Available online at www.pelagiaresearchlibrary.com

Pelagia Research Library

European Journal of Experimental Biology, 2013, 3(3):35-42

ISSN: 2248 –9215
CODEN (USA): EJEBAU

Assessment of cellular immune response to culture filtrate antigens of *M. tuberculosis* culture using *in vitro* PBMC model. Prospects to new vaccine development

Aliabbas A. Husain[1], Hatim F. Daginawala[1], Hemant J. Purohit[2], Girdhar M. Taori[1] and Rajpal S. Kashyap*[1]

[1]*Biochemistry Research Laboratory, Central India Institute of Medical Sciences, Nagpur, India*
[2]*Environmental Genomic Unit, National Environmental Engineering Research Institute (NEERI), CSIR, Nehru Marg, Nagpur, India*

ABSTRACT

In the present study, fractions of culture filtrate proteins isolated at different time periods from M.tuberculosis (MTB) culture were evaluated for T cell activity (ADA, IFN-γ, TNF-α, & IL-12) using in vitro peripheral blood mononuclear cell (PBMC) model. Our results suggest that PBMC's induced with culture filtrate proteins particularly those secreted towards late logarithmic growth phase of MTB culture have good potential T cell activity as compared to Bacillus Calmette Guerin (BCG) vaccine. On evaluation of antigen levels in cell supernants of fractions we found levels of all secretary antigens increased towards later phase fraction (fraction C).Moreover on comparing T cell activity of individual purified MTB antigens with fraction C, we found that Fraction C induced better immune response than individual antigen. In conclusion, culture filtrate proteins of MTB culture are important T-cell inducers, and may be further explored in near future for development of effective vaccination strategies for improving efficacy of currently available BCG vaccine.

Keywords: BCG vaccine, Culture Filtrate proteins, *M.tuberculosis*, PBMC model,

---

INTRODUCTION

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 variable and differs according to the form of TB [2]. For more than 80 years, no new TB vaccine has successfully been developed [3]. With introduction of TB eradication programme, new vaccines with better protection than BCG or improvement in current immunization programme 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 inducers of the T-cell response in TB [5]. In recent years, research has focused on antigens released by live MTB in culture medium; pools of such extracellular antigens have been tested in several laboratories as subunit vaccines and demonstrated to induce substantial levels of protection in animal models as these antigens are believed to be at least partially responsible for the efficacy of live vaccines [6]. The demonstration that non-living vaccines based on secreted proteins could effectively protect against subsequent MTB infection in animal models, 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 antigen expressed in culture filtrates of MTB are absent in *Mycobacterium bovis* BCG and most environmental mycobacterial species investigated [7]. These findings have increased the interest in these molecules both as potential vaccine candidates and novel specific diagnostic reagents.

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 expressions and T-cell response of different antigens that may be present in MTB culture. In our earlier studies we observed effectiveness of heterologous prime boost regimes by boosting with Ag85B molecule [Husain *et al*, 2011, unpublished data], Ag 85 B is major secretary protein present in culture filtrate. A large pool of secretary antigens present in culture filtrate, are important products of MTB growth and metabolism. The present study focused in exploring vaccine potential of these secretary antigens using *in vitro* peripheral blood mononuclear cell (PBMC) model.

A number of research groups have identified vaccine potential of CFP's. The early secretary antigenic target-6 (ESAT-6) antigen purified from strongly stimulatory and 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 adjuvants provided protection in mice challenged with MTB, and protection was mediated by gamma interferon (IFN)-producing CD4 cells [10].

In order to reduce the current burden of TB, improved vaccination strategies are needed. Novel antigens of MTB are important molecules 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 T cell potential of secretary antigens isolated at different time periods from MTB culture using *in vitro* PBMC model and also their comparison to commercially available purified antigen. In this study we have also tried an antibody detection assay protocol with aim to indirectly evaluate and identify the phase wise antigenic population secreted in MTB culture.

