

Immunogenicity Studies with Microbial Fractions of *M. tuberculosis* H37Rv Total Culture Filtrate

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Abstract

Current study investigates the whole secretory proteome of *Mycobacterium tuberculosis* as culture filtrate fractions to identify immunoprotective protein antigens on the basis of protection studies in animal (mouse and guinea pig) models. Secretory culture filtrate proteins (CFPs) of *M. tuberculosis* H₃₇Rv were fractionated into fifteen narrow molecular mass fractions in the order of increasing molecular size (F1-F15) by electroelution. Immunization studies revealed proteins in the molecular weight range of 20-24kDa (F7), 25-30kDa (F8) and 37-42kDa (F11) as key protective fractions against experimental tuberculosis in both the animal (mice and guinea pig) models. Amongst these fractions, F7 imparted even better protection as compared to BCG. Immunological studies with all the fractions demonstrated that although selected three protective fractions were able to induce significant immune responses in both short term culture filtrate (STCF) immunized and Mtb infected animals, there were number of other non-protective fractions also that were inducing higher immune responses either in immunized animals (e.g.F12-F15) or in Mtb challenged animals (e.g.F1-F6). These results demonstrate that only those mycobacterial proteins that are recognized by the host immune system both during immunization and infection can induce significant protection against experimental tuberculosis, however there is no direct correlation between the level of immune responses and degree of protective efficacy.

Keywords: tuberculosis, subunit vaccine, culture filtrate fraction, immune response, correlates of protection

Abbreviations used: Mtb, *Mycobacterium tuberculosis*; TB, tuberculosis; BCG, bacillus Calmette-Guerin; ELISA, enzyme-linked immunosorbant assay; CFU, colony-forming unit

1. Introduction

Tuberculosis (TB) is second only to HIV/AIDS as the greatest killer worldwide due to a single infectious agent. In 2013, 9 million people fell ill with TB and 1.5 million died from the disease (<http://www.who.int/mediacentre/factsheets/fs104/en/>). Currently, only vaccine available to prevent tuberculosis (TB) is the live attenuated *M. bovis* Bacille Calmette-Guérin (BCG) vaccine. BCG vaccination protects children against TB meningitis and against disseminated, miliary disease, but confers a variable protection (ranging from 0% to 80%) against pulmonary TB (Fine, 1995). In the last decade, much progress has been made and several candidates TB vaccines have entered clinical trials (Brennan & Thole, 2012). The efficacy study with a new generation TB vaccine (MVA85A) showed the absence of efficacy against TB over that conferred by BCG alone (Tameris et al., 2013). Recently, the TB vaccine, H4:IC31 demonstrated an acceptable safety profile and was immunogenic in South African adults (Geldenhuis et al., 2015). Rapid vaccine development and improvement in currently available vaccine require reliable correlates of protection against tuberculosis. In spite of intensive efforts in experimental models, there are few guidelines for rational selection of vaccine candidates for tuberculosis. Proteins secreted from live mycobacteria may have an advantage in triggering an earlier immune response as compared to those released by mycobacterial lysis (Skeiky & Sadoff, 2006).

Cell-mediated immune response, involving mainly the CD4⁺ and CD8⁺ T-cell subsets, plays an essential role in the pathogenesis of TB (Aktas et al., 2009). In mouse TB challenge studies, IFN- γ -secreting CD4⁺T cells are reported to be important mediators of protection (Kaufmann, Cole, Mizrahi, Rubin, & Nathan, 2005) and attempts to induce TB-antigens specific IFN- γ -secreting CD4⁺ T cells has been the dominant theme of most TB vaccine research studies. Various attempts have been made to identify key antigens of importance for protective immunity against *M. tuberculosis* in number of studies (Boesen, Jensen, Wilcke, & Andersen, 1995; Haslov et al., 1995; Olsen, Hansen, Holm, & Andersen, 2000; Sable, Verma, Behera, & Khuller, 2005) and demonstrated that no correlation exists between high Th1 response, at least high IFN- γ , induced during natural infection and protection imparted after immunization. Thus, still there are no immunological biomarkers that predict vaccine efficacy to tuberculosis is available. Since, culture filtrate of *Mtb* contains a mixture of proteins ranging from 3kDa to more than 100 kDa, evaluation of each and every protein present in culture filtrate for the protective efficacy in experimental TB models is not a feasible approach. Culture filtrate fractions have also been used earlier, for the identification of Immunodominant antigens in mice during infection with *Mtb* (Andersen et al., 1992) and for their ability to induce IFN- γ responses in advanced TB patients as well as healthy TB contacts and community controls (Boesen et al., 1995). The findings from our laboratory indicated that the CTL response of healthy contacts was found to be directed towards multiple antigens fractions with prominent recognition of proteins below 15 kDa and those present in the region of 30 kDa (Sable, Kaur, Verma, & Khuller, 2005). However no report is available regarding the nature of protective efficacy of *Mtb* culture filtrate fractions. Thus, for the identification of key protective antigens, the present study was initiated with the objective to evaluate the culture filtrate fractions of *Mycobacterium tuberculosis* H₃₇Rv for protective efficacy in two animal models of experimental tuberculosis i.e. mouse/guinea pig. Based on the direct evidence of protective potential against *Mtb* challenge studies in these animal models, our data suggests proteins in the region of 20-42kDa in mycobacterial secretory proteome are suitable candidates for the development of future antituberculous multivalent subunit vaccine and warrants further exploration.

2. Materials and Methods

2.1 Mycobacterial Cultures and Growth Conditions

Mycobacterium tuberculosis H₃₇Rv and *Mycobacterium bovis* BCG originally obtained from National Collection of Type Cultures (NCTC), London and being maintained on Lowenstein Jensen's (LJ) medium in the laboratory were used in the study.

2.2 Animals

Four to five weeks old female BALB/c (H-2^d) mice weighing 15-20 g each, obtained from Central Research Institute, Kasauli, India were used in this study. Mice were housed in cages contained within a negative pressure regulated animal isolator and were fed on standard pellet diet [Hindustan Lever Ltd., Mumbai] and water *ad libitum*.

Dunkin Hartley outbred female guinea pigs weighing 250-350 gm each, were procured from Haryana Agriculture University (HAU), Hisar, India. All animal experiments were carried out in accordance with the rules and regulations set forth by the Institute Animal Ethics Committee (IAEC), Postgraduate Institute of Medical Education and Research (PGIMER), Chandigarh, India.

2.3 Isolation and Fractionation of Total Short-Term Culture Filtrate Proteins (STCF) of *M. tuberculosis* H₃₇Rv in to Narrow Molecular Mass Fractions (n=15)

Culture filtrate proteins were isolated by growing *M. tuberculosis* H₃₇Rv in liquid synthetic Youman's medium for four weeks as a stationary pellicle culture at 37 °C (Haslov et al., 1995). The culture supernatant was filter sterilized and concentrated initially using tangential flow system with a cartridge of 3 kDa cut-off membrane (Millipore). The concentrated culture filtrate was desalted by extensive washings with distilled water and finally exchanged with PBS. This preparation was referred to as STCF. The protein profile was analysed by 12% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) followed by silver staining (Morrissey, 1981). Aliquots of culture filtrate proteins were finally stored at -20 °C till further use.

