CHAPTER 4

DISCUSSION
Iron acquisition systems and the regulatory role of iron on the expression of specific virulence determinants / toxins are well understood in several bacterial systems (Sritharan, 2006; Ratledge, 2004; Sritharan, 2000). Of special importance is the regulation by iron of virulence determinants and toxins, as seen in *E. coli*, *C. diphtheriae* and others. Pathogenic mycobacteria, including *M. tuberculosis* do not produce any known virulence factors / toxins. However, it is highly likely that proteins, which play an important role in the host-pathogen interactions that facilitate the survival of the pathogen in the environment of the host are influenced by iron levels. The objective of this study was to study the influence of iron levels on the secreted proteins in *M. tuberculosis* and study the immune response of the lymphocytes from tuberculosis patients to the CFPs grown under high and low iron conditions. In the first part of the study, efforts were made to identify iron-regulated culture filtrate proteins expressed by *M. tuberculosis* grown under high (8 µg Fe / mL) and low iron (0.02 µg Fe / mL) conditions. The proteins in the spent growth medium, referred to as culture filtrate proteins (CFPs), due to their low levels were initially concentrated by ammonium sulphate precipitation and ultrafiltration. Single dimension SDS-PAGE was done to separate and analyse the fractions. However, no notable differences were appreciated between the CFPs grown under high and low iron organisms. They were then analysed on 2D-PAGE, which resolved the proteins into discrete spots; several proteins were up regulated under high and low iron conditions of growth. Identification of several of these proteins was done by techniques including MALDI-TOF MS and immunoblotting. In the second part of the study, defined culture filtrate antigen pools (CF-Ag pools) were prepared and the immune response of the peripheral blood mononuclear cells from tuberculosis patients was assessed and compared with that of control healthy individuals with no evidence of the disease. Immune proliferation response and the expression of IFN-γ in response to these antigens, highlighting the role of IFN-γ in the protection against disease development is presented in this study.

There is increasing evidence to show that the mammalian host limits iron (Kochan 1976, Gold *et al.* 2001) and iron is one of the contributing factors in determining the outcome of an infection by *M. tuberculosis*. Within the intracellular environment of the macrophage, *M. tuberculosis* is exposed to conditions of iron limitation due to the presence of lactoferrin within the macrophage that can hold iron even at low pH. Iron is essential for the growth of *M. tuberculosis*. The genome
analysis shows that as many as 40 enzymes are iron-dependant, some of which play vital roles in electron transport and other metabolic processes (Sritharan, 2000). Like other bacteria, pathogenic mycobacteria face conditions of iron limitation within the mammalian host. Very little free iron is present as most of it is held as protein-bound iron. Transferrin in the human serum has been demonstrated to be tuberculostatic, and this effect can be reversed by the addition of iron (Kochan, 1976). Thus the administration of iron to tuberculosis patients should be done with caution as it can exacerbate the disease (Ratledge, 2004).

The siderophore-mediated iron acquisition machinery in mycobacteria is well delineated from studies in in vitro grown cultures. Mycobacteria are unique in that they produce two forms of siderophores, the intracellular mycobactin and the extracellular carboxymycobactin / exochelins. These siderophores have a high affinity for the ferric iron and can remove iron from the insoluble ferric hydroxide and from iron-withholding proteins. The carboxymycobactins scavenge the iron and deliver it to the inside, though the mechanism of internalization is not understood. According to Ratledge (2004), mycobactin serves as a temporary store for the acquired iron. Iron is ultimately held in bacterioferritin. Mycobactin biosynthesis by the polyketide pathway is well understood (Quadri et al. 1998). The components for the biosynthesis of the siderophore are encoded in the mbt gene cluster. De Voss et al. (2000) developed a mycobactin-deficient mbtB mutant and showed that the inability to synthesise mycobactin resulted in the poor infectivity of the macrophage and low growth of the M. tuberculosis mutant, thus establishing that this is a virulence determinant.