MATERIALS AND METHODS

Bacterial strains and culturing:
MTB bacilli isolated from sputum samples were grown in Middle brook 7H9 liquid medium along with oleic acid, Albumin, Dextrose, Catalase (OADC) enrichment and antibiotic supplements in BAC/T culture bottles (*Biomeriux, france)* and incubated at 37˚C in *BacT/Alert system(Biomerieux, France)* for 28 days.

Collection and isolation Culture Filtrate Antigens:
Fractions were collected from growth phases of MTB culture on specific days and were grouped into following three fractions:

Fraction A: Fractions collected on 3, 4, 5, 6 days and pooled.
Fraction B: Fractions collected on 10, 11,12,13,14 days and pooled
Fraction C: Fraction collected on 21, 22,23,24,25,26,27,28 days and pooled.

For isolation of culture filtrate antigens all the three fractions were separately centrifuged at 1200 rpm for 15 min and supernatants collected were stored at 4^{o} C until use

Ethical Committee Approval: Blood collection was done from healthy BCG vaccinated 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, Nagpur and in accordance with the NIH guidelines (NIH publication No. 80-23; revised 1978)

PBMC isolation and cultivation

For separation of PBMCs, 5 ml venous blood was obtained from healthy individuals in sterile EDTA vaccutainer tubes. PBMC cells were extracted from whole blood using a density gradient Ficoll histopaque method .After isolation; PBMCs were cultured keeping the concentration at 2×10^5 cells/well and were stimulated with purified protein derivative (PPD) (25ug/ml) (Span diagnostics). T cell activity of different culture filtrate antigens was evaluated by employing three experimental protocols In protocol I, cultured PBMCs were induced with 100ul of different phase fractions (25ug/ml) 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 37^0C in CO_2 incubator. Cell supernatants were collected at different time points (0, 24, 48, 72 hrs) for estimation of T-cell markers, Adenosine Diaminase (ADA), IFN-g, IL-12 and TNF-alpha. In protocol II, cell supernatants from protocol I were characterized by antibody detection assay against panel of six MTB antigens using ELISA protocol. In protocol III purified antigens with concentration equivalent to fraction C (25ug/ml) were used for assessment of T-cell activity. Procedure used was similar as used in protocol I, purified antigens and fraction C were used for induction and their T cell activity were compared.

Indirect evaluation of Culture Filtrate Antigens in cell culture supernatants

Procedure : Indirect evaluation of antigens in cell culture supernatants were done by antibody detection assay using panel of six MTB H_{37}Rv 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 microtiter 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 4^0C 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 37^0C. 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 37^0C. For color development, 100 µl of TMB/ H_2O_2 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_2SO_4 and absorbance of color in each well was read at 450 nm.

Evaluation of T-cell activity

ADA: ADA activity in the supernatant 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 1 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) 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 antibodies were 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 antibodies (IFN-γ, IL-12, TNF-α) were 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 of color was measured 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 T-cell potential of CFP's isolated from different time interval from MTB culture using *in vitro* PBMC model. To achieve our objective we divided our work in three different experimental protocols .In protocol 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. Fig 1 a. shows T cell -ADA activity, in 48 hrs cell supernatants of PBMC's induced with culture filtrate fractions A, B, C and BCG vaccine. Cell supernatants of PBMCs induced with different culture filtrate fractions showed significant ADA activity as compared BCG vaccine ($P<0.05$). Among culture fractions, PBMCs induced with Fractions C showed better ADA ($p=0.0001$) activity as compared to fraction A and B. 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 PBMCs stimulated with fraction C showed good IFN-γ response as compared to BCG vaccine which was also statistically significant ($p=0.005$) ((Fig 1b). Fractions C also showed good IL-12 levels as compared to BCG ($p=0.007$); however no IL-12 activity was reported in fraction A and B (Fig 1c.).All culture filtrate fractions showed TNF –α response, with activity increasing from fractions A to C (Fig 1d). Fraction C showed TNF–α response better than BCG vaccine and fraction A and B ($p=0.005$).

