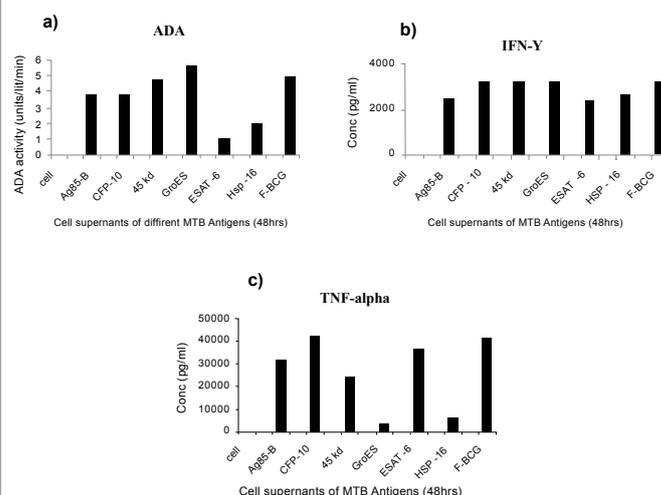
Apart from testing T-cell potential of culture filtrates of MTB culture in Phase I, this study also focused on partial identification and characterization of CFPs by antibody detection assay using panel of six MTB  $H_{37}R_V$  antigens (Ag 85B, 45 kDa, GroES, Hsp16, CFP-10 and



**Figure 1: T cell activity in culture filtrate fractions.** Figure 1 shows T cell activity a) ADA b) IFN- $\gamma$  c) IL-12 d) TNF-alpha in 48 hrs cell supernatants of short term PBMC induced with different phase MTB sputum culture filtrate fractions, A (3-6 day), B (10-14 day), C (21-28 day) (F-A, F-B, F-C) and BCG vaccine (F-BCG). Cell represents control without any induction. BCG vaccine is used as positive control to compare its T-cell activity with that of different culture filtrate fractions with that of MTB sputum culture. Each bar in the figure represents mean values of triplicates. P value <0.05 was considered statistically significant (\* significant & \*\* highly significant values). (Note: we observed T-cell activity in 48 hrs culture supernatants; hence only activity in 48 hrs is reported in figure)



**Figure 2: Antibody detection assay using selected panel of MTB  $H_{37}R_V$  antigens.** Figure 2 shows Antibody detection assay using selected panel of MTB  $H_{37}R_V$  antigens (Ag85, CFP-10, 45kd, GroEs, ESAT-6, Hsp-16) in 72 hour cell culture supernatants of short term cultures of PBMC induced with different phase MTB sputum culture filtrate fractions, A (3-6 day), B (10-14 day), C (21-28 day) (F-A, F-B, F-C). Each bar represents antibody titre against selected respective antigens in different culture filtrate fraction A, B, C (shown in different colors).



**Figure 3: T cell activity in purified MTB  $H_{37}R_V$  antigens of MTB.** Figure 3 shows T cell activity a) ADA b) IFN- $\gamma$  c) TNF-alpha in 48 hrs cell supernatants of short term PBMC induced with purified MTB  $H_{37}R_V$  antigens (Ag85, CFP-10, 45kd, GroEs, ESAT-6, Hsp-16) and BCG vaccine (F-BCG). Cell represents control without any induction. BCG vaccine is used as positive control to compare its T-cell activity with that of selected panel of MTB  $H_{37}R_V$  antigens (Ag85, CFP-10, 45kd, GroEs, ESAT-6, Hsp-16). Each bar in the figure represents mean values of triplicates. P value <0.05 was considered statistically significant (\* significant & \*\* highly significant values). (Note: we observed T-cell activity in 48 hrs culture supernatants; hence only activity in 48 hrs is reported in figure)

ESAT-6) in Phase II. Figure 2 shows antibody levels of six MTB  $H_{37}R_V$  antigens in 72 hrs culture supernatants of culture filtrate fractions, A, B & C against panel of six MTB antigens. Antibody levels against respective panel of MTB antigens was found in all culture filtrate fractions of MTB culture, with increasing titre levels from fraction A to C. Fraction C showed elevated antibody levels against all six MTB  $H_{37}R_V$  antigens, which indirectly showed us that levels of these six MTB antigens may be elevated in fraction C, and also indicated us that levels of these six secretory antigens increases with growth phase of MTB culture.

After partial characterization of culture fractions, in third phase we

used commercially available purified MTB H<sub>37</sub>R<sub>v</sub> antigens, (used earlier in characterization phase) for determining T-cell activity. Purpose was to check T-cell activity of individual purified antigens, with that of BCG vaccine and also to compare its activity with previously evaluated different phase MTB CFPs. Figure 3 shows a) ADA b) IFN- $\gamma$  c) IL-12 d) TNF- $\alpha$  in 48 hrs cell culture supernatants of individual MTB H<sub>37</sub>R<sub>v</sub> antigens (Ag 85B, 45 kDa, GroES, Hsp16, CFP-10 and ESAT-6). All antigens showed ADA activity (except ESAT-6 and Hsp-16) comparable to BCG vaccine. GroES showed was found to give ADA activity better than BCG but was not statistically significant (Figure 3a). Similarly we found that all MTB antigens induced good levels of IFN- $\gamma$  and TNF- $\alpha$  but again results obtained were not statistically significant when compared to BCG vaccine (Figure 3b). We also checked IL12 levels, but to our surprise we didn't find any of the IL-12 activity in any of antigenic fractions except BCG (data not shown). Result of these phase showed us that individual antigens may also have the potential to stimulate T-cell activity and produce protective cytokines. However pattern of secretion of protective cytokine was not as consistent and significant as observed in later phase Culture filtrate fraction (Fraction C).