A panel of molecular weight fractions was obtained from STCF by electro-elution (Boesen et al., 1995). Briefly, Five-milligram of the above STCF was separated on 16% preparative SDS-PAGE overnight on protein II xi cell (Bio-Rad) having gel dimensions of 16/20 cm inner plate and 18.3/20 cm outer plate. Constant current of 10mA was applied for stacking of protein samples followed by resolution at 30 mA. After the electrophoresis run, the gel was pre equilibrated in tris borate buffer pH 8.7 (50mM tris and 250 mM boric acid) and transferred to whole gel elutor (Bio-Rad) and electroeluted at 250 mA for 30 min and the current was reversed for 10 seconds at the end of the elution period to loosen molecules sticking to the cellophane membrane at the bottom of the wells. Fractions were harvested from the unit with the help of vacuum pump and adjacent fractions were pooled to make easily

manageable numbers and designated as F1 to F15 in the order of increasing molecular size and kept frozen at -20°C until further use. All fractions were analysed by separation on SDS-PAGE (16%) followed by silver staining (Figure 1). The proteins in the fractions were identified on the basis of reactivity with available monoclonal / polyclonal antibodies by ELISA as represented in Table 1.

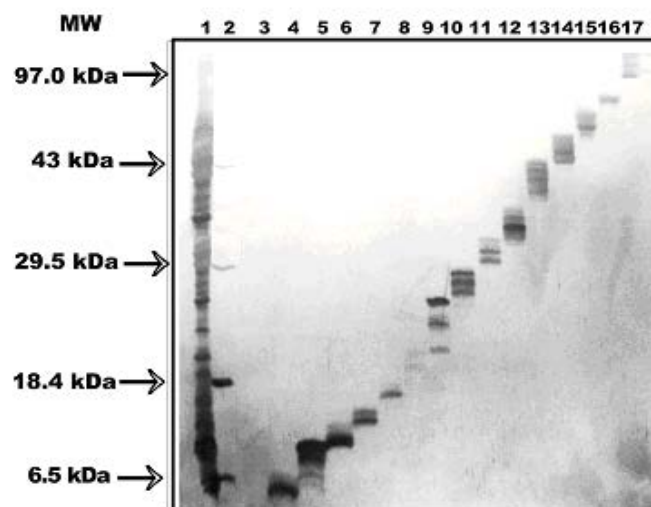


Figure 1. Fractionated culture filtrate of *M. tuberculosis* H₃₇Rv

The culture filtrate was separated by 16% SDS-PAGE and fractionated into narrow molecular mass fractions by electroelution. The fractions were analysed by SDS-PAGE and silver staining. Lane 1- STCF, Lane 2- molecular weight markers (kilo dalton) and lane 3 to 17- fifteen culture filtrate fractions

Table 1. Panel of monoclonal/polyclonal antibodies used to identify purified polypeptides/antigens from protective fractions of *Mtb* H₃₇Rv by ELISA

S.No	Protein fraction	Apparent Mol. weight. (kDa)	Specific MoAb/PAb ^a	O.D. at 492 nm	Reported corresponding antigen ^b
1.	F7	20	IT-69 (HBT-11)	0.386	20kDa (Rv1392)
2.		20.5	IT-10(F29-47-3)	0.428	20.5 kDa
3.		21	K8483	1.248	21kDa CFP-21 (Rv1984c)
4.		23	CS-28	0.387	23kDa, Superoxide dismutase (SOD)
5.		24	IT-67 (L24-b4)	0.689	24kDa MPT-64 (Rv1980c)
6.	F8	25	IT-52 (HBT4)	0.458	25kDa MPT-51 (Rv3803c)
7.		28	α -28(MC-9246)	0.368	28kDa
8.		29-30	IT-49 (HYT27)	0.379	Ag85B (Rv1886v)
9.		30-31	IT-49 (HYT27)	0.318	Ag85A (Rv3804c)
10.	F11	37	NA		Uncharacterized
11.		38	IT-23 (TB71)	1.375	Pst-S (Rv0934)
12.		40	NA		Uncharacterized
13.		42	NA		Uncharacterized

^a Antibodies against CFPs were provided by Dr John T. Belisle, Colorado State University, Fort Collins, Colorado, USA or kind gift from Dr. Peter Andersen and Dr. I. Rosenkrands, Statens Serum Institute, Copenhagen, Denmark.

The purified proteins were also analysed for reactivity with other antibodies (corresponding to other proteins) and the OD at 492nm was found to be below 0.1.

NA. Not available.

^b Ref; [Sonnenberg et al., 1997; Sable et al., 2005].

2.4 Immunizations

2.4.1 Mice

BALB/c mice (5 animals per group) were immunized with Mtb culture filtrate fractions (F1 to F15 individually in parallel) emulsified with 250 µg of dimethyl dioctadecyl ammonium bromide (DDA) as adjuvant. DDA emulsion was prepared as described previously (Brandt, Elhay, Rosenkrands, Lindblad, & Andersen, 2000). Immunizations were performed with 25 µg of each culture filtrate fraction individually (F1 to F15) or 25 µg of total short term culture filtrate (STCF) protein adjuvanted with 250 µg of DDA in a volume of 0.2ml/dose/animal. Animals in each group were injected subcutaneously with three biweekly doses of the above-mentioned immunogens. For comparison, group of mice were also immunized with BCG (10^5 CFUs/animal) subcutaneously as a single dose. Control animals were sham-immunized with adjuvant only.

2.4.2. Guinea Pigs

Guinea pigs (5 animals per group) were immunized with F7, F8 and F11 fractions individually along with 500 µg of DDA adjuvant. A group of animals were also immunized with a single dose of BCG (10^5 CFUs/animal) administered subcutaneously in parallel. Immunization was carried out with 100 µg of each culture filtrate fractions or with 100 µg total short term culture filtrate (STCF) protein. . A group of guinea pigs were also immunized with combination of F7 and F11 (50 µg each fraction, total 100 µg) culture filtrate fraction. The guinea pigs were immunized three times with the designated dose per animal, at biweekly interval, using 500 µg of DDA as adjuvant in a volume of 0.2ml subcutaneously on the back. Control animals were sham-immunized with adjuvant only.

2.5 Evaluation of Cellular and Humoral Responses

2.5.1 Splenocyte Proliferation Assay

Spleen cells from mice and guinea pigs immunized with STCF were suspended in RPMI-1640 medium supplemented with 10% heat inactivated fetal bovine serum. The concentration of splenocytes was adjusted to $\sim 2 \times 10^5$ cells/well in 96 well culture plates and cultured in the presence of 2 µg/ml of STCF or culture filtrate fractions. The plates were incubated for 4 days at 37 °C in 5% CO₂ followed by incubation with [³H]-thymidine (0.25 µCi/well) for 18 hour. The cells were harvested using a cell harvester, and the incorporated radioactivity was measured and stimulation indices (SI) were calculated by dividing mean counts per minute in antigen-stimulated wells by mean counts per minute in unstimulated wells.

2.5.2 IFN-γ and IL-12 (p40) Assay

Levels of IFN-γ and IL-12 induced in culture supernatants of mice splenocytes in response to antigens were estimated after 72 hours by ELISA using commercially available kits (Opt EIA™ Set BD Pharmingen, CA, and USA) following manufacturer's instructions.