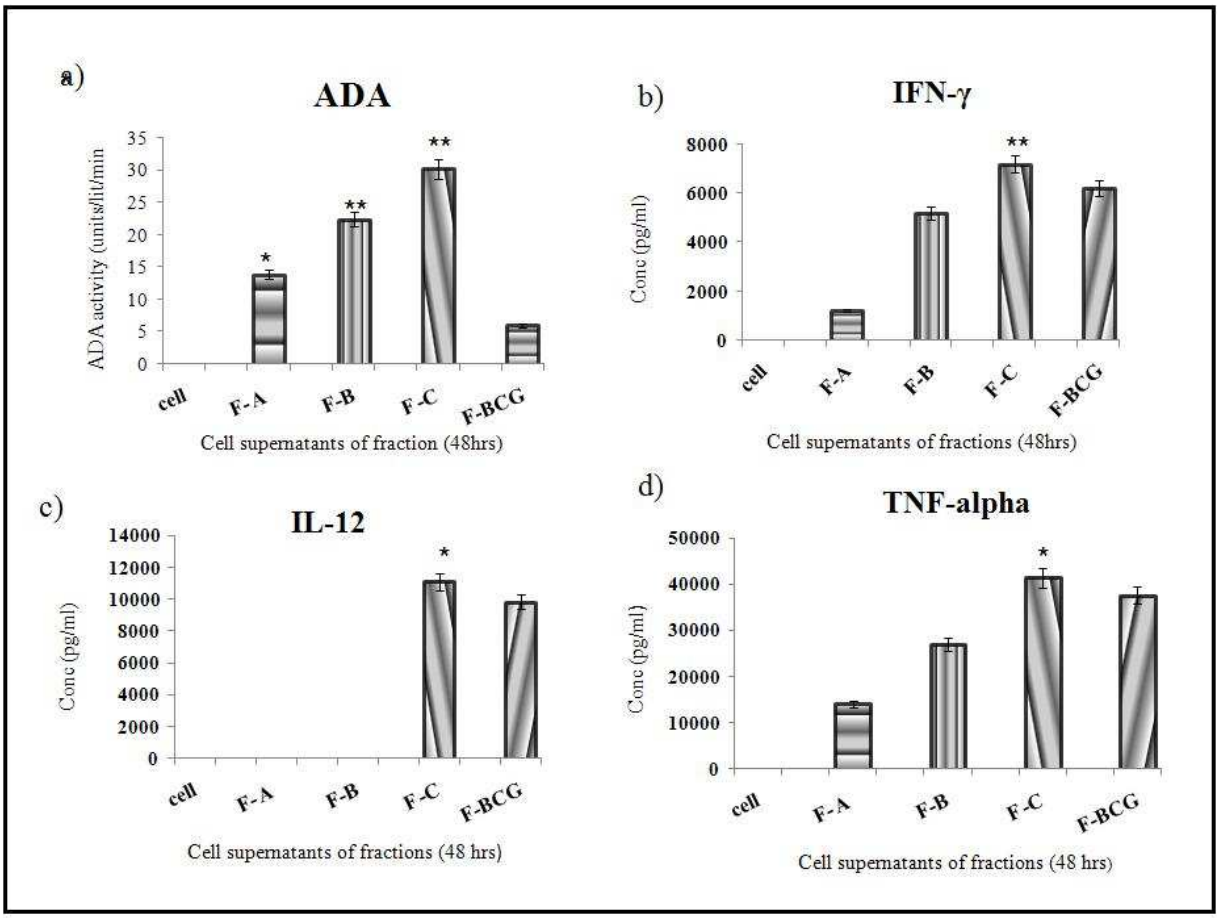

Figure 1. T cell activity a) ADA b) IFN-γ c) IL-12 d) TNF-alpha in 48 hrs cell supernatants of short term PBMC induced with different phase MTB sputum culture filtrate fractions, A (3-6 day), B (10-14 day), C (21-28 day) (F-A, F-B, F-C) and BCG vaccine. (F-BCG). Cell represents control without any induction. 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. Statistically significant (*) and highly significant values (**)
(Note: we observed T-cell activity in 48 hrs culture supernatants; hence only activity in 48 hrs is reported in figure)