Iron regulation is perhaps complex in mycobacteria, as more than one regulator is seen. The genome shows four regulators, among which the IdeR (iron dependant regulator) is well characterised. Based on its homology to DtxR, IdeR was initially predicted to function as an iron-dependant transcriptional repressor. Transcripational profiling of the mutant vs the parental strain showed that about one third of the iron-regulated genes in M. tuberculosis are regulated by IdeR (Rodriguez et al., 2002). IdeR controls the genes involved in siderophore production and iron storage and in addition, controls genes encoding putative transporters, transcriptional regulators, proteins involved in general metabolism, members of the PE/PPE family of conserved mycobacterial proteins. The furA gene (encoding the FurA regulator) is located upstream of the katG gene and its expression is negatively regulated. The
katG gene expression is regulated by iron levels, as shown by other studies in our lab (Yeruva et al., 2005; Sritharan et al., 2006).

The influence of iron levels on cell surface proteins was first studied in *M. smegmatis* (Hall et al., 1987). At least five IREPs were detected in envelope extracts from *M. smegmatis* grown in iron-deficient conditions, which also included the 29 kDa protein. This protein can associate directly *in vitro* with ferri-exochelin, and the addition of a polyclonal antiserum generated against it to *M. smegmatis* cells significantly inhibits ferri-exochelin mediated iron uptake. Based on these observations, the 29 kDa protein has been postulated to be a ferri-exochelin receptor in *M. smegmatis*. IREPs were also identified in other mycobacterial species not only under defined lab conditions of established iron status but also under *in vivo* conditions. The 21 kDa IREP was expressed in *M. leprae* and *M. avium* recovered from experimentally infected animals (Sritharan & Ratledge, 1990). Recent studies on *M. tuberculosis* in our lab showed the coordinated expression of mycobactin, carboxymycobactin and Irep-28 upon iron limitation (Yeruva et al., 2006). The latter was identified as HupB, the DNA-binding HU homologue. The clinical correlation of the detection of anti-HupB antibodies with culture and radiological confirmation showed the significance of this protein.

In the light of the observations on the influence of iron on several proteins, the effect of iron on the expression of CFPs was studied. As established earlier (Yeruva et al., 2006), maximal levels of carboxymycobactin and mycobactin were found to be expressed in *M. tuberculosis* grown under low iron conditions (0.02 µg Fe / mL), which was about 5 - 6 times higher than organisms grown with iron added at 8 µg Fe / mL (high iron). The spent growth medium, recovered by centrifugation followed by filtration through 0.45 µm membrane to ensure the complete removal of all bacterial cells was processed further. The CFPs were concentrated by precipitation with ammonium sulphate followed by ultrafiltration. Electrophoretic separation by single dimension SDS-PAGE did not reveal any differences in the protein profile of the CFPs of high and low iron grown organisms, harvested after about 14 days of growth, when the cells were in the log phase of growth. However, when allowed to grow till day 20, differences in the protein profile was observed. Proteins of approximate molecular mass of 60, 27, 17 and 8 kDa proteins were expressed by low iron
organisms. Further analysis of these proteins was not pursued as they could be cytoplasmic proteins released from dead cells and/or stress proteins.

2D-PAGE analysis of CFPs of *M. tuberculosis* was attempted by Young and Garbe (1991); they identified DnaK, GroES, GroEL and four novel proteins, recognized as heat shock proteins. Simultaneously, Nagai *et al.* (1991), using 2D-PAGE and N-terminal sequencing identified the 12 most abundant proteins from the spent growth medium of *M. tuberculosis*. Subsequently, 2D–PAGE coupled with peptide mass printing identified ORFs, which are not revealed by genome sequencing. A number of techniques can be used for the identification of proteins from 2D-PAGE gels; they include N-terminal sequence analysis, mass spectrometry, amino acid composition analysis, co-migration studies and immunodetection, the latter techniques being applicable for small concentration of the protein samples, while the first three techniques were considered applicable for large-scale samples (Rosenkrands *et al.*, 2000; Malen *et al.*, 2007). 2D maps have been generated by these groups and it is now possible to assign, with certain degree of accuracy the details of the protein(s) by comigrational analysis, based on the pI and molecular mass of the protein(s).