2.5.3 IgG ELISA

IgG titers were determined in the sera of STCF immunized animals by ELISA. Briefly, the wells of 96 well ELISA plates were coated with proteins antigens (STCF/ culture filtrate fraction) at a concentration of 2 µg/ml in carbonate buffer (pH 9.6) overnight at 4 °C. Plates were washed with PBS containing 0.05% Tween-20 (PBS-T) and blocked with 3% BSA at 37 °C for 2h. Diluted sera (1:100 dilution in PBS-T having 1% BSA) were added and bound antibodies were detected using HRP-conjugated anti-mouse/anti-guinea pig IgG. The absorbance was read at 492 nm.

2.6 Protection Studies

Mice were challenged with 0.1 ml (10^5 bacilli) suspension of *M. tuberculosis* H₃₇Rv per animal intravenously at 3 weeks p.im, while guinea pigs were challenged with 0.1 ml (10^5 bacilli) suspension of *M. tuberculosis* H₃₇Rv per animal intramuscularly (i.m) in the thigh muscle at 6 weeks p.im. Four weeks after the challenge, the mice were sacrificed and lungs and spleen were aseptically isolated. Guinea pigs were sacrificed after 6 weeks of challenge and spleen and the right lung were aseptically isolated. The organs were homogenized and appropriate serial dilutions were spread on Middlebrook 7H11 agar plates supplemented with oleic acid–albumin–dextrose–catalase (OADC). To selectively inhibit the growth of the residual BCG bacteria in the test organs, 2-thiophenecarboxylic acid hydrazide (2 µg/ml) was added to media. Colonies were counted after 3-4 weeks of incubation at 37 °C and bacterial counts per organ were determined.

2.7 Statistical Methods

Two-way comparison between test and control groups was performed using Student's *t*-test. Multiple comparisons amongst different groups were performed by (one way-Anova with tukey's multiple comparison test.). The statistical analyses were made using GraphPad prism 5. A *p* value of <0.05 was considered significant.

3. Results

3.1 Protective Efficacy of Culture Filtrate Fractions in Mouse Model

The \log_{10} CFUs in the lungs of BCG and STCF immunized animals were significantly lower ($p \leq 0.001$) as compared to unimmunized animals (Figure 2A) whereas; no significant difference in CFUs was observed in animals immunized with BCG and STCF+DDA. The \log_{10} CFUs in the lungs of animals immunized with fractions F2, F3, F7, F8, F10, and F11 were significantly lower ($p < 0.001$) as compared to the unvaccinated animals. When the experimental subunit vaccine preparations based on culture filtrate fractions were compared with BCG, amongst all the fractions, F7 showed significantly higher ($P < 0.05$) reduction in CFUs in the lungs by an average of 0.52 logs followed by F11 and F8 respectively (Figure 2A).

Further evaluation of CFUs in the spleens of *Mtb* challenged mice indicated that \log_{10} CFUs in animals immunized with F2, F3, F6, F7, F8, F10, F11, STCF and BCG vaccinated groups were significantly lower as compared to unvaccinated animals (Figure 2B). However, similar to that observed in the lungs, in the spleens also, animals immunized with F7 and F11 fractions showed significant reduction ($p < 0.05$) in CFUs as compared to that observed in BCG vaccinated group. Taken together, these findings indicate that culture filtrate fractions comprising F7, F8 and F11 are the most immunoprotective and suggested their further evaluation in another animal model i.e. guinea pig.

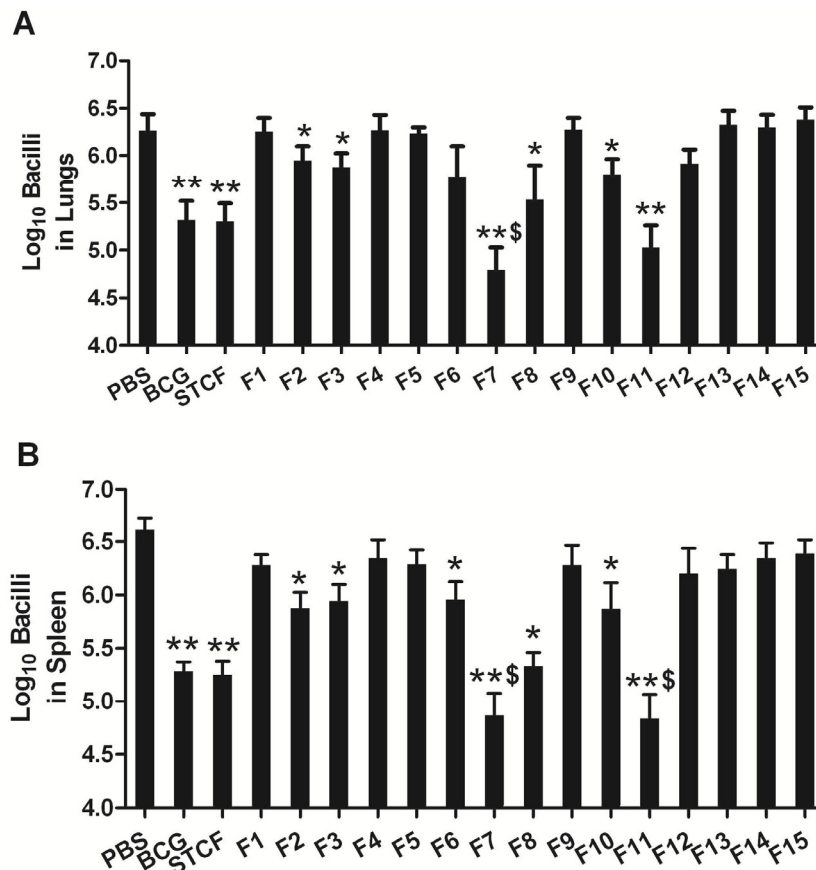


Figure 2. Comparative account of viable *M.tuberculosis* H₃₇Rv (Log₁₀ units) observed in the organs of Balb/C mice vaccinated with BCG or different experimental subunit vaccines at 4 weeks post infection

The results are expressed as mean Log₁₀ CFUs \pm SD of five animals per group. Significant differences were determined by ANOVA and student 't' test. *** $p < 0.001$, ** $p < 0.01$ w.r.t naive controls. \$ $p < 0.05$ w.r.t. BCG immunized group.

3.2 Culture Filtrate Fractions, F7, F8 and F11 Induced Significant Protection against TB in Guinea Pig Model

BCG immunized animals had a significantly ($p < 0.001$) lower bacterial burden both in the lungs and spleens as compared with sham-immunized guinea pigs (Figure 3A, 3B). When the vaccine potential of experimental subunit vaccines were compared, it was observed that all the vaccine formulations consisting of STCF, F7, F8 and F11 showed significant reduction ($p < 0.001$) in terms of \log_{10} CFU as compared with sham immunized group. Similar to mouse model, F7 immunised guinea pigs also showed significant reduction of bacterial burden in the lungs ($p < 0.01$) and the spleen ($p < 0.05$) as compared to BCG immunized animals and the protection induced by F7 was even better than that produced by STCF consisting of whole secretory proteome.

On the whole, culture filtrate fraction, F7 adjuvanted with DDA induced significantly higher protection followed by F11 and F8 in both mouse and guinea pig models of experimental tuberculosis.