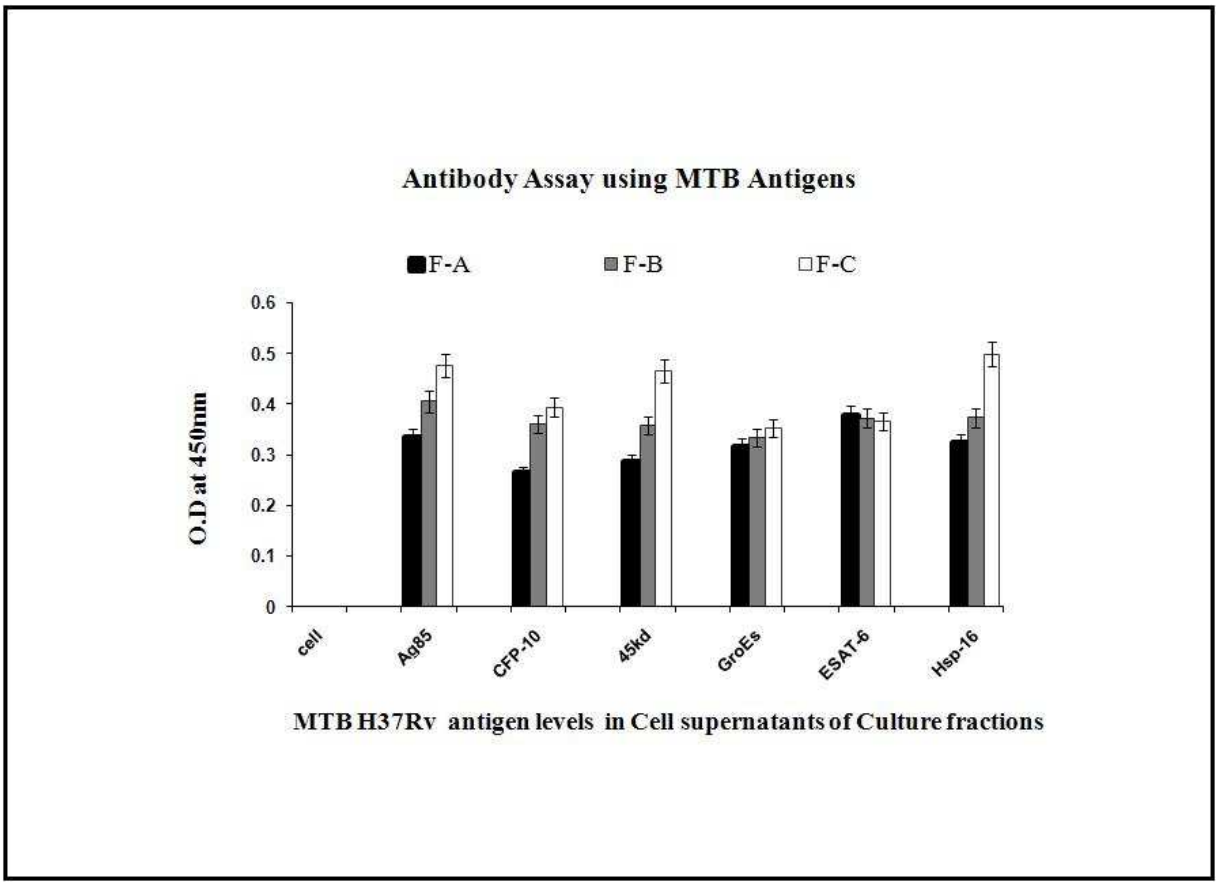

Figure 2. **Antibody detection assay using selected panel of MTB $H_{37}R_V$ antigens (Ag85, CFP-10, 45kd, GroEs, ESAT-6, Hsp-16) in 72 hour cell culture supernatants of short term cultures of PBMC induced with different phase MTB sputum culture filtrate fractions, A (3-6 day), B (10-14 day), C (21-28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