In this study, several iron-regulated proteins were identified by 2D–PAGE. They are represented as CFP with their respective molecular mass written as superscript. Iron induced the expression of several proteins (Table 9), including CFP$^{65}$, CFP$^{44}$, CFP$^{40a, 40b}$, CFP$^{35}$, CFP$^{30c}$, CFP$^{23}$, CFP$^{22b}$, CFP$^{13}$, CFP$^{12.5}$, CFP$^{9a}$ and CFP$^{9b}$. The CFP$^{30c}$ and CFP$^{23}$ protein were subjected to MALDI-TOF analysis and shown to be MCE1B (Rv0170) and Rv0660 respectively. The former also reported by Malen *et al.* (2007) is a member of the MCE family of proteins, with a putative role in host cell invasion. The latter is a conserved hypothetical protein, possibly an antitoxin that is part of toxin-antitoxin (TA) operon along with Rv0659c, encoding the toxin. Iron limitation induced the expression of CFP$^{70}$, CFP$^{55}$, CFP$^{30a, 30b}$, CFP$^{31}$, CFP$^{28a, 28b}$, CFP$^{22a, 22c}$, CFP$^{11a, 11b}$, CFP$^{10}$ and CFP$^{8}$. The CFP$^{70}$ is DnaK, as also seen in the 2D maps of Statens Serum Institute and Max Plank Institute. It is a chaperone protein with ATPase activity and thought to be induced by stress conditions. Of the three proteins spots of about 22 kDa, CFP$^{22a}$ and CFP$^{22c}$ were induced upon iron limitation; the functions of these proteins are unclear with the former belonging to rRNA methylase family of proteins while the latter is a hypothetical protein. The Sec pathway is highly conserved and is the primary pathway for protein transport across
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the cytoplasm of mycobacteria. Proteins transported by the Sec pathway are synthesized as precursors with sec sequence at their amino terminal. Sec precursor proteins are transported in an unfolded state through the core of the translocase, which is the membrane embedded channel, composed of SecY and SecE proteins (Braunstein et al., 2001). CFP\textsuperscript{10}, identified as the pre-protein translocase SecE1 subunit and CFP\textsuperscript{8} as a probable 50 S ribosomal proteins L10RP need further analysis to understand the influence of iron on their expression as well as their role under in vivo conditions of growth. Kitaura et al. (1999), in their studies on PPD showed that two of the secretory proteins were the ribosomal proteins L7 and L12. Further, a combination of these two proteins L7/L12 induced a strong delayed-type hypersensitivity reaction in guinea pigs. Another subunit of the 50S ribosome is L22 (Rv0706), which is an IdeR- independent iron induced gene (Rodriguez et al., 2003).

As mentioned earlier, regulation by iron is complex in mycobacteria with more than one regulator and it is likely that the iron machinery and oxidative stress machinery are associated and gene regulation in these bacteria are so finely tuned to adapt them to the intracellular hostile environment of the macrophage. CFP\textsuperscript{55} and CFP\textsuperscript{11a} were recognized as glutamine synthetase and the GroES respectively by comigrational analysis. Of special significance is the ESAT-6 family of proteins, some members of which are found to iron-regulated.