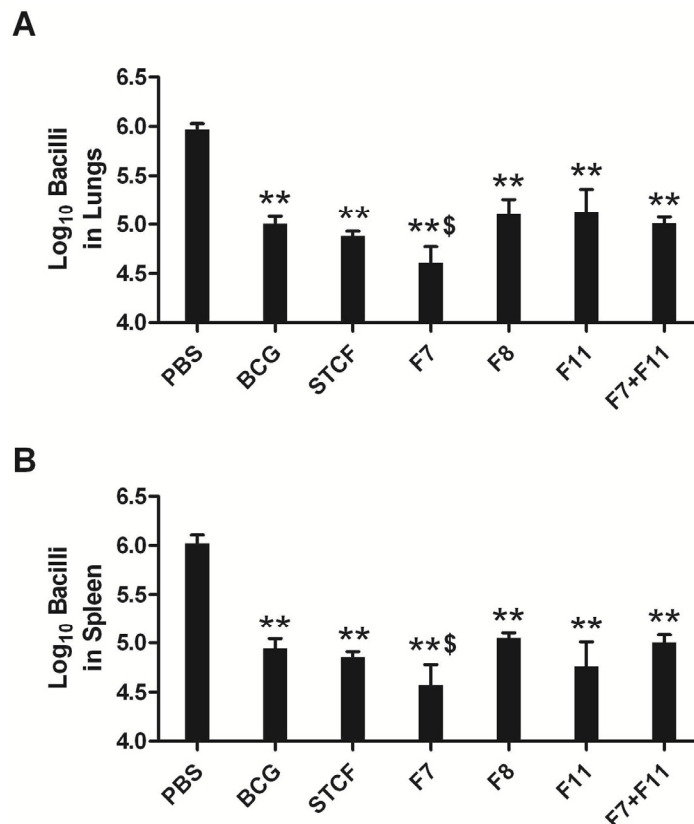


Figure 3. Comparative account of viable *M. tuberculosis* H₃₇Rv bacilli (\log_{10} units) observed in the lungs (A) and spleen (B) of guinea pigs vaccinated with BCG or different experimental subunit vaccines at 6 weeks post infection. Results are expressed as \log_{10} CFUs (mean \pm SD) of five animals per group. The experiment was repeated two times with similar results. Significant differences were determined by ANOVA and student 't' test. *** $p < 0.001$ as compared to naive controls. \$ $p < 0.05$ as compared to BCG immunized group.

3.3 Immunoreactivity of Culture Filtrate Fractions in STCF Immunized Mice

The specificity of the T-cell responses induced by the immunization with STCF was evaluated by stimulating splenic T-cells with narrow molecular mass fractions obtained by the multielution technique. Immune responses in terms of T cell proliferation, cytokine release and serum total IgG were studied at three weeks post immunization. The proliferation represented as stimulation indices (Figure 4A) of splenocytes from STCF immunized animals were found to be significantly higher ($p \leq 0.001$) than those of sham immunized group ($S.I < 1$ and data not shown) indicating a broader recognition of secreted antigen fractions. When the lymphocyte proliferation induced by protective fractions F7, F8 and F11 were analysed, higher stimulation indices ($S.I > 6$) were observed thus indicating high immunogenicity of these fractions.

As observed in case of stimulation indices, culture filtrate fractions elicited significantly higher ($p < 0.001$) levels of IFN- γ (Th1 cytokine) as compared to unvaccinated animals. The levels were notably higher (≥ 500 pg/ml) in all the fractions except fraction number F1 (Figure 4B). Another Th1 cytokine important for the protective immunity against Mtb is IL-12p40 and its secretion was also found to be significantly higher ($p < 0.001$) in the immunized animals as compared to unvaccinated animals. However, prominent secretion of IL-12p40 (> 500 pg/ml) was observed with proteins in the molecular weight range of 21-45 kDa comprising fraction F7 to F12 (Figure 4C). IFN- γ and IL-12p40 secretion in response to STCF was higher as compared to the individual culture filtrate fractions in the immunized group. Further, amongst fractions also, F12-F15 were although not providing significant protection against experimental tuberculosis but were able to induce prominent cytokine release (Figure 3b, 3c). Titers of antigen-specific IgG were evaluated in the sera of immunized as well as control mice by indirect ELISA to study the humoral responses. The animals immunised with STCF elicited substantial levels of IgG response to all the culture filtrate fractions as compared to the sham-immunized animals (data not shown). However, higher levels of IgG in terms of absorbance (OD 492 nm ≥ 0.5) were observed with protein fractions F7 to F15.

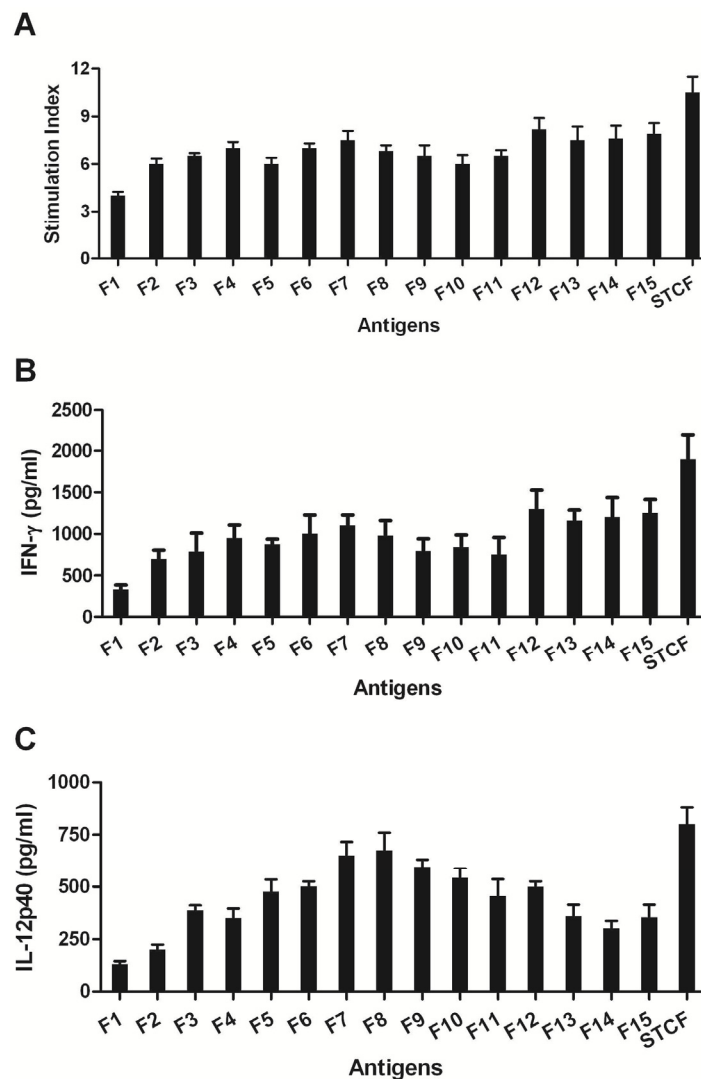


Figure 4. Immune responses to narrow molecular mass culture filtrate protein fractions in STCF immunized mice after three weeks of immunization

A) Splenocyte proliferation response. Results are presented as stimulation indices and each value has been calculated as mean \pm standard deviation (error bars) of stimulation indices obtained in all the five animals in each group. B) IFN- γ levels and C) IL-12p40 levels. Results represent mean \pm S.D values of five mice tested individually. The IFN- γ and IL-12p40 levels in unstimulated wells (culture without antigens) were below 100 and 50 pg/ml, respectively.