In addition the study was also focused on evaluation of antigenic levels in cell supernatants of PBMCs induced with different culture filtrate fractions by antibody detection assay using panel of six MTB $H_{37}R_V$ antigens (Ag 85B, 45kDa, GroES, Hsp16, CFP-10 and ESAT-6). Fig 2 shows antibody levels of six MTB H_{37}Rv 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 were found in all cell supernatants of PBMCs induced with culture filtrate fractions of MTB culture, with increasing titre levels from fraction A to C. Fraction C showed high antibody titres against all MTB antigens, which indirectly showed us that levels of these six MTB antigens may be elevated in fraction C, and also indicate us that levels of these six secretory antigens increases with growth phase of MTB culture.

In the third protocol, T-cell response of PBMCs induced with commercially available purified MTB H_{37}Rv antigens was compared to BCG vaccine and previously evaluated MTB fraction C. Fig 3 shows a) ADA b) IFN-γ c) IL-12 d) TNF-α in 48 hrs cell culture supernatants of individual MTB H_{37}Rv antigens (Ag 85B, 45kDa, GroES, Hsp16, CFP-10 and ESAT-6) and Fraction C (fig 3a).Fraction C showed comparably good ADA activity as compared to all individual MTB antigens, which was also very statistically significant (p=0.0003).Based on IFN-γ results we found that although MTB antigen induced sufficient IFN-γ response their levels were still much less than that observed in fraction C (p=0.0001). (Fig 3b). On the contrary antigens such as ESAT-6, and CFP-10 induced good TNF-α response comparable that fraction C and BCG, However we didn't find any of the IL-12 activity in any of antigenic fractions except BCG and Fraction C (data not shown). Result of this protocol 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 cytokines was not as consistent as observed in later phase culture filtrate fraction, (Fraction C).