There is a wealth of information on the ESAT-6 family of proteins (Brandt et al., 2000, 1996, Brodin et al., 2006). The significance of these proteins stems from the observation that they are restricted to the pathogenic \textit{M. tuberculosis} and they are lost in the attenuated \textit{M. bovis} BCG, as a part of the RD1 region not seen in the latter as well in a majority of environmental mycobacteria (Maharis et al., 1996). Genome analysis data and 2D map data, coupled with immuno-detection using specific monoclonal antibodies have shed light on this important group of mycobacterial proteins. Certain isoforms of the ESAT-6 molecule from \textit{M. tuberculosis}, separated by two-dimensional electrophoresis, were identified as carrying a post translational modification, namely an acetylation of the threonine residue at position 2, and it was observed that only the unacetylated form of ESAT-6 interacted with CFP-10 (Okkels et al., 2004). The implications of ESAT-6 in virulence and the diagnostic potential of these proteins for sero-diagnosis are gaining strength with experimental evidence from several reports. It is useful as a diagnostic marker to differentiate between the BCG vaccinated healthy individuals and the tuberculosis patients, with high
specificity and sensitivity (Ravn et al., 1999). Also, it is used to evaluate the T cell immune response as it stimulates T cells from tuberculosis patients to proliferate and produce IFN-\(\gamma\). It is thus used in the IFN-\(\gamma\) commercial kits currently used in several clinical laboratories.

The first preparation of ESAT-6 available for biochemical characterization was purified from \(M.\, tuberculosi\)s culture filtrate and the protein was expected to have a pI of 4.48 and a mass of 9.8 kDa. Initially studies by Sorensen et al. (1995) had identified 2 reactive spots with ESAT-specific monoclonal antibodies HYB 76.8 between the pI 4 - 4.8. Modification of the protocol by Okkels et al. (2004) identified as many as 8 reactive spots with HYB76.8. In our study here, we used HYB 76.8 for immunoblotting analysis. Two 9 kDa protein spots, recognized by HYB 76.8 were expressed constitutively while the 8 kDa protein was repressed upon iron limitation. The time course study also showed increased expression of ESAT-6 (it was possible to identify only one band of 9 kDa) in high iron organisms when compared to low limited cells. These observations need further analysis to understand the role of iron in this important class of proteins implicated in virulence. It may shed light on post-translational modifications, if any, of some of the members that perhaps could play a significant role in host-pathogen interactions.

In this study, we also analysed the different strains of \(Mycobacterium\, bovis\) BCG, which was first obtained by serial passages as an attenuated strain of the virulent \(M.\, bovis\) in 1921. Since then, it has moved to several countries around the world, resulting in several strains. Reports on the variations among these strains are available. IS6110-based restriction-fragment-length- polymorphism (RFLP) showed two patterns for BCG strains (Bher et al., 1999). Some BCG strains contain two copies of this transposable element and other strains of BCG only have one copy. The strains Japan Russia, Moreau contain two copies while the others contain one copy of IS6110. MPT64 was another genetic variant in BCG. Genetic analysis documented that the gene encoding this antigen, \(mpt64\), was absent in certain strains of BCG. Using the historical record of strain dissemination as reference, it was noted that two copies of IS6110 are only seen in strains obtained before 1925 (strains Russia, Moreau and Japan) and that absence of the \(mpt64\) gene coincides exactly with BCG strains obtained from the Pasteur Institute after 1927.

In this study, we included \(M.\, bovis\) BCG Danish, Birkhaug, Phipps, Moreau, Sweden, Phipps and Pasteur to analyse the effect of iron concentration on the CFP
profile of the different BCG strains. All the BCG strains responded to iron deprivation and expressed significant levels of both mycobactin and carboxymycobactin. Maximal level of mycobactin was expressed by Danish strain, with the lowest expression in Russia. Several iron-regulated CFPs were seen, as mentioned in results. However, further work is required to understand the role of these proteins.