3.4 Immunoreactivity of Culture Filtrate Fractions in STCF Immunized Guinea Pigs.

Immune responses in terms of T cell proliferation and serum IgG levels were also studied at three weeks post immunization of guinea pigs with STCF. The stimulation indices of splenocytes of immunized groups were found to be significantly higher ($p \leq 0.001$) than those of sham immunized group ($SI < 1$) (Figure 5A). However, as observed in mouse model, higher responses ($SI \geq 6$) were also observed with proteins in the molecular weight range of 21-45kDa comprising fractions F7 to F12 in STCF immunized guinea pigs. The immunization also resulted in higher IgG response ($OD_{492nm} \geq 0.2$) for all the culture filtrate fractions as compared to the sham-immunized animals ($OD_{492nm} < 0.1$). However, among the fifteen fractions, F6-F15 showed higher IgG responses ($OD_{492nm} \geq 0.5$) (Figure 5B).

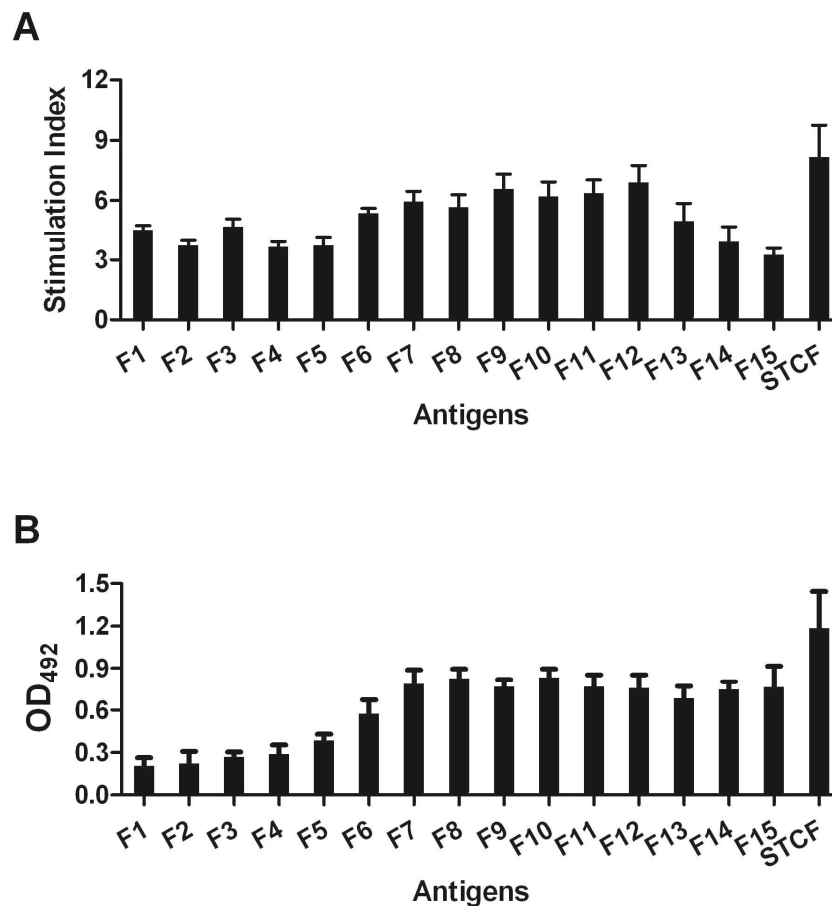


Figure 5. Lymphocyte proliferation (A) and serum IgG (B) responses towards narrow molecular mass culture filtrate fractions of STCF in guinea pigs immunized with STCF

a) The results are expressed as SI (mean \pm SD) of five immunized animals per group. b) Results represent antigen specific IgG in term of OD at 492nm (mean \pm S.D) in the sera of five animals tested individually

3.5 Immunoreactivity of Culture Filtrate Fractions in *Mtb* Infected Mice

To evaluate the total secretory proteome for its recognition by the immune system during experimental mycobacterial infection, unimmunized mice were challenged with 0.1 ml (10^5 bacilli) suspension of *M. tuberculosis* H₃₇Rv intravenously, and two weeks post infection splenocytes were isolated and IFN- γ and IL-12 cytokine secretions were measured in response to culture filtrate fractions. The response was directed to multiple secreted antigens and significantly higher ($p < 0.001$) levels of IFN- γ were elicited by culture filtrate fractions in infected animals as compared to uninfected animals (≤ 500 pg/ml) (Fig. 6A). IL-12p40 secretion (Fig. 6B) was also significantly ($p < 0.001$) higher in the infected animals as compared to uninfected animals (undetectable). However, amongst all the fractions, F1 to F11 having proteins in the molecular weight range of 3-42kDa induced both high IFN- γ and IL-12p40 levels. On contrary, the proteins corresponding to fractions F12-F15, which were inducing

strong cytokine, release in STCF immunized animals were not being recognized by immune system during experimental infection.

Thus, based on protection studies in two experimental models (mice and guinea pigs), three culture filtrate fractions of STCF i.e. fraction F7, F8 and F11 comprising the proteins in the molecular weight range of 20 to 42kDa (Table 1) were found to be most protective. Further, analysis of the immune responses to all the fifteen fractions of STCF indicated that although these three protective fractions were also able to elicit prominent immune responses both during Mtb challenge and after immunization in two different animal models, there were other fractions also which were inducing strong Th1 immune responses, not correlating with the protection induced by these fractions..

4. Discussion

Recent advances in mycobacterial genome characterization, identification of protective antigens, understanding the molecular basis of protective immune response, adjuvant design and development have reinforced the idea of a feasible subunit vaccine for tuberculosis (TB). The development of new antituberculous vaccine(s) requires an understanding of the protective immune response against TB. Despite recent advances, relatively little is known regarding the parameters involved in the protective immunity to tuberculosis in animal species. Based on the experimental studies in mice infected with Mtb, the cytokines considered to be involved in resistance to tuberculosis are interferon- γ produced by T cells (Orme, 2003) and IL-12 from antigen presenting cells that further leads to the induction of IFN- γ (Flynn & Chan, 2001). However, it has been demonstrated that no correlation exists between high Th1 response, at least high IFN- γ , induced during natural infection and protection imparted after immunization (Hovav et al., 2003; Olsen et al., 2000; Sable et al., 2005). As there is no good *in vitro* correlate of protective immunity, one is left with the daunting task of screening each potential mycobacterial antigen for its protective efficacy.