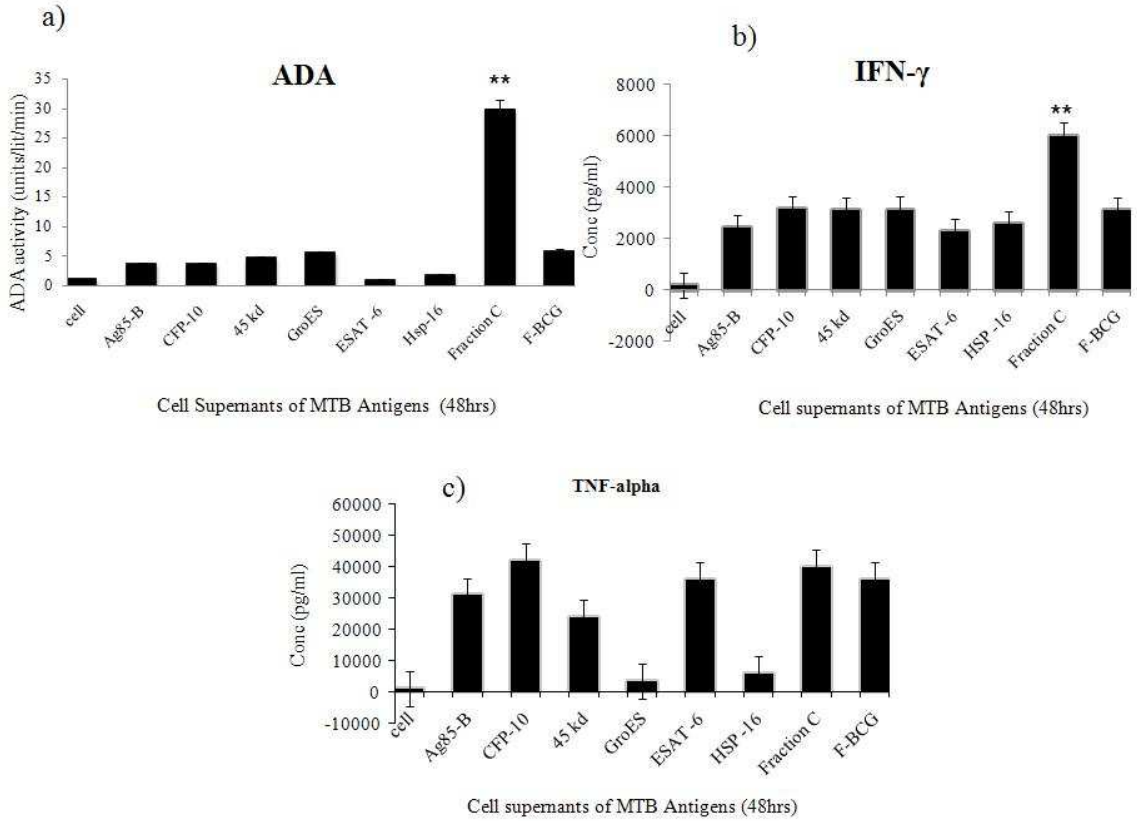

Figure 3. T cell activity a) ADA b) IFN-γ c) TNF-alpha in 48 hrs cell supernatants of short term PBMC induced with purified MTB $H_{37}R_V$ antigens (Ag85, CFP-10, 45kd, GroEs, ESAT-6, Hsp-16), fraction C and BCG vaccine. (F-BCG). Cell represents control without any induction. BCG vaccine is used as positive control to compare its T-cell activity with that of selected panel of MTB H37RV antigens (Ag85, CFP-10, 45kd, GroEs, ESAT-6, Hsp-16)

Each bar in the figure represents mean values of triplicates. Statistically significant (*) and highly significant values (**)

DISCUSSION

Despite its widespread use in newborn babies, BCG does not totally 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 novel 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 antigens of MTB.

In our current study we have evaluated T cell activity (ADA, IFN-γ, IL-12, TNF-α) of CFPs isolated at different time periods from MTB 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 $H_{37}R_v$ antigens (Ag 85B, 45kDa, GroES, Hsp 16, CFP-10 and ESAT-6) by ELISA protocol. All six antigens selected are major secretary proteins of MTB. We found that levels of all secretary 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 secretary antigens in that fraction. Further we also evaluated T-cell activity in all these six secretary antigens of MTB and found that antigens particularly although antigens CFP-10 and ESAT-6 induce good T-cell response (TNF-α) comparable to that of fraction C but ADA and IFN-γ response was significantly less than fraction C.

Cellular immune responses are critical for control of MTB infection (6). Culture filtrate antigens secreted during growth phase of MTB culture act has important inducers for T-cell response. The recent identification of novel secreted proteins of MTB and their characterization has given us new insights for incorporating 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. 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-γ 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 that 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 secretary antigens for T-cell response, we found that antigens Ag85B,CFP-10, ESAT-6 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 culture. Ag85B is major secretary protein secreted in culture filtrate and strongly recognized antigen by T-cell during early phase of infection. It also contains numerous well-characterized T-cell epitopes which makes it an important candidate for vaccine development. Similarly CFP-10 is major early secretaty protein secreted by RD 1 region which is absent in currently available BCG vaccine. Use of such antigens for vaccine development is area on which many scientists are focusing their research. Based on studies in mice, Dietrich *et al* reported that CFP-10 show predominant T-cell response (2). In another study Huygen et *al* showed DNA vaccine encoding Ag85A& B produce elevated IL-12 and IFN-g response (13). A striking feature observed in our study was that none of the secretary antigens separately evaluated produced IL-12 response which was observed 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 for boosting existing immune response developed by BCG in prime boost regimes. The present discussed strategy will in turn also help to minimize cost of existing TB vaccine development programmes which has till now invested millions of dollars in improvement or replacement of BCG.