The objective in the second half of this study was to look at the immune response of the lymphocytes from tuberculosis patients to the CFPs of *M. tuberculosis*. Due to the low levels of these iron-regulated proteins, and also due to the fact that antigen pools are better candidates for study, we prepared culture filtrate antigen (CF-Ag) pools from high and low iron grown organisms of *M. tuberculosis*. The selection of these antigen pools was done based on molecular weight cut-offs. The CFPs were separated by preparative gel electrophoresis and the gel slices between specified markers were cut out and the proteins eluted, as detailed in methods. The eluted antigen pools were re-run, both by single dimension PAGE and 2D – PAGE and compared with the crude CFPs. Our protocols were designed based on published data and strategies followed by other researchers to identify CFPs for studies in T-cell proliferation. In some of the methods, the CFPs were initially eluted from gel slices (carrying specific protein bands / spots) using SDS-containing buffer followed by the removal of SDS by high-pressure dialysis. In the T-cell Western blot method, nitrocellulose membrane carrying the CFPs (after transfer from gel subjected to SDS-PAGE) was cut into small pieces carrying closely associated proteins and then adding these nitrocellulose strips to T cell cultures (Abou-Zeid *et al.*, 1987). This technique minimised the SDS in the cell cultures, but it had the disadvantage that proteins added could not be quantified and characterized. Mehra *et al.* (1989) used the T-cell western blot method to identify a low molecular mass fraction of *M. leprae* sonicate recognised by a high frequency of reactive *M. leprae* reactive human T-cell lines. Another method included the elution of the protein spots from 2D gels and the subsequent stimulation of lymphocytes using these gel-eluted proteins (Wallis *et al.*, 1993). This technique helped to identify a 58 kDa protein in *M. tuberculosis* which induced the production of TNF-α in cultures of human monocytes. This technique however had the disadvantage of low recovery of protein(s), thereby limiting quantitative and qualitative analysis. Recent studies have adopted the procedure of Andersen and Heron (1993), in which the CFPs were separated by SDS-PAGE and the gel was cut into slices and the proteins eluted from them. A modification of this
procedure is to electrophoretically elute out the protein fractions. The net objective of these procedures is to obtain a panel of antigens, representing proteins over a narrow molecular mass range.

All these efforts are being made to identify potential diagnostic antigens and proteins that are immuno-protective, with the objective of preparing effective vaccines (Abebe et al., 2007; Demisse et al., 1999). Till date, BCG is the only available tuberculosis vaccine. Since its discovery in 1921 by Calmette and Guerin in France, it has been distributed to several countries and is widely used for the control of tuberculosis, with more than three billion people receiving the vaccine. However, there is controversy about its efficacy, with the 10 year old trial in Chingleput, India (Tuberculosis Research Centre, 1999) showing it is ineffective in the population studied.

The outcome of an infection by M. tuberculosis is determined by a complex interplay of both the host and pathogen related factors. In tuberculosis, the cell-mediated immunity plays an important role. This involves a varied population of immune cells, including the macrophages, different subset of T-cells and interplay among them mediated by several cytokines. Phagocytes are activated to produce pro-inflammatory cytokines, including TNF-α, IL-1β, and IL-12. TNF-α is a monocyte-activating cytokine which stimulates anti-mycobacterial activity and helps to maintain the integrity of the tuberculous granulomas in which M. tuberculosis is contained (Tufariello et al., 2003). IL-12 links innate and adaptive immunity, drives T-cells and NK cells to produce Th1 pro-inflammatory cytokines, including IFN-γ and TNF-α (Hoeve et al., 2006). In synergy with TNF-α, IFN-γ activates infected macrophages and tries to eliminate intracellular pathogens as a major effector mechanism of CMI. T-lymphocytes, sensitized by an antigen undergo blastic transformation and produce IFN-γ when stimulated by the same antigen. The profile of secreted cytokines in vitro is taken as an indication of T-lymphocyte function in vivo, but the possible relationships between antigen specificity and T-lymphocyte function have so far not been examined. Murine CD4+ T cells have been divided into at least two different subsets (Th1 and Th2), based on the cytokine profiles that they secrete upon antigen stimulation. The Th1 cells characteristically secrete IL-2 and IFN-γ whereas the Th2 lymphocytes produce typically IL-4, IL-5 and IL-10, which enhance antibody synthesis of B cells. In vitro stimulation of leukocytes with mycobacteria or their products induces synthesis and release of several cytokines, including IL-1, IL-2, IL-
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6, TNF-α and IFN-γ. Two approaches have been taken to identify the cytokines that mediate protective immunity: characterization of the cytokine milieu at a local site of effective immunity and identification of cytokines that activate intracellular killing by mononuclear phagocytes. The pleural fluid and the mononuclear phagocytes show an over expression of IFN-γ, IL-2, and IL-12 with lowered expression of IL-4 (Sable et al., 2007).