## Discussion

Despite its widespread use in newborn babies, BCG does not prevent adult pulmonary disease satisfactorily and therefore has not reduced the global burden of TB [11]. A great deal of research has been directed to either replace BCG or improve its immunogenicity using approaches. Fact that BCG provides good protection rates against childhood forms of TB, it can be postulated that BCG may be immunogenic to protect against childhood TB but not sufficient enough for protection against pulmonary disease. Another fact that cannot be ruled out is that for more than 80 years, not a single molecule has been developed which can replace BCG vaccine for immunization against TB [12], however immunogenicity of BCG may be increased using prime boost approaches with novel CFPs of MTB.

In our current study we have evaluated T cell activity (ADA, IFN- $\gamma$ , IL-12, TNF- $\alpha$ ) of CFPs isolated at different time periods from MTB sputum culture using *in vitro* PBMC model. We found that Fraction C (21-28 day fraction) particularly induced good ADA activity and cytokine response as compared to BCG vaccine. Fraction B (10-14 day fraction) also induced good ADA activity but somehow cytokine response induced by it was lower than that of BCG vaccine. These results suggest us that CFPs particularly those secreted in later phase of growth curve of MTB may have good potential to induce T-cell response. From this study we have also tried to partially characterize CFP fractions by antibody detection assay using panel of six MTB H<sub>37</sub>R<sub>v</sub> antigens (Ag 85B, 45kDa, GroES, Hsp 16, CFP-10 and ESAT-6) by ELISA protocol. All six antigens selected are major secretory proteins of MTB. We found that levels of all secretory antigens increased with respect to increase in growth period of MTB, with maximum titre levels found in fraction C (21-28 day fraction). These results correlated with our earlier results in fraction C, suggesting us that good cytokine response in that fraction was may be due to elevated levels of secretory antigens in that fraction. Further we also evaluated T-cell activity in all these six secretory antigens of MTB and found that antigens CFP-10, Ag85B and ESAT-6 particularly CFP-10 induces good T-cell response. These results suggest us that these antigens form a major part of culture filtrate antigens secreted during growth phase of MTB.

Cellular immune responses are critical for control of MTB infection [6]. Culture filtrate antigens secreted during growth phase

of MTB culture act has important targets for T-cell response. The recent identification of novel secreted proteins of MTB and their characterization has given us new insights for implementing these proteins for better vaccine design [4]. In the current study our results suggested us that culture filtrate proteins particularly those secreted towards later phase of MTB culture induces good T-cell response. Moreover on partial characterization we found that in later phase fractions these culture filtrates have good levels of major secretory antigens such Ag85B, ESAT-6, CFP-10, GroES, Hsp-16, and 45kd antigens. Several laboratories subsequently reported the protective effect of vaccination with culture filtrate proteins (CFP's) prepared from log-phase *M. tuberculosis* cultures in mice and guinea pigs and demonstrated that the protection was transferable by CD4+ T cells [10]. Henriette Boesen et al. using an *in vitro* approach showed that secreted antigens of MTB produce good IFN- $\gamma$  response [1]. Similarly Fonseca et al. showed that combination of culture filtrates with CpG oligonucleotide produces reduced histopathology and elevated cytokine response in mice model [5]. These findings are consistent with our results and suggest us combination of such culture filtrates of MTB (particularly of later phase) with BCG in future may increase the efficacy of vaccine.

On evaluating panel of individual secretory antigens for T-cell response, we found that antigens Ag85B, CFP-10, 45kd induced sufficient levels of T-cell response which were more or less comparable to BCG vaccine but not as consistent when compared with Fraction C of MTB sputum Culture. Ag85B is major secretory protein secreted in culture filtrate and strongly recognized T-cell antigen during early phase of infection. It also contains numerous well-characterized T-cell epitopes which makes it attractive candidate for vaccine development. Similarly CFP-10 is major early secretory protein secreted by RD 1 region which is absent in currently available BCG vaccine. Use of such antigen for vaccine development is area on which many scientists are focusing their research on. Based on studies in mice, Dietrich et al. reported that CFP-10 has predominant T-cell response [2]. In another study Huygen et al. showed DNA vaccine encoding Ag85A, B produces elevated IL-12 and IFN-g response [13]. A striking feature we observed in our study was that none of the secretory antigens separately evaluated produced IL-12 response which was consistent with Fraction C. Thus instead on focusing on individual purified antigens of MTB, fractions of CFP's isolated from later phase of MTB culture can be used as candidates for boosting existing immune response developed by BCG in prime boost regimes.

Our studies with culture filtrates of MTB sputum culture showed us that they are important targets for vaccine design and may be used with BCG vaccine in prime boost approaches. However lot of studies, involving concentration of antigen to be used, use of effective adjuvant formulation and effective characterization of culture filtrates, proper studies in animal models are need before implementation of such antigens molecules in vaccination strategies in near future.

## Conclusion

Culture filtrate proteins of MTB sputum culture are important T-cell targets, particularly those secreted towards later phase and thus potential of such Culture Filtrate proteins may be further explored in near future for development of effective vaccination strategies for improving efficacy of currently available TB vaccine.

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### Conflict of interest

All the authors declare that they have no conflict of interest.

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