Our studies with culture filtrates of MTB culture showed us that they are important molecules 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, effective characterization of culture filtrates and proper studies in animal models are need before incorporation of such antigens molecules in vaccination strategies in near future

In conclusion, culture filtrate proteins of MTB culture are important T-cell targets, particularly those secreted towards later phase. The potential of such culture filtrate proteins must be explored for development of effective vaccination strategies than commercially used antigens to improve efficacy of currently available TB vaccine.

Acknowledgement
A.A.H acknowledges award of Senior Research Fellowship by Indian Council of Medical Research, New Delhi .All Authors would like to thank Central India Institute of Medical sciences for funding the study.

REFERENCES

[1] H. Boesen, B.N. Jensen, T. Wilcke, P. Andersen, *Infect Immun*., **1995,** 63:1491-1497.
[2] J. Dietrich, K. Weldingh, P. Andersen, *Vet Microbiol*., **2006,** 112:163–169.
[3] C. Martín, Tuberculosis, Brazil: TuberculosisTextbook.com, **2007**.
[4] U.D. Gupta, V.M. Katoch, D.N. McMurray, *Vaccine.*, **2007**, **25**: 3742-3751.
[5] D.M. Fonseca, C.L. Silva, P.F. Wowk, M.O. Paula, S.G. Ramos, C. Horn, G. Marchal, V.L.Bonato, *Infect Immu.*, 2009, **77**: 5311–5321.

[6] P. Ravn, A. Demissie, T. Eguale, H. Wondwosson, D. Lein, H.A. Amoudy, A.S. Mustafa, A.K. Jensen, A. Holm, I. Rosenkrands, F.Oftung, J, Olobo, R.F. Von, P. Andersen, *J Infect Dis.*, **1999**, 179: 637–45.
[7] A.L. Sorensen, S. Nagai, G. Houen, P. Andersen, A.B. Andersen, *Infect Immun.*, **1995**, 63: 1710–7.
[8] A.A. Husain, R.S. Kashyap, D.R. Kalorey, S.R. Warke, H.J. Purohit, G.M. Taori, H.F. Daginawala, *Indian J Exp Biol*., **2011**, 49:7-10.
[9] Andersen P, *Infect Immun.*, **1994**, 62: 2536–44.
[10] E.B. Lindblad, M.J. Elhay, R. Silva, R. Appelberg, P. Andersen , *Infect Immu*., **2007,** 65 : 623-9
[11] S.H. Kaufmann, G. Hussey, P.H. Lambert, *Lancet.*, **2010** 375: 2110–19
[12] C. Martin, B. Gicquelb, *Enferm Infecc Microbiol Clin.*, **2011,** *29:* 57-62
[13] K. Huygen, J. Content, D.L. Montgomery, A.M. Yawman, R.R. Deck, M.D. Corrille, I.M. Orme, S. Baldwin, C. D'Souza, A. Drowart, E. Lozes, P. Vandenbussche, J.P. Vooren, A.L. Margaret, J.B. Ulmer, *Nature Med.*, **1996**, 2: 893 – 898.
[14] R.N Sharma, K.P. Sharma, S. N. Dixit, *Der Chemica Sinica*., **2010**, 1: 57-66
[15] A.J. Kundawala, A.P Vishnu, H.V. Patel, D. Choudhary, *Der Pharmacia Sinica*., **2011**, 5:88-97
[16] M. Saleshier, S. Suresh, N. Anitha, J. Karim, M.C. Divakar, *Euro. J. Exp. Bio*., **2011**, 1: 150-159
[17] J.A. Alli, B.E. Boboye, I.O.Okonko, A.F. Kolade, J.C. Nwanze, *Adv. Appl. Sci. Res*., **2011**, 2:25-36
[18] B. Josephin Sheeba, T. Selva Mohan, *Asian J. Plant Sci. Res*., **2012**, 2:83-88