As culture filtrate proteins are recognised by the host immune system, CFPs have been evaluated, either as individual proteins or as antigen pools (Abebe et al., 2007, Pai et al., 2004; Surekha et al., 2005). In this study, the CF-Ag pools (mentioned above) were used to study the T cell immune response of peripheral blood mononuclear cells (PBMCs) from three categories of tuberculosis patients, namely the smear positive (SP), smear negative (SN) and extrapulmonary (EP) cases respectively and compared with normal healthy (NH) individuals. Both immuno-proliferation and IFN-γ levels were analysed to assess the immune response of T cells. The study population is from Hyderabad, Andhra Pradesh and consists of patients being screened and treated in the Government Chest Hospital, Hyderabad. As indicated in methods, specific exclusion criteria were adopted and clear cases of smear positive (n = 25) and smear negative (n = 20) confirmed cases of pulmonary tuberculosis with no prior treatment for tuberculosis and a group of extra pulmonary tuberculosis (n = 20) were major components of the study. The normal healthy individuals (n = 20) included student donors who were not associated with the hospital area.

Lymphocyte proliferation in the four study groups was done with the whole CFPs from high and low iron grown *M. tuberculosis* and compared with that of human PPD. The latter is used as a positive control in several studies, due to the high stimulation achieved with this mixture of culture filtrate proteins (Kori et al., 2000, Van Crevel et al., 1999). The extrapulmonary group did not show notable proliferative response. In this study also, PPD evoked a strong proliferative response in cases of pulmonary tuberculosis, including both smear positive (SP) and negative (SN) group, unlike the control normal healthy (NH) individuals whose lymphocytes showed poor response. However, no differences were discerned between high and low iron preparations. This expected observation could be attributed to the large number of antigens in the crude preparations, resulting in a cumulative effect on proliferative response. Appreciable differences were however seen with defined CF-Ag pools, not only among the different pools from the respective high / low iron cells but also
between the corresponding CF-Ag pools from high and low iron grown cells. Statistical analysis of the data was done using SPSS software. The values are represented as medians since the data did not approximate normal distributions. This is consistent with the hypothesis that all the groups contain antigen responsive and antigen un-responsive individuals (Demissie et al., 1999).

The individual antigen pools, like the crude CFPs induced low levels of proliferation of the lymphocytes from the controls (NH). However, these values are relatively higher than expected, as this is an endemic area and most of the individuals are exposed to mycobacterial antigens. The extrapulmonary samples showed a similar effect, with a slightly higher response to PPD. Analysis of the different antigen pools on pulmonary tuberculosis cases showed significant findings. The stimulation index of the PBMCs of SP group in response to H1 and L1 CF-Ag pools showed a median SI of 2.3 and 2.47 respectively. This is close agreement with that seen with PPD (median SI of 2.5). Using PPD as antigen, case recognition in the SP group was 76%. A closer percentage of case recognition was seen with LI (68%) with a much lower percentage with H1 (60%), with case recognition of 60% as compared to 55% with both H1 and PPD. As mentioned above, low proliferation and poor recognition was observed with these two antigen pools; PPD showed a relatively significant response with 60% recognition of the extra-pulmonary cases. This first set of antigen pools contained proteins in the molecular weight range of 66 – 116 kDa.