Excretory/secretory polypeptides of mycobacteria released in culture filtrates have raised much interest as a source of protective antigens owing to their ability to induce a protection at a similar level as did vaccination with BCG (Brandt et al., 2000; Demissie et al., 1999; Sable et al., 2005). So, to identify protective antigen(s) from Mtb culture filtrate with potential use as TB vaccine candidates, a simple and effective method was employed. The Mtb total culture filtrate was fractionated into fifteen non-overlapping narrow molecular mass fractions consisting of 2-5 protein bands by whole gel elutor (Figure 1). The elutor acts as an electro-dialyzer and removes SDS from proteins, leaving culture filtrate protein fractions in a non-toxic physiological buffer, which can be used, directly in a various cellular assays. Till date, various narrow molecular mass fractions obtained by similar fractionation technique have been evaluated for immunological reactivity and shown to stimulate lymphocytes of humans (Demissie et al., 1999; Wilkinson, Belisle, Mincek, Wilkinson, & Toossi, 2000). Studies based on human immune recognition have shown that narrow molecular mass culture filtrate fractions of Mtb are recognised by healthy tuberculosis contacts and not by TB patients (Boesen et al., 1995). Successful protection against Mtb infection in an animal model has long been regarded as the best test of a vaccine's efficacy. Therefore, a direct comparative analysis of individual narrow molecular mass fractions adjuvanted with Dimethyl dioctyldecylammonium bromide (DDA) was carried out. To the best of our knowledge, this study is the first report in which the protective efficacy of culture filtrate fractions of whole secretory proteome of Mtb was carried out against experimental tuberculosis in two different animal models using DDA as adjuvant. The adjuvant DDA was used because it is known to induce strong cell mediated immune responses to various mycobacterial antigens without any toxic effects (Brandt et al., 2000; Hilgers & Snippe, 1992; Leal, Smedegard, Andersen, & Appelberg, 1999; Lindblad, Elhay, Silva, Appelberg, & Andersen, 1997). Out of the fifteen fractions of mycobacterial secretory proteome, the mice immunized with fraction F7 (20-24kDa) showed highest protection followed by fraction F11 (37-42kDa) and then by F8 (25-30kDa) (Fig. 2). The mouse is the most commonly used experimental animal model to study human tuberculosis. Initial screening is usually done in the mouse, which has the advantage of being relatively inexpensive, infection with *Mtb* induced lesions like those seen in the natural disease in humans and availability of a wide variety of immunological reagents such as monoclonal antibodies against surface markers and cytokines (Gupta & Katoch, 2009; Orme, McMurray, & Belisle, 2001).

To further evaluate the protective potential of fractions F7, F8 and F11, the guinea pig model of tuberculosis was tested. The guinea pig model is especially relevant to human tuberculosis clinically, immunologically and pathologically (Grover et al., 2009; Orme et al., 2001). In addition, guinea pigs are outbred and, therefore, successful vaccination reduces concerns that protective immunity could be mouse strain specific or MHC specific (Skeiky et al., 2005). Significant protection was observed against intramuscular challenge with Mtb H₃₇Rv by immunization with fraction F7 followed by fraction F11 and F8 as compared to sham-immunized animals (Figure 3). These results are in concordant with earlier studies in which immunization with secretory proteins of Mtb have

been shown to induce protective immunity in guinea pigs (Jain et al., 2008). These studies clearly demonstrate that in the mycobacterial secretory proteome, there are certain regions (21-42kDa), which are able to induce protection both in mouse and guinea pig models even better than BCG. Therefore, subunit vaccine development should be focused on the proteins of this region only.

The induction of Type-1 response dominated by IFN- γ and IL-12 following subunit vaccine immunization is usually considered essential for protection against tuberculosis (Leal et al., 1999; Wu et al., 2008). Experimentally, mice deficient in IFN- γ or in IL-12 have been reported to be highly susceptible to challenge with Mtb (Cooper, Magram, Ferrante, & Orme, 1997). Screening of narrow molecular mass fractions in mice immunized with STCF representing the whole secretory proteome of Mtb revealed highest release of IFN- γ by antigens with molecular masses >42kDa comprising fractions F12 to F15 whereas, higher secretion of IL-12 was elicited by fraction F7 and F8 (20-30kDa) (Fig. 4B and C). Taking the cut off 500pg/ml for both IFN- γ and IL-12, it was observed that these cytokines were mainly induced by the proteins in the molecular weight range of 20-42kDa comprising fractions F7 to F11 (Fig. 6). Surprisingly, the negligible protection observed in the case of animals immunized with fraction F12 to F15 was associated with high concentration of IFN- γ secretion in response to in vitro stimulation with these proteins. These observations can be explained on the findings of Majlessi *et al* (Majlessi et al., 2006), who recently demonstrated that induction of strong Th1 response (IFN- γ) to Ag85A and ESAT-6 did not confer any protection against Mtb challenge in C₅₇BL/6J mice. Moreover, various researchers have also found a positive correlation between the levels of the IFN- γ and the extent of pathology (Hovav et al., 2003; Turner et al., 2000; Vordermeier et al., 2002; Yermeev et al., 2000). The possibility of the fractions F12 to F15 comprising decoy proteins diverting immune responses towards biased high Th1 type resulting in pathology cannot be ruled out. Since histopathological studies were not carried out in the present study, pathological changes in the vaccinated animals cannot be commented upon. Screening of culture filtrate fractions for “built-in” immunogenicity in guinea pigs immunized with total culture filtrate also yielded predominant recognition of proteins in the molecular weight range of 18-45kDa, comprising fractions F6 to F12 (Figure 5A and B). Due to the lack of specific reagents for use in guinea pigs, immune responses only in terms of splenocyte proliferation and humoral IgG only were monitored.

Considering the similar immune recognition of STCF fractions in both mice and guinea pigs following immunisation with STCF, all the fifteen fractions of secretory proteome were evaluated for their immunoreactivity during early stage of mycobacterial infection in mouse model only. In contrast to immunogenicity studies, the cytokine release in infected animals was found to be directed towards F1-F11 indicating that there are certain proteins in the mycobacterial secretory proteome which have although good antigenicity but have poor in-built immunogenicity. Earlier we have suggested that it is important to consider the in-built immunogenicity for the vaccine design (Sable, Kalra, Verma, & Khuller, 2007). The protective fractions F7, F8 and F11 were found to be recognized by host immune system both during infection as well as immunization (Figure 4B and 4C; Figure 6A and 6B). On the other hand, there were certain non-protective fractions, which were being either recognized during infection (F1) or during immunization (F15). The poor protective potential of F1 comprising low molecular weight proteins is supported by earlier studies demonstrating that in spite of unique ability to produce marked levels of IFN- γ , majority of low molecular mass proteins identified as suitable vaccine candidates in T cell assays, failed to impart significant protection against experimental tuberculosis as compared to BCG (Kamath, Feng, Macdonald, Briscoe, & Britton, 1999; Morris, Kelley, Howard, Li, & Collins, 2000). If such an assumption is true, the progress of these low molecular weight polypeptides as useful vaccine candidates for new prophylactic interventions in long run, from animal experiments to clinical trials and for actual human use seems doubtful. The observations of the present study suggest that one cannot solely rely on the identification of candidate molecules on the basis of T cell antigenicity in existing infection models. Our initial characterizations on the basis of available monoclonal / polyclonal antibodies against well-characterised Mtb proteins reactivity (Sable et al., 2005; Sonnenberg & Belisle, 1997) with purified protein from these protective fractions are represented in Table 1.

Hence, Mtb antigens comprising protein fractions F7, F8 and F11 in the molecular weight range of 20-24 kDa, 25-30 kDa and 37-42 kDa respectively, which showed protective efficacy in two animal models, can be considered as ideal candidates for the constitution of a subunit vaccine against tuberculosis. These results pinpoint the influence of antigen selection and emphasize the necessity of pursuing such kind of more studies to clearly delineate the markers of protection against TB for the selection of successful antituberculous vaccine candidates.