In the second set of antigen pools, consisting of proteins in the molecular weight range of 45 – 65 kDa, observation with the L2 panel of antigens was significant, with a median SI < 2.25 and recognizing 72% of SP cases; the H2 panel recognized only 44% of cases. Similar findings were seen with the SN cases, with 65% recognition of SP by L2 antigen pool and 40% recognition by the H2 antigen pool. These results are notable as the L2 panel recognizes only 26% of control NH group. Results were poor with EP group, with only 35% of the extra-pulmonary individuals recognised by H2 and L2. Thus the L1 and L2 CF-Ag pools are promising candidates.

The next set of antigen pools, represented as H3 and L3 in the molecular weight range of 25-45 kDa showed poor response as explained in the results section. The L4 group of antigens, with proteins in the molecular weight range of 25-14 kDa performed better than the above group though lower than L1 and L2, with L4 showing 52% recognition of cases with a median SI of 2.06 in SP cases. The corresponding H4
pool showed a low response (28% recognition). The H5 CF-Ag pool, consisting of low molecular weight proteins, including the ESAT-6 group of proteins showed stimulation of the PBMCs from SP cases with a median SI of 2.01 while L5 showed a lower stimulation. However, the L5 fraction showed good promise for the EP cases, with a much higher % of recognition seen that is comparable to H3 and PPD.

Thus, the L1, L2 and L3 CF-Ag pools performed better than the corresponding high iron CF-Ag pools, with comparable results with PPD. The H5 CF-Ag pool, containing the ESAT group of antigens showed good proliferation though the recognition was relatively lower than PPD. The L1 and L2 CF-Ag pools proved to be significant in the SN group also and in addition, there was statistical significance of both SP and SN groups when compared with normal individuals. Though most of these CF-Ag pools failed to recognize the EP group of tuberculosis cases, L5 CF-Ag pool proved to be better when compared to other antigens, though the response was still lower than PPD.

It is now well established that higher levels of IFN-γ is promising as it correlates with lower incidence of the establishment of the disease. Individuals in an endemic area get infected but not all develop the disease. There are reports that suggest that activation pathways and induction of Th-1-like immune response pathways leading to the generation of IFN-γ is critical for protection against the disease (Cooper et al., 1993). IFN-γ is a powerful activator of macrophages, thereby enabling the latter to kill the residing mycobacteria. Thus, the level of IFN-γ correlates with protection against disease development by the pathogen. However, as other studies have shown, it must be acting in concert with other cytokines and not on its own in affording protection to the host (Sahiratmadja et al., 2007). In a study by Demisse et al. (1999), aimed at studying the immune response of tuberculosis patients and their close contacts, the latter showed high levels of this cytokine in comparison with the tuberculosis patients. In our study here, several fold higher levels of IFN-γ is seen in the healthy controls, with much lower levels of expression in patients with disease. This adds confirmatory evidence to the protective nature of this molecule. Among the antigen pools used in this study, the L5 CF-Ag pool (containing the ESAT-6) evoked the maximal response with markedly high levels of IFN-γ, higher than that triggered by PPD. The findings that ESAT-6 is present in members of *M. tuberculosis* complex but absent in *Mycobacterium bovis* BCG strains and most environmental mycobacterial species enable this molecule to be used both as a
potential vaccine candidate and a novel specific diagnostic reagent. ESAT-6 was consistently found to be a very important target for the T-cell response in the first phase of infection in humans, cattle and guinea pigs. ESAT-6 was recognized during the recall of protective immunity, and the T cells specific for ESAT-6 constituted as much as 25–35% of the mycobacterium-reactive T-cell repertoire recruited in the first phase of disease (Brodin et al., 2004). H3 and L3 CF-Ag pool that caused low level of proliferation of the PBMCs from SP cases of tuberculosis stimulated the release of high levels of IFN-γ in normal individuals, thereby raising the possibility of further studying this group of antigens as potential protective antigens. This antigen pool presumably contains the Ag85 complex, which is comprised of three proteins that have been shown to induce strong T-cell proliferation and IFN-γ production in healthy TB patients (Lim et al., 2004). Though we did not observe notable proliferation, high levels of IFN-γ were seen in the NH individuals. IFN-γ levels have also been shown to exert its influence on the cellular iron status of the macrophage. IFN-γ activation of human monocytes down-regulates (TfR) transferrin receptors numbers on the cell surface (Byrd & Horwitz, 1989) and the rate of macrophage iron acquisition from holotransferrin (Olakanmi et al., 2002). IFN-γ decreases iron availability to intracellular microorganisms that utilize transferrin iron, such as Legionella pneumophila and M. tuberculosis.