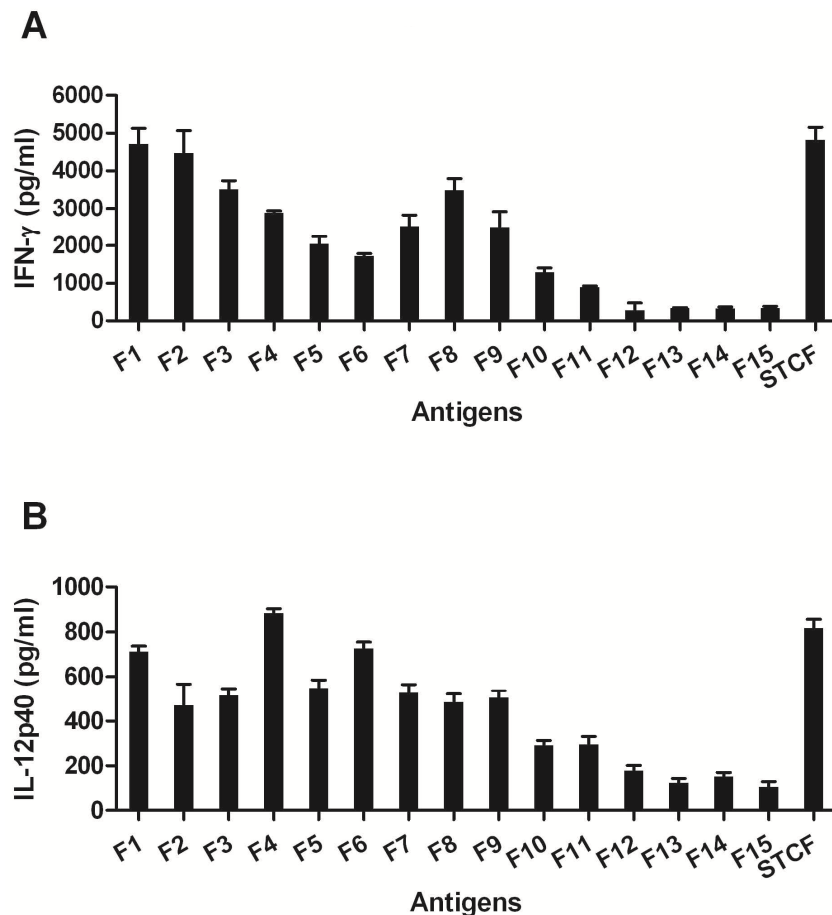


Figure 6. Cellular immune responses towards narrow molecular mass culture filtrate fractions of STCF in mice challenged with *M. tuberculosis* H₃₇Rv

IFN- γ Levels (A) and IL-12p40 levels (B) were measured in the supernatant of splenocytes *in vitro* stimulated with culture filtrate fractions or STCF. The IFN- γ and IL-12 production in unstimulated wells (culture without antigens) was below 250 and 120 pg/ml, respectively. Results represent mean \pm S.D values of five mice from each group tested individually.

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References

- Aktas, E., Ciftci, F., Bilgic, S., Sezer, O., Bozkanat, E., Deniz, O., ... Deniz, G. (2009). Peripheral immune response in pulmonary tuberculosis. *Scand J Immunol*, 70(3), 300-308. <http://dx.doi.org/10.1111/j.1365-3083.2009.02294.x>
- Andersen, P., Askgaard, D., Gottschau, A., Bennedsen, J., Nagai, S., & Heron, I. (1992). Identification of immunodominant antigens during infection with *Mycobacterium tuberculosis*. *Scand J Immunol*, 36(6), 823-831.
- Boesen, H., Jensen, B. N., Wilcke, T., & Andersen, P. (1995). Human T-cell responses to secreted antigen fractions of *Mycobacterium tuberculosis*. *Infect Immun*, 63(4), 1491-1497.
- Brandt, L., Elhay, M., Rosenkrands, I., Lindblad, E. B., & Andersen, P. (2000). ESAT-6 subunit vaccination against *Mycobacterium tuberculosis*. *Infect Immun*, 68(2), 791-795.
- Brennan, M. J., & Thole, J. (2012). Tuberculosis vaccines: a strategic blueprint for the next decade. *Tuberculosis (Edinb)*, 92 Suppl 1, S6-13. [http://dx.doi.org/10.1016/S1472-9792\(12\)70005-7](http://dx.doi.org/10.1016/S1472-9792(12)70005-7)

- Cooper, A. M., Magram, J., Ferrante, J., & Orme, I. M. (1997). Interleukin 12 (IL-12) is crucial to the development of protective immunity in mice intravenously infected with mycobacterium tuberculosis. *J Exp Med*, *186*(1), 39-45.
- Demissie, A., Ravn, P., Olobo, J., Doherty, T. M., Eguale, T., Geletu, M., ... Britton, S. (1999). T-cell recognition of Mycobacterium tuberculosis culture filtrate fractions in tuberculosis patients and their household contacts. *Infect Immun*, *67*(11), 5967-5971.
- Fine, P. E. (1995). Variation in protection by BCG: implications of and for heterologous immunity. *Lancet*, *346*(8986), 1339-1345.
- Flynn, J. L., & Chan, J. (2001). Immunology of tuberculosis. *Annu Rev Immunol*, *19*, 93-129. <http://dx.doi.org/10.1146/annurev.immunol.19.1.93>
- Goldenhuis, H., Mearns, H., Miles, D. J., Tameris, M., Hokey, D., Shi, Z., ... de Bruyn, G. (2015). The tuberculosis vaccine H4:IC31 is safe and induces a persistent polyfunctional CD4 T cell response in South African adults: A randomized controlled trial. *Vaccine*, *33*(30), 3592-3599. <http://dx.doi.org/10.1016/j.vaccine.2015.05.036>
- Grover, A., Taylor, J., Troudt, J., Keyser, A., Arnett, K., Izzo, L., . . . Izzo, A. (2009). Kinetics of the immune response profile in guinea pigs after vaccination with BCG and infection with Mycobacterium tuberculosis. *Infect Immun*. <http://dx.doi.org/10.1128/IAI.00704-09>
- Gupta, U. D., & Katoch, V. M. (2009). Animal models of tuberculosis for vaccine development. *Indian J Med Res*, *129*(1), 11-18.
- Haslov, K., Andersen, A., Nagai, S., Gottschau, A., Sorensen, T., & Andersen, P. (1995). Guinea pig cellular immune responses to proteins secreted by Mycobacterium tuberculosis. *Infect Immun*, *63*(3), 804-810.
- Hilgers, L. A., & Snippe, H. (1992). DDA as an immunological adjuvant. *Res Immunol*, *143*(5), 494-503; discussion 574-496.
- Hovav, A. H., Mullerad, J., Davidovitch, L., Fishman, Y., Bigi, F., Cataldi, A., & Bercovier, H. (2003). The Mycobacterium tuberculosis recombinant 27-kilodalton lipoprotein induces a strong Th1-type immune response deleterious to protection. *Infect Immun*, *71*(6), 3146-3154.
- Jain, R., Dey, B., Dhar, N., Rao, V., Singh, R., Gupta, U. D., ... Tyagi, A. K. (2008). Enhanced and enduring protection against tuberculosis by recombinant BCG-Ag85C and its association with modulation of cytokine profile in lung. *PLoS ONE*, *3*(12), e3869. <http://dx.doi.org/10.1371/journal.pone.0003869>
- Kamath, A. T., Feng, C. G., Macdonald, M., Briscoe, H., & Britton, W. J. (1999). Differential protective efficacy of DNA vaccines expressing secreted proteins of Mycobacterium tuberculosis. *Infect Immun*, *67*(4), 1702-1707.
- Kaufmann, S. H., Cole, S. T., Mizrahi, V., Rubin, E., & Nathan, C. (2005). Mycobacterium tuberculosis and the host response. *J Exp Med*, *201*(11), 1693-1697. <http://dx.doi.org/10.1084/jem.20050842>
- Leal, I. S., Smedegard, B., Andersen, P., & Appelberg, R. (1999). Interleukin-6 and interleukin-12 participate in induction of a type 1 protective T-cell response during vaccination with a tuberculosis subunit vaccine. *Infect Immun*, *67*(11), 5747-5754.
- Lindblad, E. B., Elhay, M. J., Silva, R., Appelberg, R., & Andersen, P. (1997). Adjuvant modulation of immune responses to tuberculosis subunit vaccines. *Infect Immun*, *65*(2), 623-629.
- Majlessi, L., Simsova, M., Jarvis, Z., Brodin, P., Rojas, M. J., Bauche, C., ... Leclerc, C. (2006). An increase in antimycobacterial Th1-cell responses by prime-boost protocols of immunization does not enhance protection against tuberculosis. *Infect Immun*, *74*(4), 2128-2137. <http://dx.doi.org/10.1128/IAI.74.4.2128-2137.2006>
- Morris, S., Kelley, C., Howard, A., Li, Z., & Collins, F. (2000). The immunogenicity of single and combination DNA vaccines against tuberculosis. *Vaccine*, *18*(20), 2155-2163. [http://dx.doi.org/S0264-410X\(99\)00540-X](http://dx.doi.org/S0264-410X(99)00540-X)
- Morrissey, J. H. (1981). Silver stain for proteins in polyacrylamide gels: a modified procedure with enhanced uniform sensitivity. *Anal Biochem*, *117*(2), 307-310. [http://dx.doi.org/0003-2697\(81\)90783-1](http://dx.doi.org/0003-2697(81)90783-1)
- Olsen, A. W., Hansen, P. R., Holm, A., & Andersen, P. (2000). Efficient protection against Mycobacterium tuberculosis by vaccination with a single subdominant epitope from the ESAT-6 antigen. *Eur J Immunol*, *30*(6), 1724-1732. [http://dx.doi.org/10.1002/1521-4141\(200006\)30:6<1724::AID-IMMU1724>3.0.CO;2-A](http://dx.doi.org/10.1002/1521-4141(200006)30:6<1724::AID-IMMU1724>3.0.CO;2-A)
- Orme, I. M. (2003). The mouse as a useful model of tuberculosis. *Tuberculosis (Edinb)*, *83*(1-3), 112-115. <http://dx.doi.org/S1472979202000690>
- Orme, I. M., McMurray, D. N., & Belisle, J. T. (2001). Tuberculosis vaccine development: recent progress. *Trends Microbiol*, *9*(3), 115-118. [http://dx.doi.org/S0966-842X\(00\)01949-1](http://dx.doi.org/S0966-842X(00)01949-1)
- Sable, S. B., Kalra, M., Verma, I., & Khuller, G. K. (2007). Tuberculosis subunit vaccine design: the conflict of antigenicity and immunogenicity. *Clin Immunol*, *122*(3), 239-251. <http://dx.doi.org/10.1016/j.clim.2006.10.010>