Thus, in this study, we tested the efficacy of the CFPs of M. tuberculosis grown under high and low iron conditions to elicit appropriate immune response, so as to enable their application as diagnostic and vaccine candidates. As we could demonstrate differences only by 2D – PAGE, we could not obtain sufficient quantities of the individual antigens for immune proliferation studies. Hence, pools of antigen had to be used. The promising antigen pools have been identified and future work includes the scaling up of these antigen preparations and testing them on a bigger population of samples to establish their applicability as candidate antigens for both diagnosis and vaccine preparation. CFPs have been tested in various animal models of tuberculosis (Andersen et al., 1991). One of the requirements of a candidate vaccine antigen is recognition by the immune system during the course of infection by the majority of individuals of the target population (Dietrich et al., 2006). The parameters used as indicators of T-cell responses include antigen specific proliferation and the measurement of IFN-γ production to identify what are likely to be CD4+ lymphocytes
of the Th1 phenotype that are considered to play an important role in the response against virulent *M. tuberculosis*.

Though antibody-based diagnostic tests have not shown much promise, there is increasing interest in the development of ELISA based tests, specifically with antigens like ESAT-6 that are expressed only by *M. tuberculosis*. Several CFP genes have been cloned, expressed and evaluated for their sero-diagnostic potential (Weldingh *et al.*, 2005). Results with several different recombinant *M. tuberculosis* CFPs suggest that immune recognition varied randomly from patient to patient and there is no definite antigen or set of antigens that is recognized by all or a majority of patients (Gennaro, 2000). No commercially available serological test has so far shown useful levels of sensitivity and specificity, which may be due to the great heterogeneity of the antibody response in TB patients (Weldingh *et al.*, 2005), and it is therefore generally accepted that it will be necessary to include several antigens in a future serodiagnostic assay (Lyashchenko *et al.*, 1998). Through the use of both cocktails of single proteins and genetically engineered fusion molecules containing several antigens, it has been demonstrated that the necessary improvements in sensitivity can be achieved by combining the best antigens (Houghton *et al.*, 2002).

In this study, the CF-Ag sets were used as antigens in ELISA to detect antibodies in the serum of tuberculosis patients. PPD was used as control, which however showed low reactivity. Sero-reactivity was seen only with the SP group with negligible reactivity in the SN and EP cases. In the SP group, the H1, L2, H3 and L3 showed prominent sero-reactivity. The results obtained with ELISA were correlated with Mycotest, a lateral flow device which was also useful only in the SP cases. The CF-Ag H1 showed 60% specificity and 90% sensitivity. Laal *et al.* (1997) showed that the high molecular weight proteins (88 kDa) elicited strong humoral response. Our data is in agreement with their studies that H1 and L2 had given prominent sero-reactivity with the smear positive group. The H3 and L3 antigen pools, containing the Ag85 complex had given about 60% and 50% positivity with the smear positive individuals; this was promising as earlier studies by Sable *et al.* (2005) showed substantial levels of antibodies against Ag85A and Ag85B were found in tuberculosis contacts.

In conclusion, this study has given significant observations on the influence of iron on the culture filtrate proteins. They have been shown to play a significant role in host-pathogen interactions when analysed with the PBMCs of tuberculosis patients.
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Future work will focus on the further characterization and screening of a wider group of patients both for cellular immunity and humoral immunity and identify antigens of diagnostic and vaccine potential.