- Sable, S. B., Kaur, S., Verma, I., & Khuller, G. K. (2005). Immunodominance of low molecular weight secretory polypeptides of *Mycobacterium tuberculosis* to induce cytotoxic T-lymphocyte response. *Vaccine*, *23*(41), 4947-4954. <http://dx.doi.org/10.1016/j.vaccine.2005.04.046>
- Sable, S. B., Kumar, R., Kalra, M., Verma, I., Khuller, G. K., Dobos, K., & Belisle, J. T. (2005). Peripheral blood and pleural fluid mononuclear cell responses to low-molecular-mass secretory polypeptides of *Mycobacterium tuberculosis* in human models of immunity to tuberculosis. *Infect Immun*, *73*(6), 3547-3558. <http://dx.doi.org/10.1128/IAI.73.6.3547-3558.2005>
- Sable, S. B., Verma, I., Behera, D., & Khuller, G. K. (2005). Human immune recognition-based multicomponent subunit vaccines against tuberculosis. *Eur Respir J*, *25*(5), 902-910. <http://dx.doi.org/10.1183/09031936.05.00105404>
- Skeiky, Y. A., Alderson, M. R., Ovendale, P. J., Lobet, Y., Dalemans, W., Orme, I. M., . . . Campos-Neto, A. (2005). Protection of mice and guinea pigs against tuberculosis induced by immunization with a single *Mycobacterium tuberculosis* recombinant antigen, MTB41. *Vaccine*, *23*(30), 3937-3945. <http://dx.doi.org/10.1016/j.vaccine.2005.03.003>
- Skeiky, Y. A., & Sadoff, J. C. (2006). Advances in tuberculosis vaccine strategies. *Nat Rev Microbiol*, *4*(6), 469-476. <http://dx.doi.org/10.1038/nrmicro1419>
- Sonnenberg, M. G., & Belisle, J. T. (1997). Definition of *Mycobacterium tuberculosis* culture filtrate proteins by two-dimensional polyacrylamide gel electrophoresis, N-terminal amino acid sequencing, and electrospray mass spectrometry. *Infect Immun*, *65*(11), 4515-4524.
- Tameris, M. D., Hatherill, M., Landry, B. S., Scriba, T. J., Snowden, M. A., Lockhart, S., ... Team, M. A. T. S. (2013). Safety and efficacy of MVA85A, a new tuberculosis vaccine, in infants previously vaccinated with BCG: a randomised, placebo-controlled phase 2b trial. *Lancet*, *381*(9871), 1021-1028. [http://dx.doi.org/10.1016/S0140-6736\(13\)60177-4](http://dx.doi.org/10.1016/S0140-6736(13)60177-4)
- Turner, O. C., Roberts, A. D., Frank, A. A., Phalen, S. W., McMurray, D. M., Content, J., ... Orme, I. M. (2000). Lack of protection in mice and necrotizing bronchointerstitial pneumonia with bronchiolitis in guinea pigs immunized with vaccines directed against the hsp60 molecule of *Mycobacterium tuberculosis*. *Infect Immun*, *68*(6), 3674-3679.
- Vordermeier, H. M., Chambers, M. A., Cockle, P. J., Whelan, A. O., Simmons, J., & Hewinson, R. G. (2002). Correlation of ESAT-6-specific gamma interferon production with pathology in cattle following *Mycobacterium bovis* BCG vaccination against experimental bovine tuberculosis. *Infect Immun*, *70*(6), 3026-3032.
- Wilkinson, K. A., Belisle, J. T., Mincek, M., Wilkinson, R. J., & Toossi, Z. (2000). Enhancement of the human T cell response to culture filtrate fractions of *Mycobacterium tuberculosis* by microspheres. *J Immunol Methods*, *235*(1-2), 1-9. [http://dx.doi.org/S0022-1759\(99\)00200-8](http://dx.doi.org/S0022-1759(99)00200-8)
- Wu, B., Huang, C., Kato-Maeda, M., Hopewell, P. C., Daley, C. L., Krensky, A. M., & Clayberger, C. (2008). IL-9 is associated with an impaired Th1 immune response in patients with tuberculosis. *Clin Immunol*, *126*(2), 202-210. <http://dx.doi.org/10.1016/j.clim.2007.09.009>
- Yermeev, V. V., Lyadova, I. V., Nikonenko, B. V., Apt, A. S., Abou-Zeid, C., Inwald, J., & Young, D. B. (2000). The 19-kD antigen and protective immunity in a murine model of tuberculosis. *Clin Exp Immunol*, *120*(2), 274-279.

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